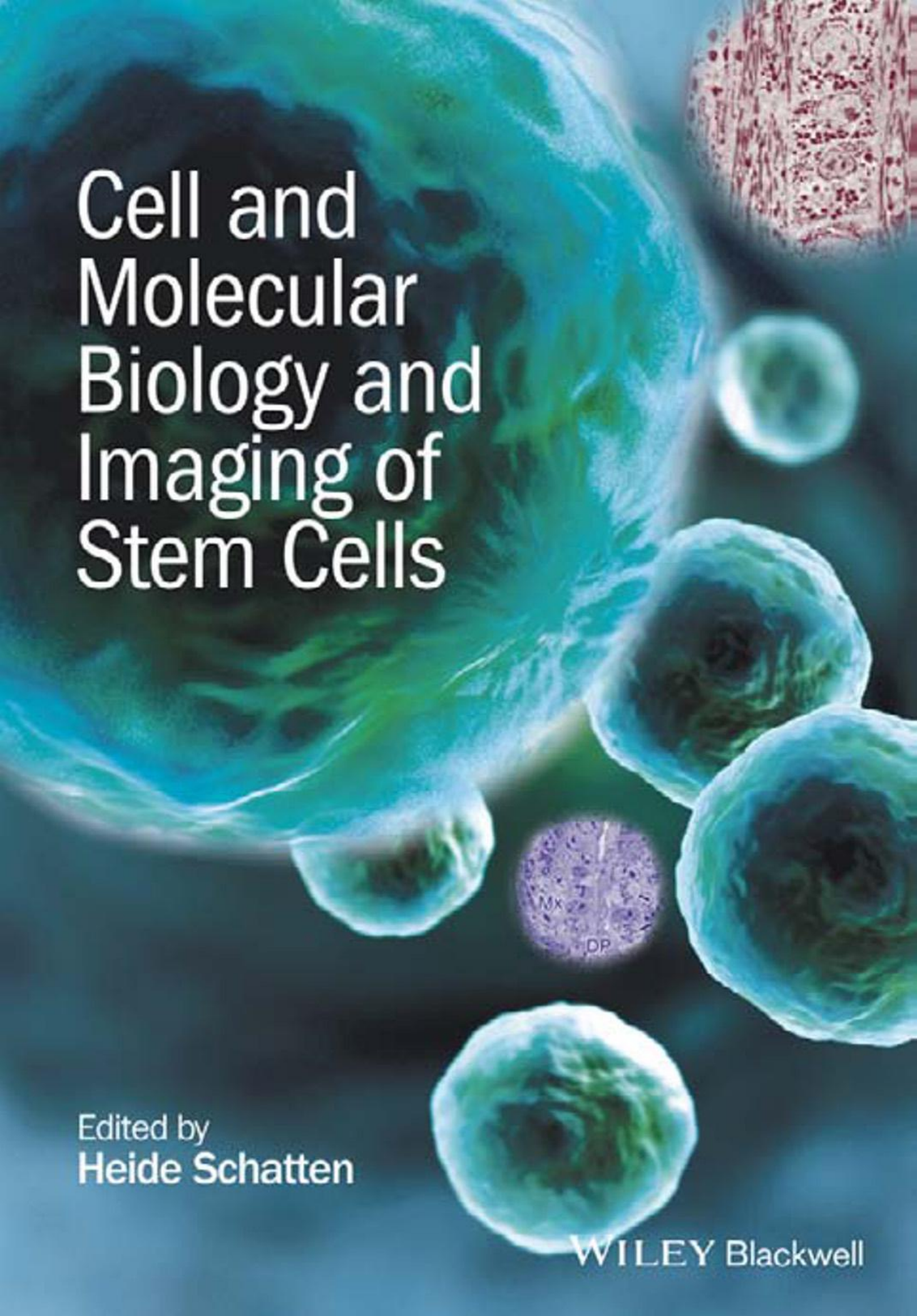
Cell and Molecular Biology and Imaging of Stem Cells



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Cell and Molecular Biology and Imaging of Stem Cells

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Preface

The enormous interest in stem cell biology has led to new research in both basic science and biomedical applications, with significant progress being reported during the past few years. Basic science experiments are focused on exploring stem cell characteristics and potential through experimental manipulations aimed at determining new possibilities for future clinical applications. Biomedical applications include growth of stem cells for tissue engineering and tissue repair, with major success using skin cells as stem cell resources for tissue-specific applications. These efforts have been highly rewarding and have led to promising new applications in regenerative medicine for treatment of organ damage, disorders, and diseases, such as heart disease, muscle diseases, and various others. Induced pluripotent stem cells (iPSCs), adult stem cells and peri-natal stem cells have been utilized to generate multiple cell types including hematopoietic, osteogenic, chondrogenic, adipogenic, endothelial, myogenic, neural, and lung cells that can be utilized for the regeneration of the blood and immune system, bone, cartilage, heart muscle, neural and lung tissue. Using iPSCs to create patient-specific cell lines for autologous cell therapies has generated enormous enthusiasm for translational potential and new cell-based therapies.

Furthermore, new knowledge about cancer stem cells has resulted in new approaches for cancer therapy when it was recognized that this small population of cells within tumors could be the cause for enhanced drug resistance and for recurrence of tumors that had been in remission. Significant new information on cancer stem cells has generated new ideas for targeted tumor treatment strategies.

New journals have been created to specifically address stem cell research and several books have been dedicated to various aspects of stem cell biology; yet, based on the rapid progress in the field, new books are in demand to address new aspects of this exciting and expanding area in the life sciences.

Cell and Molecular Biology and Imaging of Stem Cells features chapters written by experts in their specific areas of research who have been invited for their significant contributions to stem cell biology and imaging of stem cells. Topics include stem cells from the amniotic fluid and placenta, biomaterials as artificial niches for pluripotent stem cell engineering, low-intensity ultrasound in stem cells and tissue engineering, mammalian neo-oogenesis from ovarian stem cells *in vivo* and *in vitro,* Oct4-EGFP transgenic pigs as a new tool for visualization of pluripotent and reprogrammed cells, regulation of adult intestinal stem cells through thyroid hormone-induced tissue interactions during amphibian metamorphosis, stem cell therapy for veterinary orthopedic lesions, sex steroid combinations in regenerative medicine for brain and heart diseases (the vascular system cell niche and a clinical proposal), hair follicle stem cells, the potential of using induced

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pluripotent stem cells in skin diseases, mitochondrial differentiation in early embryo cells and pluripotent stem cells, the role of centrosomes in cancer stem cell functions.

It has been a great pleasure to edit this book on new aspects of stem cell biology and I am most grateful to all contributors for their outstanding chapters and for sharing their unique and specific expertise with the stem cell community. I would like to express my sincere thanks to all for their most valuable contributions and I hope that this book will stimulate further advances in stem cell research to deepen our understanding toward biomedical applications and regenerative medicine.

Heide Schatten

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Chapter 1

Cell and Molecular Biology and Imaging of Stem Cells: Stem Cells from the Amniotic Fluid and Placenta

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Introduction

The promise of stem cell technology has mainly focused on the potential of pluripotent or multipotent cells to differentiate into a variety of different cell types representative of all three germ layers (Dushnik-Levinson and Benvenisty, 1995). Embryonic stem cells (ESCs) were the first truly pluripotent stem cell type described and subsequent research has provided a wealth of knowledge relating to stem cell development and lineage commitment (Reubinoff et al., 2000; Brivanlou et al., 2003). The main disadvantages of using ESCs for cell therapy is the potential for immune rejection of the allogeneic source of cells and their potential to form tumors *in vivo*. In the past few years, researchers have described methods to induce somatic cells into pluripotent stem cells by a process known as reprogramming (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Wernig et al., 2007; Stadtfeld et al., 2008; Zhou et al., 2009). This involves introducing transcription factors, proteins or small molecules into terminally differentiated cells to induce reprogramming into a stem cell phenotype. These induced pluripotent stem cells (iPSCs) possess many of the same properties as ESCs in terms of self-renewal and pluripotency. The advantage of using iPSCs is that patient-specific cell lines can be generated for autologous cell therapies. However, like ESCs, iPSCs possess the potential to form tumors *in vivo*, so are likely to be most useful for *in vitro* applications.

Adult stem cells are stem cell populations that have been identified in organs and tissues of adults such as the bone marrow (Ballas et al., 2002), blood (Lewis and Trobaugh, 1964) brain (Taupin, 2006), lung (Giangreco et al., 2002), and heart (Kinder et al., 2001). Adult stem cells are tissue-specific progenitors and are mainly involved in repair of their corresponding organ. They possess the ability to self-renew like ESCs and are more appealing to use as a replacement therapy, as they circumvent ethical concerns. In addition, autologous transplantation is possible with adult stem cells. The main disadvantage is that adult stem cells often require invasive biopsies for cell isolation, and the resultant cell populations are generally restricted to generating cell

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lineages corresponding to their organ of origin (Weiner, 2008). An attractive alternative is stem cells that can be isolated from gestational tissue such as the placenta, amnion membrane, and the amniotic fluid (De Coppi et al., 2007; Troyer and Weiss, 2008; Serikov et al., 2009; Murphy et al., 2010; Galende et al., 2010). Gestational tissue is usually discarded after birth and usage of this tissue involves minimal ethical or legal concerns. Amniotic fluid is frequently obtained in the second trimester during amniocentesis to detect any chromosomal abnormalities, malformations, and also to determine the sex of the fetus (Joo, 2011). Gestational tissue such as the placenta, placental membranes, and amniotic fluid are untapped reserves of stem cells. In the past decade, research groups have isolated and characterized stem cell populations that are highly multipotent, with the ability to differentiate into hematopoietic, osteogenic, chondrogenic, adipogenic, endothelial, myogenic, neural, and lung cells, among other cell lineages (In 't Anker et al., 2003, Portmann-Lanz et al., 2006; De Coppi et al., 2007). These cells also possess potent immunomodulatory properties, such as production of anti-inflammatory factors as well as interacting with immune cells to modulate the immune response (Murphy et al., 2010, 2011). These properties make peri-natal stem cells an attractive alternative for cell therapy. Therefore, the use of peri-natal stem cells for regeneration or replacement of damaged or diseased tissue such as blood and immune system (Ottersbach and Dzierzak, 2005; Ditadi et al., 2009), bone defects (Fan et al., 2012), myocardial infarction (Zhao et al., 2005; Bollini et al., 2011), neural degeneration (Kakishita et al., 2003; De Coppi et al., 2007), lung disease (Carraro et al., 2008), and diabetes would be valuable (Wei et al., 2003; Chang et al, 2007).

Gestational Tissue Development

Fetal placental tissue is derived from the trophoblast layer of the blastocyst, and functions to provide nutrients, eliminate waste, and provide gas exchange via the mother's blood supply. The placental membrane provides a protective sac for the embryo and its contents. The inner cell mass of the developing embryo forms the epiblast and hypoblast layers. The hypoblast gives rise to extraembryonic tissue, while the epiblast gives rise to the ectoderm, mesoderm and endoderm, germ cells and extraembryonic mesoderm of the yolk sac, amnion, and allantois (Gardner and Beddington, 1988; Loebel et al., 1988; Downs and Harmann, 1997; Downs et al., 2004). Cells of the epiblast collected before implantation are the source of ESCs and can generate the entire fetus (Benitah and Fyre, 2012). The allantois tissue forms the umbilical cord and the mesenchymal part of the mature placenta (Downs and Harmann, 1997; Moser et al., 2004).

Around day 8.5 of gestation the hypoblast and epiblast form a bilayered disc and separate the blastocyst into two chambers; the yolk sac and the fluid-filled amniotic sac (Parameswaran and Tam, 1995; Kinder et al., 1999) (Figure 1.1). The amniotic sac consists of a pair of thin membranes: the outer membrane, the chorion, which envelops the inner membrane and forms a part of the placenta; the inner membrane, the amnion, which envelops the developing fetus and amniotic fluid (Kinder et al., 1999; Robinson et al., 2002).

The amniotic fluid is initially an isotonic fluid consisting of nutrients promoting fetal growth: carbohydrates, proteins, lipids, phospholipids, urea, and electrolytes. As the fetus develops, urine is excreted, which changes the composition and volume of the amniotic fluid (Heidari et al., 1996; Srivastava et al., 1996; Sakuragawa et al., 1999;

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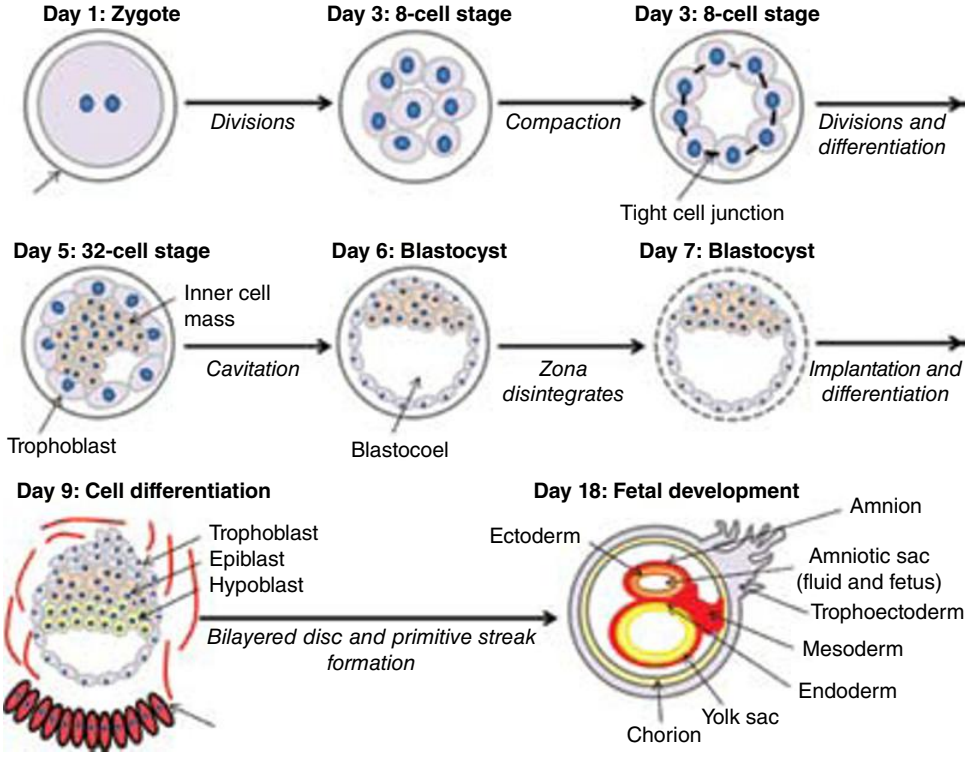


Figure 1.1. The initial stages in the development of an embryo. A single-celled zygote undergoes multiple cell divisions, at the eight-celled stage the cells undergo compaction and bind tightly to one another. Further cell divisions and cell differentiation lead to the formation of the morula (32-celled stage), followed by cavitation resulting in the development of a blastocyst. The zona pellucida disintegrates, followed by implantation. The trophoblast layer of the blastocyst gives rise to the fetal placenta. Further divisions and differentiation occurs giving rise to the primitive streak and hence the three layers of the embryo. The epiblast and hypoblast form a bilayered disc that leads to the formation of amnion and chorion membranes.

Bartha et al., 2000). Amniotic fluid ensures movement of the fetus and symmetrical structural fetal development and growth. Amniotic fluid is inhaled and swallowed by the fetus contributing to lung development and gastrointestinal tract development respectively. Amniotic fluid contains a variety of cell types present during development, such as cells sloughed off the developing fetus, the alimentary tract, respiratory tract, urogenital tract, fetal amnion membrane, and skin (Medina-Gómez and del Valle, 1988).

The mature human placenta has both a fetal as well as a maternal component. Much of the placenta originates from the inner cell mass of the morula (chorion, amnion, and mesenchymal core of the chorion), with the trophoblast layer contributing towards the outer layer of the placenta. As the origin of gestational tissue or extraembryonic tissue takes place during the very early stages of embryonic development, these cells possess multipotent differentiation potential and hence are a rich and valuable source of stem cells for cell therapy.

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Isolation and Characterization of Peri-Natal Stem Cells

Gestational tissue is usually discarded after birth so obtaining tissue is non-invasive and resourceful. Multipotent cells can be isolated from gestational tissue: placenta (Steigman and Fauza, 2007), amnion membrane (Alviano et al., 2007), chorion membrane (Bailo et al., 2004), and amniotic fluid (Kaviani et al., 2001) (Figure 1.2). Mesenchymal stromal cells (MSCs) can be isolated from the placenta by mechanically separating the fetal placental specimen from the maternal decidua (Steigman and Fauza, 2007). The fetal placental tissue is minced and digested with a collagenase/dispase mixture, filtered, and plated on cover slips. Placental MSCs display a typical spindle-shaped fibroblastic morphology. Placental MSCs express the typical MSC markers (CD44, CD73, CD29, CD90 and CD105) and do not express hematopoietic markers such as CD14, CD34, and CD45, the stem-cell markers CD133, or the endothelial marker CD144 (C. D. Li et al., 2005; Kern et al., 2006). The cells also possess the ability to adhere to plastic and can be expanded *ex vivo*. On the other hand, placental MSCs expand quicker *in vitro* than adult MSCs and appear to be less immunogenic and more immunosuppressive than adult MSCs (C. D. Li et al., 2005; Kern et al., 2006). Although placental MSCs do not possess the pluripotent capacity of ESCs, they can give rise to multiple cell types representative of all three

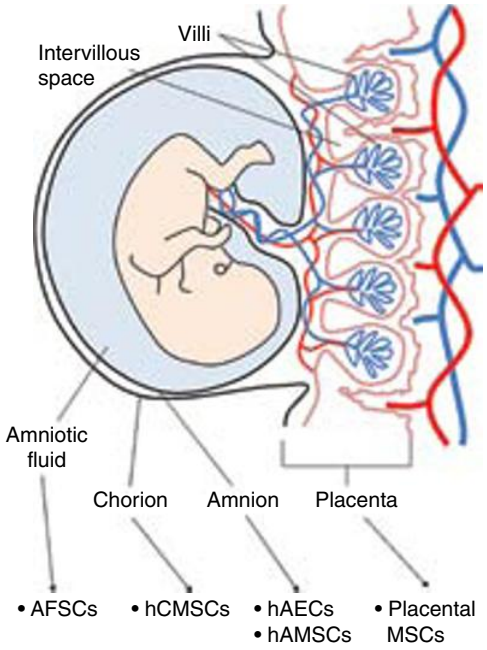


Figure 1.2. Source of peri-natal stem cells. Highly multipotent amniotic-fluid-derived stem cells (AFSCs), chorion mesenchymal stromal cells (hCMSCs), amnion epithelial stem cells (hAECs), mesenchymal stromal cells (hAMSCs), and placental mesenchymal stem cells can be isolated from gestational tissue and have a large range of potential applications for the treatment of disease.

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germ layers (Battula et al., 2007). The properties of MSCs that can be derived from the placenta hold promise for inflammatory conditions and cell therapy.

Epithelial cells from the amnion membrane have been found to be multipotent (Ilancheran et al., 2007) with low immunogenicity (Bailo et al., 2004) and immunosuppressive functions (H. Li et al., 2005). Recently, our group demonstrated the isolation of human amniotic epithelial cells (hAECs) using methods suitable for clinical applications (Murphy et al., 2010). The isolation procedure first requires the term human amnion membrane to be separated from the chorion membrane and washed in a saline solution to remove the blood. This is followed by enzymatic digestion of the amnion membrane with TrypZean – an animal-component-free recombinant trypsin, which generates epithelial cells that can be maintained in serum-free conditions. Approximately 120 million viable epithelial cells can be isolated from a single amnion membrane. The hAECs express a variety of epithelial markers, including cytokeratins, EpCAM (epithelial markers) and CA125 (differentiation antigen of fetal coelomic epithelium) (Regauer et al., 1985; Nanbu et al., 1989; Murphy et al., 2010). With subsequent divisions, epithelial cells retain a normal karyotype and cell cycle distribution while maintaining their telomere lengths. Although hAECs do not proliferate as well as placental MSCs *in vitro,* they are highly multipotent and give rise to cell types from all three germ layers (Ilancheran et al., 2007; Murphy et al., 2010). In addition they possess immunosuppressive properties that have been well characterized *in vitro* (Hori et al., 2006) as well as *in vivo* (H. Li et al., 2005). The desirable properties of hAECs hold promise for the treatment of inflammatory conditions and regenerative therapies.

Mesenchymal stromal cells can also be isolated from the amnion membrane that are multipotent and give rise to multiple cell lineages: adipogenic, chondrogenic, endothelial, osteogenic, and myogenic (Alviano et al., 2007). The term amnion membrane is separated from chorion membrane by blunt dissection. The amnion is minced and digested with 0.25% trypsin– ethylenediaminetetraacetic acid (EDTA) solution for 15 min. The supernatant is discarded and the remaining amnion membrane fragments are further digested with 0.25% trypsin–EDTA solution, collagenase and DNaseI in Dulbecco's modified Eagle's medium (DMEM). This process generates human amnion membrane mesenchymal stromal cells (hAMSCs), which can be cultured and expanded up to 15 passages *in vitro* without any noticeable morphological alterations. The hAMSCs express CD105, CD73, CD29, CD44, and CD166, and are negative for CD14, CD34, and CD45, similar to adult MSCs (Alviano et al., 2007). Similarly multipotent MSCs from human chorion membrane stem cells (hCMSCs) can also be isolated (Bailo et al., 2004). The chorion fragment is mechanically separated from the maternal decidua, minced and enzymatically digested with dispase and collagenase. The fragments are thrice digested with trypsin and the remaining tissue fragments are further treated with collagenase. The hCMSCs can be successfully propagated *in vitro.* Human chorion membrane stem cells express the mesenchymal markers CD166, CD105, CD90, CD73, CD49e, CD44, CD29, and CD13, but are negative for the hematopoietic stem-cell markers CD14, CD34, and CD45 (Portmann-Lanz et al., 2006). Both hAMSCs and hCMSCs are found to have low immunogenicity and immunosuppressive properties (Bailo et al., 2004), making them an attractive source of cells for cell therapy and treatment for inflammatory diseases.

Amniotic fluid is routinely obtained during amniocentesis and can also be obtained at term following birth. Two millimeters of amniotic fluid contains approximately

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20,000 cells (Kaviani et al., 2001), of which 1% are c-kit positive multipotent stem cells (De Coppi et al., 2007). During the initial passage, amniotic-fluid-derived stem cells (AFSCs) maintain a round shape when cultured in non-treated culture dishes. After a week, these cells adhere to the plate and adopt a spindle-shaped morphology with a high proliferative capacity needing to be passaged every 48–72 h. The AFSCs maintain self-renewal capacity up to 250 doublings, exceeding Hayflick's limit of 50 doublings for somatic cells. The karyotype does not change when early and late passages are compared and proceed through the normal cell cycle checkpoints as somatic cells. Amniotic-fluid-derived stem cells express some markers similar to ESCs: they express SSEA-4 (stage-specific embryo antigen 4) and the stem-cell marker Oct-4 (Mihu et al., 2008), but they do not express SSEA-1/-3, CD34, CD133, and BMP4 (bone morphogenetic protein 4), or alkaline phosphatase, which is expressed by ESCs (Mihu et al., 2008). The AFSCs also do not express hematopoietic stem-cell markers: CD45, CD34, and CD133 (Mihu et al., 2008). The AFSCs can be characterized by their expression of certain mesenchymal and neuronal markers: CD29, CD44, CD73, CD90, and CD105. The expression profile of AFSCs suggests that AFSCs are like ESCs in some aspects and not in others. Amniotic-fluid-derived stem cells can form embryoid bodies in vitro (Valli et al., 2010), but do not form teratomas when implanted in immunodeficient mice (Siddiqui and Atala, 2004), which is favorable. The AFSCs, like ESCs, can differentiate and give rise to cells from all three germ layers.

Cell Types Derived from Gestational Tissue

Cells isolated from gestational tissue possess self-renewal capacity and have the potential to give rise to multiple cell lineages. These properties allow researchers to generate large numbers of cells that can be differentiated into functional lineage committed cell types (Table 1.1). The following section provides a brief overview of the various specialized cell types that can be derived from peri-natal stem cells.

Hematopoietic Cells

The ability to regenerate the entire hematopoietic system would be extremely beneficial to cancer patients and patients receiving transplants. Cancer patients and patients receiving an organ transplant are often immuno-compromised and are hence susceptible to infections. In leukemia patients, bone marrow transplants are carried out to repopulate the blood system and this is an invasive and painful procedure. The promise of peri-natal stem cells to repopulate the hematopoietic system would overcome these problems. The potential of murine and human AFSCs to differentiate into cells of the hematopoietic linage has been reported (Ditadi et al., 2009). From the amniotic fluid, cells expressing c-Kit are found to possess hemopoietic differentiation potential. Hematopoietic stem cells (HSCs) and subsequent progenitors can be classified as lineage negative (Lin^-) if they do not express mature blood cell markers such as those found on mature lymphoid or myeloid cells. c-Kit^+Lin^- AFSCs can give rise to cells from all three hematopoietic lineages *in vitro*; erythroid, myeloid and lymphoid. When cultured in semi-solid media consisting of MethoCult Methycellulose colony assay medium supplemented with human stem cell factor (SCF), granulocyte colony stimulating factor

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Table 1.1. *In vitro* differentiation of peri-natal stem cells.

Differentiation potential	Multipotent cell type	Differentiation media
Hematopoeitic	AFSCs	MethoCult Methycellulose colony assay medium, SCF, G-CSF, GM-CSF, IL-3, Il-6, and erythropoietin (erythrocytes, granulocytes, monocytes) OP9 stroma cell in RPMI/alpha medium, Glutamax I, 20% FBS, SCF, IL-15, IL-7, and Flt3 (lymphocytes)
Osteogenic	AFSCs, placental MSCs	DMEM supplemented with dexamethasone, β-glycerophosphate, and ascorbic acid 2-phosphate
	hAECs, hAMSCs, hCMSCs	DMEM-H medium supplemented with 10% FBS, dexamethasone, 1α,25-dihydroxyvitamin D3 ascorbic acid, and β-glycerophosphate
Chondrocytic	AFSCs	DMEM with 1% ITS Premix, L-glutamine, supplemented with dexamethasone, L-proline, 50 μg/mL ascorbic acid-2-phosphate,TGF-β1, BMP2 and IGF-1 in 3-D alginate hydrogels and pellet cultures
	hAMSCs	DMEM containing insulin, transferrin, selenous acid, linolenic acid, BSA, proline, sodium pyruvate, dexamethasone, L-ascorbic acid-2-phosphate, supplemented with TGF-β3 in pellet cultures
Adipogenic	AFSCs, placental MSCs, hAECs, hAMSCs, hCMSCs	DMEM supplemented with 10% FBS, isobutyl-methyl xanthine, indomethacin, dexamethasone, and insulin
Endothelial	AFSCs, hAMSCs	DMEM supplemented with 10% FBS, VEGF, hFGF-b, EGF, IGF-1, heparin, ascorbic acid
Myogenic	AFSCs (cardiomyocytes)	Low-glucose DMEM supplemented with 10% horse serum, 10% FBS, and 1% PS
	hAMSCs	DMEM supplemented with 5% of FBS, 40% MCDB-201, ITS-LA*BSA, dexamethasone, ascorbic-acid-2-phosphate, bFGF, VEGF, IGF-1
	hAECs	DMEM-H supplemented with 10% FBS/5% horse serum, hydrocortisone, dexamethasone
Neurogenic	hAECs	RPMI 1640 medium supplemented with 10% fetal calf serum
	Placental MSCs, hAMSCs, hCMSCs	DMEM-H supplemented with 10% FBS, all-trans retinoic acid
Pulmonary cells	AFSCs	hAECs microinjected into explant lungs placed on pore filters laid on the surface of (DMEM)/Ham's F-12 medium (F12) containing 10% FBS
	hAECs	SAGM

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(G-CSF), granulocyte macrophage colony stimulatory factor (GM-CSF), IL-3, Il-6, and erythropoietin for 3 weeks, the c-Kit$^+$Lin$^-$ AFSCs differentiate into granulocyte, monocyte, erythrocyte, and megakaryocyte colony-forming units (CFU). When AFSCs are co-cultured with OP9 stroma cells in Roswell Park Memorial Institute (RPMI) or alpha medium with Glutamax I supplemented with 20% fetal bovine serum (FBS), SCF, IL-15, IL-7, and Flt3 ligand, they give rise to CFUs that express markers found on T-cells (CD3, CD4, and CD8), B-cells (CD19), and NK cells (CD56 and CD16). Hematopoietic progenitors can be found in the amniotic fluid before the 12th week of gestation (Torricelli et al., 1993). When AFSCs are cultured on a layer of bone marrow stromal cells (feeders), these cells give rise to erythroid CFU. Due to the differing origin of cells found in the amniotic fluid, early progenitors of various cell lineages can be found along with multipotent stem cells. These studies suggest the potential of peri-natal stem cells for use in regenerating the hematopoietic system.

Osteocytes

Regeneration of bone tissue would be advantageous in treating many types of bone defects or damage such as spinal cord injuries. Amniotic-fluid-derived stem cells cultured in osteogenic induction media consisting of dexamethasone, beta-glycerophosphate, and ascorbic acid 2-phosphate give rise to an osteogenic phenotype. Induction toward an osteocyte lineage is characterized by the loss of the spindle-shaped morphology and development of an osteoblast-like appearance with finger-like excavations into the cytoplasm (De Coppi et al., 2007). The AFSC-derived osteoblasts present key osteoblast features such as formation of cell aggregates resembling lamellar-like structures, production of alkaline phosphatase, and precipitation of calcium. The AFSC-derived osteoblasts also exhibit expression of specific bone development genes such as alkaline phosphatase, core-binding factor A1, and osteocalcin. In addition, AFSC cultured on three-dimensional fibrin scaffolds in osteogenic induction media can differentiate into osteoblasts with extracellular matrix (ECM) production and deposition on scaffolds (Maraldi et al., 2011).

Placental MSCs also differentiate into an osteogenic phenotype when cultured in osteogenic induction media (Zhong et al., 2012). After 6 days in culture, the placental-derived MSCs lose their spindle-shaped morphology and appear more flat with a polygonal morphology. After 12–15 days in culture, mineralized nodules can be observed. By 16 days in culture, the cells appear cuboidal with stabilization in mineralized nodule density indicating a stably differentiated state of osteoblasts. The nodules stain red with Alizarin red S, confirming calcium content in them. The placental MSC-derived osteoblasts show lineage-specific function, including: secretion of growth factors involved in bone development; vascular endothelial growth factor (VEGF); insulin-like growth factor 1 (IGF-1); and hepatocyte growth factor (HGF).

The hAECs, hAMSCs, and hCMSCs can be induced towards an osteogenic lineage (Portmann-Lanz et al., 2006). When cultured in Dulbecco's modified Eagle's medium, high glucose (DMEM-H) supplemented with 10% FBS, dexamethasone, 1α,25-dihydroxyvitamin D3 ascorbic acid, and β-glycerophosphate, these cells differentiate into osteocytes. Within 18–21 days in culture, the cells express alkaline phosphatase, which is followed by the expression of osteocalcin and collagen I. The cells stain red with Alizarin red S, indicating calcium deposition and mineralization. Although hCMSCs

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were found to favor differentiating into osteocytes more than the other two cell types, all cell types have the potential to differentiate into osteocytes. These results point toward the potential of gestational tissue in regenerating bone defects and spinal and bone injuries.

Chondrocytes

Regeneration of the hyaline cartilage is a major challenge in treating cartilage damage as native chondrocytes are bound by lacunae and cannot migrate to sites of damaged cartilage. The AFSCs can be differentiated towards a chondrogenic phenotype *in vitro*, in the presence of transforming growth factor beta 1 (TGF-β1), bone morphogenic protein 2 (BMP2), and IGF-1 both in three-dimensional alginate hydrogels and pellet cultures (Kolambkar et al., 2007). The AFSCs cultured in this fashion for 3 weeks produce sulfated glycosaminoglycan (sGAG) and collagen II (ColII). Other research groups have made similar observations. Amniotic-fluid-derived stem cells encapsulated in a fibrin hydrogel containing TGF-β3 are able to give rise to chondrocytes, both *in vitro* and *in vivo*, resembling normal cartilage structure and development (Park et al., 2011). The AFSC-derived chondrocytes express markers that are associated with cartilage development: aggrecan, COLII, SOX9, and cartilage oligomeric matrix protein (COMP). These cells are also able to form a collagen matrix with collagen content.

Similarly, hAMSCs can differentiate into chondrocytes when cultured in media supplemented with TGF-β3 after 3 weeks (Alviano et al., 2007). The hAMSC-derived chondrocytes stain positive for ColII production, indicating abundant ECM formation. These findings suggest the important role of the TGF-β family of proteins in chondrogenic development and the potential to be used for cartilage repair.

Adipocytes

Peri-natal stem cells possess the ability to differentiate into adipocytes under specified conditions. The AFSCs, placental MSCs, hAECs, hAMSCs, and hCMSCs cultured in media supplemented with indomethacin, dexamethasone, insulin, and methylxanthine for 2–3 weeks can be induced towards an adipogenic phenotype (Portmann-Lanz et al., 2006; Alviano et al., 2007; De Coppi et al., 2007; Soncini et al., 2007;Chen et al., 2011;). These cells contain a high density of vacuoles containing oil droplets and hence stain red with oil red O. The AFSC-derived adipocytes also secrete considerable amounts of lipid drops and express markers such as peroxisome proliferation-activated receptor γ (PPARγ), lipoprotein lipase (LPL), and CCAAT/enhancer binding protein (C/EBPα) specific to adipose cells.

Endothelial Cells

The AFSCs and hAMSCs can be induced towards an endothelial phenotype *in vitro* in culture media supplemented with VEGF, human fibroblast growth factor basic (hFGF-b), epidermal growth factor (EGF), IGF-1, heparin, ascorbic acid, and 10% FBS (Alviano et al., 2007; De Coppi et al., 2007; Zhang et al., 2009). Endothelial cell induction has been observed on semi-solid media, gelatin, or matrigel-coated surfaces. Within a week, the cells undergo morphological changes including the formation of

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capillary-like structures. Within 2 weeks, the induced endothelial cells are able to form cord-like structures and take up acetylated low-density lipoprotein (LDL)/lectin when plated on matrigel. Uptake of LDL/lectin is a characteristic of functional endothelial cells. These cells express common endothelial markers such as CD31, human-specific endothelial cell surface marker (P1H12), factor VIII (FVIII), kinase insert domain receptor (KDR), von Willebrand factor (vWF), and vascular cell adhesion molecule (VCAM). When stimulated by physiological levels of shear force (12 dyne/cm^2), the expression of CD31 and vWF is upregulated and the length of cords increases. Under hypoxic conditions, VEGF and placental growth factor (PGF) expression increase. These observations point toward the use of peri-natal stem cells for vascular therapies.

Myocytes

The ability to regenerate muscle tissue would be advantageous in treating cardiac disease. Amniotic-fluid-derived stem cells in their undifferentiated state express many cardiac-specific genes such as transcription factor mef2 and the gap-junction proteins connexin 43, H- and N-cadherin (Guan et al., 2011). When cultured in media supplemented with low-glucose DMEM supplemented with 10% horse serum, 10% FBS and 1% penicillin–streptomycin (PS), AFSCs give rise to cardiomyocyte-like cells. The AFSCs when cultured under these conditions upregulate expression of cardiac specific genes, troponin I (TnI) and troponin T (TnT), redistribute connexin 43, and downregulate transcription factor SRY-box 2 (SOX2), which is a marker expressed by progenitors (Guan et al., 2011). In addition, AFSCs co-cultured with neonatal rat cardiomyocytes (NRCs) are able to form mechanical and electrical connections with the NRCs. The co-culture stimulates upregulation of the cardiomyocyte markers cTnT, cTnI, and α-actinin in AFSCs (Guan et al., 2011; Bollini et al., 2011). The connections formed by AFSCs with beating NRCs induce differentiation of AFSCs into functional beating cardiomyocytes.

Under similar conditions, hAMSCs with the addition of modified Ham's nutrient mixture F-12 (MCDB-201) express myosin D (MyoD) after 1 week of culture, and by 2–3 weeks express myogenin and desmin, which coincide with expression patterns during normal muscle development (Alviano et al., 2007). The hAECs when cultured with DMEM-H supplemented with 10% FBS or 5% horse serum, hydrocortisone, and dexamethasone differentiate into myocytes (Portmann-Lanz et al., 2006). The hAEC-derived myocytes after 18–21 days of culture formed long multinucleated cells that are precursors of myotubes. The cells express MyoD1 and a skeletal muscle myosin heavy chain, indicating a skeletal muscle phenotype.

The differentiation of peri-natal stem cells into myocytes and cardiomyocytes, in the case of AFSCs, suggests its potential use for skeletal muscle and cardiac muscle regeneration.

Neuronal Cells

The generation of neural tissue would be valuable for treatment of neurodegenerative diseases and spinal cord injuries that are currently irreparable. The hAECs when cultured in RPMI 1640 medium supplemented with 10% fetal calf serum give rise to neuronal progenitors (Sakuragawa et al., 1996). The cultured hAECs stain positive for

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microtubule associated protein 2 (MAP-2), mitogen-activated protein kinase 2 (MAPK2), vimentin, neurofilament proteins, GFAP, 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase), myelin basic protein, and galactocerebroside. The hAEC-derived neuronal progenitors also produce neurotransmitters such as catecholamines (Elwan et al., 1998) and dopamine (Kakishita et al., 2000). Another group has shown that when placental MSCs, hAECs, hAMSCs, and hCMSCs are cultured in DMEM-H supplemented with 10% FBS and all-trans retinoic acid they give rise to neuron-like structures (Portmann-Lanz et al., 2006). These structures have bodies with long processes and growth cones. The placenta-derived cells stain positive for neural stem markers (CD133), neural progenitor markers (nestin), and mature neuron marker (NF200). These results indicate the neurogenic differentiation potential of peri-natal stem cells.

Pulmonary Cells

Regeneration or replacement of lung tissue would be beneficial in respiratory disorders such as cystic fibrosis, which currently has no cure. The hAECs when cultured in small airway growth medium (SAGM) for 28 days (without passaging) give rise to cystic fibrosis transmembrane conductance regulator (CFTR) expressing cells (Murphy et al., 2012a). The SAGM is a medium that induces differentiation of embryonic stem cells into type II pneumocytes. These hAEC-derived CFTR-expressing cells have polarized CFTR distribution on their plasma membrane and the CFTR iodide/chloride ion channels are functional, indicating the potential of hAECs to be used as a cell therapy for cystic fibrosis.

The AFSCs have shown pulmonary differentiation potential following intratracheal injection into explanted embryonic mouse lungs (E11.5-12.5). Within 1 week of injection, AFSCs integrate into the lung epithelium and differentiate into cells expressing thyroid transcription factor (TTF-1), which is a marker of an early lung phenotype (Carraro et al., 2008). After injection, these explant lungs were placed on pore filters that were laid on the surface of (DMEM)/Ham's F-12 medium (F12) containing 10% FBS for 2–10 days. These studies indicate the potential of peri-natal stem cells in cell therapy for respiratory disorders.

Immunosuppressive Properties of Peri-Natal Stem Cells

Peri-natal stem cells are a valuable source for cell therapy as they have low immunogenicity (Bailo et al., 2004) and are immunosuppressive (Wolbank et al., 2007). Amnion and chorion cells isolated from the placenta do not induce an allogenic or xenogenic lymphocyte response (Bailo et al., 2004). When amnion and chorion cells are cultured with lymphocytes from allogenic donors they do not stimulate lymphocyte proliferation. These findings indicate the low immunogenic property of peri-natal stem cells, which is advantageous for cell therapy.

Peri-natal stem cells are also able to modulate the immune response by inhibiting activated B and T-cell proliferation. Mesenchymal and epithelial cells isolated from the amnion membrane were found to inhibit the proliferation of lymphocytes, stimulated with irradiated allogenic lymphocytes or PHA, in a dose-dependant manner

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(Wolbank et al., 2007). Similarly another group has demonstrated that placental MSC-like cells can suppress peripheral-blood lymphocyte proliferation as well as cord-blood lymphocyte proliferation following stimulation (C. D. Li et al., 2005). These results indicate the immunosuppressive effects of peri-natal stem cells. The low immunogenicity and inhibition of an active lymphocyte response would be advantageous for engraftment efficiency and for treatment of inflammatory conditions. It also indicates the potential use of allogenic or even xenogenic transplants as a cell therapy. In inflammatory or autoimmune diseases such as Crohn's disease and graft versus host disease (GVHD), the ability of peri-natal stem cells to suppress the immune response may be beneficial.

Cell Therapy Applications of Peri-Natal Stem Cells

The properties of peri-natal stem cells – self-renewal capacity, multipotency, and ability to suppress the immune system – render them an attractive resource for cell therapy. This section focuses on the potential use of peri-natal stem cells in a variety of regenerative applications.

Blood and Immune System Regeneration

Peri-natal stem cells contain a small population of hematopoietic progenitors and are able to give rise to CFU of all three hematopoietic lineages *in vitro*. *In vivo*, the peri-natal stem cells are capable of regenerating the hematopoietic system. Four months following injection of AFSCs into irradiated $Rag^{-/-}$ mice, AFSC-derived NK cells, B cells, T cells, macrophages, granulocytes, and red blood cells could be detected (Ditadi et al., 2009). Secondary transplantation was partially successful, with a low engraftment rate. Cells collected following primary transplantation gave rise to all cell types of the blood and immune system. The successful engraftment of secondary transplantation confirms that AFSCs possess a small number of hematopoietic progenitors capable of self-renewal and multilineage differentiation. These studies show the long-term hematopoietic repopulation capacity of peri-natal stem cells *in vivo* and hence could be applied to treating blood and immune system disorders. In several diseases and disorders, the immune system of patients is severely compromised and repopulation of healthy normal blood cells is done using HSCs from the bone marrow. Peri-natal stem cells would provide an alternative way of repopulating blood cells and offer the possibility of an autologous transplantation where HSCs are absent or compromised.

Bone Regeneration

Mesenchymal stromal cells from the bone marrow can be used to regenerate bone tissue, however, cell isolation requires invasive biopsies and potential for *in vitro* expansion is limited. When AFSCs are embedded in an alginate/collagen scaffold and cultured in osteogenic induction media for 45 days they can be differentiated into osteoblasts that stain for Alizarin red S (calcium deposition) and form nodules (De Coppi et al., 2007). These AFSC-seeded scaffolds were implanted subcutaneously in immunodeficient mice

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(athymic nude nu/nu mice) after culture for 1 week in osteogenic induction media. Extensive mineralization was observed in seeded constructs after 8 weeks of implantation. Eighteen weeks after implantation, masses of bone-like tissue were observed near the site of implantation with greater bone densities than normal femoral bone. Similarly, placental MSCs when co-cultured with bio-derived bone materials can lead to the formation of tissue-engineered bone (TEB) (Fan et al., 2012). When implanted in sites of surgically created osteoperiosteal defects in rabbits, the placental MSC-derived TEB could repair the defect with similar results as those observed with bone-marrow-derived TEB. These results indicate the ability of peri-natal stem cells to generate bone segments and repair bone defects *in vivo* and hence use for treatment of cranio-facial defects and spinal injuries.

Myocardial Infarction

Cardiovascular disease is the leading cause of death in many developed as well as developing countries. A major target of regenerative therapy is to regenerate cardiac muscle tissue after ischemia or an infarction. The AFSCs were cardioprotective, improved myocardial cell survival, and decreased infarct size after administration in Wistar rats with ligation of the left anterior descending coronary artery (simulated ischemia) (Bollini et al., 2011). In addition, the AFSCs were found to produce the actin binding protein thymosin beta 4 (Tβ4), which is an angiogenic factor also involved in promoting cardiomyocyte survival and migration. Another group of researchers were able to successfully transplant AFSC cell aggregates into the peri-infarct areas of immuno-compromised rats by direct injection into the peri-infarct area (Lee et al., 2011). The AFSCs slowed the progression of heart failure, improved global function, and increased the motion of the heart muscle walls. Similarly, hAMSCs when co-cultivated with neonatal heart transplants and transplanted into myocardial infarcts in rat hearts were able to differentiate into cardiomyocyte-like cells and integrate into the heart muscle (Zhao et al., 2005). These experiments point towards the regeneration of heart muscle with peri-natal stem cells following a heart attack.

Neural Regeneration

Currently there are no cures for neurodegenerative diseases such as Alzheimers disease, which involve the progressive deterioration of nerve cells. The potential of peri-natal stem cells as a therapy for such conditions would be valuable. A mouse model of neurodegeneration is the twitcher mouse; these mice lack the galactocerebrosidase enzyme and hence undergo extensive neuronal deterioration (De Coppi et al., 2007). The AFSCs have been shown to integrate in various regions of the brain, the hippocampus, periventricular areas, and the olfactory bulb, after administration into the lateral cerebral side of new-born twitcher mice. The AFSCs were maintained in neural induction media for a week before transplantation. The AFSC-derived neuronal cells were morphologically similar to resident surrounding brain cells. The cells persisted for 2 months in the areas of the brain. Amnion epithelial cells have shown promise in slowing the progression of Parkinson's disease in rats (Kakishita et al., 2003). These cells were injected into the midbrain of immuno-compromised rats that were subjected to a unilateral nigrostriatal lesion (induced by

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6-hydroxydopamine infusions). The amnion epithelial cells promoted growth and survival of nigral dopamine-secreting neurons by secretion of diffusible molecules. In addition, when labeled hAECs were transplanted into the transection cavities in the spinal cord of bonnet monkeys, they were able to survive and promote the growth of host neurons (Sankar and Muthusamy, 2003). The hAECs survived in the spinal cord during the course of the study (60 days) and also prevented formation of a glial scar at the transection lesion site. These results show the promise of peri-natal stem cells in slowing the progression of neurodegenerative diseases.

Lung Regeneration

The AFSCs when injected into the embryonic mouse lung successfully integrate into the epithelium and express markers associated with lung development, such as TTF-1 (Carraro et al., 2008). The AFSCs were found to integrate into the lung epithelium and express lung associated markers: TTF-1 and surfactant protein C (SP-C). It is interesting to note that AFSCs respond in an injury-dependent manner: in mouse models of naphthalene-induced lung injury (destroys Clara cells residing in upper airways), AFSCs accumulate in upper airways; in mouse models of oxygen-induced lung injury (targets lower airways), AFSCs accumulate in lower airways. The AFSCs accumulate at the site of lung injury and have immunomodulatory effects that help in repair processes such as macrophage migration inhibitory factor (MIF) and plasminogen activator inhibitor-1 (PAI-1) (Buckley et al., 2011).

Recently, research groups have demonstrated the use of peri-natal stem cells in preventing fibrosis and improving lung function in animal models of pulmonary fibrosis (Cargnoni et al., 2009; Murphy et al., 2011, 2012b; Hodges et al., 2012). They made use of the bleomycin-induced mouse model of pulmonary fibrosis, which mimics the secondary onset of chronic inflammation and fibrosis. Human epithelial cells isolated from the amnion were injected into the peritoneal cavity of bleomycin-treated mice 24 h after bleomycin intranasal administration. Within 3 days, the bleomycin-treated mice that received human amnion epithelial cells had improved lung function and within 7 days these mice had reduced fibrosis and collagen content in their lung tissue. In addition, these mice had less α-smooth-muscle actin (αSMA)-expressing myofibroblasts in the lung tissue, which is a key indicator of fibrosis. The bleomycin-treated mice with human amnion epithelial cell administration had less expression of pro-inflammatory cytokines such as TNF-α, IL-6, TGF-β, and IFN-γ in the lung tissue compared to bleomycin-treated mice. These results were further validated by reduced infiltration of immune cells such as macrophages and neutrophils into the lung tissue of bleomycin-treated mice with human epithelial amnion cells. Similarly, hAECs have been used in a ventilation-induced lung injury sheep model (Hodges et al., 2012). The hAECs administered through the jugular vein and tracheal catheter following *in utero* ventilation (IUV) of fetal sheep alleviated the damage caused by ventilation. The fetal sheep that received hAECs during IUV had reduced collagen and elastin content in the lung tissue, indicating reduced fibrosis when compared to IUV alone. The hAECs successfully engrafted into the fetal lung tissue and morphologically resembled type I and type II alveolar epithelial cells as determined by surfactant protein C expression. The hAECs were able to attenuate inflammation by decreasing TGF-β production and pro-inflammatory cytokines such as IL-8, IL-1, and IL-6. These studies suggest that

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peri-natal stem cells have the potential to treat diseases such as cystic fibrosis and bronchopulmonary dysplasia acute respiratory distress syndrome.

Pancreatic Cell Regeneration

Diabetes mellitus is one of the most prevalent disorders in the United States. Current management of diabetes includes injection of recombinant human insulin and replacement of pancreatic tissue. However, donor tissue availability is limited, so an approach to regenerate the pancreatic islet cells (β cells of islets) of diabetic individuals would be valuable. The hAECs can be stimulated to produce insulin and express GLUT-2 transporters (insulin-dependent glucose transporter found on pancreatic β cells) (Wei et al., 2003). These cells are able to contribute to the regulation of blood glucose levels in diabetic mice after transplantation. Placenta MSCs can be stimulated to secrete insulin as well as glucagon and are able to restore glucose levels to normal in streptozotocin-pretreated nude mice, which mimics beta cell destruction observed in type I diabetes (Chang et al., 2007).

The hAECs can be stimulated to express insulin and GLUT-2 mRNA *in vitro*. *In vivo*, when hAECs are injected into streptozotocin-induced diabetic mice, they are able to normalize the blood glucose levels after several weeks of transplantation (Wei et al., 2003; Toda et al., 2007). These studies suggest the potential of peri-natal stem cells for diabetes treatment.

Immunomodulation

Inflammation, recruitment, and activation of immune cells are immune-system mechanisms in response to injury or infection to limit damage to the body. In complex chronic diseases, the immune response is constantly switched on and the inflammatory milieu promotes damage and hence the disease. Peri-natal stem cells have potent anti-inflammatory effects that can be exploited to suppress the immune response and promote tissue regeneration in such diseases.

Crohn's disease is one such condition wherein the body's immune system attacks the gastrointestinal (GI) tract resulting in severe abdominal pain among other symptoms. In this condition, the GI tract is under a chronic inflamed state. The current treatments for Crohn's disease have many undesirable side-effects and are not effective. Due to the immunosuppressive and anti-inflammatory properties of stem cells derived from the placenta (placental MSC), Celgene Cellular Therapeutics has developed a placental MSC therapy for treatment-resistant Crohn's disease (National Institutes of Health, 2011). The phase I trial was successful with no major toxic effects and appeared to be safe. In this trial, a total of 12 patients with active moderate-to-severe treatment-resistant Crohn's disease received two infusions of placental MSCs and all 12 patients showed signs of disease remission. Due to the success of the phase I trial, this cell-based therapy is now in a phase II trial.

Similarly, placental MSCs have been demonstrated to be beneficial in GVHD (Jang et al., 2012). Human placental MSCs were transplanted into BALB/c mice that had received bone marrow transplant from C57BL/6 mice; this improved the survival of the recipient BALB/c mice. These results indicate the potential of gestation stem cells as anti-inflammatory agents for cell therapy.

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Conclusion

Peri-natal stem cells are powerful agents in cell therapy for a variety of diseases and disorders: blood and immune system, bone defects, myocardial infarction, neural degeneration, lung disease, diabetes, and inflammatory conditions. The collection and use of gestation tissue would be convenient and resourceful as compared to other sources of stem cells. It is feasible to isolate cells from the placenta and amniotic fluid and expand these cells, as they possess self-renewal capacity. Peri-natal stem cells have multipotent differentiation potential and can differentiate into a variety of cell types, ranging from osteoblasts to neuronal cells. In addition, they possess anti-inflammatory properties, a characteristic that is advantageous in wound healing and repair of tissue. Due to their multipotent capacity and immunomodulatory properties, peri-natal stem cells are well accepted by the body, integrate into the tissue, and can be used in the regeneration of many tissues. Although most research is at the pre-clinical stage, several applications are in clinical trials, and the potential of peri-natal stem cells for cell therapy is powerful.

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Chapter 2

Biomaterials as Artificial Niches for Pluripotent Stem Cell Engineering

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Introduction

Pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have attracted attention as powerful cell sources for studying tissue regenerative medicine and cell-based therapies, as they have a unique capacity of self-renewal and differentiation into multiple cell types (Lukovic et al., 2012). Embryonic stem cells are derived from the inner cell mass of the developing blastocyst, and differentiate into any one of three germ layers: endoderm, ectoderm, and mesoderm (Odorico et al., 2001). The iPSCs derived from adult mature or progenitor cells are made pluripotent through induction of genes that are biologically similar to those expressed by ESCs (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Amabile and Meissner, 2009). Thus, iPSCs have the potential to develop into almost any type of cell in the body. Because of these features, PSCs are of great interest to current stem cell research for tissue engineering applications. Although PSCs are attractive due to their pluripotency, they are also limited in quantity. The development of defined, large-scale culture conditions, which can maintain the undifferentiated state of PSCs, are still ambitious challenges (Li et al., 2003). Over the past decade, a number of *in vitro* protocols have been shown to support PSC self-renewal. Conventionally, PSCs require a co-culture environment for maintaining their undifferentiated phenotype. This is carried out *via* culture on mouse embryonic fibroblasts (MEFs) as a feeder layer or *via* culture using feeder-free conditions, accomplished using defined medium containing essential cytokines and nutrients (Thomson et al., 1998; Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2012). In addition, the differentiation of PSCs prior to transplantation in the body is very critical, as transplantation of undifferentiated PSCs will spontaneously differentiate into multiple cell types, forming a benign tumor known as a teratoma (Mitjavilagarcia

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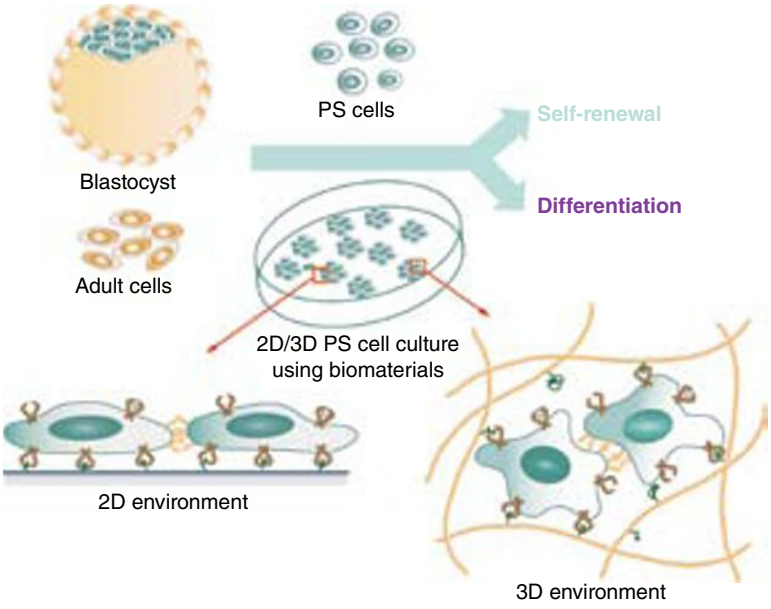


Figure 2.1. A schematic representation of the use of biomaterials to regulate the fate of pluripotent stem cells (PSCs). Pluripotent stem cells are derived from the inner cell mass of the developing blastocyst or adult stem cells and can self-renew and differentiate into any adult cell type. Biomaterials can provide a range of cues such as bioactive adhesive sites and soluble factors instrumental in directing differentiation of PS cells.

et al., 2005; Chai and Leong). In order to avoid this problem, PSCs must be guided to differentiate into specific lineages prior to implantation.

During embryonic development, PSC fate is tightly regulated by their extracellular microenvironment, which present spatially and temporally instructive cues along with specific architectures. The extracellular environment not only provides a structural framework to support cellular function but also influences cellular behavior, including self-renewal and differentiation of stem cells. Thus, to direct PSC fate, numerous studies have extensively explored the restructuring of the intricate extracellular microenvironments, referred to as the embryonic niche. With the advance in materials science combined with stem cell biology, various biomaterials have been utilized as artificial extracellular matrix (aECM) to support differentiation of PSCs *in vitro*. These biomaterials provide two- of three-dimensional artificial microenvironments that regulate cell–cell and cell–matrix communications, which in turn promote PSC self-renewal or differentiation into multiple lineages (Figure 2.1). In this chapter, we focus on the use of biomaterials, both natural and synthetic, to reconstruct extracellular microenvironments that direct PSC fate and discuss how the biomaterials can provide the instructive extracellular microenvironment that regulates PSC fate.

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Artificial Stem Cell Niche: Mimicking the Native ECM

Native ECM as Substrates

The extracellular matrix (ECM) plays a significant role in regulating cellular behavior, such as adhesion, migration, proliferation, and differentiation through cell–cell or cell–substrate interactions (Galbraith and Sheetz, 1998; Maheshwari et al., 2000; Geiger et al., 2001; Friedl, 2004). The ECM consists of proteoglycans, non-proteoglycan polysaccharides, protein fibers, and glycoproteins, which provide structural support in addition to regulating intercellular communication. The microenvironment, which presents various physicochemical milieus, can direct the dynamic behaviors of stem cells. Thus, various ECM components have been utilized as substrates to support PSC self-renewal or differentiation into specific lineages. Table 2.1 summarizes the ECM components for the PSC culture.

Matrigel, an ECM matrix extracted from mouse sarcoma cells, is used as a substrate for regulating PSC differentiation (Klienman et al., 1982; Bissell et al., 1987; Vukicevic et al., 1992; Xu et al., 2001). Matrigel has been shown to provide an effective two-dimensional environment for PSC differentiation into mesoderm and endoderm lineages (Ruhnke et al., 2003). Additionally it has been used to provide a two-dimensional microenvironment for endothelial lineage differentiation from rat ESCs (Ruhnke et al., 2003) and to improve neo-vascularization when injected with adipose-tissue-derived stem cells (ADSCs) into an ischemic animal model (Planat-Benard et al., 2004). Furthermore, Matrigel has been used in the induction of mature pancreatic insulin-producing cells from human ESCs and iPSCs. This study showed that PSCs grown on Matrigel substrates with pancreatic-cell-conditioned media developed into the mature pancreatic cells. After 20 days of the culture, the cells expressed mature β cell-specific markers such as NKX6-1 and PDX (Zhang et al., 2009). More recently, Matrigel has also been demonstrated to support hematopoietic and endothelial differentiation of human iPSCs (Choi et al., 2009).

Fibronectin, which is a large glycoprotein of the ECM that binds to integrins on the cell membrane, plays a role in cell adhesion, proliferation, migration, and differentiation (Mosher and Furcht. 1981; George et al., 1993). Pluripotent stem cell adhesion to ECM proteins is necessary for the differentiation pathway, as cell–ECM adhesions activate various intracellular signaling pathways for directing PSC fate (Pimton et al., 2011). Fibronectin has been used as a substrate for upregulating $\alpha 5\beta 1$ integrin and cell adhesion during the differentiation of mouse ESCs. Additionally, fibronectin has been implicated in enhancing the migration and differentiation of mouse ESCs. Mouse ESC migration was stimulated by fibronectin through lipid raft-dependent Na^+/H^+ exchanger-1 (NHE-1) activation (Park et al., 2012). Fibronectin has been implicated in self-renewal and differentiation of mouse ESCs. The appropriate level of fibronectin concentration (0.1–10 μg/mL) supported pluripotency of mouse ESCs, whereas either increasing or decreasing fibronectin levels led to mouse ESC differentiation (Hunt et al., 2012). Adipogenic differentiation of human iPSCs has been investigated on fibronectin-coated substrates and compared with human ESCs (Taura et al., 2009). After 22 days of embryoid body (EB) formation and differentiation on poly-l-ornithine and fibronectin-coated dishes with conditioned media, human iPSCs exhibited lipid

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Table 2.1. Extracellular matrix (ECM) substrates for pluripotent stem cells (PSCs).

ECM substrates	Application in PSCs	References
Matrigel	Self-renewal	Xu et al., 2001
	Neural differentiation	Ruhnke et al., 2003
	Hepatic differentiation	Ishii et al., 2008
	Hematopoietic differentiation	Choi et al., 2009
Fibronectin	Mesodermal and ectodermal lineages differentiation	George et al., 1993; Singh et al., 2009
	Migration stimulation	Park et al., 2012
	Adipogenic differentiation	Taura et al., 2009
	Self-renewal	Hunt et al., 2012
Collagen IV	Vascular differentiation	Nishikawa et al., 1998; Gerecht-Nir et al., 2003; Narazaki et al., 2008
	Hematopoietic lineage differentiation	Schenke-Layland et al., 2008
	Trophoectoderm differentiation	Vo et al., 2010
	Smooth muscle cell differentiation	Schenke-Layland et al., 2007
	Migration and transplantation enhancement	Li et al., 2011
Collagen I	Self-renewal	Suh and Han, 2011
	Cardiomyocyte differentiation	Sato et al., 2006
	Endothelial cell differentiation	Guo et al., 2006
Laminin	Neural differentiation	Ma et al., 2008; Hu et al., 2008
	Self-renewal	Hayashi et al., 2007; Domogatskaya et al., 2008; Rodin et al., 2010
	Pancreatic cell differentiation	Parnaud et al., 2006; Schroeder et al., 2006
	Adhesion, spreading, proliferation and extracellular signal-regulated kinase (ERK) activation	Kikkawa et al., 2004; Lam and Longaker, 2012; Suh et al., 2012
	Cardiomyocyte differentiation	Ting et al., 2012
Hyaluronic acid	Self-renewal	Khademhosseini et al., 2004
	Neural differentiation	Takahashi et al., 2009
	Osteogenic differentiation	Harkness et al., 2011

accumulation and transcription of adipogenesis-related markers (Taura et al., 2009). This study demonstrates that human iPSCs have an adipogenic potential, a property that is comparable to human ESCs. Fibronectin has also been used as a three-dimensional interpenetrate polymer network (IPN) structured hydrogel matrix to stimulate endothelial cell differentiation and vascularization of mouse ESCs (Battista et al., 2005).

Collagen, a main component of the native ECM, has been reported to direct PSC differentiation into various specific lineages (Yamashita et al., 2000; Gerecht-Nir et al., 2003; Schenke-Layland et al., 2008; Vo et al., 2010). Collagen IV has been implicated in

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enhancing migration of differentiating ESCs from EBs through $\alpha 2\beta 1$ integrin-mediated actin remodeling and promoting ESCs transplantation efficiency (Li et al., 2011b). In this study, collagen IV induced enhanced cell migration compared to other ECM molecules, such as collagen I, Matrigel, fibronectin, and laminin. Mouse ESCs cultured on collagen IV-coated substrates expressed $\alpha 2$ and $\beta 1$ integrin subunits (Li et al., 2011b). The enhancing effect of collagen IV on cell migration was significantly diminished by blocking with Arg-Gly-Asp (RGD) peptide as well as antibodies against $\alpha 2\beta 1$ integrin, indicating that the enhancing effect of collagen IV on ESC migration was mediated by $\alpha 2\beta 1$ integrin. In addition, mouse ESCs grown on collagen-IV-coated substrates exhibited well-developed actin stress fibers and extensive spreading, demonstrating accelerated migration of the cells on the collagen-IV-coated surfaces. Furthermore, the transplantation efficiency of undifferentiated mouse ESCs expanded on collagen-IV-coated demonstrated an improved engraftment and growth after subcutaneous transplantation into nude mice. Collagen IV has also been used to support EB formation and differentiation of buffalo ESCs (Taru Sharma et al., 2012). Compared to other ECM proteins, buffalo ESCs grown on a collagen IV matrix exhibited higher EB formation and a greater proliferation rate. Cardiovascular cells were directionally and systemically induced from mouse iPSCs differentiated on collagen-IV-coated dishes with vascular endothelial growth factor (VEGF) (Narazaki et al., 2008). Collagen I has also been studied as a potential substrate for regulating self-renewal of mouse ESCs via $\alpha 2\beta 1$ integrin- and discoidin domain receptor I (DDR1)-dependent Bmi-1 (Suh and Han, 2011). Mouse ESCs cultured on collagen-I-treated plates maintained Nanog and OCT-4 mRNA, indicating that mouse ESCs remained undifferentiated and collagen I stimulated the self-renewal of mouse ESCs, a process regulated by Bmi-I through the $\alpha 2\beta 1$ integrin-dependent signaling pathway.

Laminin has been implicated as the most important ECM protein for human PSCs in culture because it is the first ECM molecule to interact with cells in the early developing embryo (Dziadek and Timpl, 1985; Martin and Timpl, 1987; Klaffky et al., 2001; Ekblom et al., 2003). Thus, laminin has been investigated for its influence on directing PSC fate (Ma et al., 2008). A number of studies have demonstrated that laminin plays a critical role in self-renewal via maintenance of pluripotency (Hayashi et al., 2007; Domogatskaya et al., 2008; Rodin et al., 2010) and directed differentiation of PSCs (Reubinoff et al., 2000; Li et al., 2002; Schroeder et al., 2006). The self-renewal of mouse ESCs has been examined on laminin-coated substrates (Domogatskaya et al., 2008). Cells cultured on laminin-coated surfaces maintained their pluripotency in the absence of differentiation inhibitors or feeder cells. Interestingly, the cells cultured on a specific laminin isoform, laminin-511, maintained expression of pluripotency markers (Oct4, Sox2, Tert, UTF1, and Nanog), suggesting that the recombinant laminin isoform can provide a base material for feeder-free maintenance of undifferentiated ESCs *in vitro*. A similar result was reported by S. Rodin *et al.* (2010): human ESCs cultured on human recombinant laminin-511 maintained pluripotency, suggesting that the xeno-free and feeder-free system may be useful for the development of ESC lineages for therapeutic purposes. Laminin has been implicated in the migration and proliferation of ESCs (Kikkawa et al., 2004; Suh and Han, 2010; Suh et al., 2012). Among laminin isoforms, laminin-111 was shown to induce mouse ESC migration through the Epac1/Rap1 and Rac1/cdc42 signaling pathways (Suh and Han, 2010). Laminin has also been used as a substrate for inducing mouse ESC proliferation via a

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reduction of gap junction intercellular communication (Suh et al., 2012). Laminin has been involved in neural differentiation of human iPSCs (Hu et al., 2010). Human iPSCs cultured on a laminin substrate differentiated into neuroepithelial cells and functional neurons or glia at day 7.

Hyaluronic acid (HA), a high molecular weight polysaccharide, is well known as an important ECM component in regulating human PSC differentiation, because it is highly expressed during embryonic development and simultaneously controls cellular behaviors as well as morphogenesis of human PSCs (Toole, 2004). Consequently, HA substrates have been utilized to support PSC differentiation in combination with exogenous factors, including growth factors and other ECM components (Khademhosseini et al., 2006; Takahashi et al., 2009). For instance, human ESC colonies isolated from the HA-coated substrates exhibited upregulation of osteogenic differentiation markers, and the cells were implanted either subcutaneously or in the cavarial bone defect to confirm osteogenic differentiation potential suitable for bone tissue regeneration (Harkness et al., 2011). *In vivo* study resulted in new bone formation and partial recovery of the defect, demonstrating that human ESCs grown on the HA substrates represent great potential for tissue regenerative medicine.

Mimicking the ECMs: Designing Aspects of aECM

In order to accurately design aECMs for stem cell culture, we must know all of the necessary components and compositions of the *in vivo* stem cell microenvironment, referred to as the stem cell niche. The ECM is an essential component of the PSC niche that plays a role in regulating PSC fate. The ECM retains PSCs in their niche and also serves to initiate signal transduction events (Dellatore et al., 2008). Current stem cell research aspires to reconstruct the multifaceted embryonic niche to recapitulate development *in vitro* and extensive research is focused on recreating and mimicking the critical microenvironment conditions found *in vivo*. Engineering aECMs that recapitulate the necessary elements has been widely explored through biomaterials design. These materials can provide dynamic microenvironments in regulating PSC fate. Although various kinds of engineered aECMs have been developed to control PSC fate, these materials are still being developed to mimic elaborately the *in vivo* microenvironment surrounding PSCs. Basically, the engineered aECM environment has been composed of three-dimensional hydrophilic network structure, referred to as a hydrogel, and decorated with insoluble cell-adhesive ligands and proteolytic degradable sites (Figure 2.2).

The native ECMs are the hydrophilic three-dimensional network structure, which are composed of various protein fibrils and fibers interwoven within a hydrated network of GAGs. The hydrophilic network allows the matrix to imbibe a large quantity of water as well as biological fluids that together enable nutrient transport and protection from external stimuli (Alsberg, 2002). Synthetic hydrogels are utilized as a three-dimensional microenvironment for stem cell research due to their tissue-like tunable properties. These three-dimensional networks can be fabricated using various natural and synthetic polymers and their physicochemical properties can be controlled easily. Additionally, the hydrogels are easily modified with bioactive molecules and deliver soluble biochemical factors to improve their biological activity. Accordingly, the three-dimensional synthetic matrix has attracted much attention as it is proven to be an adequate three-dimensional microenvironment for stem cell research.

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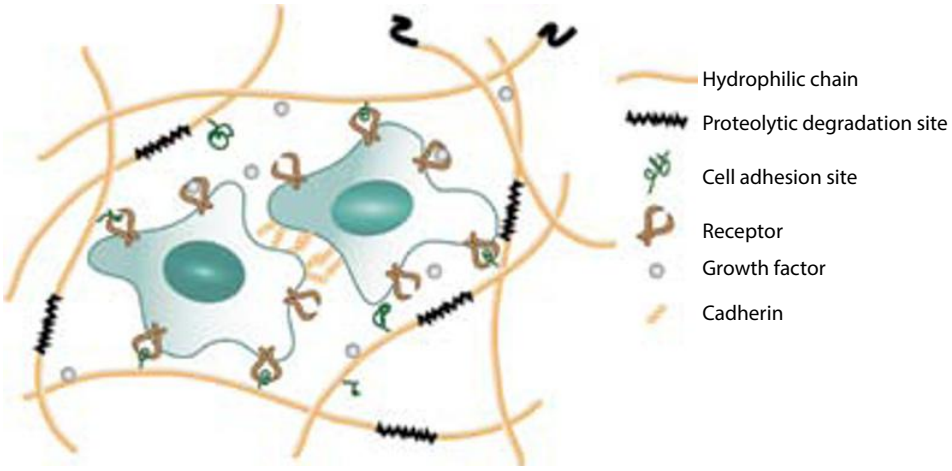


Figure 2.2. Illustration of the artificial extracellular matrix (aECM) for PSC culture. The aECMs have been composed with a three-dimensional hydrophilic chain and decorated with insoluble cell-adhesive ligand and proteolytic degradable sites.

To design the aECM for stem cell culture, the scaffolds should provide cell adhesive domains in a spatial and temporal manner to control cellular behavior. The cell–matrix interactions can mediate specific receptor–ligand interactions leading to activation of receptor-mediated signaling pathways to control cellular behaviors, including differentiation (Ruoslahti, 1996; Aplin et al., 1999; Giancotti and Ruoslahti, 1999). Various cell adhesion ligands derived from the native ECM, such as RGD, Lys-Gln-Ala-Gly-Asp-Val (KQAGDV), Arg-Glu-Asp-Val (REDV), Ile-Lys-Val-Ala-Val (IKVAV), Arg-Asn-Ile-Ala-Glu-Ile-Ile-Lys-Asp-Ile (RNIAEIIKDI) and Tyr-Ile-Gly-Ser-Arg (YIGSR), have been incorporated into synthetic matrices to enhance the cell–material interaction or cellular activities (Shin et al., 2003). Numerous studies have demonstrated that the insoluble ligands influence maintenance and differentiation of stem cells (Hwang et al., 2006c; Li et al., 2006; Derda et al., 2007; Dellatore et al., 2008; Bratt-Leal et al., 2009). For example, RGD-immobilized poly(ethylene glycol) (PEG)-based hydrogels have been shown to induce chondrogenic differentiation of human ESCs through enhanced cell–matrix interactions (Hwang et al., 2006c; Derda et al., 2007) and the optimized RGD concentration promoted osteogenic differentiation (Yang et al., 2005). The RNIAEIIKDI sequence found in the lamimin γ-chain has been implicated in self-renewal of human ESCs (Derda et al., 2007). The peptide was arrayed onto a gold substrate via self-assembled monolayers (SAMs). Human ESCs were cultured on the functionalized substrates. Embryonic stem cells cultured on matrices containing the RNIAEIIKDI peptide conjugated substrate showed a high level of Oct-4 expression, demonstrating that the biomimetic substrate promotes the self-renewal of human ESCs.

Extracellular matrix remodeling is an important aspect of tissue morphogenesis because the degradation rate influences ECM production and cellular activities. The biomacromolecular components of the ECM are degraded via matrix metalloproteinases (MMPs) mainly secreted from the cells. This produces a dynamic reciprocal

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response, with the ECM stimulating the cells within it and cellular proteases remodeling the ECM and releasing bioactive components from it. The cell-induced proteolysis is often required for three-dimensional cell migration and invasion since the porosity of the ECM may lead to barrier function and thus impede migration (Basbaum and Werb, 1996). The aECM microenvironments have been decorated with protease-sensitive sites along with cell adhesive sites and subsequently encapsulated with stem cells. Several novel methods to fabricate synthetic hydrogel matrices with sensitivity to proteases, including plasmin (Ulbrich et al., 1980; Halstenberg et al., 2002; Pratt et al., 2004) and MMPs (West and Hubbell, 1999; Kim and Healy, 2003; Rizzi et al., 2006) have been developed. These hydrogels, which are responsive to MMPs or other cell-mediated enzyme activities, have also been used to support PSC differentiation.

Biomaterials as a Three-Dimensional Microenvironment for PSC Culture

Most stem cell studies have been performed on two-dimensional plates coated with various biomaterials, which can mimic precise spatial and temporal presentation of factors directing stem cell fate. The two-dimensional environment does not entirely reconstruct physiological *in vivo* environments. The differentiation profile of PSCs was significantly different when cultured in two- or three-dimensional environments (Garcia et al., 1999; Mimeault and Batra. 2006). Synthetic three-dimensional microenvironments fabricated using various biomaterials provide mechanical support while regulating cell adhesion, migration, proliferation, and differentiation (Tibbitt and Anseth, 2009). Current research in directing PSC differentiation using biomaterials is focused on such three-dimensional microenvironments. A wide variety of natural or synthetic biomaterials have been evaluated as three-dimensional scaffolds to support differentiation of PSCs.

Natural Polymers for PSC Culture

Natural biomaterials, which can consist of components found in the ECM and are similar in molecular structure of the ECM, have been widely investigated in three-dimensional PSC encapsulation and differentiation studies. Various natural polymers have been extensively used to support the PSC differentiation, including collagen, fibrin, HA, alginate, dextran, chitosan, silk, heparin, etc.

Collagen, the most widely distributed ECM component in the human body, has been utilized as an attractive biomaterial for stem cell engineering (van der Rest and Garrone, 1991; Friess. 1998; Gordon and Hahn, 2010; Parenteau-Bareil et al., 2010). Collagen hydrogels have been used as three-dimensional microenvironments for regulating PSC fate. The three-dimensional collagen matrix fabricated through multiple cross-linking methods provides a fibrous architecture similar to native ECM for supporting PSC differentiation (Battista et al., 2005; Narazaki et al., 2008; Duan et al., 2011). Chondrogenic differentiation of mouse ESCs has also been evaluated in collagen gels. In this study, mouse ESC-derived chondrocytes morphologically resembled mature chondrocytes and expressed a number of chondrogenic differentiation markers, such

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as GAGs and collagen type II (Ng et al., 2011). Collagen gel has been shown to support osteogenic differentiation of ESCs. Collagen matrix cross-linked with β-glycerol-phosphate-induced osteogenic differentiation *in vitro* with an efficiency of over 80% without purification and prevented tumorigenic potential of ESCs when implanted *in vivo* (Krawetz et al., 2012). Collagen gel was also used as a three-dimensional matrix for enhancing the proliferative ability and neuronal differentiation of neuronal progenitors isolated from embryonic rat cortical or subcortical neuroepithelium (Ma et al., 2004).

Fibrinogen is a large glycoprotein found in plasma and plays a role in blood clotting, fibrinolysis, cellular and matrix interactions, the inflammatory response, wound healing, and neoplasia (Mosesson, 2005; Wolberg, 2012). Fibrin gels formed by polymerization of fibrinogen with thrombin provide a nano-/microfibrous architecture mimicking the native ECM. This fibrous gel matrix has been used to regulate PSC differentiation into specific lineages such as vascular and neuronal differentiation (Liu et al., 2006; Willerth et al., 2006, 2007). It has also been used as a mechanically tunable three-dimensional matrix to regulate ESC differentiation (Jaramillo et al., 2012; Zhang et al., 2012). The three-dimensional fibrillar structured matrix with soft substrate stiffness (4–72 Pa) enhanced the mouse ECM cell proliferation and induced endodermal differentiation (Jaramillo et al., 2012).

Hyaluronic acid plays an important role in regulating PSC fate (Toole, 2004). In our previous study, we investigated HA hydrogels as a three-dimensional microenvironment for maintaining the pluripotency and undifferentiated state of human ESCs (Figure 2.3a–d; Gerecht et al., 2007). Hyaluronic acid hydrogels provided a three-dimensional microenvironment capable of maintaining the pluripotency and undifferentiated state. Moreover, the HA hydrogels encouraged vascular differentiation of human ESCs in the presence of VEGF. The human ESCs were sprouted and elongated with expression of endothelial and smooth muscle markers, demonstrating their vascular differentiation (Figure 2.3e–h) (Gerecht et al., 2007). Recently, HA hydrogels tailored with MMP-sensitive and RGD peptides have also been reported as a three-dimensional microenvironment for supporting vasculogenesis from early endothelial progenitors (Hanjaya-Putra et al., 2011).

Other natural polymers used as three-dimensional scaffolds for studies of PSC differentiation include alginate, chitosan, heparin, and silk. Alginate, a natural linear polysaccharide with 1,4-linked β-d-mannuronate and α-l-guluronate residues, has been used as a three-dimensional microenvironment for PSC differentiation *in vitro* due to their biocompatibility and easy fabrication (Gerecht-Nir et al., 2004; Imamura et al., 2004; Maguire et al., 2006; Fang et al., 2007; Li et al., 2011c; Siti-Ismail et al., 2008; Wang et al., 2009; Kuo and Chung, 2012; Lee and Mooney, 2012; Malpique et al., 2012; Pawar and Edgar, 2012; Tang et al., 2012). Encapsulation of undifferentiated human ESCs in alginate microbeads in combination with macroporous calcium-phosphate-cement-induced osteogenic differentiation, resulted in effective bone tissue regeneration (Tang et al., 2012). Alginate has also been investigated for mouse ESC differentiation into insulin-producing cells (Wang et al., 2009). Chitosan, composed of a relatively simple glucosamine and *N*-acetylglucoamine unit, is well known as a biocompatible and biodegradable biopolymer, and its chemical structure is analogous with diverse GAGs. Photocross-linkable chitosan hydrogels have been used to control mouse ESC differentiation into the vascular lineage in the presence of $VEGF_{165}$ (Chiang et al., 2010). Additionally, it has the ability to control PSC fate through

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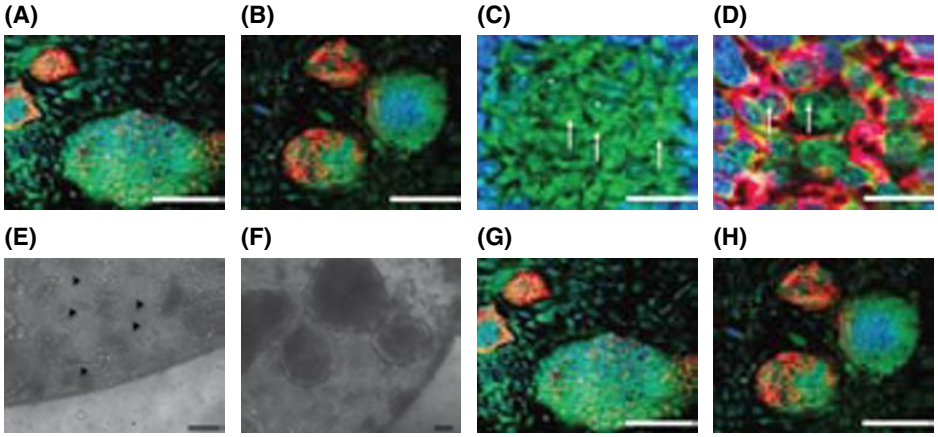


Figure 2.3. Hyaluronic acid (HA) hydrogels for maintaining human ESC pluripotency and vasculogenesis. Staining of undifferentiated human ESCs for HA binding site (green), undifferentiated membrane marker TRA-1-81 (red), and nuclei (blue): intracellular localization of HA (**A** and **B**), including perinuclear area (arrows) **(C)** and nuclei (*), and nucleoli (arrowheads) **(D)**. Differentiation of human ESCs cultured in conditioned medium for 1 week followed by the replacement of medium containing VEGF (**E** and **F**). Cell sprouting was observed after 48 h in HA hydrogel matrix transferred to medium containing VEGF (arrows) **(E)** compared with hydrogels continuously cultured in conditioned medium **(F)**. After 1 week of differentiation, sprouting elongating cells were mainly positive for vascular α-smooth muscle actin **(G)**, whereas some were positive for early stage endothelial marker **(H)**. (Scale bars: **(A)**, **(B)**, **(E)**, and **(F)**, 100 μm; **(C)**, **(G)**, and **(H)**, 25 μm; **(D)**, 10 μm.). (Adapted from S. Gerecht, J. A. Burdick, L. S. Ferreira, S. A. Townsend, R. Langer, G. Vunjak-Novakovic. 2007. Hyaluronic acid hydrogel for controlled self-renewal and differentiation of human embryonic stem cells. *Proc Natl Acad Sci U S A* **104**(27): 11298–11303.)

topography-mediated cell differentiation. The chitosan nanofiber, 400 nm and 1.1 μm diameters, supported increased expression of neural differentiation markers, while the matrix of 200 nm diameter fibers increased expression of osteogenic and hepatic markers indicative of endodermal and mesodermal commitment (Cooper et al., 2012). Heparin sulfates are highly polymorphic GAGs found on the cell surface and in the ECMs, and are a critical cell surface coreceptor in embryogenesis (Sasisekharan and Venkataraman, 2000; Johnson et al., 2007). Heparin-based materials have been utilized to support PSC growth, self-renewal and differentiation in combination with various exogenous factors or other ECM components (Furue et al., 2008; Lanner et al., 2010; Holley et al., 2011; Pickford et al., 2011; Toh and Voldman, 2011; Forsberg et al., 2012). Silk is a versatile natural polymer that is derived from silkworm cocoons. It is an insoluble polypeptide including up to 90% of the amino acids, glycine, alanine, and serine (Nazarov et al., 2004). Porous silk scaffolds have been used as a composite for cartilage tissue engineering from human ESC-derived mesenchymal stem cells (MSCs) (Tigli et al., 2007).

Despite their weak mechanical strength, natural biomaterials have demonstrated distinctive effects on cellular attachment and production of biological signals for PSC culture. Table 2.2 summarizes the various natural polymers utilized for PSC studies.

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Table 2.2. Application of natural polymers in pluripotent stem cell (PSC) culture.

Natural polymers	Application in PSC culture	References
Collagen	Hepatic differentiation	Wang et al., 2011; Nagamoto et al., 2012
	Chondrogenic differentiation	Ng et al., 2011
	Osteogenic differentiation	Krawetz et al., 2012
Fibrinogen (fibrin gel)	Neural lineage differentiation	Willerth et al., 2006
	Vascular differentiation	Liu et al., 2006
Hyaluronic acid	Vascular differentiation	Gerecht et al., 2007; Hanjaya-Putra and Gerecht, 2009
	Self-renewal	Gerecht et al., 2007
	Chondrogenic differentiation	Hwang et al., 2006b; Toh et al., 2010
	Cardiomyocyte differentiation	Chan et al., 2010
Alginate	Hepatic differentiation	Imamura et al., 2004; Maguire et al., 2006; Fang et al., 2007
	Neural lineage differentiation	Li et al., 2011; Kuo et al., 2012
	Pancreatic differentiation	Wang et al., 2009
	Vascular differentiation	Gerecht-Nir et al., 2004
	Cryopreservation	Malpique et al., 2012
	Self-renewal	Siti-Ismail et al., 2008
Chitosan	Vascular differentiation	Chiang et al., 2010
	Osteogenic and hepatic differentiation	Cooper et al., 2012
	Ciliated cell differentiation	Wang et al., 2010
	Self-renewal	Li et al., 2010
Heparin	Hematopoietic differentiation	Holley et al., 2011
	Self-renewal and cell growth	Furue et al., 2008; Toh and Voldman, 2011
	Neural lineage differentiation	Pickford et al., 2011

Synthetic Polymers for Three-Dimensional PSC Culture

Synthetic polymers have been used as three-dimensional artificial microenvironments to support PSC differentiation (Levenberg et al., 2003, 2005; Liu and Roy, 2005; Hwang et al., 2006a). The most commonly used synthetic polymers include PEG, poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA), polyacrylamide (PAM), poly(hydroxyl ethyl methacrylate (PHEMA), and poly(vinyl alcohol) (PVA). Among these materials, hydrophilic PEG and polyester-based materials have been extensively utilized as three-dimensional scaffold for PSC culture (Table 2.3).

PEG-based hydrogels have been extensively used as synthetic three-dimensional scaffold for PSC differentiation due to their high water content, tunable elastic modulus, and biocompatibility. However, PEG hydrogels lack bioactivities such as cell adhesiveness and degradability, therefore PEG hydrogels are decorated with various cell adhesive

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Table 2.3. Application of synthetic biomaterials in pluripotent stem cell (PSC) culture.

Synthetic biomaterials	Application in PSC culture	References
Poly(ethylene glycol) (PEG)	Chondrogenic differentiation	Hwang et al., 2006a,b
	Hematopoietic differentiation	Liu and Roy, 2005
	Endothelial cell differentiation	Moon et al., 2009
	Microwell array system	Karp et al., 2007; Hwang et al., 2009
	Neural lineage differentiation	Soman et al., 2012
Poly(lactic acid) (PLA)	Hematopoietic differentiation	Taqvi and Roy, 2006
Polyglycolic acid (PGA)	Hepatic differentiation	Sharma et al., 2010
Poly(lactic-co-glycolic) (PLGA)	Neural, chondrogenic, and hepatic differentiation	Levenberg et al., 2003; Liu et al., 2010
Poly(ε-caprolactone) (PCL)	Adipogenenic differentiation	Kang et al., 2007

ligands (i.e. RGD) and proteolytic degradable sites (i.e., MMP-sensitive peptide sequences) to provide an artificial microenvironment with improved biological activities. These engineered microenvironments have been investigated for supporting the PSC differentiation into vascular and chondrogenic lineages (Hwang et al., 2006a,b; Moon et al., 2009). Murine ESCs encapsulated within acrylated PEG hydrogels have been shown to upregulate the expression of chondrogenic markers in the presence of exogenous growth factors (Hwang et al., 2006a,b). Injectable MMP-responsive PEG hydrogels with thymosin β4 along with human ESC-derived vascular cells have been used as an injectable scaffold in rat heart ischemic injuries (Kraehenbuehl et al., 2011). The bioactive PEG hydrogels promoted structural organization of native endothelial cells, resulting in decreased infarct size as compared to control groups treated with phosphate buffer solution (PBS) injection. Microengineered PEG hydrogel microwells have been used to direct PSC differentiation in a size-dependent manner (Hwang et al., 2009). Endothelial cell differentiation was enhanced in smaller EBs, whereas cardiogenesis was increased in larger EBs. Biomimetic three-dimensional PEG hydrogels fabricated by a digital micromirror-device-based projection-printing system (DMD-PP) have been utilized as a three-dimensional organoid structure to support cellular adhesion of human ESC-derived neural cells (Soman et al., 2012). The complex hexagonal and log-pile three-dimensional structure promoted growth and differentiation of neurons derived from human ESCs. The mechanical properties of the hydrogel scaffold were adjusted by controlling porous architecture, resulting in long-term human ESC-derived neural cell culture.

Other synthetic materials suitable for supporting PSC differentiation are polyester-based materials (i.e. PLGA, PLA, PGA (poly(glycolic acid)), PCL (poly(caprolactone)), and their copolymers). These have been extensively used as synthetic three-dimensional

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scaffolds or drug carriers for tissue engineering and drug delivery due to their biodegradability and biocompatibility. These synthetic materials were fabricated using standard methods such as salt leaching, porogen melting, and electrospinning (Borden et al., 2002; Lin et al., 2003; Mondrinos et al., 2006) and their physicochemical features can be easily controlled by varying the molecular weight of the polymer, composition ratio, or salt/porogen size. Mesenchymal cells derived from human ESCs encapsulated in the PLGA/PLA (poly(lactide acid)) with hydroxyapatite differentiated toward the osteogenic lineage *in vivo* (Hwang et al., 2013). The hybrid three-dimensional microenvironment induced faster bone formation through intramembranous ossification compared to the microenvironment without hydroxyapatite. Although the synthetic polyester-based materials provide the three-dimensional microenvironment for supporting ESC differentiation, acidic by-products are a critical drawback.

Recapitulating Physical Cues for Controlling PSC Fate

During embryogenesis, PSCs sense and respond to the spatial heterogeneity of the extracellular microenvironment and cellular communication between cells and the ECM occurs through a number of pathways that direct their fate. Recapitulating the physical cues of the native ECM using biomaterials has been involved in an alternative method to regulate PSC fate. The physical constraints, such as matrix stiffness, surface topographical architecture, and control of EB size, influence PSC fate.

The inherent mechanical strength of three-dimensional scaffolds is of significant interest because they can regulate differentiation of PSCs. Recently, Evans et al. (2009) demonstrated that PSC differentiation could be modulated by varying the mechanical properties of substrates. Therefore, the inclusive mechanical integrity of the biomaterials is a key parameter that needs to be addressed when evaluating material properties that affect differentiation pathways of PSCs. A study examined the matrix stiffness of polydimethylsiloxane (PDMS) surfaces on the osteogenic differentiation of murine ESCs (Evans et al., 2009). The osteogenic differentiation of murine ESCs was enhanced on stiff substrates compared to soft substrates, suggesting that the mechanical properties should be taken into consideration when engineering a scaffold to support PSCs differentiation. Substrate stiffness was found to also profoundly influence self-renewal of mouse ESCs through downregulating cell-matrix tractions (Chowdhury et al., 2010). The cells seeded on the soft substrates generated homogeneous, undifferentiated colonies maintaining high levels of Oct3/4, Nanog, and alkaline phosphatase (ALP), and formed EBs for 5 days in the absence of exogenous leukemia inhibitory factor (LIF). This result suggested that soft substrates could be applied to long-term ESC culture with pluripotency. Another study examined the mechanical stiffness of PAM hydrogels on the neurogenic differentiation of human iPSCs (Keung et al., 2012). The cells seeded on the substrate with different matrix stiffness (100–75,000 Pa) expressed a high level of neurogenic differentiation marker after 9 days, demonstrating that the softer hydrogel substrate with stiffness similar to that of neural tissue enhances the generation of early neural ectoderm and neurogenic differentiation of human PSCs.

Fate decisions of PSCs are influenced by topographical architecture within their extracellular microenvironment (Li et al., 2011a). Recent technologies in micro- and nano-fabrication have been developed, which allow for the possibility of creating

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spatially and temporally patterned structures that can control PSC fate. Many studies have investigated PSC differentiation in response to the engineered micro- and nano-substrates. Silica colloidal crystal (SCC) substrates with different diameters (100–600 nm) have also been employed to support murine ESC differentiation (Ji et al., 2012). Culturing the cells on collagen coated SCC substrates with nanogratings induced morphological changes. Murine ESC colonies on the substrates developed a central pit and expressed proteins related to epithelialization, demonstrating that the control of surface topographical cues influence both the shape and fate of differentiating colonies of murine ESCs *in vitro*.

Differentiation induced by EBs is a common approach to mimic some of the spatial and temporal aspects of embryonic development *in vivo* (Thomson et al., 1998; Schuldiner et al., 2001; Xu et al., 2001). Numerous micro- and nanoengineering methods have been developed to regulate the size and shape of EBs (Karp et al., 2007; Park et al., 2007; Bauwens et al., 2008; Bratt-Leal et al., 2009). One of the approaches to culturing human PSCs is to create size-specified cell colonies on micropatterned extracellular matrix islands. The single-cell suspensions were seeded onto the patterned polystyrene slides with different areas, resulting in a higher ratio of expression of endoderm-associated markers to neural-associated markers when the colony size decreased (Bauwens et al., 2008). Mohr et al. (2010) reported that microwells formed by photolithography and plasma leaching created uniform EBs for maintaining the undifferentiated human ESCs without the use of MEFs. By creating a PEG microwell array system using soft lithography, Karp et al. (2007) were able to fabricate spatially uniform EBs. The human ESCs were seeded onto the microfabricated cell-repellant PEG well with controlled sizes and shapes. Results indicated that the EB size and shape could be controlled and easily harvested from the well. The microwells fabricated using photocurable PEG hydrogels have been used as a template to regulate ESC differentiation. Hwang *et al*. (2009) reported that controlling EB size using microwells regulated ESC differentiation through differential expression of WNT5a and WNT11. The cells were cultured on the PEG hydrogel microwell with different diameters (150–450 μm) to form ESC colonies and EBs and to differentiate into directed cell lineages. The results indicated that cardiogenesis was enhanced in larger EBs (450 μm in diameter), whereas endothelial cell differentiation was increased in the smaller EB (150 μm). Various microfabrication techniques such as microcontact printing, photolithography/plasma etching techniques, and soft lithography could be useful for directing ESC fate and studying ESC differentiation in a controlled manner.

Conclusion

Biomaterials might be potential tools to reconstruct the native extracellular microenvironment for regulating PSC differentiation toward various specific lineages or self-renewal. Numerous biomaterials have been investigated to provide artificial microenvironments for directing PSC fate decision. In addition, the multiple parameters of the surrounding milieu influence PSC differentiation through orchestrated actions of various critical cues, such as scaffold composition, ligand presentation, spatial topographical architecture, and physicochemical properties. While the specific molecular mechanism in the cellular microenvironment and the signaling pathways are not fully

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understood, numerous seminal studies have demonstrated that there is a great potential in using biomaterials for stem cell biology. It may be possible to incorporate these complex factors into the design of new biomaterial to provide better microenvironments for PSC fate.

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Chapter 3

Low-Intensity Ultrasound in Stem Cells and Tissue Engineering

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Introduction

Living cells respond to both biochemical and mechanical forces, and transduce them into the cytoplasm and nucleus for execution of diverse cellular functions (Wang et al., 2009). Biochemical and mechanical stimulation can also change the behavior of stem cells, including self-renewal ability and differentiation into specific cell lineages (Engler et al., 2006; Wingate et al., 2012; Liu et al., 2013). The stem cell niche is believed to provide them with a complex array of biochemical signals, such as growth factors and biomechanical signals via cell–cell contacts and cell–matrix interactions in a temporal and spatial manner. Soluble factors include various growth factors, morphogenetic factors, cytokines, and small molecules, such as transforming growth factors (TGF), bone morphogenetic protein (BMP), and retinoic acids (RAs). When added to the cell culture, or secreted by stem cells or niche cells, these factors diffuse and bind to cell-membrane receptors, activating cellular signaling pathways capable of altering stem cell gene expression.

Cells and tissues are always subjected to external mechanical stimulation, which can highly influence their growth and development. Mechanical stimulation, such as compressive forces, shear stress, and tensile forces can also play a critical role in stem cell adhesion, migration, proliferation, self-renewal, and differentiation (Sumanasinghe et al., 2006; Haudenschild et al., 2009; Kim et al., 2011). The ability of stem cells to respond to mechanical stimulation is generally governed by mechanosensitive receptors that sense and convert them into biochemical signaling events, the phenomenon referred to as mechanotransduction.

Embryonic stem cells (ESCs) and adult stem cells, including mesenchymal stem cells (MSCs), are also known to be sensitive to mechanical stimulation. When ESCs were exposed to fluid sheer stress they showed increased gene expression specific to the cardiovascular lineage, such as vascular endothelial growth factor (VEGF) receptor 2,

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smooth muscle actin, and platelet-derived growth factor-β (PDGF-β) (Yamamoto et al., 2005). Compressive forces were shown to alter the phenotype of MSCs. When MSCs were subjected to dynamic compression or hydrostatic pressure they showed an increase in chondrogenic markers and ECM deposition (Haudenschild et al., 2009;). Cyclic stretch was shown to commit MSCs to a myogenic phenotype and mouse ESCs to a vascular smooth muscle cell phenotype (Haghighipour et al., 2008; Shimizu et al., 2008). Shear stress has shown a significant impact on the fate of stem cells as well (Yamamoto et al., 2005). In two-dimensional systems, it induced osteogenic differentiation of MSCs by activation of multiple intracellular signaling pathways (Liu et al., 2010). Although the exact mechanism of the mechanical stimulation is not yet clear, these reports reinforce its importance in stem cell fate and behavior.

In this review, we summarize the effect of low-intensity ultrasound (LIUS), a special type of mechanical stimulation, on phenotypes and activities of stem cells. Particular emphasis is placed on its effects on chondrogenic differentiation of MSCs, its utility in cartilage tissue engineering, and the possible signaling pathways involved (also reviewed briefly by Park et al., 2007).

What is Ultrasound?

Ultrasound (US) is known as an oscillating sonic pressure wave with a high frequency beyond the range of human audibility (20 Hz to 20 kHz). As a propagating pressure wave, US is capable of transferring mechanical energy into the tissues. Depending on its frequency, the US energy is absorbed, propagated, or reflected. Ultrasound can be used for medical imaging and treatment, detection, measurement, and cleaning in many different fields, and is generally divided into three types: diagnostic, disruptive, and therapeutic (Figure 3.1). Diagnostic US uses an US frequency of around 3–10 MHz and low intensity (1–50 mW/cm^2). Since its introduction in the late 1950s, diagnostic US has been studied extensively for its clinical applications and has become one

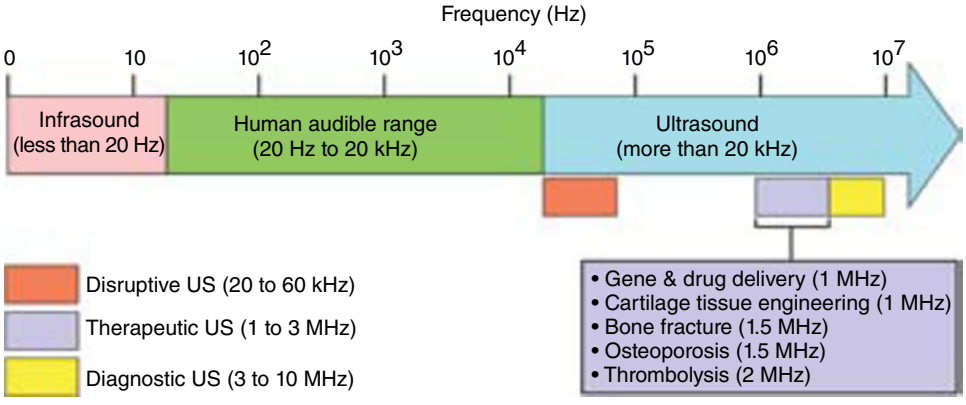


Figure 3.1. Diagram of sound frequency range. Ultrasound has a high frequency beyond the range of human audibility, and can be divided into three types: disruptive, therapeutic, and diagnostic ultrasound.

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of the most commonly used diagnostic tools besides X-ray. Diagnostic US is used for visualization of various internal organs, in order to observe their shape, structure, and pathological lesions with tomographic images. Disruptive US with low frequency (20–60 kHz) and high intensity above 8 W/cm^2 is used in ultrasonic cleaning devices, and for removal of bladder stones and treatment of solid cancers, etc. Therapeutic US uses frequencies ranging from 1 to 3 MHz and intensities ranging between 0.1 and 2 W/cm^2. It uses diverse frequencies and intensities depending on the purpose of application, such as physiotherapy, destruction of blood clots, and transdermal drug delivery. Therapeutic US can be subdivided into two broad groups depending on its main biological effects: thermal and nonthermal (Dalecki, 2004). Early therapeutic US applications investigated several conditions using the effect of tissue heating. The thermal effects are exerted by high-intensity US encompassing generation of heat energy (Merino et al., 2003). The thermal effects in therapeutic uses are well understood, particularly in physical therapy for treatment of tendinitis, osteoarthritis, and pain relief. On the other hand, nonthermal effects of US are known to be associated with LIUS of less than 1 W/cm^2 (Feril and Kondo, 2004). Its therapeutic effects appear to involve cavitation, shear stresses, and radiation forces. Many studies have shown that LIUS can regulate synthesis of DNAs and proteins in cells (Liu et al., 2001; Parvizi et al., 1999), changes in cell membrane permeability (Deng et al., 2004), and can help in recovery of various diseases in connective tissues, including bone (Pilla et al., 1990; Heckman et al., 1994), tendon (Enwemeka et al., 1990), and cartilage in animals and humans (Nieminen et al., 2004). Of particular interest, LIUS has also been reported to increase the viability, proliferation (Yoon et al., 2009; Choi et al., 2011), and differentiation (Xu et al., 2012) of stem cells. The representative biological activities of nonthermal LIUS are summarized in Table 3.1. These effects of LIUS may be useful for a broad range of tissue engineering and regenerative medicine applications utilizing stem cells. Figure 3.2 shows representative images of LIUS equipment for research purposes, which has a control panel for adjustment of intensity and duty cycle and multiple transducers for culture dishes and animal use.

Bioeffects of LIUS

Cell Adhesion

Mesenchymal stem cells have generally been known as a promising therapeutic tool for use in stem-cell-based therapy and tissue engineering for replacement or repair of the function of injured tissues (Deans and Moseley, 2000; Quarto et al., 2001), because they have ability for self-renewal and multipotency for differentiation into a variety of cell types (Pittenger et al., 1999). However, they still have a limitation in obtaining large amounts of MSCs for clinical applications compared with ESCs, which have unlimited proliferation potential and consistently high telomerase activity. Indeed, MSCs in bone marrow (BM) exist at a low-end ratio of approximately 1/10^5 in BM mononuclear cells (Short et al., 2003). In addition, their abilities for self-renewal and multipotent differentiation show a gradual decrease with increased donor age and/or passage numbers *in vitro* (D'Ippolito et al., 1999; Bianco et al., 2001). Thus, numerous studies have investigated methods for improvement of the replicative ability of MSCs, in order to obtain a large amount of cells by controlling various culture environments. For instance,

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Table 3.1. Bioeffects of low-intensity ultrasound.

Bioeffects	Cell type	Frequency	Intensity (mW/cm^2)	Sonication time	Wave type	References
Anti-apoptosis	Mesenchymal stem cells	1 MHz	200	10 min every 12 h for 1 or 2 weeks	Continuous	Lee et al., 2007
Cell adhesion	Mesenchymal stem cells	40 kHz	25–35	100, 300 or 600 s	Pulsed	Yoon et al., 2009
	Mesenchymal stem cells	1 MHz	100	10 min/day for 6 days	Continuous	Choi et al., 2011
Chondrogenesis	Mesenchymal stem cells	1 MHz	15–120	20 min/day	Continuous	Ebisawa et al., 2004
	Mesenchymal stem cells	1 MHz	200	20 min/day for 7 days	Continuous	Cui et al., 2007
Extracellular matrix synthesis	Mesenchymal stem cells	1 MHz	200	10 min/12 h for 1 or 2 weeks	Continuous	Lee et al., 2006
	Mesenchymal stem cells	0.8 MHz	200	10 min/day up to 4 weeks	Continuous	Cui et al., 2006
Osteogenesis	Pluripotent mesenchymal cell line (C2C12)	1.5 MHz	70	20 min	Pulsed	Ikeda et al., 2006
	Mouse adipose stem cells	1.0 kHz	100	10 min/day for 3–7 days	Pulsed	Yue et al., 2013
	Normal osteoblast	1 MHz	30	20 min	Pulsed	Wu et al., 2009
Viability and proliferation	Gingival fibroblasts, Mandibular osteoblasts, and monocytes	45 kHz or 1 MHz	15–30 or 100–400	5 min	Pulsed or continuous	Doan et al., 1999
	Hematopoietic stem/ progenitor cells	1.5 MHz	30–100	10 min/day for 4 days	Pulsed	Xu et al., 2012

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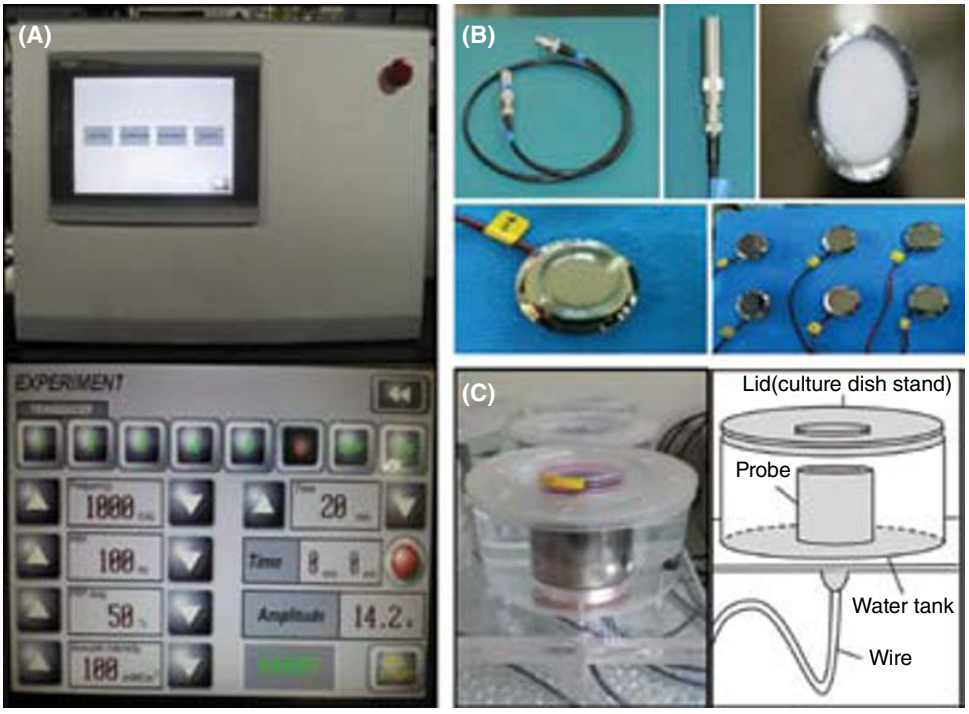


Figure 3.2. Ultrasound devices for research use. Low-intensity ultrasound (LIUS) devices have a controller for control of the condition of LIUS, including intensity and time in front of the main body **(A)**. The LIUS generators have various types of probes for use of animals in research **(B)**. Experimental setup showing LIUS stimulation of cells taken in the 60 mm culture dish placed on the probe. The water tank is completely filled with distilled water **(C)**.

basic fibroblast growth factor (bFGF) has been used in MSCs culture media and found to prolong the life span of MSCs to more than 50 doublings, thus maintaining their multipotent differentiation capability and the size of telomeres (Bianchi et al., 2003). Extracellular matrix (ECM) molecules, such as laminin, fibronectin, and collagens, have also been used as a precoating factor to increase the yield of MSCs (Matsubara et al., 2004; Ogura et al., 2004). The ECM molecules are known to enhance cell proliferation, attachment, and migration through interaction with cell membrane receptors like integrins (Giancotti and Ruoslahti, 1999). Thus, ECM molecules can enhance the yield of MSCs during primary culture by promoting their early attachment and proliferation.

Similar to the biochemical factors, LIUS stimulation is able to influence stem cell adhesion activity and matrix interaction. Stimulation of BM mononuclear cells with LIUS for 10 min daily during the first 6 days following initial plating of cells resulted in an increase in the number of MSCs colonies compared with nonstimulated cells (Figure 3.3) (Choi et al., 2011). In addition, no differences in proliferation rate, expression patterns of CD29, CD45, CD90, and CD106 (cell surface markers), and multipotent differentiation capability were observed in LIUS-stimulated MSCs compared with untreated cells. Stimulation with LIUS also induced an increase in expression of

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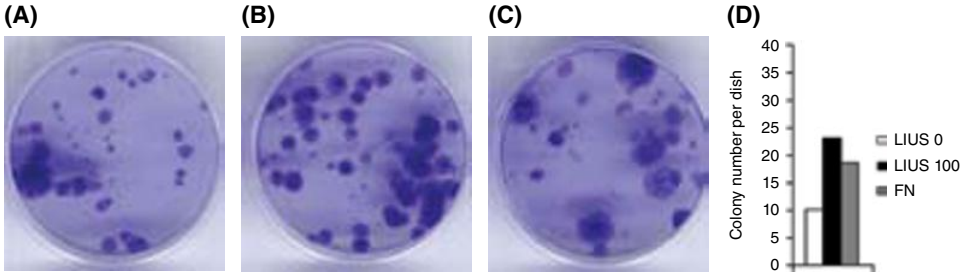


Figure 3.3. The colony-forming unit-fibroblasts assay using bone marrow mononuclear cells. Colonies formed at 12 days were stained with crystal violet and the number of colonies > 2 mm in diameter was counted in each dish of control **(A)**, LIUS treatment **(B)**, and fibronectin-coated **(C)**. Average values from three independent experiments are shown in the histogram **(D)**.

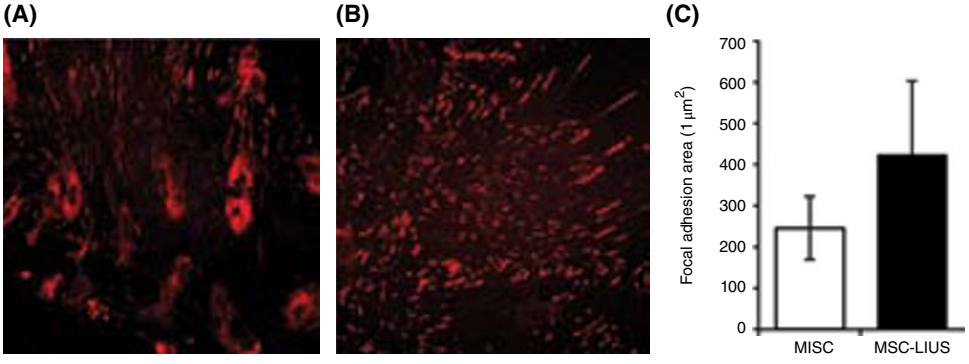


Figure 3.4. Immunostaining of MSCs with a cell adhesion-related protein, paxillin. Rat MSC colony was stained with paxillin for MSCs alone **(A)** and MSCs stimulated with LIUS **(B)**. The area of focal adhesion was measured quantitatively in the 10 microscopic fields of the images from each group **(C)**. The data represent the mean ± standard deviation of focal adhesion area for three separate experiments. (Modified from Low-intensity ultrasound increased colony forming unit-fibroblasts of mesenchymal stem cells during primary culture. Choi WH, Choi BH, Min BH, Park SR. *Tissue Eng Part C Methods.* 2011 May; **17**(5): 517–26.)

cell-adhesion-related proteins such as integrins, fibronectin, and paxillin, and induced formation of focal adhesions in MSCs. Similarly, pulsed LIUS (PLIUS) stimulation resulted in an increase in the yield of MSCs from whole human umbilical cord (hUC) without enzymatic digestion or growth factor supplementation, and increased the capacity of isolated hUC-MSCs to proliferate (Yoon et al., 2009). These results demonstrate that stimulation with LIUS leads to activation of the cell adhesion process and increases the colony-forming ability of MSCs during the early stage of primary culture, without affecting their phenotypes and multipotency. The mechanism of the effect of LIUS on adhesion of MSCs also appears to involve activation of integrin signals (Figure 3.4). These findings suggest that LIUS could be a beneficial tool for obtaining large amounts of MSCs for use in therapeutic applications.

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Cell Proliferation

A number of studies have shown that LIUS can stimulate proliferation of fibroblasts, osteoblasts, monocytes, and tendon cells (Doan et al., 1999; Tsai et al., 2005). Low-intensity ultrasound was also shown to increase proliferation of chondrocytes; however, its effect is rather controversial depending on the different experimental conditions. For instance, Huang et al. (1997) reported that stimulation with LIUS resulted in an increase in the total cell density in osteoarthritic rat cartilage compared with the nonstimulated one. Zhang et al. (2003) also demonstrated that stimulation with LIUS influenced chondrocyte proliferation in three-dimensional alginate culture in an intensity- and time-dependent manner. However, another study reported that stimulation with LIUS (100 mW/cm^2) resulted in enhanced chondrocyte proliferation in a monolayer culture, but not in the three-dimensional alginate system (Choi et al., 2006). In the case of stem cells, the PLIUS stimulation was shown to enhance proliferation of fresh hematopoietic stem/progenitor cells (HSPCs) and maintain the viability of cryopreserved HSPCs *in vitro*. In addition, LIUS stimulation does not affect the percentage of CD34-positive (HSPC marker) and CD14-positive (monocyte/macrophage differentiation marker) cells, indicating that LIUS-stimulated HSPCs maintain their phenotype, as in nonstimulated cells (Xu et al., 2012). However, its effect on proliferation of MSCs is not clearly understood.

Cell Viability

Stem cells are generally placed under harsh culture conditions in order to induce cellular differentiation *in vitro*. Actually, a harsh microenvironment for chondrogenic differentiation of MSCs using three-dimensional culture and soluble factors, including transforming growth factor-β (TGF-β), appears to be harmful to cells and induces cell death during long-term culture. However, LIUS stimulation can enhance the viability of both chondrocytes and MSCs during formation of cartilage tissue in three-dimensional alginate culture (Choi et al., 2006; Lee et al., 2007). Stimulation of chondrocytes cultured in three-dimensional alginate beads with LIUS at 100–200 mW/cm^2 for 10 min every day over 15 days resulted in significantly higher cell viability, compared with untreated cells. In addition, LIUS induced significant repression of apoptosis of human MSCs under chondrogenic differentiation in the three-dimensional alginate layer (Lee et al., 2007). In the gene expression analysis, LIUS was shown to decrease the expression of proapoptotic genes such as p53 and Bax, whereas it induced anti-apoptotic genes such as Bcl-2 and proliferating cell nuclear antigen (PCNA) in human MSCs under chondrogenic differentiation. The anti-apoptotic effect of LIUS can also be observed in monolayer culture of MSCs treated with staurosporine, an initiator of apoptosis in many different cell types (Figure 3.5). Therefore, LIUS appears to have a protective effect on MSCs during chondrogenic differentiation by inhibiting apoptosis of cells and regulating expression of apoptosis-related genes.

Cellular Differentiation and Tissue Engineering

Several studies have reported on the potential of mechanical stimulations to influence the fate of MSCs. When MSCs are isolated from bone marrow and perichondrium they express chondrogenic markers, such as collagen type II and aggrecan, and have a

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Figure 3.5. Effect of LIUS stimulation on staurosporin-treated MSCs. The apoptotic cells were examined using the terminal deoxynucleotidyl transferase dUTP nick end labeling assay for MSCs alone **(A)** and MSCs stimulated with LIUS (200 mW/cm^2) **(B)** at 5 days after treatment with staurosporin (1 μm). Average values at each time point from three independent experiments are shown in the histogram **(C)**.

chondrogenic potential (Ma et al., 2003). For effective chondrogenic differentiation of MSCs *in vitro*, they must be incubated in specific culture conditions, including growth factors such as TGF-β, BMPs, and fibroblast growth factor 2 (FGF2), and three-dimensional culture methods using scaffolds such as collagen and alginate scaffolds (Mastrogiacomo et al., 2001; Ma et al., 2003; Indrawattana et al., 2004). In addition to growth factors and three-dimensional cultures, mechanical stimulations are also effective in inducing differentiation of MSCs toward the chondrogenic lineage. Angele et al. (2003) reported that application of cyclic hydrostatic pressure induces chondrogenic differentiation of BM-derived MSCs in pellet culture. Huang et al. (2004) also reported that cyclic compressive loading can promote chondrogenesis of MSCs by inducing endogenous TGF-β and maintains viability of cells in fibrin gel scaffolds as well (Pelaez et al., 2009).

In addition, LIUS stimulation enhances chondrogenic differentiation of MSCs in three-dimensional culture. Its effect was first observed in pellet culture with TGF-β (Ebisawa et al., 2004) and in alginate culture without TGF-β (Lee et al., 2006). In both cases, stimulation with LIUS resulted in induction of chondrogenic differentiation of human-BM-derived MSCs by enhancing the synthesis of cartilage matrix and expression of chondrogenic markers such as type II collagen, aggrecan, and Sox-9 (Figure 3.6). In addition, in the alginate culture, treatment with LIUS resulted in increased expression of TIMP-2, but not MMP-3, which might enhance the matrix integrity. The positive effect of LIUS on chondrogenic differentiation of MSCs has also been observed in other studies using rabbit MSCs cultured in poly(glycolic acid) (PGA) or fibrin/hyaluronic acid (HA) scaffolds (Cui et al., 2006, 2007; Choi et al., 2013). After rabbit MSCs were seeded on PGA scaffold, the cells were stimulated with LIUS at 200 mW/cm^2 for 10 min every day for 1 week *in vitro* before implantation into the back of nude mice for 4 weeks (Cui et al., 2007) or for 4 weeks during the implantation without preconditioning *in vitro* (Cui et al., 2006). In both cases, gross observation and total collagen and glycosaminoglycans (GAGs) content indicated a clear increase in the LIUS stimulation group compared with the control group. In addition, the MSCs in PGA scaffolds pretreated with LIUS showed much better results in regenerating an articular cartilage defect in rabbits (Figure 3.7) (Cui et. al., 2010). A similar

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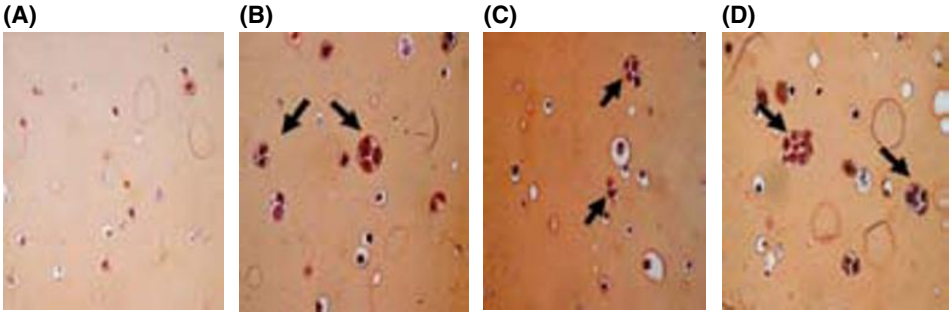


Figure 3.6. Low-intensity ultrasound (LIUS) stimulation enhances chondrogenesis of MSCs. Human MSCs cultured in alginate scaffold for 2 weeks were stained with Safranin O/Fast Green for examination of chondrogenesis for control **(A)**, TGF **(B)**, LIUS stimulation **(C)**, and co-treatment with LIUS and TGF **(D)**. Arrows show Safranin O/Fast-Green-positive cells in cartilage lacuna. TGF, transforming growth factor-β. (Modified from Low-intensity ultrasound stimulation enhances chondrogenic differentiation in alginate culture of mesenchymal stem cells. Lee HJ, Choi BH, Min BH, Son YS, Park SR. *Artif Organs.* 2006 Sep; **30**(9): 707–15)

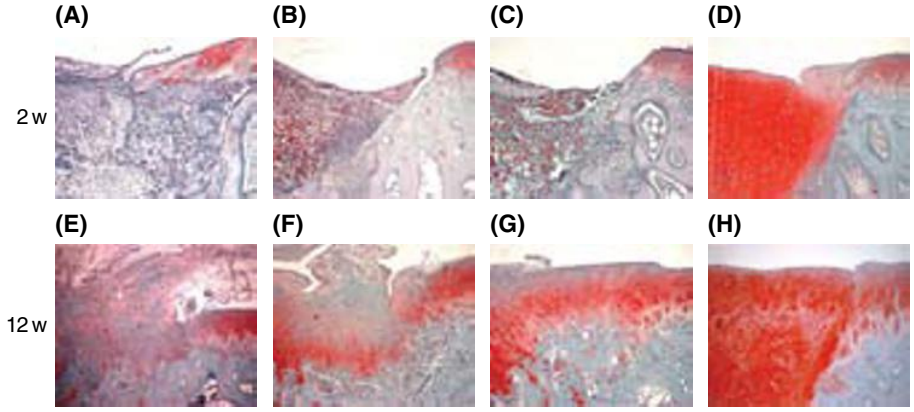


Figure 3.7. Cartilage tissue engineering. Mesenchymal stem cells (MSCs) or chondrocytes seeded on PGA scaffold were implanted in a rabbit model of osteoarthritis. Histologic features of Safranin O/Fast Green staining at 2 and 12 weeks post-implantation were observed for the injury only (**A** and **E**), MSCs (**B** and **F**), MSCs stimulated with LIUS (**C** and **G**), and chondrocytes (**D** and **H**). Specimens in MSCs stimulated with LIUS showed better chondrogenesis in terms of accumulation of cartilage-specific extracellular matrix compared with MSCs alone. PGA, poly(glycolic acid). (Modified from Preconditioning of rabbit mesenchymal stem cells in polyglycolic acid (PGA) scaffold using low-intensity ultrasound improved regeneration of cartilage in rabbit articular cartilage defect model. Cui JH, Choi BH, Min BH, Park SR. *Tissue Eng Regen Med.* 2010 Feb; **7**(1): 24–31.)

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study using fibrin-HA hydrogel in vitro again showed that treatment with LIUS resulted in significant enhancement of the amounts of GAGs, collagen, and mechanical strength (Choi et al., 2013). Of particular interest in these studies is that the chondrogenic effect of LIUS was demonstrated, even without exogenous TGF-β, a well-known inducer of chondrogenesis, in the chondrogenic differentiation media. Similar results were also reported on chondrogenic differentiation of MSCs using other mechanical stimulations of an electromagnetic compression (Park et al., 2006) and cyclic compression (Angele et al., 2003), which collectively suggest that the mechanical stimulations activated the TGF-β signal or utilized a TGF-β-independent mechanism in order to exert their effects. Another notable effect of LIUS treatment is that MSCs differentiated using LIUS maintain their chondrogenic phenotype for a longer period of time, even when transferred to two-dimensional culture. Mesenchymal stem cells derived from human synovial membrane when differentiated into chondrocytes in pellet culture using TGF-β lost their chondrogenic phenotype after being replated in monolayer culture (De Bari et al., 2004). By contrast, when cells differentiated in three-dimensional alginate culture are replated on the monolayer culture, the chondrogenic phenotypes are maintained well until passage 2 in LIUS-pretreated cells (Lee et al., 2006). Therefore, LIUS stimulation appears to be effective in maintaining the chondrogenic phenotypes. Taken together, all these findings suggest that LIUS stimulation can be a useful tool for induction of chondrogenic differentiation of MSCs both *in vitro* and *in vivo*, and for tissue engineering of cartilage using MSCs for regeneration of injured cartilage.

In addition to the chondrogenic effect of LIUS on stem cells, stimulation with LIUS can also induce osteogenic differentiation of a variety of cell types. One study on osteogenic differentiation of adipose-derived stem cells (ASCs) stimulated by LIUS reported that LIUS induced mRNA and protein levels of osteogenic genes such as runt-related transcription factor 2 (Runx 2), osteopontin (OPN), and osterix (Osx) in the presence of osteogenic medium (Yue et al., 2013). Stimulation with LIUS also induced osteogenic differentiation of BM-derived human MSCs, resulting in increased levels of expression of osteogenic markers, Runx 2, and alkaline phosphatase (ALP). No additive or altered effect was observed when LIUS and BMP-2, an osteoinductive factor, were combined, suggesting again that the effect of LIUS might be independent of and nonadditive with biochemical signaling pathways (Lai et al., 2010). In addition, treatment with LIUS resulted in acceleration of osteogenic differentiation of a normal human osteoblast cell line and promoted bone formation in a rat osteoporosis model (Wu et al., 2009). The activity of LIUS in induction of osteogenesis of MSCs supports its therapeutic effect on fracture healing, which will be discussed later.

Signaling Pathways

Control of stem cell fate is based on various components of the surrounding microenvironment, such as soluble factors, ECM molecules, cell-to-cell interactions, and mechanical stimulations. Depending on their spatio-temporal context, these components can stimulate stem cells to either proliferate or differentiate into a particular cell lineage. Unlike soluble factors, including growth factors and cytokines that bind their receptors with specificity, mechanical stimulations appear to have low specificity.

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However, significant evidence has shown potential candidates capable of transducing the mechanical stimulations into cells, such as integrins, focal adhesions (Chen et al., 1999; Matthews et al., 2006; Schwartz and DeSimone, 2008), and ion channels (Ingber, 2006). Integrins and stretch-activated channels (SACs) are among the most well known mechanoreceptors. Integrins have been shown to play key roles in cell adhesion and cytoskeletal remodeling by binding with various ECM proteins (Miyamoto et al., 1995; Giancotti and Ruoslaht, 1999). Subsequently, they induce an assembly of focal adhesion-related factors, including paxillin, vinculin, and focal adhesion kinase (FAK), and then activate the downstream signaling pathways that involve small G proteins, Ras, phosphoinositide 3-kinase (PI3K), and mitogen-activated protein kinases (MAPKs) (Guan, 1997; Giancotti and Tarone, 2003). Integrins can also interact with ion channels and growth factor receptors to form complexes and regulate their signaling events. The activity of SACs was also shown to be necessary for various cellular responses to mechanical stimulations, which involves tyrosine phosphorylation of paxillin and FAK and cytoskeletal remodeling. In the stretch-induced morphologic changes of human umbilical endothelial cells, SACs have been shown to regulate the stretch-specific tyrosine phosphorylation of paxillin and FAK (Malek and Izumo, 1996; Naruse et al., 1998). Thus, SACs appear to mediate the mechanotransduction signals to diverse downstream pathways. Cytoskeleton by itself is also one of the important cellular mechanical components and was shown to mediate mechanical signals to intracellular signaling pathways, including Rho family GTPases (Ingber, 2006; Orr et al., 2006). The underlying mechanism appears to involve its structural rearrangement upon mechanical stimulations, such as substrate stiffness, external compression, and shear stress (Janmey and McCulloch, 2007; Wang et al., 2007). These potential molecular mediators of mechanotransduction triggered by mechanical stimulations induce activation of downstream target proteins and changes in the gene transcription profiles, and eventually affect a diverse array of cell functions, including cell adhesion, proliferation, differentiation, ECM metabolism, and metabolic activity. For example, mechanical strain induces generation of reactive oxygen species (ROS), which activates integrins and, subsequently, the PI3K/Akt signaling pathway. The PI3K/Akt signal in turn induces the translocation of β-catenin into the nucleus, which increases the expression of connexin (Cx) 43 and homeobox protein Nkx 2.5 required for cardiomyocyte differentiation (Hoo and Lee, 2007). Shear stresses by fluid flow also enhance proliferation of bone-marrow MSCs via activation of the calcium-sensitive protein phosphatase calcineurin and extracellular signal-regulated kinase (ERK)-1/2 (Riddle et al., 2006).

Little is known about the LIUS signal transduction pathways in stem cells. Early studies on LIUS signaling pathways were performed in chondrocytes and osteocytes having continuous exposure to a variety of different mechanical forces, such as shear, stretch, or compressive forces *in vivo*. These cells express various transmembrane receptors, including integrins, SACs, and anchorin CII, known as annexin V (a collagen receptor) in transduction of mechanical signals (Loeser, 2000). The effects of LIUS on the expression of type II collagen and aggrecan in chondrocytes are also known to be mediated by these mechanoreceptors. Low-density lipoprotein induced phosphorylation of FAK and paxillin, and selectively activated the PI3K/Akt pathway, but not the MEK/MAPK pathway in chondrocytes (Takeuchi et al., 2008). Upon treatment with gadolinium (a potent blocker for SACs) or Gly-Arg-Gly-Asp-Ser-Pro

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(GRGDSP; a peptide inhibitor of integrins), the LIUS-stimulated expression of type II collagen and aggrecan is significantly blocked depending on the incubation time. In addition, stimulation with LIUS induces phosphorylation of c-Jun N-terminal kinase (JNK) and ERK in a chondrocyte cell line (C-28/I2 cells) but not p38 kinase among members of the MAPKs. Phosphorylation of ERK is reduced by the specific inhibitors of the ERK pathway and integrin (Choi et al., 2007). These results demonstrate that stimulation with LIUS induces production of ECM through canonical mechanoreceptors, integrins, and SACs, and subsequently through the JNK and ERK pathways.

In rat osteocytes, application of PLIUS induced expression of osteocalcin and insulin-like growth factor I (IGF-1) without involvement of calcium ion influx, which differed from the mechanism of stretch stress (Naruse et al., 2003). The PLIUS induced an increase in expression of COX-2 and oteocalcin in murine BM-derived cells (ST2), which was blocked by inhibitors of PI3K and p38 kinase in the same study (Naruse et al., 2003). In addition, stimulation with LIUS resulted in transiently increased expression of a2, a5, and b1 subunits of integrins, and reorganization of the actin cytoskeleton in both an osteoblastic cell line (MC3T3-E1) and primary osteoblasts. When these cells were cultured in osteogenic differentiation medium, stimulation with LIUS resulted in increased formation of mineralized nodules, alkaline phosphatase activity, and collagen content (Yang et al., 2005). Findings of another study showed that LIUS stimulation directed the differentiation of C2C12, a murine myoblast cell line, into osteoblasts and chondroblasts via phosphorylation of ERK1/2 and p38 kinase (Ikeda et al., 2006). Of particular interest, treatment with LIUS resulted in increased mRNA levels of Runx2, Msx2, Dlx5, AJ18 (osteoblasts-specific markers), and Sox-9 (chondroblasts-specific marker), while decreasing those of MyoD (myoblasts-specific marker), C/EBP, and PPAR gamma (adipocytes-specific markers) (Ikeda et al., 2006). A similar result showed inhibition of myogenic and adipogenic differentiation of rat MSCs by LIUS (Kim et al., 2011). Taken together, these findings indicate that LIUS has a regulatory effect on integrin expression and focal adhesion complex and activates ERK1/2 and p38 kinase, which occurs at least in part in the osteogenic differentiation of a number of progenitors.

Detailed role(s) of LIUS in the integrin signal pathways have been reported only in human fibroblasts. Stimulation with LIUS resulted in increased formation of stress fibers and focal adhesions, and enhanced proliferation of fibroblasts via activation of Rho/ Rho-associated protein kinase (ROCK) and ERK signaling pathways. Treatment of cells with Y27632 (ROCK inhibitor) resulted in LIUS-induced blockade of ERK1/2 activation, demonstrating that the Rho/ROCK signaling pathway was an upstream regulator of ERK activation in response to stimulation with LIUS. In addition, the integrin blocking antibody and RGD peptide (integrin inhibitor) decreased LIUS-induced DNA synthesis (Zhou et al., 2004). Another study using fibroblasts without syndecan-4 expression, a co-receptor of integrin, showed that stimulation with LIUS complemented its loss and supported the normal adhesion of fibroblasts in its absence (Mahoney et al., 2009). Taken together, these data suggest that LIUS can directly activate integrin and downstream cascade of Rho/ROCK–ERK or PI3K signals in fibroblasts and in other cell types, including stem cells, in order to exert its various cellular functions (Figure 3.8).

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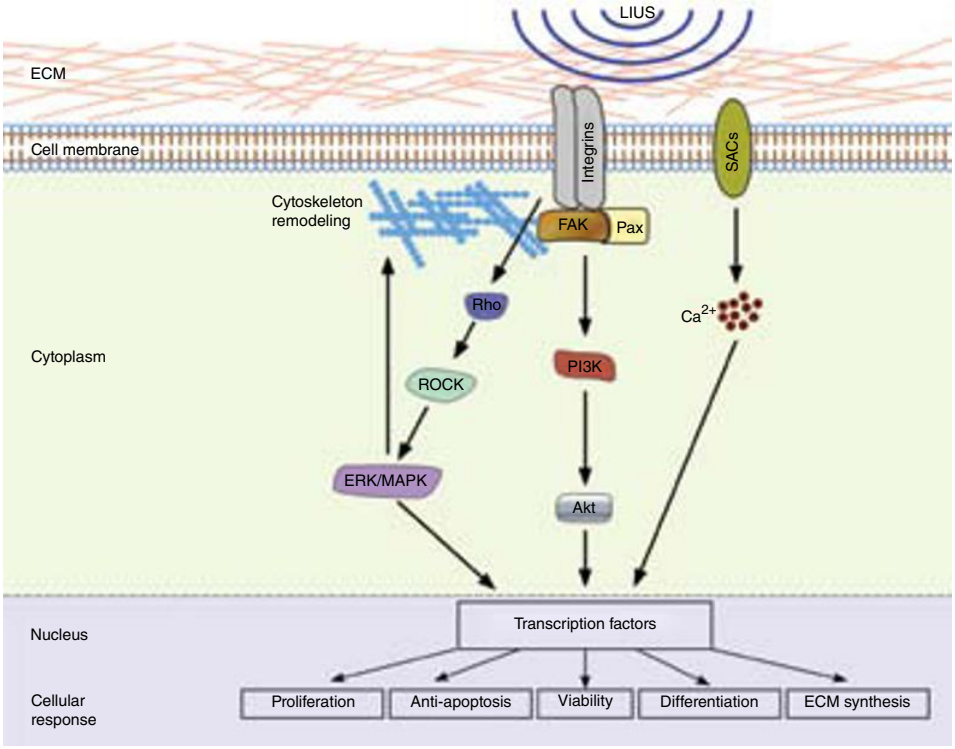


Figure 3.8. Signaling pathways by low-intensity ultrasound. Low-intensity ultrasound stimulation causes activation of integrins and SACs, and assembly of some focal adhesion proteins, including FAK and paxillin. Phosphorylation of the assembled proteins selectively activates the PI3K/Akt and Rho/ROCK pathways. Subsequently, these pathways induce various cellular responses such as proliferation, anti-apoptosis, viability, differentiation, and ECM synthesis depending on cell types and microenvironmental changes. ECM, extracellular matrix; SACs, stretch-activated channels; FAK, focal adhesion kinase; Pax, paxillin; PI3K, phosphoinositide 3-kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; ROCK, Rho-associated protein kinase.

Therapeutic Application of LIUS

Osteoarthritis

Articular cartilage is an avascular tissue with a low self-healing ability and is difficult to repair following damage. Therefore, without adequate treatment, traumatic injuries and degenerative diseases in the articular cartilage can easily lead to development of osteoarthritis (OA) (Mankin, 1982). Many surgical techniques have been developed for treatment of cartilage defects, such as autologous osteochondral transplantation and drilling through the subchondral bone. However, several limitations still exist and should be overcome for the effective clinical use of these methods (Peterson et al., 2000). Cell therapy using chondrocytes and MSCs is widely used for treatment of cartilage

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defect, and tissue engineering of cartilage is under extensive investigation. Using the tissue engineering approach, MSCs are cultured in three-dimensional conformation using scaffolds and differentiated into chondrocytes to artificial cartilage tissue. Growth factors, including TGF-β, BMPs, and FGF-2 are known to be important factors for cartilage tissue engineering using MSCs (Mastrogiacomo et al., 2001; Indrawattana et al., 2004).

Articular cartilage is maintained by balance between synthesis and degradation of ECM. Proteoglycan and type II collagen are two major components of cartilage ECM. Thus, degradation or decreased synthesis of these components has been shown to induce a significant loss of cartilage function in OA (Hollander et al., 1995). Matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) are key regulators of matrix integrity and remodeling (Mott and Werb, 2004). The TIMPs inhibit MMPs involved in degradation of cartilage matrix in OA. Therefore, regulation of their levels could be a target for treatment of cartilage diseases (Mix et al., 2004). Articular cartilage is always exposed to complex mechanical stimuli and can play an important role in maintenance of the normal structure and function of cartilage (Garcia et al., 1996; Hung et al., 2000). An appropriate mechanical stimulation from the surrounding environment promotes the metabolism of chondrocytes (Grandolfo et al., 1998; Zhang et al., 2003) and recovery of injured cartilage tissues (Huang et al., 1997; Feril and Kondo, 2004). As mentioned above, LIUS can increase the viability of chondrocytes, proliferation, and ECM synthesis, and effectively induce chondrogenic differentiation of MSCs *in vitro* and *in vivo* (Cui et al., 2006; Lee et al., 2006; Min et al., 2006). Of particular importance, LIUS also directly inhibits the expression of MMP-1 and induces an increases in the TIMP-2 levels in chondrocytes and during chondrogenic differentiation of MSCs (Min et al., 2007). Apart from cartilage tissue engineering, LIUS has been used successfully in treatment of injured cartilage without implantation of cells. Low-intensity ultrasound enhanced the repair of arthritic cartilage in papain-induced arthritis in rats. It also facilitated production of stress protein in arthritic chondrocytes, which is consistent with its therapeutic effects on chondrocyte preservation and repair of arthritic cartilage (Huang et al., 1999). In addition, combined treatment of LIUS with hyaluronic acid (HA) resulted in a reduction in total synovial fluid volume and accretion of proteoglycan and prostaglandin E2 in synovial fluid. Histological examination also showed that the combined treatment reduced the severity of OA-induced structural damage in cartilage and synovium (Park et al., 2005). The role of stem cells in repair of OA is not yet clear. However, recent studies have suggested the existence of stem cells in cartilage, which play a role in the integrity of cartilage structure and function (Redman et al., 2005). Therefore, it is plausible that the therapeutic effect of LIUS involves activation of resident stem cells in modulation of ECM synthesis and cartilage regeneration. Thus, LIUS could be another useful tool for treatment of cartilage defect and OA.

Bone Fracture

Bone healing has been investigated extensively in various research fields, such as stem cells, gene therapy, drug development, and mechanobiology. The healing process of a fractured bone involves inflammation, soft and hard callus formation, and bone remodeling. Several *in vitro* and *in vivo* studies have demonstrated the biological effects

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of LIUS in the bone healing process. Stimulation by LIUS in rabbit fracture models significantly accelerated the recovery of mechanical strength of intact bone (Pilla et al., 1990). Similarly, in clinical trials, treatment with LIUS resulted in an acceleration of fracture repair in both the tibia and distal radial fractures (Heckman et al., 1994; Kristiansen et al., 1997). Currently, LIUS is generally used for treatment of fractures. However, the precise mechanisms underlying the therapeutic effects of LIUS on bone fracture are still unknown. Several biological mechanisms have been proposed to explain the influence of LIUS on promotion of fracture healing. Studies have demonstrated the potential effect of LIUS in acceleration of fracture healing by changing cellular and molecular pathways involved in each step of the healing process. Integrins are known to play an important role in modulation of cellular signaling involved in fracture healing (Pounder et al., 2008). They react to the mechanical stimulation generated by LIUS in the cellular environment. Subsequently, the induced intracellular signaling activates COX-2 enzymes in osteoblasts (Kokubu et al., 1999). This in turn results in increased production of prostaglandin E2, which is critical to effective mineralization during endochondral ossification of the soft callus (Tang et al., 2006). Endochondral ossification stimulated by LIUS also results in a larger area and greater extent of bony callus formation by enhanced mineral deposition. In addition, angiogenesis is also enhanced by LIUS stimulation through an increase in production of VEGF in both osteoblasts and periosteal cells (Uchida et al., 2003; Leung et al., 2004).

Low-intensity ultrasound induces changes in stem cell functions, which may accelerate healing of bone fractures. After the inflammation phase during the process of fracture healing, the callus is invaded by MSCs and blood vessels, while mechanical stimuli will sensitize these cells to differentiate to osteoblasts for bone formation. Most recently, one study reported that combined treatment with MSCs and LIUS is beneficial to fracture healing. That study investigated the effects of combined treatment with exogenous MSCs and LIUS on fracture healing by comparison with MSCs alone. Microcomputed tomography showed the highest bone volume/tissue volume values for the combined treatment (MSC–LIUS). Collectively, these findings suggest that the combined treatment of MSCs with LIUS as well as LIUS alone could be a therapeutic tool for achievement of success of fracture healing (Cheung et al., 2013).

Conclusion

Stem cells represent the most promising cell source for repair of numerous tissues. Although many studies have demonstrated their therapeutic potential, a better understanding and control of their proliferation and differentiation is required in order to realize their clinical applications. One of the important points is to encourage and maintain the appropriate phenotype and cell metabolism. It is clear that stem cells respond sensitively to various biochemical and mechanical microenvironments. However, in order to do so, most of the studies conducted so far have focused on biochemical stimulations. Recently, accumulating data have shown that the mechanical environment influences stem cell proliferation and differentiation. Therefore, mechanical stimulations represent an additional and efficient tool for augmentation and maintenance of stem cell proliferation and differentiation. Providing proper mechanical stimulations

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both *in vitro* and *in vivo* will lead to improvement of engineered tissues and to ultimate success in stem-cell-based therapies.

In this review, we have introduced current research on LIUS in stem cells and tissues. Therapeutic potential of LIUS has become evident in various animal models of musculoskeletal system diseases, and its application is being widely expanded, particularly in the repair of cartilage and bone diseases. In summary, LIUS has several bioeffects on: (i) cell adhesion of MSCs; (ii) viability of cells, including MSCs in three-dimensional cultures; (iii) MSC differentiation, particularly into chondrocytes and osteoblasts; (iv) ECM synthesis; and (v) repair of cartilage and bone injuries *in vivo*. In particular, LIUS stimulation is a non-invasive, efficient, and cost-effective tool for cartilage tissue engineering, because it enhances the viability of cells, ECM protein synthesis, and matrix integrity without TGF-β treatment in the process of cell culture. Together with its non-invasive and cost-effective properties, a variety of bioeffects of LIUS on stem cells and tissues will make it an innovative tool for use in stem cell research and tissue engineering. An in depth understanding of the mechanisms of LIUS that allow regulation of stem cell behavior will undoubtedly lead to more effective development of therapeutics for use in tissue engineering and regenerative medicine.

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Chapter 4

Mammalian Neo-Oogenesis from Ovarian Stem Cells *In Vivo* and *In Vitro**

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Introduction

It is still widely believed that oocytes in adult ovaries of higher vertebrates (birds, monotremes, and with few exceptions all eutherian mammals) persist from the early stage of ovarian development, that is, from the fetal period in humans and perinatal period in mice and rats (Pearl and Schoppe, 1921; Zuckerman, 1951; Zuckerman and Baker, 1977). However, this 60-year-old storage doctrine was generally accepted after 100 years of discussion on whether or not adult mammalian females form new eggs. From the evolutionary point of view the essential question is: what is the advantage of egg storage from the early period of life for several decades in large mammals, including humans, compared to the continual formation of new eggs known in less evolved animal species. Our opinion is that the egg renewal exists during the prime reproductive period (PRP) throughout the animal kingdom (PRP theory) (Bukovsky and Virant-Klun, 2007), and ceases after PRP termination, when the remaining eggs are stored until exhausted by continuing ovarian function, that is, cyclic follicular selection and ovulation.

Among invertebrates (flies and worms) and lower vertebrates (fishes and frogs), germ cells develop in the same manner in both sexes. Large numbers of eggs and sperm are developed and ripened at the same rate and all are "discharged" either at once or within a very brief interval. In the males of higher vertebrates there is no significant variation in this process. In females of higher vertebrates, including

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mammals, however, a different state exists. Only several of numerous resting primordial ovarian follicles are induced to grow during each cycle, and by the process of follicular selection only one or a few are selected to ovulate. In addition, ovarian ovulatory function in long-living species, for example humans, stops at a certain age.

Why would such differences exist between lower and higher vertebrates? The difference between frogs and birds or humans is in the presence or absence of the maturation and discharge of all available oocytes. While frog progeny develops outside of the female body, independently of the parental care, birds produce a certain (limited) number of eggs with respect to the need to take care for the development of limited progeny after hatching. Mammalian females ovulate a limited number of eggs, due to the limited capacity of the uterus to develop multiple fetuses. With respect to age, ovulation also ceases earlier in mammals compared to birds of similar size (Holmes et al., 2003), probably because the hatching of the eggs is outside of the body. Mammalian females appear to be naturally precluded from pregnancy at an advanced age.

The oocyte storage doctrine proposal by Solomon Zuckerman and collaborators in the 1950s (Zuckerman, 1951; Mandl and Zuckerman, 1951a,b; Green et al., 1951) was essentially unopposed until 1995, when neo-oogenesis and formation of new granulosa cells for primordial follicle renewal was described for the first time ever in adult human females (Bukovsky et al., 1995a). Almost 10 years later, the neo-oogenesis in adult mice described by Allen (1923) was confirmed by Johnson et al. (2004) The origin of human germ cells and follicular renewal from ovarian stem cells (OSC) in adult human females was elaborated further in 2004 by Bukovsky et al., and the origin of germ cells from ovarian surface epithelium in human fetuses was described in 2005 (Bukovsky et al., 2005a). The development of new eggs in ovarian surface epithelium cultures lacking oocytes was also demonstrated in 2005 (Bukovsky et al., 2005b) and the first clinical human trial reported by Bukovsky and Virant-Klun in 2007. Studies show that clinical trials should utilize intracytoplasmic sperm injection (ICSI), since eggs developed *in vitro* lack expression zona pellucida (ZP) proteins and do not attract sperm (reviewed by Bukovsky and Caudle, 2012). Neo-oogenesis was described in normal adult rats in 2007 as well as alternative pathways for neo-oogenesis in adult rats lacking ovarian surface epithelium cells (Bukovsky et al., 2007). Work in mice significantly advanced in 2009, when germ-line stem cell cultures derived from 5-day-old and adult mouse ovaries were shown to produce functional oocytes when transplanted into the ovaries of infertile mice (Zou et al., 2009). A lightly modified study with human germ-line stem cell cultures from fertile women has shown the ability to produce new primordial follicles when injected into functional human ovarian tissue and transplanted into immunodeficient mice (White et al., 2012). This study is the first direct confirmation of the report by Bukovsky et al. (1995a) that functional adult human ovaries contain germ cells capable of follicular renewal.

Terminology

In this article, the term "primordial germ cell" is used to determine extragonadal cells migrating into gonads during the early embryonic period, where they play their important role in the gonadal development, and then degenerate (see references to Waldeyer (1870), Rubaschkin (1909), Firket (1914) and Reagan (1916) below).

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The term "secondary germ cell" denotes cells originating from somatic stem cells at the ovarian surface: formerly known as germinal epithelium in vertebrates and nonvertebrates, and since 1970 called ovarian surface epithelium (Fathalla, 1971). However, the term germinal epithelium persists in invertebrates, such as medusae, where cells are gradually transformed into large oocytes (Adonin et al., 2012). In vertebrates, the term germinal epithelium persists in teleosts (the ray-finned fishes) where, in the differentiated ovary, the active germinal epithelium is the source of germ cells (Mazzoni et al., 2010). Also, in developing birds, the germinal epithelium persists and contains primordial germ cells (Ishiguro et al., 2009). In human fetuses, the surface epithelium contains germ cells and contributes to the development of granulosa cells (Motta and Makabe, 1982). It has been suggested that "The existing dogma that the former term ovarian 'germinal epithelium' resulted from a mistaken belief that it could give rise to new germ cells is now strongly challenged" (Nishida and Nishida, 2006, p. 1). Since in mammalian species the ovarian surface epithelium cells are a bipotent source of new female germ and granulosa cells (Bukovsky et al., 1995a, 2004), including human fetuses (Bukovsky, 2006a; Bukovsky et al., 2005a), from 2004 they have been called the OSC in humans (Bukovsky et al., 2004, 2006a,b; 2009a; Bukovsky and Virant-Klun, 2007; Bukovsky, 2008, 2011a; Virant-Klun et al., 2008, 2011a; Bhartiya et al., 2012a,b; Bukovsky and Caudle, 2012; Hosni and Bastu, 2012; Telfer and Albertini, 2012) as well as in other mammals (Gandolfi et al., 2005; Hutt and Albertini, 2006), and in the *Drosophila* ovary (Jin and T. Xie, 2007; Kirilly and Xie, 2007; Bakhrat et al., 2010).

In addition, it must be noted that the earlier rarely used term "oogonial stem cells" (Faddy et al., 1992; Gosden, 1992; Nandedkar and Narkar, 2003), now considered as a new term (White et al., 2012), is misleading, since such cells are in reality well known germ cells capable of proliferating, and not persisting in adult ovaries from the fetal period but originating in fetal and adult human ovaries by asymmetric division of their progenitors, that is, OSC (see below).

The primordial follicles can be divided into "fetal primordial follicles," formed during the second trimester of intrauterine life from OSC-derived secondary germs and granulosa cells and persisting until the perimenarcheal period, and "adult primordial follicles," formed by follicular renewal during the PRP (from menarche till 38 ± 2 years of age) and persisting thereafter until menopause.

Historical Overview of Oogenesis

Gametes represent a unique type of cell, since they are capable of undergoing meiotic division, which apparently does not occur in somatic cells. Hence, scientists have questioned whether gametes can originate from somatic cell precursors or only from a germ-line type of cells set apart during the earliest stage of embryonic development.

In 1843, Sir Richard Owen studied development of invertebrates ("apterous larva" of Aphis), and discovered a recognizable distinction between the general body mass of the embryo and a cluster of cells which he designated the "germ mass." He suggested that the creative cells were for the next generation grown from this "germ mass." Owen (1843) wrote "… the essential condition of the development of another embryo in this larva is the retention of part of the progeny of the primary impregnated germ-cell"

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(reviewed by Bukovsky, 2007a, p. 231). Hence, no epigenetic mutations in somatic cells were expected to be transmitted to subsequent generations of derived individuals. This indicates an extragonadal origin of germ cells but conflicts with theories of evolution stating that surviving individuals are those who reproduce, mutate, and are capable of transmiting these mutations to subsequent generations. Owen's observations were developed further by Goette (1875) and Nussbaum (1880), and resulted in the "continuity of germ plasm" theory.

The "Origin of Germ Cells from the Somatic Cells" Theory

Contrary to Owen's view, Wilhelm von Waldeyer-Hartz in his book "Eierstock und Ei" in 1870 presented evidence from mammalian females for successive stages of differentiation of germ cells from somatic coelomic epithelium of the developing gonad (Waldeyer, 1870), that is, from OSC cells. He also felt that such differentiation of germ cells was reserved for the period of early ovarian development and did not occur thereafter.

The "Continuity of Germ Plasm" Theory

In 1875, Goette indicated that he had evidence supporting an extragonadal origin of the large germ cells in the toad. He indicated that these cells migrate into the developing gonads. Nussbaum in 1880 discussed Waldeyer's (1870) opinion regarding the origin of germ cells from somatic cells and maintained that germ cells have a different function compared to somatic cells, as somatic cells serve as nurse cells for germ cells and cannot be considered progenitors of these cells.

In 1883, a series of papers from Weismann began dealing with the theory on the continuity of germ plasm (Weismann, 1885). Weismann's theory of germinal continuity, however, gave little difference to the previous cytological observations of Goette and Nussbaum (reviewed by Simkins, 1923). Weismann's view did, however, stimulate a series of investigations showing the physical continuity of primordial germ cells in invertebrates and the extragonadal origin of primordial germ cells in vertebrates.

Extragonadal Origin of Primordial Germ Cells

Studies by Eigenmann (1891), Nussbaum (1880), and others showed an extragonadal origin of primordial germ cells, which migrate into developing gonads. They presumed that in gonads, these primordial germ cells divide and produce definitive gametes for progeny.

Two Kinds of Germ Cells

Until the work of Dustin (1907), few questioned the fate of primordial germ cells within developing ovaries. He demonstrated two kinds of cells in the germ line of amphibians: (i) primordial germ cells, which populated the developing gonad, differentiated into gonocytes, and degenerated; and (ii) secondary germ cells originating from the OSC, which differentiated into definitive oocytes.

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The Germ Cell Route

At about the same time, Fedorov (1907) and Rubaschkin (1907) first described the appearance of primordial germ cells in the endoderm of the posterior part of the intestine (hindgut). Based on studies of embryos from several mammalian species, Rubaschkin (1909) suggested division of the history of the germ cell route (Keimbahn) into three periods. The first period begins with the differentiation of primordial germ cells, which, however, do not have the potential to become definitive gametes (Urgeschlechtszellen). Gonadal development is associated with the establishment of the so-called germinal epithelium (Keimepithel). The second period is associated with the appearance of female or male sex-specific cells (Ureier or Ursamenzellen), and the third deals with development of the sex-specific glands.

The germ cell route described by Rubaschkin again raised the question of the fate of primordial germ cells (Dustin, 1910). Winiwarter and Sainmont (1908) suggested that these cells degenerate after reaching the sex gland, and that definitive germ cells arise from the OSC. An alternative possibility considered was that the primitive germ cells are destined from the time of cleavage to become definitive female or male germ cells (Spuler, 1910).

In Firket's (1914) opinion, the primordial germ cells originate from the blastomeres, before the development of the genital ridge. They have an ability to migrate into sexual gland anlages, where they differentiate into sex cells, and then degenerate. The secondary germ cells do not descend from the primordial germ cells, but from the OSC. Swift reported that the primordial germ cells are found among the cells of the OSC from its very beginning. They are easily recognized, as they are quite different from the surrounding cylindrical cells. They are large, oval or round, have a large excentric nucleus, which appears vesicular and devoid of chromatin. The cytoplasm stains faintly, and germ cells of certain stages in the development of the chick scatter over the entire embryo, even in blood vessels (Swift, 1914, 1916).

Although extragonadal primordial germ cells may degenerate after entering the gonad, they nevertheless play an important role in gonadal development. In the chick, the germ cells are first recognizable in the crescentic area of the germ-wall endoderm, as early as 24 h of incubation (Swift, 1914). Reagan (1916) cut out this crescentic area, in which the primordial germ cells were supposedly located. Operated chicks were then further incubated and killed for examination after varying lengths of time. In no instance where the removal of this sex-cell area was complete did germ cells arise from somatic cells of the gonads, even after establishment of the OSC. In the normal chick, the OSC is well formed on day 4 of incubation, and primitive ova are clearly recognizable in it (Lillie, 1908). But in Reagan's operated chicks, even after 5 days of incubation, no germ cells were recognized (Reagan, 1916).

The "Continuity of Germ Plasm" Theory Does Not Fit for the Mammals

Application of the "continuity of germ plasm" theory across the animal kingdom persisted until the late 1970s (Baker, 1972; Zuckerman and Baker, 1977). At this time studies of mouse embryos, in which genetically marked cells were introduced at the 4- and 8-cell stage blastomere, showed that such cells could either become germ cells or somatic cells (Kelly, 1977). This suggested that no specific germ cell commitment exists prior to implantation.

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In nonmammalian species, removing either pole of the undivided egg prevents normal development: embryos may arrest early, lack organs, or the adults may be sterile. Such experiments have shown that spatial patterning of the egg is essential for subsequent development. However, when a substantial amount of material either from the animal (polar-body associated) or the vegetal (opposite) pole of the fertilized mouse egg is removed, the development of blastocysts is not affected, and after transfer to the uteri of pseudopregnant foster mothers they can produce viable offspring. Furthermore, these develop into fertile adult mice. Hence, mouse eggs have no essential components that are localized uniquely to the animal or the vegetal pole and, therefore, do not rely on maternal determinants that are localized in the fertilized egg for their axial development. Thus mammalian eggs appear to be unusual in the animal kingdom in that they establish the embryonic axes after the zygote has begun development (Zernicka-Goetz, 1998).

During the postimplantation period, mouse germ cells are not identifiable before ~7 days after fertilization (Ginsberg et al., 1990). The germ cells differentiate from somatic lineage (Lawson and Hage, 1994). It has also been shown that cellular differentiation of grafted embryonic cells does not depend on where the grafts are taken, but where they are placed (Tam and Zhou, 1996).

Additional studies suggest an important role in the development of germ cells for bone morphogenetic protein 4 (BMP4), a member of the transforming growth factors (TGF) superfamily, as null BMP4 mouse embryos fail to develop primordial germ cells (Lawson et al., 1999). Newer techniques have shown that Weissmann's theory may fit invertebrates (*Caenorhabditis elegans* and *Drosophila*) and some lower vertebrates (zebra fish and frogs; Wylie, 200) and birds (Tsunekawa et al., 2000), although not mice, and possibly mammals in general (McClaren, 1999; Raz, 2000).

An alternative is that migrating extragonadal primordial germ cells are, in reality, totipotent stem cells, which become tissue specific stem cells after seeding in the gonads (Simkins, 1923). Hence, totipotent stem cells of any origin, if available during adulthood, might develop into germ cells and oocytes within the gonads. Indeed, oogenesis has been demonstrated in cultured mouse embryonic stem cells. Such oogonia entered meiosis, recruited adjacent cells to form follicle-like structures, and later developed into blastocysts (Hubner et al., 2003; Toyooka et al., 2003). Cultured mouse embryonic stem cells have also been reported to differentiate into haploid male gametes capable of fertilizing eggs and developing into blastocysts (Geijsen et al., 2004). Human embryonic stem cells expressing STELLAR and DAZL genes originate from the inner cell-mass cells of blastocysts expressing STELLAR only, and may differentiate either into germ cells expressing the VASA gene or somatic cells (Clark et al., 2004).

In vivo, mouse primordial germ cells are induced to form around the proximal part of the epiblast adjacent to the extraembryonic region, and induction occurs through cellular interactions during gastrulation: they then move through the primitive streak and invade the definitive endoderm, parietal endoderm, and alantois, and are next incorporated into the hindgut pocket, either by a passive or active process. Finally, primordial germ cells emerge from the dorsal side of the hindgut and migrate towards and colonize the developing genital ridges. The primordial germ cells not reaching the genital ridge till a later stage of embryonic development are subjected to apoptosis and die (Raz, 2000; Molyneaux and C. Wylie, 2004). Human primordial germ cells

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expressing VASA and c-kit markers of germ cells are found to migrate into the region of the gonadal ridge between week 6 and 7 of the embryonic age (Castrillon et al., 2000; Hoyer et al., 2005).

After primordial germ cells enter the developing embryonic gonad, they commit to a developmental pathway that will lead them to become either eggs or sperm, depending not on their own sex chromosome constitution but on whether the gonad has begun to develop into an ovary or a testis. Sex chromosomes in the gonadal somatic cells determine which type of the gonad will develop. A single Sry gene on the Y chromosome can redirect a female embryo to become a male (reviewed by Alberts et al., 2002). These observations indicate that mammalian primordial germ cells originate from uncommitted (totipotent) stem cells, and their sex commitment is determined by local gonadal environment – signals produced by neighboring somatic cells.

During adulthood, the ovarian environment is essential, since germ cells must assemble with granulosa cells to form the new primordial follicles needed for oocyte survival, development, and maturation. Although germ cells can utilize vascular transportation for distant destinations (Bukovsky et al., 1995a, 2004), granulosa cells are only available in the ovary. If granulosa cell precursors are eliminated, such as by radiation, germ cells may not have a chance to establish new primordial follicles and rejuvenate the ovaries (Bukovsky and Caudle, 2012).

Summary on the Origin and Fate of Primordial Germ Cells in Mammalian Females

Altogether, two opinions regarding the origin of definitive eggs in ovaries of higher vertebrates emerged. The first view argues that somatic cells are never involved in gametogenesis. Extragonadal primordial germ cells are set apart at an early stage of ontogenesis in the endoderm of the hindgut. They migrate into a developing germ gland, where, after multiplication, they are directly transformed into the definitive oocytes, which persist in the gonads until and during the sexual maturity. This opinion is the prevailing current view.

The second opinion contends that extragonadal primordial germ cells migrate into developing gonads, where they pass through the typical phases of the sex-cell differentiation and degenerate. By so doing, the primordial germ cells somehow stimulate the production of definitive germ cells from somatic cells of the OSC. This is our belief.

Origin of Oocytes in Adult Gonads

In addition to study of the origin of oocytes from extragonadal primordial germ cells or from the OSC somatic stem cells in lower animals, some attention has been directed at the origin of oocytes in ovaries of higher vertebrates, including mammals.

Definitive Oocytes May Emerge Prior To or During Sexual Maturity

In 1917, based on the studies of the white mouse, Kingery found that the definitive oocytes were derived from the OSC prior to sexual maturity. This observation was extended by Edgar Allen and Herbert M. Evans, who concluded their studies in adult

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ovaries of mice and other species as follows: "A cyclical proliferation of the germinal epithelium (OSC) gives rise to a new addition of young ova to the cortex of the adult ovary at each normal oestrous period." (reviewed by Bukovsky, 2007a, p. 235). They defined the "continued formation" of oocytes theory (Allen, 1923) and stated that "New oocytes are formed throughout life, and in phase with the reproductive cycle, from germinal epithelium of the adult mammal, at the same time as vast numbers of already-formed oocytes become eliminated through atresia" (Evans and Swezy, 1931, p. 184).

Definitive Oocytes May Originate From Primordial Germ Cells in Developing Gonads

The current belief that in mammals there is never any increase in the number of oocytes beyond those differentiating during fetal or perinatal ovarian development ("storage" theory) was stated by Pearl and Schoppe (1921). During the 1950s to 1960s this dogma that all primordial follicles in adult mammalian females are formed during the period of ovarian development (Franchi et al., 1962; Baker, 1972) prevailed, primarily because of the diminution of primordial follicle numbers with age reported by Block (1952). However, Erik Block also wrote: "In the age range eighteen to thirty-eight, the relation between the patient's age and the number of primordial follicles cannot be established statistically" (Block, 1952, p. 119). This suggests that during the 20 years of the human female PRP, there is no significant change in the number of primordial follicles. Nonetheless, this important part of Block's conclusions has not been appreciated by his interpreters (Franchi et al., 1962; Baker, 1972).

Meiotic Prophase Arrest

There are other observations contradicting the prevailing "storage doctrine." During embryonic and fetal development, primordial germ cells multiply, and some of them pass into the meiotic prophase, consisting of leptotene, zygotene, pachytene, and diplotene stages, with a characteristic arrangement of chromosomes (Baker, 1972). The principal milestone of the current dogma is the belief that all oocytes in developing ovaries pass into the meiotic prophase and persist in the diplotene stage, preserving chromosomes in a resting stage till adulthood and thereafter (Zuckerman and Baker, 1977). However, in mouse and rat females, meiotically arrested oocytes disappear after postnatal day 3. In the male gonads no fetal meiotic prophase is apparent, but meiosis is initiated at puberty (primary spermatocytes) and progresses without a resting phase (Baker, 1972).

In addition, human fetuses show a peak in oocyte numbers during the sixth month of intrauterine life (7×10 cells), and 5×10 oogonia undergo degeneration until birth. This degeneration affects most oocytes at pachytene, but also at the diplotene stage, especially oocytes in primordial follicles (Baker, 1972). Chromosomes seen in the nuclei of human fetal oocytes are not apparent in adult human ovaries until the initiation of meiosis in Graafian follicles (Baker, 1972).

This suggests that entering the meiotic prophase during fetal development is a characteristic for female but not for male mammalian gametes. An important aspect of this unique meiotic activity of female germ cells appears to be the prevention of mesonephric cell migration and testis cord formation in developing ovaries (Yao et al., 2003). Hence, arrest of some oocytes of developing ovaries in the meiotic prophase appears to

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determine ovarian structure, preventing a tendency toward the development of testicular morphology. Most of the fetal primordial/primary oocytes are not preserved till adulthood, but undergo perinatal demise. Nevertheless, fetal differentiation of oocytes and primordial/primary follicles, which are not functionally required until at least puberty, may play an important role in programming the timespan for existence of periodical follicular renewal (FR) during the PRP in adulthood (Bukovsky, 2006b).

Follicular Pool

The evaluation of the follicular pool in adult mammalian ovaries reveals that 90% are primordial follicles and 10% are follicles in certain stages of development (Zuckerman, 1951), however, 50–70% of primordial follicles are undergoing atresia (Ingram, 1962). It is well known that primordial follicles may persist for a long time, but growing follicles should develop into Graafian follicles and ovulate, or degenerate. Assuming that at least 50% of the total follicular pool persist unaffected, and 10% enter growth phase during each cycle and ovulate or degenerate later, and that follicular development from the primordial follicle toward ovulation is about three cycles (Gougeon, 1996), the follicular pool should be exhausted within about seven cycles. Yet, during the PRP, that is, before the beginning of a significant diminution of primordial follicle numbers in the mouse (Kerr et al., 2006) and human ovaries (Block, 1952), there are about 35 and 300 ovarian ovulatory cycles, respectively.

Clinical Evidence for Follicular Renewal During the Prime Reproductive Period

It is widely believed that all primordial follicles in adult human ovaries originate from the fetal period of life, since their numbers decline in aging ovaries and fetal genetic abnormalities increase with the advancing maternal age. However, during more than 20 years of the PRP, from 18 to 38 ± 2 years of age, genetic abnormalities of the progeny are constantly rare (0.5%). The incidence of fetal trisomy and other genetic alterations begin to increase exponentially within a few years thereafter, that is, 6% in 40–44-year-old mothers and 25% in the 45–49-year-old age group (reviewed by Bukovsky et al., 2004).

We have attempted to unify the storage (lack of follicular renewal) and continued formation (presence of follicular renewal) theories by proposing the "PRP theory" (Bukovsky, 2007a). According to this proposal, the "storage" doctrine fits two periods in humans, that between the termination of fetal formation of new primordial follicles and the advanced puberty or premenarcheal period (about 10–12 years, when the primordial follicles are not renewed), and that following the end of the PRP at 38 ± 2 years of age (termination of follicular renewal), until the menopause. On the other hand, the "continued formation" doctrine accounts for the follicular renewal during the PRP, which ensures the availability of fresh oocytes for healthy progeny. During the PRP, the number of primordial follicles does not show a significant decline (Block, 1952; Kerr et al., 2006) due to the replacement of aging primordial follicles by follicular renewal (Bukovsky et al., 1995a, 2004; Johnson et al., 2004). In adult ovaries, 50–70% of primordial follicles degenerate (Ingram, 1962). Great individual variations in the number of primordial follicles during PRP (Block, 1952) may reflect waves of their

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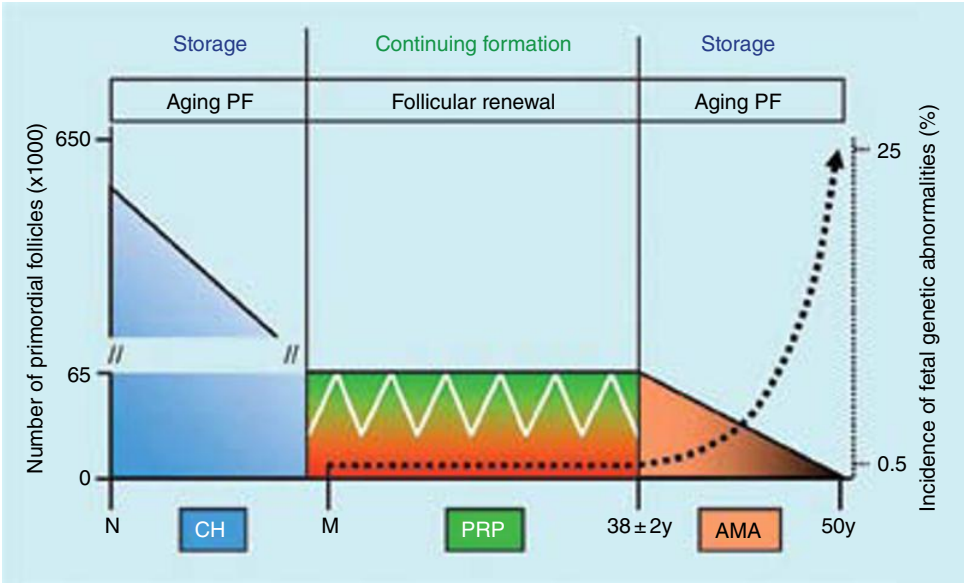


Figure 4.1. The prime reproductive period (PRP) doctrine. The incidence of trisomic fetuses (dotted line) exponentially increases after 38 ± 2 years of age, that is, after the termination of follicular renewal during the PRP. White line indicates fluctuation of primordial follicle numbers due to their cyclic atresia and renewal during the PRP. PF, primordial follicles; N, neonate; CH, childhood; M, menarche; AMA, advanced maternal age. (Adapted from A. Bukovsky and I. Virant-Klun, "Adult stem cells in the human ovary," in Stem Cells in Reproductive Medicine: Basic Science & Therapeutic Potential, ed. C. Simon and A. Pellicer (London: Informa Healthcare, 2007), 53–69. © Informa Healthcare, London, UK.)

atresia followed by waves of follicular renewal. Atresia of primordial follicles declines during the premenopausal period (Gougeon et al., 1994), allowing a reduced number of persisting primordial follicles to remain functional in humans for another 10–12 years beyond the end of follicular renewal. While there are no consequences of oocyte aging for the progeny during childhood, the oocytes persisting after termination of the PRP accumulate genetic alterations, resulting in an exponentially increasing number of fetal trisomies and other genetic abnormalities with advanced maternal age (Figure 4.1; reviewed by Bukovsky et al., 2004).

Summary on the Origin of Oocytes in Adult Gonads

The advantage of the "storage" theory is that it is easy to understand: extragonadal primordial germ cells migrate into developing ovaries, achieve sex-specific properties, multiply, and complete the meiotic prophase of oocytes. These form primordial follicles serving a reproductive function up to menopause in humans. The main disadvantage, however, is the requirement for storage of female gametes for up to several decades prior to utilization for the development of new progeny. Under such conditions, there is a higher probability of accumulation of genetic alterations in

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stored oocytes, possibly due to the long influence of environmental hazards. On the other hand, the "storage" doctrine supports evidence that in invertebrates and lower vertebrates the oogenesis continues throughout reproductive life. The problem here is a lack of advantage of oocyte storage in higher vertebrates from a Darwinian evolutionary theory point of view.

The "continued formation" theory holds that primordial germ cells degenerate and new oocytes originate during adulthood from cyclic proliferation of ovarian germinal epithelium cells (Kingery, 1917; Allen, 1923), and that new oocytes are formed throughout life, in phase with the reproductive cycle, from germinal epithelium of the adult mammal, while vast numbers of previously formed oocytes are eliminated through atresia (Evans and Swezy, 1931). The advantage of the "continued formation" theory is that it suggests that there is a uniform ability of oocyte and follicular renewal in all adult females throughout the animal kingdom. The disadvantage is that it is not easy to discern a distinct pattern of this process among species, for example, apparent formation of new oogonia in adult prosimian primates (Gerard, 1920; Rao, 1928; Zuckerman and Weir, 1977), versus a more cryptic process in adult human females (Bukovsky et al., 1995a, 2004).

The Dogma on the Fixed Pool of Oocytes in Higher Vertebrates

During the first half of the twentieth century, there was disagreement as to whether definitive oocytes are formed before or around the birth (Pearl and Schoppe, 1921), or whether they are renewed in adult animals (Allen, 1923; Evans and Swezy, 1931; Allen and Creadick, 1937; Stein and Allen, 1942), as noted above. The second view prevailed until the 1950s, when the dogma on the fixed pool of oocytes was promoted by Solomon Zuckerman (Zuckerman, 1951; Mandl and Zuckerman, 1951a,b; Green et al., 1951). This currently prevailing dogma indicates that the process of oogenesis in the animal kingdom follows a uniform pattern, with two main variants. One variant is that the oogenesis appears to continue either uninterruptedly or cyclically throughout the reproductive life – for example, most teleosts, all amphibians, most reptiles, and conceivably a few mammals. The other variant is that oogenesis occurs only in the fetal gonads, and oogonia neither persist nor divide mitotically during sexual maturity – for example, cyclostomes, elasmobranchs, a few teleosts, perhaps some reptiles, all birds, monotremes, and with a few possible exceptions, all eutherian mammals (Franchi et al., 1962; Zuckerman and Baker, 1977).

However, Zuckerman (1971) indicated that his conclusion was not based on any experiment demonstrating that mammalian females are incapable of producing new eggs during adulthood, and Mossman and Duke (1973a,b) emphasized that misconceptions of the general nature of mammalian ovaries were widespread and proposed that the occurrence of neo-oogenesis in adult mammals should be seriously investigated.

We had raised questions about the origin of "definitive" oocytes in the adult mammalian ovaries (Bukovsky and Presl, 1977), but until the mid-1990s there were no new developments. In a recent article in Science–Business eXchange (SciBX) of the Nature Publishing Group, the Senior Editor Tracey Baas (2012, p. 4) noted:

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"Up until the 1990s, the central dogma of reproductive biology was that female mammals have a restricted capacity for generating oocytes before birth, and once born the ovaries cannot renew egg cells that die because of aging or disease. Consequently, infertility resulting from oocyte loss had been considered irreversible.

However, multiple papers now cast doubt on that belief through the identification of a population of stem cells that give rise to functional oocytes.

First, Antonin Bukovsky and colleagues at The University of Tennessee Knoxville published in the American Journal of Reproductive Immunology in 1995 that a subpopulation of human germline stem cells, now known as oogonial stem cells (OSCs), could be collected from the ovaries of women undergoing surgery and used to generate what were perceived as oocytes in cell culture, based on detection of oocyte markers [Bukovsky et al., 1995a, 2004, 2005b].

Almost a decade later, Jonathan Tilly and colleagues at Massachusetts General Hospital (MGH) and Harvard Medical School (HMS) produced multiple datasets that ran counter to the belief that germline stem cells disappear from ovaries at birth [Johnson et al., 2004]."

The Immune System Role in Regulation of Ovarian Function

Until the late 1970s, regulation of ovarian function was considered limited to interactions between the hypothalamo-pituitary axis and the ovary. Such concepts, however, did not explain a number of experimental data in rats and mice, such as ovarian "dysgenesis" after neonatal thymectomy (Nishizuka and Sakakura, 1969, 1971; Sakakura and Nishizuka, 1972a,b), prevention of steroid-induced sterility in neonatal rats by thymocytes from fertile females (Kincl et al., 1965), and superovulation after cyclophosphamide (Russell et al., 1973) and X-ray treatment (Hahn and Morales, 1964).

Our observations indicated that intraperitoneal treatment of adult rats with anti-thymocyte serum causes anovulation with the persistence of corpora lutea (CL) and a diestrus (Bukovsky et al., 1976). We also found a migration of immune-system-related cells among granulosa cells of atretic rat follicles (Bukovsky et al., 1978a, 1979a), and delayed ovarian maturation and a shortened reproductive period in nude mice with congenital absence of the thymus (Bukovsky et al., 1978b).

In 1979, we proposed a role for the immune system in ovarian function Bukovsky and Presl, 1979), as recently indicated by Joy Pate et al. (2010). Ovarian structures present during the fetal adaptive period of immune system development, such as primordial follicles, are tolerated by the immune system during adulthood, while those structures which are absent, such as antral follicles and CL, have a limited functional life unless pregnancy occurs. Under normal conditions, the antral follicles and CL should be cyclically destroyed in order to maintain the cyclic character of ovarian function. A lack of atresia of aged antral follicles results in their persistence and the polycystic ovary syndrome. In species with cyclic ovarian function, the length of the ovarian cycle (about 4 days in the rats and mice and 28 days in humans) is determined by the length of the immune cycle. We also proposed that age-dependent impairment of ovarian function is caused by a conceding age-dependent impairment of the

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immune system (Bukovsky and Presl, 1979). Actually, impairment of immune system function with age precedes that of the ovary.

In 1980, Espey hypothesized that ovulation of ovarian follicles involves an inflammatory reaction. The connective tissue layers of the ovarian tunica albuginea (TA) and theca externa must be weakened to allow the follicle wall to dissociate and break open under modest intrafollicular pressure. Such changes may be caused by thecal fibroblasts transformed into proliferating cells, similar to tissue responses to inflammatory reactions (Espey, 1994).

The Tissue Control System Theory

Our studies in the late 1970s (Bukovsky et al., 1977,1978b, 1979b; Bukovsky and Presl, 1979) and early 1980s (Bukovsky et al., 1981, 1982) resulted in a proposal for a wider role of the immune system (immune cells and vascular pericytes), the so-called tissue control system (TCS), in regulation of ovarian function (Bukovsky et al., 1983). The TCS theory was further refined when the role of autonomic innervation in the regulation of "quantitative aspects in tissues," including follicular selection, was added (Bukovsky et al., 1991, 1995b, 1997; Bukovsky and Caudle, 2002). More recently, a role for the immune system in the regulation of ovarian aging and the regulation of asymmetric cell division has been described (Bukovsky et al., 2000a, 2002; Bukovsky, 2007b). In addition, we proposed a role of the TCS in immune maintenance of the self-antigens, related to tissue morphostasis, tumor growth, and regenerative medicine. For more detailed information about the TCS components and their functions see (Bukovsky et al., 2008a,b, 2009a,b; Bukovsky, 2011b; Bukovsky and Caudle, 2012).

Immune System and In Vivo Regulation of Ovarian Function

Ovarian compartments are among those structures showing the most pronounced morphological (cellular proliferation, differentiation, and regression) and functional changes in the body. Regulation of ovarian function is complex, involving interactions between follicular compartments (oocyte, granulosa, and theca cells), as well as of sex steroids produced by follicles, CL, and interstitial tissues originating from theca of degenerating follicles. Additionally, communication of the hypothalamo-pituitary system and the influence of gonadotropins, autonomic innervation, growth factors, and cytokines produced by mesenchymal cells of the immune system all regulate functions of ovarian compartments. While gonadotropins are essential for follicular maturation and ovulation (Hillier et al., 1980), autonomic innervation is necessary for the regulation of follicular selection (Dominguez et al., 1981; Nakamura et al., 1992). Interactions between the immune system and ovary are numerous, as immune cells are associated with regulation at every level of the hypothalamo-pituitary–ovarian axis, including growth and regression of both follicles and CL (Vinatier et al., 1995; Chryssikopoulos, 1997).

Ovarian ovulatory function during adulthood requires the presence of oocytes and granulosa cells, the origin of which and the mechanisms required for their appearance in higher vertebrates remained completely enigmatic until 1995 (see above and below).

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Ovarian Stem Cells Contribute to Follicular Renewal in Adult Human Ovaries

First Evidence of Follicular Renewal from OSC in Adult Human Ovaries

For follicular renewal to exist, newly developed oocytes originating from germ cells must associate with uncommited primitive granulosa cells, giving rise to new primordial follicles. Morphological similarities among OSC-derived ovarian epithelial cords, granulosa cells, and interstitial glands led to speculation that the OSC may serve as a source of several different somatic cells in the mature ovary (Motta et al., 1980).

Bukovsky et al. (1995a) reported that in normal adult human ovaries the OSC-derived cytokeratin (CK) positive epithelial cords fragment into small epithelial nests migrating into the lower ovarian cortex (close to the medulla), where they accompany primordial follicles. Some of such nests in the lower cortex exhibited a crescent shape and contained CK negative oocyte-like structures.

Investigation of the major histocompatibility class I (MHC-I) antigens revealed its expression in the OSC, which, however, contained unstained germ-like cells. Further observations have shown that the OSC contained primitive monocyte-derived cells (MDCs) expressing CD14 interacting with epithelial cells (arrows, Figure 4.2A). Some of such interactions resulted in an asymmetric division (arrowheads) giving rise to the smaller OSC daughter (white asterisk) and larger daughter resembling a germ cell (black asterisk), which was also accompanied by the CD8 T lymphocyte (Figure 4.2B).

Primitive MDCs (m, Figure 4.2C) were also associated with symmetric division of germ cells (white arrowheads) in TA and with germ cells entering (black arrowhead) the upper cortex (ct). Activated MDCs (HLA-DR+) were associated (arrow, Figure 4.2D) with migrating germ cells showing HLA-DR expression in the perinuclear envelope (arrowhead), and these cells associated (arrow, Figure 4.2E) with the cortical vasculature (cv) expressing MHC-I, and utilized (arrowhead Figure 4.2 F) vascular transportation expressing Thy-1 glycoprotein of pericytes (arrow) to reach distant destinations.

We concluded that primordial follicle renewal may occur in adult human ovaries. This process may be restricted to the small portions of the ovary and it may be dependent on a precise interplay of morphoregulatory molecules, immune-system-related cells, and other factors. Age-induced depletion of such factors may account for the dramatic decrease in the number of primordial follicles in premenopausal (climacteric) ovaries and their lack in postmenopausal women (Bukovsky et al., 1995a).

Origin of New Granulosa and Germ Cells from Bipotent OSC

Follicular renewal in adult human ovaries from bipotent OSC, that is, precursors of germ and granulosa cells, is a two-step process, consisting of the new formation of epithelial cell nests (primitive granulosa cells) and the formation of new germ cells (Bukovsky et al., 2004).

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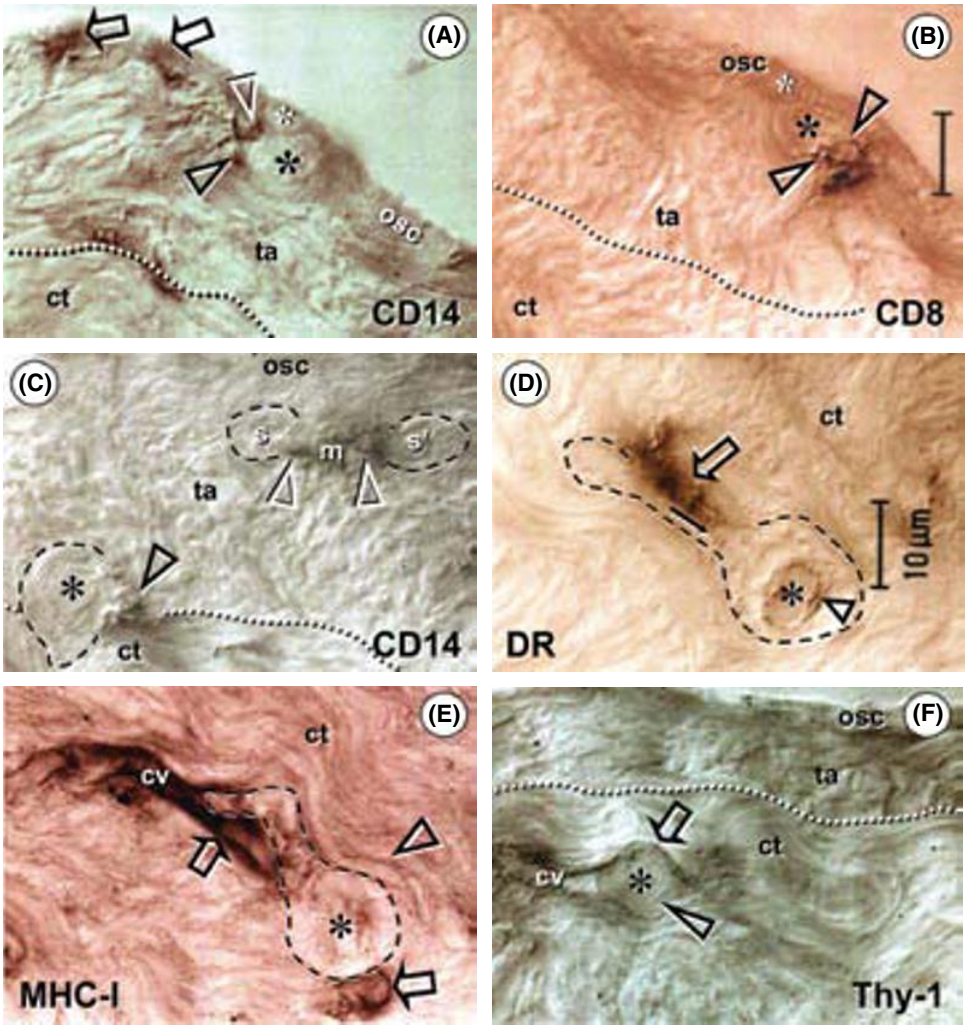


Figure 4.2. Immune type cells influence commitment of ovarian stem cells (OSC). Staining of the adult human OSC (osc), tunica albuginea (ta), and an adjacent cortex (ct) for CD14 of primitive MDCs and HLA-DR of activated MDCs, CD8 of cytotoxic/suppressor T cells, MHC-I heavy chain, and Thy-1 glycoprotein of pericytes, as indicated in panels. Large asterisks and dashed lines indicate putative germ cells. **(A)** Primitive MDCs associate with OSC (arrows) and accompany (arrowheads) origination of germ cells by asymmetric division of OSC (asterisks). **(B)** Asymmetric division is also accompanied by extensions from a T cell (arrowheads) into a putative germ cell daughter. **(C)** Primitive MDCs accompany (white arrowheads) symmetric division (s-s′) of germ cells in tunica albuginea and their migration into the adjacent cortex (ct). **(D)** Migrating tadpole-like germ cells are accompanied by activated MDCs (open arrow), and HLA-DR material is apparent in the cytoplasm (solid arrow) and in the nuclear envelope (arrowhead). **(E)** The germ cells associate with cortical vasculature (cv) strongly expressing MHC-I (arrows vs. arrowhead), enter and are transported by the bloodstream **(F)**. (Adapted from Bukovsky et al., 1995a with permission, © Blackwell Publishing, Oxford, UK.)

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Source of Bipotent OSC in Adult Human Ovaries

Bipotent OSC are present in human fetal ovaries (see below), but they are converted by epithelial–mesenchymal transition into ovarian TA by the end of pregnancy (reviewed by Bukovsky et al., 2004). The TA is a thick fibrous subepithelial layer of loose connective-tissue cells. It does not begin to form until the end of intrauterine life (Motta and Makabe, 1986) or several months after the birth (Simkins, 1932).

Origin of New Granulosa Cell Nests

The initiation of granulosa cell nest formation consists of an extension of TA (ta, Figure 4.3A) over the ovarian surface, the so called TA flap (taf arrowhead): F29 indicates the age of the female ovaries that were studied. This TA flap contains CK + fibroblast-like cells (+fb, Figure 4.3B, detail of TA flap from 4.3A), which are converted into OSC precursors (fb/osc) and OSC (rounded arrow and osc). For the emergence of granulosa cells, this process is associated with numerous DR + MDCs (asterisks, Figure 4.3C). Early OSC show weak DR expression (arrow). The inset in Figure 4.3A shows formation of the bilaminar OSC channel (osc-ch). The channel collapses into a bilaminar OSC cord (white arrow, Figure 4.3A) and is overgrown by CK unstained TA (white arrowhead). Detail of CK + OSC cord adjacent to the ovarian cortex (ovc) is shown in Figure 4.3D. Dashed line in Figure 4.3A indicates the TA and ovarian cortex interface – note that the formation of the OSC cord begins on the ovarian cortex lacking the TA.

An ovary from another female in the PRP (F28) shows (Figure 4.3E) an upper ovarian cortex (uc) with fragmentation of OSC cords (black arrows) into primitive granulosa cell nests (arrowheads). Figure 4.3F shows the lower ovarian cortex (lc, close to the ovarian medulla – 1000 μm from the ovarian surface) containing primordial follicles. The dashed box indicates a primordial follicle shown in the inset. New primitive granulosa cell nests move to this level during each periovulatory phase in the PRP and associate with intravascular or migrating germ cells to form new primordial follicles (see below).

Origin of New Germ Cells and Follicular Renewal

For the formation of secondary germ cells in adult human ovaries during the PRP in the periovulatory phase, a single layer of OSC is formed at the ovarian surface from CK + TA fibroblasts (Figure 4.4A). The granulosa cell nests (n, Figure 4.4B) associate with ovarian vessels (v) lined by endothelial cells (e) in the lower ovarian cortex to catch the circulating oocytes (o) from the blood. During follicle formation, extensions of granulosa cells penetrate the ooplasm (arrowheads, Figure 4.4C) like a sword in the shield to form a single paranuclear Balbiani body (asterisk). It contains additional organelles which the oocyte needs to develop later into the mature egg (Motta et al., 1994). Formation of adult primordial follicles is verified by double color staining for CK of the nest cells (CK brown color, Figure 4.4D) and the zona pellucida (ZP blue color) glycoprotein staining of assembling oocytes (Bukovsky et al., 2004).

Germ cells originate by asymmetric division of OSC differentiating above the TA (Figure 4.4E). Germ cells showing PS1 meiotically expressed oocyte carbohydrate

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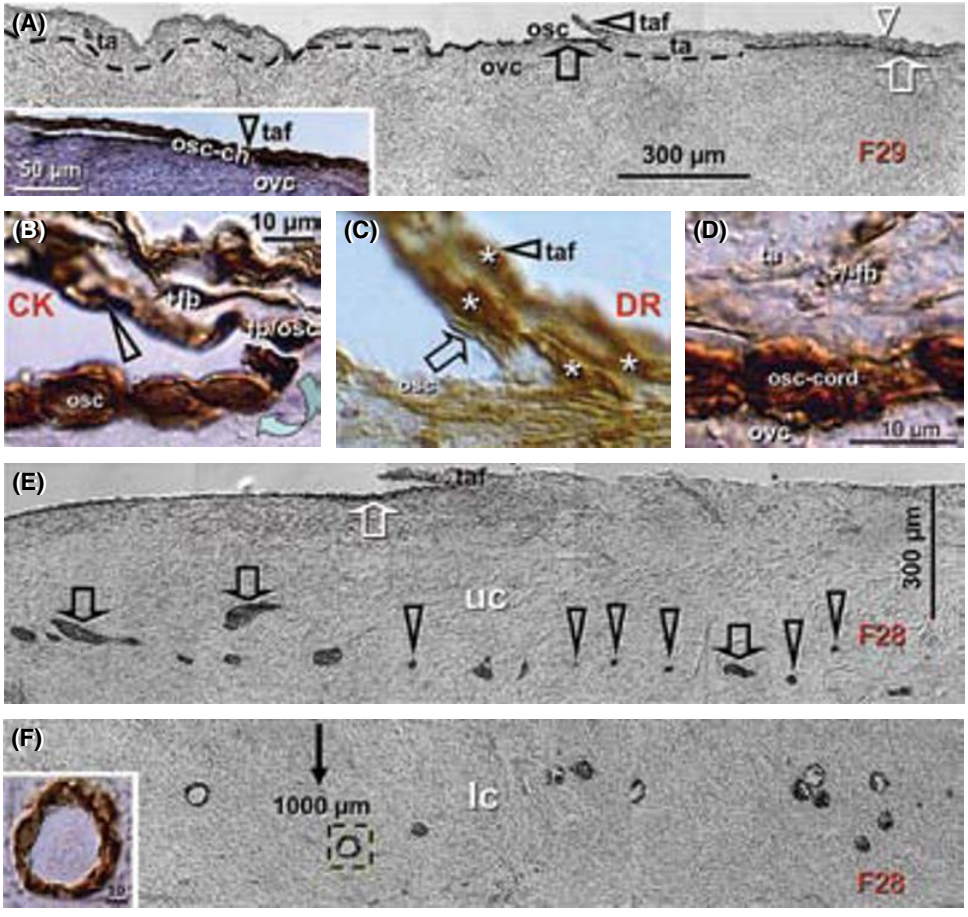


Figure 4.3. Origin of new granulosa cells from OSC during the PRP in adult human ovaries. **(A)** Panoramic view of ovarian surface and adjacent cortex. Dashed line indicates interface between TA and stroma of the ovarian cortex: osc and black arrow – ovarian stem cells; taf and black arrowhead – TA flap; white arrowhead – a lack of OSC above the TA; white arrow – bilaminar epithelial cord. **(B)** Detail from (A) shows association of CK + (brown color) fibroblasts (+fb,) with the TA flap surface (arrowhead), transition from mesenchymal to epithelial morphology (fb/se), and ovarian stem cells (osc, arched arrow). **(C)** A parallel section to (B) showing numerous DR + MDCs (asterisks) in the TA flap. Note DR expression also in early OSC (arrow). **(D)** Detail from (A) shows CK + epithelial cord consisting of two layers of epithelial cells and lying between the ovarian cortex (ovc) and TA (ta). Note diminution of CK immunoexpression in TA fibroblasts (+/-fb). **(E)** Epithelial cords (black arrows) fragmenting into granulosa cell nests (arrowheads in the upper ovarian cortex (uc). White arrow CK + OSC associated with the TA with flap. **(F)** Lower ovarian cortex (lc) with primordial follicles. Arrow indicates distance from the ovarian surface, dashed box indicates follicle shown in the inset. F29 indicates female age in years. Bar in (D), for (B–D). (Panels A, B, and D–F adapted from Bukovsky et al., 2004 © Antonin Bukovsky; panel C from Bukovsky, 2006a with permission, © Wiley-Liss, Inc.)

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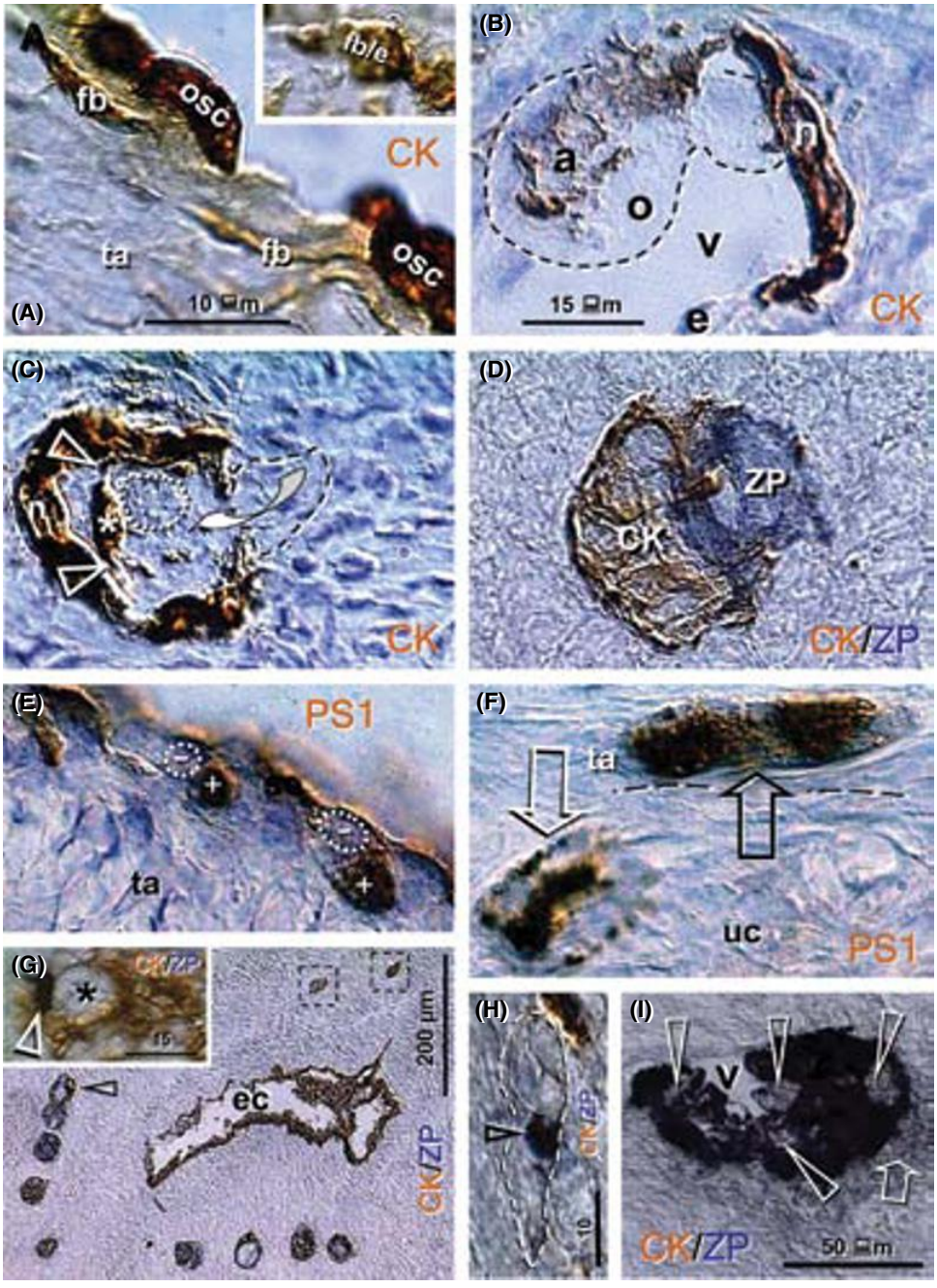


Figure 4.4. Follicular renewal in adult human ovaries. **(A)** Cytokeratin (CK) positive (brown color) cells of fibroblast type (fb) in tunica albuginea (ta) exhibit mesenchymal–epithelial transition into OSC (osc). Inset shows a transitory stage (fb/e). **(B)** The CK + epithelial nest (n) inside of the venule (v) in lower ovarian cortex, which extends an arm (a) to catch the oocyte (o, dashed line) from the blood circulation: e – endothelial cell. **(C)** The nest body (n) and closing "gate." A portion of the oocyte (dashed line) still lies outside the complex, and is expected to move inside (arched arrow). The oocyte contains intraooplasmic CK + extensions from the nest

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antigen [(+) vs. (−) sign] enter the TA, where they undergo a symmetric division (black arrow, Figure 4.4F) required for crossing over, and then enter (white arrow) the adjacent upper cortex (uc) and cortical vessels. During vascular transport the germ cells increase in size, and are picked up by epithelial nests (Figure 4.4B).

An alternative source of germ cells in human ovaries is from OSC crypts originating from OSC invaginations into the lower cortex. Such epithelial crypts (ec, Figure 4.4G) show transformation of OSC into ZP+(arrowhead in inset, Figure 4.4G) germ cells (asterisk), capable of saturating neighboring nests of primitive granulosa cells (dashed boxes, Figure 4.4G) to form new primordial follicles (arrowhead). Hence, if OSC are not available at the ovarian surface (or are destroyed) the cortical epithelial crypts are an alternative source of germ cells. For adult primordial follicles, however, nests of primitive granulosa cells should be available. When the epithelial nests are not available locally, the ZP+tadpole-like germ cells resembling sperm (Figure 4.4H) migrate to reach cortical vessels, using vascular transport to reach distant targets (Figure 4.4B). Finally, intravascular oocytes not utilized in the formation of adult primordial follicles due to the lack of granulosa cell nests, degenerate (arrowheads, Figure 4.4I) in the medullary vessels (v).

Localization of SCP3 in Adult Human and Monkey Functional Ovaries During the Ovarian Cycle

Liu et al. (2007) compared the expression of meiotic entry synaptonemal complex protein-3 (SCP3) in fetal and in functionally undefined adult human ovaries. The authors maintained that SCP3 protein was not detectable in the TA, OSC, or in oocytes of adult primordial follicles in adult human ovaries, and concluded that no meiotic oocytes are present in ovaries during adulthood. In a subsequent commentary, Tilly and Johnson (2007) stated that the lack of evidence in this study of neo-oogenesis in adult human females was not convincing, and that some data of Liu et al. (2007) actually supported the existence of neo-oogenesis in adult women.

We studied SCP3 expression using the same SCP3 antibody, and found immunoreactivity with segments of TA, OSC, and in oocytes of one-third of adult primordial follicles in functional mid-cycle adult human and monkey ovaries

Figure 4.4. (*Continued*) wall (arrowheads), which contribute to the formation of CK+paranuclear (Balbiani) body (asterisk). The oocyte nucleus is indicated by a dotted line. **(D)** The occupied "bird's" nest type indicates a half-way oocyte–nest assembly. CK indicates cytokeratin staining of primitive granulosa cells and ZP indicates zona pellucida expression in the assembling oocyte. **(E)** Segments of OSC show cytoplasmic PS1 (meiotically expressed carbohydrate) expression. Asymmetric division of OSC gives rise to cells exhibiting nuclear PS1 (+nuclei vs. − cell daughters) and descending from the OSC into tunica albuginea (ta). **(F)** In tunica albuginea, the putative germ cells increase in size, show a symmetric division (black arrow) and exhibit development of cytoplasmic PS1 immunoexpression when entering (white arrow) the upper ovarian cortex (uc). **(G)** Association of new primordial follicles (arrowhead) with the cortical epithelial crypt (ec). Dashed boxes indicate unassembled epithelial nests. Inset shows origination of germ-like cells among CK+cells (CK) in epithelial crypt. Note ZP+segment (white arrowhead) associated with unstained round cell nucleus (asterisk). **(H)** Migrating germ cells with tadpole shape (dashed line), unstained nucleus (dotted line) and ZP+staining of the intermediate segment (arrowhead). **(I)** Some medullary vessels (v) show accumulation of ZP+(dark color) degenerating oocytes with unstained nuclei (arrowheads). Arrow indicates ZP release. (Adapted from Bukovsky et al., 2004 © Antonin Bukovsky.)

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(Bukovsky et al., 2008c). Functional ovaries without evidence of ongoing follicular renewal (mid-follicular, mid- and late luteal phases) indeed lacked staining for SCP3 in TA, OSC, and in adult primordial follicles.

Functional ovaries with ongoing follicular renewal, that is, with unoccupied nests of primitive granulosa cells (often occupying a portion of the vascular lumen), oocyte/nest assemblies, and degenerating superfluous oocytes in ovarian medullary vessels (see Figure 4.4I), were observed during the postovulatory (early luteal) phase of the menstrual cycle. In these ovaries a strong SCP3 expression was shown in some segments of TA. Such TA cells exhibited a mesenchymal pattern, characteristic of the OSC precursors. The differentiated OSC layer also showed SCP3 immunostaining. Synaptonemal complex protein-3 immunoexpression was also evident in differentiated OSC of postovulatory monkey ovaries.

Moreover, the SCP3 immunostaining was observed in the nucleoli of oocytes in some adult primordial follicles in human and monkey ovaries. Laura L. Tres (2005) has reported that male germ cells exhibit nucleolar SCP3 expression during early stages of meiotic prophase. In addition, SCP3+ synapsis of two chromosomes was detected in human primordial follicle oocytes, possibly representing XX chromosomal synapsis, since sex chromosomes start synapsis during early zygotene, before autosomes synapse. Rare SCP3+ oocytes (less than 10%) were detected in the mid-follicular phase ovaries. The most frequent expression (10–30% of primordial follicle oocytes) was found in postovulatory ovaries during the early luteal phase in younger (till 38 years of age) women. However, by age 42, postovulatory ovaries showed no SCP3 expression. No staining of oocytes was observed in three younger women studied during the mid- and late luteal phases and in polycystic ovary syndrome (PCOS) ovaries (Bukovsky et al., 2008c). Synaptonemal complex protein-3 expression was also reported in juvenile and adult mouse ovaries (Zou et al., 2009).

These observations indicate that SCP3 is expressed in adult human, monkey, and mouse ovaries. The SCP3 expression detected in TA stem cells indicates that preparation for meiotic activity may occur at the level of TA OSC progenitors, and meiotic prophase activity may continue and terminate in the oocytes of newly formed adult primordial follicles. Synaptonemal complex protein-3 expression in about 30% of primordial human follicles during follicular renewal indicates that in each menstrual cycle about 30% of adult primordial follicles are replaced. These data also confirm that follicular renewal occurs during the PRP, but not thereafter, and is not present in PCOS ovaries.

Summary on Follicular Renewal in Adult Human Ovaries

The origin of new epithelial nests and germ cells and their assembly in adult human ovaries is schematically depicted in Figure 4.5. Under the influence of cellular and other local signaling (CS & LS, Figure 4.5A), including immune-system-related activated MDCs (Bukovsky, 2006a) and neural signals and molecules (Bukovsky et al., 1995a), the TA overgrows the upper ovarian cortex (uc, Figure 4.5A) and its mesenchymal cells attain CK expression and transform into OSC. In this way, the bilaminar OSC layer is formed, which descends into the cortex (arrow), and fragments into epithelial nests (en) of primitive granulosa cells. The epithelial nests move through stromal rearrangements into the lower cortex (lc) (Bukovsky et al., 2004), where they associate with cortical vessels (venules) to pick up circulating oocytes.

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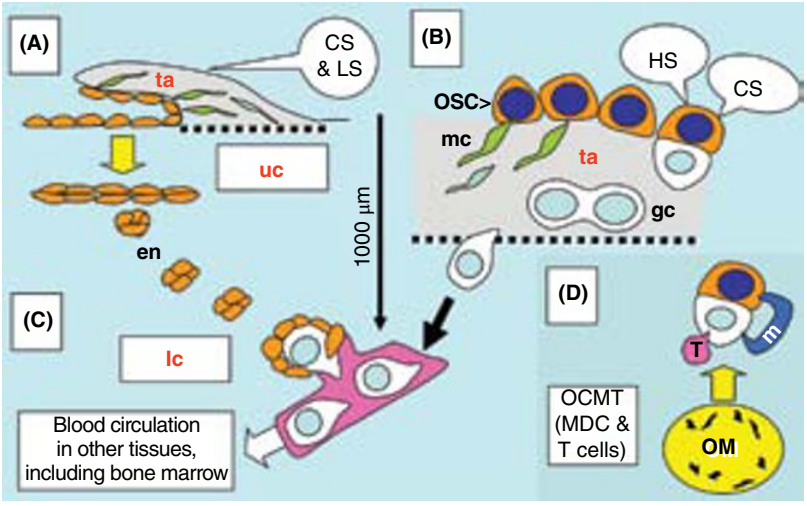


Figure 4.5. Survey of follicular renewal in adult human ovaries. Follicular renewal in adult human ovaries is a two-step process based on mesenchymal–epithelial transition of tunica albuginea (ta) bipotent progenitor cells into OSC. **(A)** Epithelial nests: segments of the OSC directly associated with the upper ovarian cortex (uc) are overgrown with tunica albuginea, which forms a solid epithelial cord that fragments into small epithelial nests (en) descending into the lower ovarian cortex (lc) and associating with the blood vasculature. Initiation of this process may require cellular and other local signaling (CS & LS), possibly neural (Bukovsky et al., 1995a). **(B)** Germ cells: under the influence of cellular signaling (CS) of ovary-committed MDCs & T cells (OC-BMCs; bone marrow cells) and hormonal signaling (HS), some OSC covering the tunica albuginea undergo asymmetric division and give rise to new germ cells (gc). The germ cells subsequently divide symmetrically and enter adjacent cortical blood vessels. During vascular transport they are picked up by epithelial nests associated with vessels. **(D)** The ovary-committed BMCs originate from BM (MDCs) and from lymphoid tissues (T cells) carrying "ovarian" memory (om), which diminishes with utilization; when spent, the follicular renewal ceases, in spite of persisting hormonal signaling (Table 4.1). (Reprinted from Bukovsky and Caudle, 2012, © Antonin Bukovsky.)

Under the influence of hormones and cellular signaling (HS, CS, Figure 4.5B) of ovary-committed bone marrow (BM) cells (arrow, Figure 4.5D) derived from immune-system-related structures presumed to carry an "ovarian memory" (om; Bukovsky et al., 2006b), the mesenchymal OSC precursors (mc, Figure 4.5B) differentiate into the OSC covering not the cortex, as above, but the TA layer (ta, Figure 4.5B), and produce germ cells (gc) by asymmetric division (Bukovsky et al., 2004). This is followed by a single symmetric division of germ cells required for crossing over. Subsequently, the germ cells enter the upper cortex, associate with blood vessels, and enter the circulation (arrow, Figure 4.5B). They assemble with epithelial nests of primitive granulosa cells in the lower cortex (lc, Figure 4.5C). The circulating germ cells may contaminate other tissues, including BM, and eventually degenerate in the ovarian medullary vasculature (Figure 4.5I), to which they appear to have an affinity for homing. For a schematic description of an alternative origin of germ cells from epithelial crypts in adult ovaries see Figure 4.4G and (Bukovsky et al., 2004).

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Human Prenatal Oogenesis

After studying follicular renewal in adult human ovaries (Bukovsky et al., 1995a, 2004), we were interested in the role of primordial germ cells and whether or not OSC also contribute to the formation of germ and granulosa cells in human fetal ovaries (Bukovsky et al., 2005a).

Primordial Germ Cells

It is now well known that mammalian primordial germ cells originate from uncommitted (totipotent) somatic stem cells, known as embryonic stem cells (ESCs) in the inner cell mass of the blastocyst expressing STELLAR and deleted azoospermia-like (DAZL) proteins of human germ cells, oocytes, and ESCs (Clark et al., 2004; Cauffman et al., 2005). The sex commitment of germ cells is determined by the local gonadal environment – signals produced by neighboring somatic cells (Alberts et al., 2002). Studies of mouse embryos, in which genetically marked cells were introduced at the 4- and 8-cell stage blastomere, have shown that such cells can either become germ or somatic cells (Kelly, 1977). This suggests that no specific germ cell commitment exists prior to implantation. During the postimplantation period, mouse germ cells are not identifiable before 7 days after fertilization (Ginsberg et al., 1990), suggesting that germ cells differentiate from somatic lineage (Lawson and Hage, 1994). It has also been shown that cellular differentiation of grafted embryonic cells does not depend on where the grafts were taken, but rather where they are placed (Tam and Zhou, 1996).

As indicated above, after primordial germ cells enter the developing embryonic gonad, they commit to a developmental pathway that will lead them to become either eggs or sperm, depending not on their own sex chromosome constitution but on whether the gonad has begun to develop into an ovary or a testis. The sex chromosomes in the gonadal somatic cells determine which type of the gonad will develop, as a single Sry gene on the Y chromosome can redirect a female embryo to become a male (reviewed by Alberts et al., 2002).

Human Embryonic Ovaries

Ovarian differentiation begins before follicles form. It is characterized by the evolution of the OSC from coelomic (peritoneal) mesothelium in the region of the gonadal ridge, organization of the rete ovarii developing from mesonephric ducts, and the development of oocytes from germ cells. In human embryos, primordial germ cells arise outside the urogenital ridge, in the dorsal endoderm of the yolk sac at 24 days of embryonic age. They migrate by amoeboid movements into indifferent gonadal primordial tissue at 28–35 days (Peters and McNatty, 1980). After reaching the urogenital ridge, the primordial germ cells express VASA, a protein required for germ cell maintenance and function, and initially accumulate among OSC of the developing gonads (Castrillon et al., 2000). Differentiation of an indifferent gonad into an ovary or a testis takes place during the second month (Simkins, 1932). At the age of 9 weeks, female gonads show a marked development of rete cords with lumen formation. The rete reaches the center of the ovary at 12 weeks, when meiosis of oocytes begins. The nuclei of the germ cells lie

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close together in clusters without clearly defined cell membranes. These syncytia are surrounded by slender stromal (mesenchymal) cells (Peters and McNatty, 1980).

Human Fetal Ovaries

Developing fetal ovaries are filled with numerous germ cells and maturing oocytes expressing VASA at 15 weeks of age, but at the same age the developing testes contain only scattered VASA positive germ cells in seminiferous tubules (Castrillon et al., 2000). Numerous germ cells 10 μm in diameter are present in human fetal OSC, and they often exhibit a tadpole-like shape, suggesting their ability to migrate (Motta and Makabe, 1982; 1986).

The first primordial follicles are formed in the human fetus after month 4. This is substantially later than the early embryonic occurrence of primordial germ cells. The delayed appearance of primordial follicles may be due to a requirement for activated MDCs with formation of primitive granulosa cells from bipotent OSC, and also the association of activated MDCs with formation of primordial follicles (see below). Activated MDCs are detected in second trimester human fetuses (Olweus et al., 1997).

Follicle formation always begins in the innermost part of the cortex, close to the rete ovarii, which is essential for follicular development. Follicles will not form if the rete ovarii is removed before formation of primordial follicles has started (Byskov et al., 1977). Formation of the follicle requires attachment of granulosa cells to the oocyte surface and closure of the basement membrane around this unit. At 5 months of fetal age the population of oocytes in the ovary peaks. In human fetal ovaries at the age 5.5 months, newly differentiated oogonia are found to lie within and just below the OSC (Van Wagenen and Simpson, 1965, plate 17C). At 7 months of intrauterine life the last oogonia enter meiosis (reviewed by Peters and McNatty, 1980).

Origin of Secondary Germ Cells and Granulosa Cells from Fetal OSC

The OSC have been implicated in the formation of oocytes in mice and humans (Allen et al., 1923; Evans and Swezy, 1931; Simkins, 1928, 1932; Everett, 1943; Bukovsky et al., 1995a, 2004, 2005a), and it also has been suggested that the OSC are a source of granulosa cells in fetal and adult mammalian ovaries (Brambell, 1927; Motta et al., 1980; Motta and Makabe, 1982; Bukovsky et al., 1995a, 2004; Sawyer et al., 2002). The formation of germ cells from OSC is, however, a selective process. In a given time, only some OSC are transformed into germ cells (Bukovsky et al., 1995a).

Figures 4.6–4.8 show morphological and immunohistochemical observations in mid-pregnancy human fetal ovaries. The germ cells within OSC are smaller (white arrowheads, Figure 4.6A; Papanicolaou (PAP) staining) when compared to oocytes deeper in the cortex (black arrowhead). Oocytes with well-defined cytoplasm (black arrowhead) lie among smaller cells with round or elongated nuclei (black arrow). Beneath the well-defined germ cells lies a nuclear cluster (nc) or syncytium of germ cells, and the entire area is surrounded by mesenchymal cell cords (mcc), that is, extension of the rete cords into the cortex.

Figure 4.6B–E shows fetal primordial follicles and associated mesenchymal cells. In the innermost part of the cortex, follicles (asterisk, Figure 4.6B) develop in close vicinity to the microvasculature (v). Note the strong major histocompatibility heavy chain class I antigens (MHC-I) expression by endothelial cells, and also the moderate expression by granulosa cells. Figure 4.6C shows secretion of Thy-1+ intercellular vesicles

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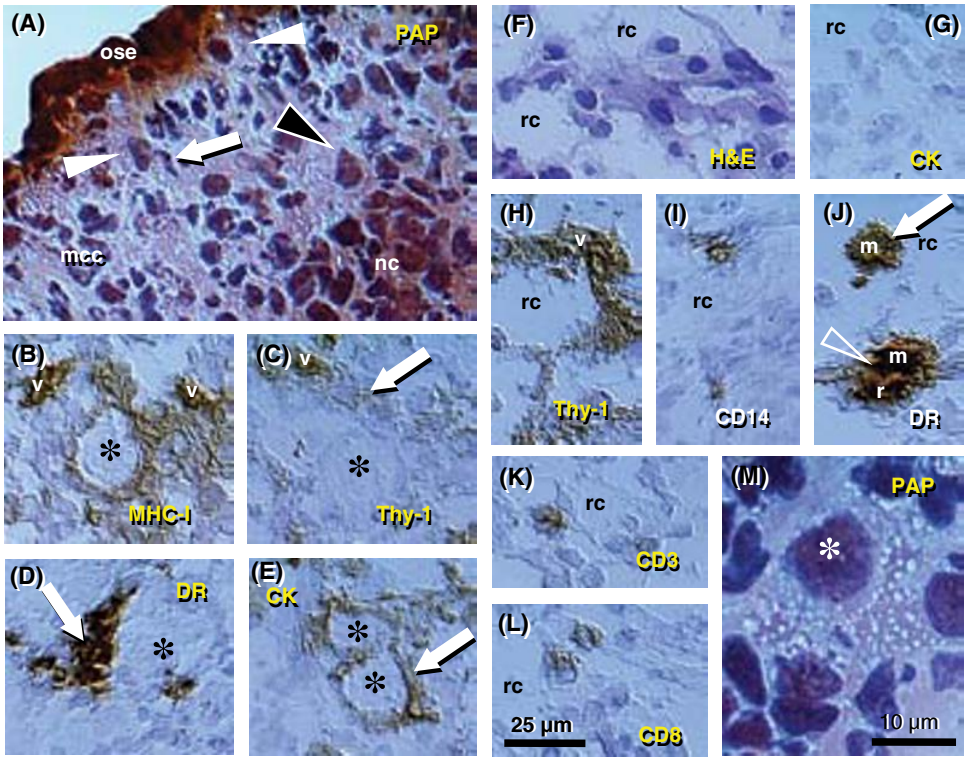


Figure 4.6. The human fetal ovary (24 weeks). Papanicolaou (PAP) staining and immunohistochemistry as indicated in panels. **(A)** Secondary germ cells descend (white arrowheads) from the OSC between mesenchymal cell cords (mcc), enlarge within the cortex (black arrowhead) above the nuclear cluster (nc) or syncytium of germ cells. The arrow indicates a mesenchymal type cell. Expression of MHC-I **(B)**, Thy-1 **(C)**, HLA-DR of activated MDCs **(D)**, and cytokeratin (CK) accompany primordial and primary follicles (asterisks) and vessels (v). **(F)** hematoxylin and eosin (H&E) staining of rete ovarii showing rete channels (rc). The rete shows no CK expression **(G)** but shows high Thy-1 staining **(H)**. The presence is indicated of CD14+ primitive MDCs **(I)**, DR + MDCs **(J)** and CD3 **(K)**, and CD8 T cells **(L)**. **(M)** Numerous oocytes exhibit degenerative changes (vacuolization). A scale bar in (L) for panels A-L. See text for details. (Adapted in part from Bukovsky et al., 2005a with permission, © Humana Press.)

(arrow) from vascular pericytes (v) to the fetal primordial follicle (asterisk). An arrow in Figure 4.6D demonstrates the large activated MDCs (HLA-DR$^+$) associated with a growing primary follicle (asterisk). Figure 4.6E shows moderate CK expression by granulosa cells (arrow).

Rete Ovarii Channels Contain Immune System-Related Cells

Figure 4.6F–L shows the rete at the center of the fetal ovary. Staining with hematoxylin and eosin (Figure 4.6F) shows the loose character of the rete, containing spacious lumina or rete channels (rc). Cells forming the rete ovarii do not express CK (Figure 4.6G),

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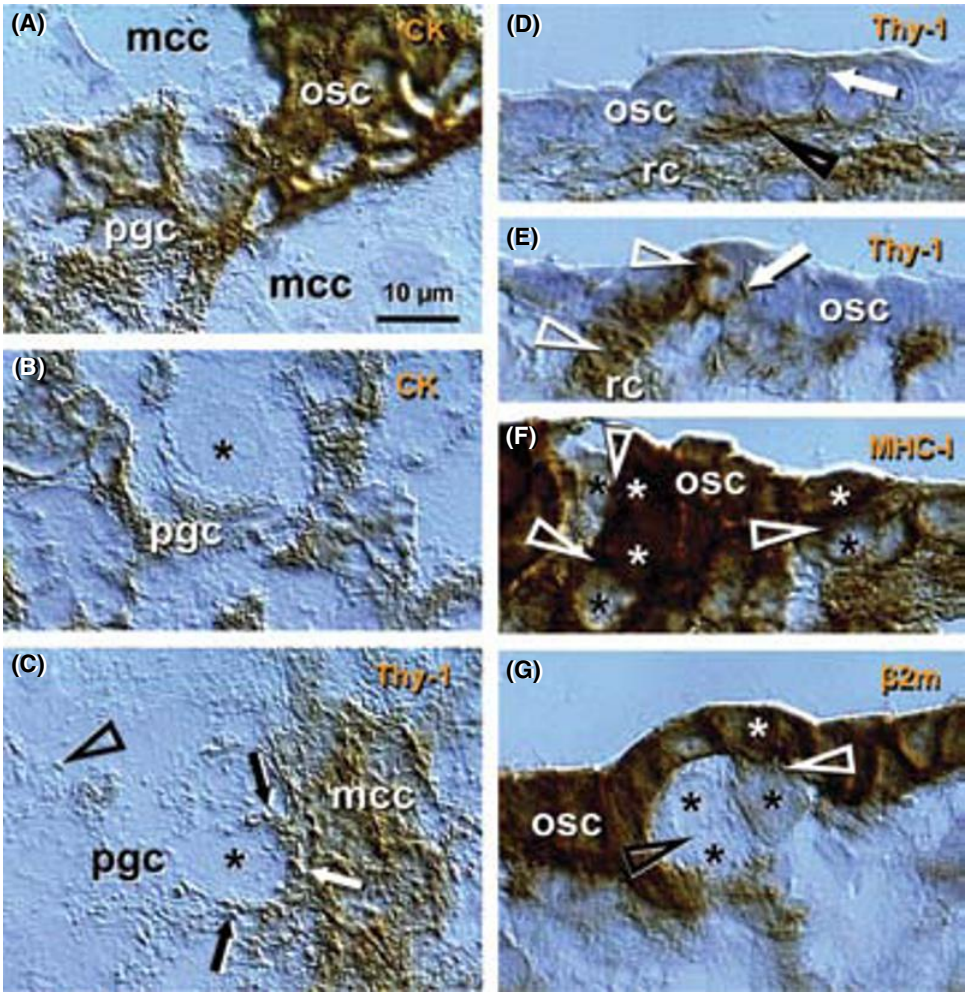


Figure 4.7. The human fetal ovary (24 weeks). **(A)** Sprouts of primitive granulosa cells (pgc) originating from OSC between adjacent mesenchymal cell cords. **(B)** In the cortex the primitive granulosa cells associate with available oocytes (asterisk). **(C)** Pericytes (white arrow) in mesenchymal cell cords release large quantities of Thy-1 (black arrows and arrowhead) among adjacent oocytes and primitive granulosa cells. (**D** and **E**) Rete cord (rc) extensions underline OSC and secrete Thy-1 (arrowheads) collapsing into spikes (arrows). (**F** and **G**) Secondary germ cells (black asterisks) originating by asymmetric division of OSC (white asterisks) show depletion of MHC heavy (F) and light chain (G). Staining as indicated in panels (see Figure 4.6 caption). **(G)** β2 microglobulin (β2m) – MHC-I light chain. Asterisks indicate germ cells/oocytes. Abbreviations and arrows/arrowheads are explained in the text. (Adapted in part from Bukovsky et al., 2005a with permission, © Humana Press.)

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but do express Thy-1 differentiation protein, and the strongest Thy-1 expression is characteristic of pericytes accompanying rete vessels (v, Figure 4.6H). The rete ovarii also contains $CD14^+$ (primitive) small MDCs (Figure 4.6I) differentiating into large activated [class II major histocompatibility antigens (HLA-DR)+] MDCs (m, Figure 4.6J), which migrate through the channels (arrow) and interact (arrowhead) with resident MDCs (r). In addition, T cells expressing CD3 (Figure 6K) or CD8 (Figure 4.6L) of cytotoxic/suppressor T cells are also present in the rete channels.

Degeneration of Fetal Oocytes

Many germ cells and oocytes within the developing ovary degenerate (Peters and McNatty, 1980). The maximum number of oocytes in human fetal ovaries (5–7 million) is present in the fetal month 6, and they are reduced to 1 million at birth. Figure 4.6M shows a degenerating oocyte (asterisk) accompanied by an irregular layer of granulosa cells. Note the extensive oocyte cytoplasmic vacuolization.

Origin of Primitive Granulosa Cells

Sprouts of primitive granulosa cells (pgc, Figure 4.7A) originate from OSC migrating into the ovary: individual sprouts of granulosa cells are surrounded by mesenchymal cell cords (mcc). Primitive granulosa cells show a decrease in CK expression compared to OSC (osc, Figure 4.7A). Primitive granulosa cells associate with available oocytes (asterisk, Figure 4.7B) to form fetal primordial follicles. Pericytes accompanying the microvasculature in mesenchymal cell cords release large quantities of $Thy\text{-}1^+$ intercellular vesicles (arrows, Figure 4.7C), which then migrate between adjacent oocytes and primitive granulosa cells. These intercellular vesicles collapse into the characteristic empty "spike-like" structures (arrowhead, Figure 4.7C) after reaching their targets, indicating the release of their vesicular content.

Rete cords (rc, Figure 4.7D and E; rete extensions) consisting of mesenchymal cells, underlie segments of the OSC. Pericytes adjacent to the OSC secrete $Thy\text{-}1^+$ material among OSC. This material consists of intercellular vesicles (arrowheads, Figure 4.7D and E) converted into empty "spikes" (arrows).

Secondary Germ Cells Originate by Asymmetric Division of OSC

Some cells within the OSC show asymmetric division (white arrowheads, Figure 4.7 F and G) accompanied by a diminution of MHC-I and light chain (beta2m) expression in one of the daughter cells (black vs. white asterisks, Figure 4.7 F and G). The size of these cells substantially increases compared to typical OSC. Such cells resemble intraepithelial germ cells (Motta and Makabe, 1986), and subsequently divide symmetrically (black arrowhead and black asterisks, Figure 4.7G).

Monocyte-derived Cells and T Cells Accompany Origin of Secondary Germ Cells

Why are only some OSC transformed into germ cells? It has been suggested that to become a germ cell, the OSC receive an impulse from ovary-committed BM cells, such as monocytes and T cells, in a milieu of favorable systemic (hormonal) conditions

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(Bukovsky et al., 2005a; Table 4.1). During ovarian development the immune-system-related cells migrate through the rete ovarii and interact with resident MDCs (Figure 4.6I–L), and this may result in their ovarian commitment.

In the upper cortex adjacent to the OSC, primitive CD14+ MDCs exhibit extensions among some OSC (arrowheads, Figure 4.8A), and accompany (arrowheads, Figure 4.8B) the symmetric division of germ cells (asterisks). Activated MDCs exhibit the morphology of mature dendritic cells (DC) (Figure 4.8C). Focal HLA-DR staining is seen among OSC (Figure 4.8D), suggesting that DC may undergo apoptosis similar to that observed in the normal stratified epithelia of adults (Bukovsky, 2000; Bukovsky et al., 2001), and/or some OSC may express HLA-DR when activated. Strong binding of Ig-κ (most probably fetal IgM) is apparent in OSC but not the germ cells (asterisk, Figure 4.8E). The T cells that migrate through the rete channels and cords associate (arrowhead, Figure 4.8F) with the emerging germ cells (black vs. a white asterisk) and exhibit an activation (HLA-DR expression) pattern (Figure 4.8G). The association of MDCs and T lymphocytes with the OSC suggests that they are ovary-committed, possibly occurring during their migration through the rete ovarii channels (Figure 4.6I–L).

Together, the origin of the germ cells from the OSC is a process driven by ovary-committed BM cells. The number of ovary-committed BM cells interacting with the OSC may determine the number of germ cells actually originating in the ovaries. High binding of immunoglobulins to OSC may prevent them from spontaneous (not driven by ovary-committed BM cells) transformation into germ cells. Heat-inactivated serum in media of OSC cultures lacks immunoglobulins, and, therefore, the OSC proliferate and differentiate into oocytes without the need for MDCs and T cells (see below).

Table 4.1. Working model of age-associated changes of ovary-committed bone marrow cells (OC-BMCs) and hormonal signals (LH/hCG and E2) required for the initiation and resumption of oogenesis in human ovaries.

Period of life	OC-BMCs[3]	LH/hCG[4]	E2[5]	Oogenesis
First trimester to midpregnancy	Yes	Yes	Yes	Yes[6]
Last trimester to newborn	Yes	No	Yes	No[6]
Postnatal to menarche	Yes	No	No	No[7]
The prime reproductive period[1]	Yes	Yes	Yes	Yes[6]
Premenopause[2]	No	Yes	Yes	No[7]
Postmenopause	No	Yes	No	No[6]

[1]From menarche till 38 ± 2 years of age.
[2]From 38 ± 2 years till menopause.
[3]MDC and T cells with commitment for stimulation of OSC to germ cell transformation.
[4]Levels corresponding to the mid-cycle LH peak, or more (hCG levels should be 10 times more, since it has only a 10% affinity to the LH receptor compared to that of LH – see Bousfield et al. (1996).
[5]Levels corresponding to the preovulatory E_2 peak, or more.
[6]Confirmed.
[7]Predicted.
(*Source:* Adapted from Bukovsky et al., 2005a with permission, © Humana Press Inc.)

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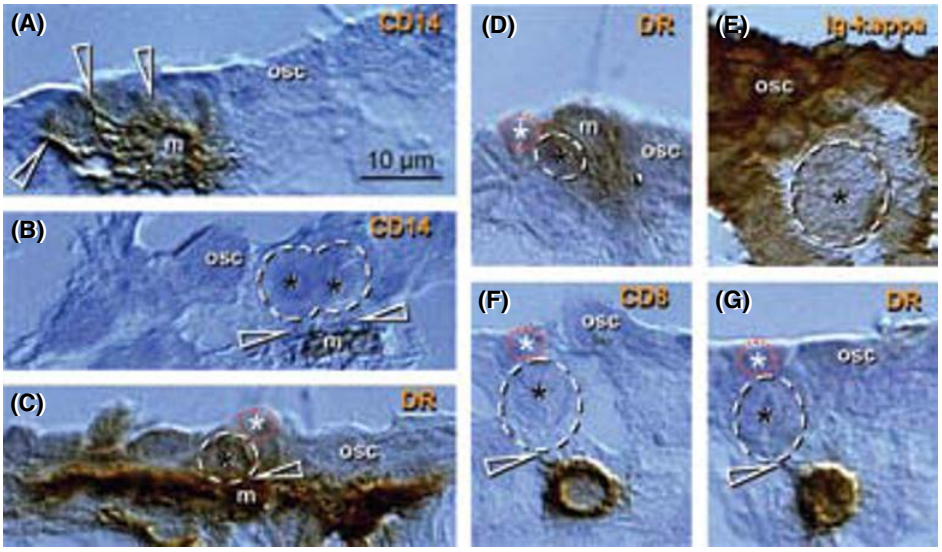


Figure 4.8. The human fetal ovary (24 weeks). CD14+ MDCs (m, **(A)** exhibit extensions (arrowheads) among some OSC, and accompany **(B)** symmetrically dividing (arrowheads) secondary germ cells. (**C** and **D**) Germ cells (black asterisks) originating by asymmetric division from OSC (white asterisks) are accompanied by DR + MDCs (m), and also by CD8+ (F) and DR + (G) T cells. **(E)** Ig-κ light chain of immunoglobulins (Ig-κ) is depleted in emerging germ cells. (Adapted in part from Bukovsky et al., 2005a with permission, © Humana Press.)

In contrast to the association of T cells with the origin of OSC-derived secondary germ cells *in vivo*, T cells were not found accompanying (i.e., to be required for) follicular growth. Both processes were accompanied by activation of Thy-1+ pericytes and association of MDCs, however.

Conclusions on the Origin of Secondary Germ Cells

These observations indicate that secondary germ cells originate by asymmetric division of the OSC. This is quite complex, since it requires a sequential involvement of the immune system and TCS-related cells and molecules. This includes the involvement of primitive MDCs, activation of Thy-1+ vascular pericytes, the interaction of activated (HLA-DR+) MDCs, and involvement of activated (HLA-DR+) T cells. Once formed, germ cells undergo symmetric division required for crossing over of chromosomes. Next, they attain a tadpole-like shape, enabling them to leave the OSC and enter the ovarian cortex, where they differentiate into oocytes. The oocytes then associate with OSC-derived granulosa cells to form fetal primordial follicles. Hence, OSC have the dual potential to differentiate either into somatic granulosa cells or female secondary germ cells, depending on local cellular signaling and hormonal conditions (Bukovsky et al., 2005a).

An interesting question is whether or not OSC are committed to production of secondary germ cells per se, once differentiation from peritoneal mesothelium in

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developing embryonic gonads begins. On the other hand, such commitment may require the presence of extragonadal primordial germ cells. Although extragonadal primordial germ cells may degenerate after entering the gonad, they nevertheless play an important role in gonadal development.

As indicated above, in the chick, the germ cells are first recognizable in the crescentic area of the germ-wall endoderm as early as 24 h of incubation (Swift, 1914). Reagan (1916) cut out this crescentic area where the primordial germ cells were supposedly located. The operated chicks were then further incubated and examined after varying lengths of time. In no instance where the removal of this sex-cell area was complete did germ cells arise from gonadal somatic cells, even after establishment of the OSC. In the normal chick, the OSC are well formed on day 4 of incubation, and the primitive ova are clearly recognizable among them (Lillie, 1908). But in Reagan's operated chicks, even after 5 days of incubation, no germ cells were recognized (Reagan, 1916). These observations indicate that the production of secondary germ cells from the OSC require OSC commitment induced by primordial germ cells.

A proposed model of OSC commitment for the production of secondary germ cells is shown in Figure 4.9. Uncommitted ovarian surface (coelomic) epithelium cells (u-OSC, Figure 4.9A) are present in human embryos during week 4 of development, prior to arrival of primordial germ cells. The primordial germ cells (pgc, Figure 4.9B) invade the OSC during week 5 and commit the OSC (c-OSC) to production of secondary germ cells. The primordial germ cells degenerate and secondary germ cells (sgc, Figure 4.9C) are produced from OSC under influencing hormonal and cellular signaling. This hormonal signaling includes estradiol (E2) and hCG, and cellular signaling includes MDCs, Thy-1 pericytes, and T cells. Secondary germ cells enter the ovarian cortex and differentiate into definitive oocytes (do). All OSC are influenced by hormonal signaling, but only those influenced by cellular signals undergo asymmetric division (ad, Figure 4.9D) followed by the symmetric division (sd) required for crossing over (co). Tadpole-like migrating secondary germ cells (m-sgc) enter the ovarian cortex.

The involvement of cellular signaling in the origin of secondary germ cells by asymmetric division of OSC (ad, Figure 4.9E) is complicated. It requires primitive and activated MDCs, activated pericytes [(P), with permissive autonomic neural signaling (NS-), that is, a lack of neural inhibition], and activated T cells (T). The thymus-derived ovary-committed T cells may eventually diminish with the age-related thymic regression. This may be why the development of new germ cells ceases by the end of the third decade of life (Figure 4.1 and Table 4.1). The symmetric division of germ cells follows (sd, Figure 4.9E) and appears to require primitive MDCs (see Figure 4.8B and above). Migration of secondary germ cells (m-sgc, Figure 4.9E) is accompanied by activated MDCs, which contribute HLA-DR to germ cells (see above).

Another question is why the developing ovary exhibits so high a number of germ cells developing into oocytes, and why oocytes enter meiotic prophase at the time when the rete ovarii developing from mesonephric ducts reaches the center of the ovary. This contrasts with the scarcity of male gametes and a lack of meiotic prophase in the developing male gonads (Baker, 1972). The unique meiotic activity of female germ cells and oocytes appears to be dependent on the prevention of mesonephric cell migration and testis cord formation in developing ovaries (Yao et al., 2003). Hence, arrest of some oocytes of developing ovaries in the meiotic prophase appears to impact ovarian structure and prevent development of testicular morphology.

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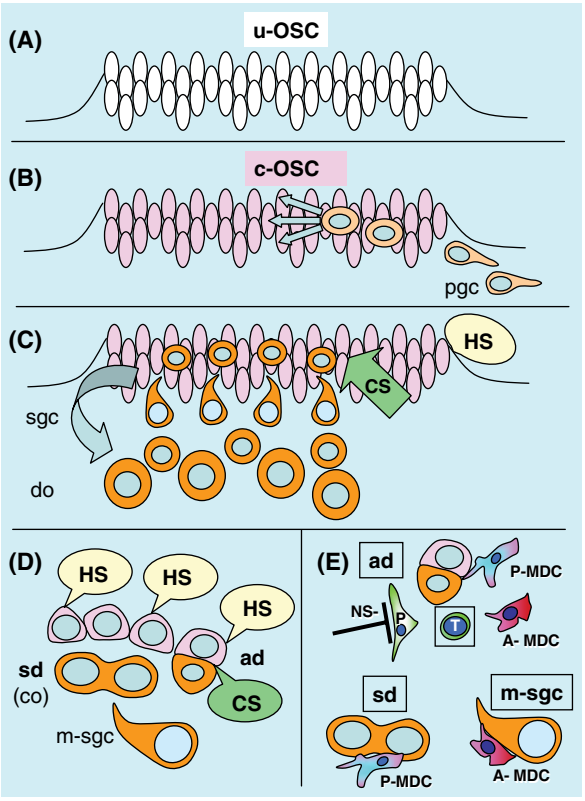


Figure 4.9. Model of OSC commitment for production of secondary germ cells. **(A)** The uncommitted OSC (u-OSC) is present during week 6 of gestational age, prior to the arrival of primordial germ cells (pgc). **(B)** Primordial germ cells invade OSC during week 7 and cause commitment of OSC (c-OSC) for production of secondary germ cells (sgc). **(C)** The primordial germ cells degenerate and secondary germ cells are produced from OSC influenced by hormonal signaling and cellular signaling (MDCs, Thy-1 pericytes, and T cells). The secondary germ cells enter ovarian cortex and differentiate into definitive oocytes (do). **(D)** All OSC are influenced by systemic hormonal signals (HS), but only those influenced by CS undergo asymmetric division (ad) followed by symmetric division (sd) required for crossing over (co). Tadpole-like migrating secondary germ cells (m-sgc) leave OSC and enter the ovarian cortex. **(E)** Origination of secondary germ cells from OSC by asymmetric division appears to require primitive (CD14+) MDCs (P-MDCs), activated pericytes (P) with a lack of suppressive neural signaling (NS-), activated (DR+) MDCs (A-MDCs) and activated (DR+) T cells (T). (Adapted from Bukovsky et al., 2008a with permission, © Transworld Research Network.)

Most fetal oocytes are not preserved undergoing the process of perinatal demise. Nevertheless, it is possible that fetal differentiation of oocytes and primordial follicles, not functionally required until at least puberty and during sexual maturity, may still play an important role in programming the timespan for periodical follicular renewal during the PRP of adults (Bukovsky, 2006b; Bukovsky and Caudle, 2012).

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Cessation of Oogenesis in Prenatal Human Ovaries

Human fetus formation of new oocytes and primordial follicles ceases after the second trimester of fetal intrauterine life, possibly due to the diminution of circulating human chorionic gonadotropin in the fetal blood (Bukovsky et al., 2005a). Thereafter (perinatally), the layer of loose mesenchymal cells forming ovarian TA develops by mesenchymal conversion of OSC, exhibiting some features of the OSC (CK expression), possibly originating from epithelial–mesenchymal conversion of the OSC (Bukovsky et al., 2004, 2006b)., as described in OSC cultures (Auersperg et al., 2001). These TA mesenchymal cells may be converted back into the OSC by mesenchymal–epithelial conversion, that is, into bipotent stem cells capable of differentiating into granulosa and secondary germ cells in adult human ovaries (Bukovsky et al., 1995a, 2004; Bukovsky, 2006a). This may not happen prior to puberty or menarche, due to a lack of hormonal signaling (Table 4.1; Bukovsky et al., 2005a).

The Working Hypothesis on the Regulation of Oogenesis During Prenatal, Prime Reproductive, and Aging Ovary Periods

Our working hypothesis on the role of gonadal environment in the regulation of human oogenesis (Bukovsky, 2006b) is presented in Figure 4.10. After the indifferent gonad is populated with primordial germ cells (Figure 4.10A), the rete ovarii stimulates differentiation of oocytes from secondary germ cells (Figure 4.10B). During developmental immune adaptation, the rete is populated by uncommitted MDCs and T cells (UMT), from which the MDCs differentiate into the veiled cells. The veiled cells transmit information on oocytes from the rete into the developing lymphoid tissues (curved arrowhead, Figure 4.10B). The MDCs in the rete ovarii then become ovarian memory cells capable of converting UMT passing through the rete channels into ovary-committed BM cells. These ovary-committed BM cells, with appropriate hormonal stimulation, induce the development of germ cells from the OSC (Figure 4.10C). The number of veiled cells populating lymphoid tissues increases further.

When developmental immune adaptation is terminated, the rete ovarii degenerates and oogenesis ceases, due to diminution of hormonal signaling (fetal hCG barrier; Bukovsky et al., 2005a). The ovarian TA develops from OSC (epithelial–mesenchymal transformation), and the number of ovarian memory cells (om; the transformed veiled cells) in lymphoid tissues is set (Figure 4.10D).

Around menarche and during the PRP, hormonal signaling and ovary-committed BM cells resume cyclic oogenesis to replace aging primordial follicles undergoing atresia (Figure 4.10E). Follicular renewal during adulthood requires a cyclic supply of ovary-committed BM cells. Their generation in lymphoid tissues (ovary-committed T cells in particular) causes depletion of the pool of memory cells. Hence, the pool of ovarian memory cells in lymphoid tissues, but not the pool of primordial fetal follicles, is what is set during mammalian fetal development.

Once the available pool of ovarian memory cells is consumed, oogenesis and follicular renewal cease, despite the presence of hormonal signaling (Figure 4.10F). Adult primordial follicles remain and are utilized until gone. However, aging oocytes accumulate genetic alterations and may become unsuitable for ovulation and

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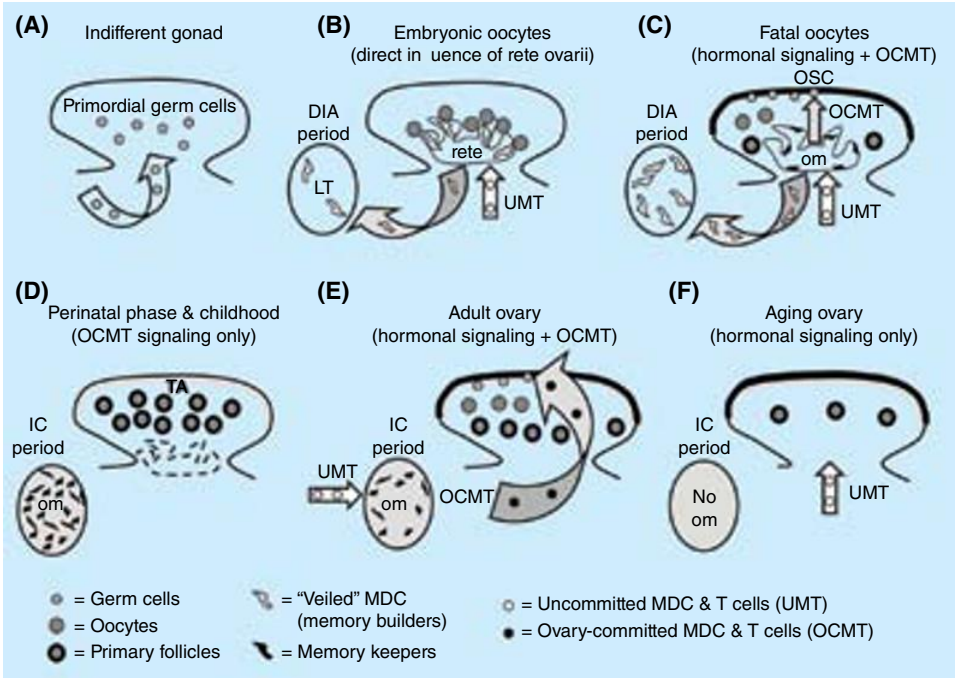


Figure 4.10. Evolution of ovaries during developmental immune adaptation and their behavior during immune competence. **(A)** Primordial germ cells imprint the OSC for production of secondary germ cells (see Figure 4.9B vs. 4.9A). **(B)** Development of rete ovarii and lymphoid tissue (LT). Uncommitted MDCs and T cells (UMT) saturate rete ovarii to be converted into ovary-committed MDCs and T cells (OCMT). **(C)** Secondary germ cells originate by asymmetric division of OSC under the influence of rete-derived OCMT and hormonal signaling. The ovary commitment is also transferred into draining lymphoid tissues (arched arrows). During the perinatal period immune competence (ic) is initiated, the ovarian memory (om) is built and the rete ovarii regresses. During childhood the OCMT is available but hormonal signaling is absent until menarche. **(E)** During the PRP (from menarche to 38 ± 2 years of age) OCMT and cyclic hormonal signaling cause cyclic formation of germ cell and renewal of primordial follicles. **(F)** After the PRP the hormonal signaling persists but follicular renewal ceases due to the lack of OCMT. (Reprinted with permission from A. Bukovsky, "Oogenesis From Human Somatic Stem Cells and a Role of Immune Adaptation in Premature Ovarian Failure," *Curr. Stem Cell Res. Ther*. **1** (2006): 289–303. © Bentham Science Publishers, Ltd.)

fertilization. Postmenopausal ovaries are reported to carry occasional follicles with degenerated oocytes (reviewed by Talbert, 1968).

The initial pool of ovarian memory cells in lymphoid tissues may be reduced due to retardation of normal ovarian development during embryonal and fetal immune system adaptation (Bukovsky, 2011b). If the period for which the primordial follicles differentiate during developmental immune adaptation is shorter (delayed or terminated earlier), the period of follicular renewal after menarche ceases earlier compared to normal ovaries, and premature ovarian failure (POF) with secondary

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amenorrhea results. Restriction of primordial follicle development during adaptation may result in a lack of follicular renewal after menarche and primary amenorrhea (early POF). Primordial follicles and ovarian memory in the lymphoid system can also be depleted by cytotoxic chemotherapy. Interestingly, the incidence of POF after such chemotherapy increases with age. In women < 20 years old the incidence is 13%, in 20–30 years is 50%, and > 30 years is 100% (Lo Presti et al., 2004). This suggests a diminution of ovarian memory in the lymphoid system with age and increasing sensitivity to chemotherapy.

Together, we speculate that a lack of follicular renewal may be caused by age-associated exhaustion of memory cells in the lymphoid system, which are required to generate effector cells that migrate to ovaries and stimulate the transformation of OSC into primitive granulosa and germ cells. Premature ovarian failure may be caused by delayed ovarian development during developmental immune adaptation (Bukovsky, 2011b), by earlier termination of developmental immune adaptation, or by cytostatic chemotherapy affecting both the existing pool of primordial follicles and ovary-committed mesenchymal cells required for follicular renewal. Patients with POF have been found to have abnormalities in the function of circulating monocytes, activated lymphocytes, natural killer (NK) cells, and exhibited other immune system abnormalities (Hoek et al., 1993, 1995; Rebar, 2000), including anti-ovarian autoantibodies (Edassery et al., 2010). This suggests a role for immune system alteration in the pathogenesis of POF.

Premature Failure of Ovaries with Primordial Follicles and Animal Models

Premature ovarian failure is the result of a lack of adult primordial follicles within the ovaries of women less than 35–40 years of age (Kumar et al., 2012). However, POF is often clinically associated with follicular resistance to gonadotropins, called "hypergonadotropic amenorrhea." Here, ovaries contain normal primordial or even antral follicles not responding to gonadotropins by the production of estrogens. No woman with primary amenorrhea has been reported to ovulate or conceive with her own oocytes, but in one study more than one-third of the women with secondary amenorrhea were pregnant at least once before developing hypergonadotropic POF. A quarter of them had evidence of ovulation after the diagnosis was established, and 8% of those with secondary amenorrhea later conceived (Rebar, 2000).

Animal models (rats and mice) indicate that there are two types of POF with primordial and antral follicles within ovaries. The first, persistent ovarian immaturity, can be induced by inhibition of ovarian development (temporary inhibition of androgen receptor expression) with estrogens during developmental immune adaptation, while the second, premature ovarian aging, can be induced by acceleration of ovarian development (premature expression of an androgen receptor) with androgens (Bukovsky et al., 2000a, 2002).

The Tissue Control System Theory and a "Stop-effect" of Monocyte-Derived Cells

The TCS theory (Bukovsky et al., 1991, 1995c, 1997, 2000b, 2001, 2002) addresses the role of vascular pericytes, MDCs, and T and B cells in the regulation of tissue function. It proposes that MDCs stimulate early differentiation of tissue-specific (epithelial,

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parenchymal, neural, and muscle) cells. Monocyte-derived cells also regulate expression of epitopes of tissue-specific cells, and in this way control their recognition by circulating tissue-committed T cells and antibodies. Such T cells and antibodies promote the advanced differentiation of tissue cells, which ultimately results in the aging and apoptosis of these cells (Bukovsky et al., 2001).

By the end of the developmental immune adaptation in early ontogeny, the MDCs encounter the most differentiated tissues in a tissue-specific manner, and prevent them from differentiating beyond the encoded state by the so-called "stop effect." The nature of the "stop effect" may reside in the inability of monocyte-derived cells to stimulate differentiation of tissue cells beyond the encoded stage. Retardation or acceleration of certain tissue differentiation during developmental immune adaptation causes a rigid and persisting alteration of this tissue function. The ability of monocytes to preserve tissue cells in a functional state declines with age. This is accompanied by functional decline of various tissues within the body, including the ovary, resulting in menopause and an increased incidence of degenerative diseases in humans.

In large mammals, including primates, developmental immune adaptation is terminated during intrauterine life, while in small laboratory rodents it continues for several postnatal days, ending about 1 week after birth (Klein, 1982). Estrogens given to neonatal rats and mice inhibit ovarian development. During adulthood such rat females exhibit persisting ovarian immaturity, characterized by retardation of follicular development (Bukovsky et al., 1997) despite normal levels of gonadotropins (Nagasawa et al., 1973; Matsumoto et al., 1975). This shows that suppression of early ovarian development results in persistent ovarian immaturity, resembling POF and gonadotropin resistence of ovarian follicles. Injection of estrogens in neonatal mice (days 0–3) caused permanent anovulation, but mice injected later (days 3–6; closer to the end of developmental immune adaptation) resume ovulatory cycles after initial anovulation (Deshpande et al., 1997). Hence, persisting ovarian immaturity can result in a delay of normal ovarian function. Since the incidence of degenerative diseases increases with age, one would expect a tendency of the "stop-effect" to shift up with age (Bukovsky, 2011b). This could explain how persistent ovarian immaturity might resolve with subsequent normal ovarian function.

On the other hand, injection of androgens causes premature ovarian aging, which persists throughout adulthood. Androgen-induced anovulation can be prevented by neonatal injection of a thymic cell suspension from immunocompetent prepubertal normal female donors, but not if given from animals prior to completion of developmental immune adaptation (Kincl et al., 1965). This suggests that certain thymic cells (thymocytes, or thymic MDCs) of normal immunocompetent females carry information on the differentiation of ovarian structures, and that information can be transferred to immunologically immature neonatal rats.

However, when a low dose of androgens is injected during developmental immune adaptation, the rats exhibit a delayed anovulatory syndrome. Ovaries exhibit the onset of normal function after puberty (~40 days of age), but premature aging of the ovary occurs between 60 and 100 days (Swanson and van der Werff ten Bosch, 1964). This delayed manifestation of ovarian dysfunction resembles human POF with secondary amenorrhea, as well as some human degenerative diseases with autoimmune character, which also occur after a shorter (juvenile diabetes mellitus) or longer (Alzheimer's disease) period of normal tissue function.

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An application of the TCS theory (see above) on the regulation of tissue function via the "stop effect" was recently described in detail (Bukovsky, 2011b). In normal tissues, the functional cells are present during developmental immune adaptation and the tissue-specific cells are "parked" in the functional state during adulthood. Retardation of cell differentiation during adaptation results in persisting immaturity (POF with primary amenorrhea) and an acceleration of premature aging (POF with secondary amenorrhea, degenerative diseases). If the tissue was absent during adaptation, like CL, it is handled as a "graft" (Bukovsky et al., 1995c).

Note that the functional stage of cell differentiation differs among distinct tissue types, being very low in the vagina (in the absence of hormonal stimulation only the basal/parabasal cells are present), low in the brain, skeletal muscle, and pancreatic beta cells (lack of intraepithelial lymphocytes), moderate in the gut (presence of intraepithelial lymphocytes), and high in the skin (apoptosis of surface keratinocytes) (Coghlan, 2005).

The Immune System Memory and Aging of the Body

The "ovarian memory" built within the lymphoid system can be viewed as a charged battery, which is drained by periodic follicular renewal. The higher the charge during the developmental immune adaptation, the longer it will last, and vice versa. The involvement of developmental immune adaptation in the programming of ovarian function and various types of POF can be extrapolated to other tissues in the body as well. From this perspective, degenerative changes of the immune system with advancing age may have a role in the aging of other tissues and the body in general.

Former and Current Views on Ovarian Oogenesis and Follicular Renewal

Milestones of the Oocyte Storage Theory

The prevailing current belief that all oocytes in adult mammalian gonads come from the fetal period was originally based on the "continuity of germ plasm" theory. It declared that an essential condition for the development of another embryo is the retention of a part of the progeny of the primary impregnated germ cell, and that germ cells have a different character compared to the somatic cells, since somatic cells serve as nurse cells for the germ cells and cannot be considered as their progenitors (Nussbaum, 1880; Zuckerman and Baker, 1977). This theory assumes that the primary impregnated cell determines subsequent individuals. This, however, contrasts with the tenant of evolutionary theory that surviving species are those who reproduce themselves, mutate, and are capable of transmitting these favorable mutations to subsequent generations.

Oogenesis in Adult Prosimians

The original report by Gerard (1920) on the unique oogenic activity from germinal cords in the cortex of the ovary of adult *Galago* was followed by studies of ovaries

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of adult South Indian prosimians, where the origin of multiple oocytes from deep invaginations of OSC was reported (Rao, 1928). The occurrence of oogonia in adult ovaries of other prosimian species was confirmed subsequently by many investigators (reviewed by Zuckerman and Weir, 1977). Although the fate of such oogonia is a matter of dispute (Telfer, 2004), these observations indicate that new oocytes are formed in adult prosimians. Primate species appear evolutionary much more developed than mice in this regard.

Rodent Ovaries

Mouse and Guinea Pig Ovaries

Edgar Allen studied neo-oogenesis in adult mice and suggested the possibility of follicular renewal during sexual maturity (Allen, 1923). These mouse studies were confirmed by Johnson et al. (2004). Evans and Swezy (1931), in studies of oogenesis in adult guinea pigs, showed that cells move from the OSC into the ovarian stroma, usually as a solid cord, and one or more cells become enlarged into oocytes; the remaining cells form the granulosa cells. Another possibility is that oocytes are formed along the cord and other cells group themselves around these, forming primordial follicles (Evans and Swezy, 1931).

Rat Ovaries

We observed similar mechanisms in normal adult rat ovaries and showed that a group of cells within the descending OSC cord express ZP proteins. Some of these ZP cells are transformed into tadpole-like cells capable migrating (Bukovsky et al., 2005a). This enables the development of adult primordial follicles either directly in adjacent areas, or via the blood stream in more distant sites.

In an additional study of rat ovaries (Bukovsky et al., 2007), by using double color immunohistochemistry we found that BM-derived cells (MDCs and T cells) are also involved in the OSC-niche triggering of origin of germ cells from the OSC in adult rats. The T cells (red asterisk Figure 4.11A) accompanied asymmetric division of OSC (white arrowhead) giving rise to an OSC daughter (black asterisk) and ZP + germ daughter (white asterisk) descending into the solid epithelial cord (epc), a source of granulosa cells under the OSC layer. Large ZP + oogonia (asterisks, Figure 4.11B and C) divided symmetrically (crossing over) in the solid epithelial cords. Such division was accompanied by activated (Ia+) MDCs (yellow arrowhead, Figure 4.11C). Divided oogonia separated and resulting oocytes formed adjacent new primordial follicles (asterisks, Figure 4.11D). Note the association of primitive granulosa cells derived from solid epithelial cords with oogonia and oocytes (blue arrowheads, Figure 4.11A–E). Monocyte-derived cells also accompanied (yellow arrowhead, Figure 4.11E) the growth of primary follicles.

In adult rats (ages 45–60 days) lacking OSC after neonatal estrogen treatment, the germ cells originated in the ovarian hilar region and formed primordial follicles in the juxtaposed (lower) ovarian cortex (Bukovsky et al., 2007).

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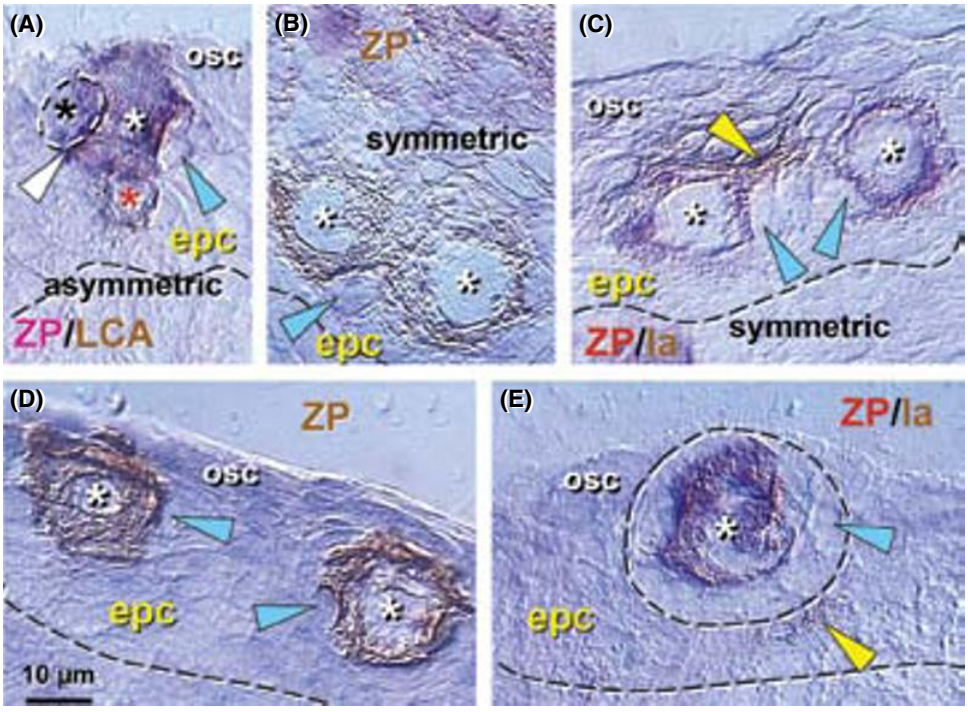


Figure 4.11. Oogenesis in adult rat ovaries. **(A)** Asymmetric division of OSC (white arrowhead) accompanied by a lymphocyte-type cell (red asterisk). White asterisks indicate ZP + emerging germ cell, oogonia, and oocytes. **(B)** Symmetric division of oogonia. **(C)** This is accompanied by a MDC (yellow arrowhead). **(D)** Adjacent primordial follicles. **(E)** Growing primary follicle is accompanied by a MDC (yellow arrowhead). Immunostaining with ZP and leukocyte common antigen (ZP/LCA), ZP alone, and ZP/class II MHC (ZP/Ia) as indicated in the panels. Scale in (D) for (**A–E**). Blue arrowheads indicate granulosa cells derived from epithelial cords (epc); osc, ovarian stem cells; ZP, zona pellucida. (Adapted with permission from A. Bukovsky et al., "Bone Marrow Derived Cells and Alternative Pathways of Oogenesis in Adult Rodents," *Cell Cycle* **6**, no. 18 (2007): 2306–2309., © Landes Bioscience.)

Functional Repair of Anovulatory Mouse Ovaries with Cultured Germ-Line Stem Cells

Former observations indicating that neo-oogenesis occurring in both adult mouse and human ovaries (Allen, 1923; Bukovsky et al., 1995a, 2004, 2008c; Johnson et al., 2004; Kerr et al., 2006) were confirmed by Zou et al. (2009) from evidence that cultured mouse germ-line stem cells transplanted into the ovaries of sterilized mice could produce new oocytes and offsprings. Ji Wu and colleagues studied germ-line stem cell cultures derived from 5-day-old and adult mouse ovaries, and their ability to produce functional oocytes when transplanted into the ovaries of infertile mice (Zou et al., 2009). The cultured cells were infected with the green fluorescent protein (GFP) virus and, when transplanted, they were transformed into oocytes and produced offspring expressing GFP transgene (Zou et al., 2009).

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These observations suggest the possibility of restoring fertility in women with POF (Gougeon, 2010; Parte et al., 2011).

Oocyte Formation by Mitotically Active Germ Cells Purified from Ovaries of Reproductive-Age Women – Repowering the Ovary

In a recent article in Science–Business eXchange (SciBX) of the Nature Publishing Group, the Senior Editor Tracey Baas (2012, p. 4) noted:

> "In 2009, Ji Wu and colleagues at Shanghai Jiao Tong University used a unique biomarker of murine OSCs-dubbed DEAD box polypeptide 4 (Ddx4) to isolate oogonial stem cells (OSCs) from adult mouse ovaries. The team used the marker to purify OSCs, which were transplanted into ovaries of infertile mice to generate functional oocytes capable of producing offspring.
>
> Those data were published in Nature Cell Biology [Zou et al., 2009]. The open question was whether OSCs purified from humans had a similar ability to generate functional ovarian follicles.
>
> To answer that question, researchers at MGH [Massachusetts General Hospital] and HMS [Harvard Medical School] started by modifying Wu's Ddx4-based purification protocol to improve its selectivity for OSCs. The team, which was once again led by Tilly, used fluorescence-activated cell sorting (FACS), which is less likely to be contaminated by oocytes than Wu's technique of magnetic bead sorting.
>
> His group isolated DDX4-positive cells from murine ovarian tissue as well as from human ovarian tissue derived from whole ovaries of reproductive-age women undergoing elective surgery. *In vitro*, both human and murine DDX4-positive cells expressed primitive germ-line markers but not oocyte markers, suggesting the procedure had indeed purified OSCs. To determine whether the OSCs could differentiate into viable oocytes, the team cultured the cells on mouse embryo fibroblasts used as feeder cells, which supplied a cellular matrix upon which the stem cells grew. The human and mouse OSCs spontaneously differentiated into oocytes, as assessed by morphology, gene expression patterns and progression through meiosis.
>
> The next step was to look at whether the OSCs could generate oocytes *in vivo*. To do so, the team engineered the mouse and human OSCs to express GFP, which made it possible to visualize and track the cells. When GFP-expressing mouse OSCs were injected into the ovaries of mice, primary ovarian follicles developed that contained GFP-positive oocytes. Moreover, those oocytes led to the development of GFP-expressing mouse embryos following fertilization.
>
> Finally, GFP-expressing human OSCs were injected into human ovarian tissue and transplanted into immunodeficient mice. The result was primary ovarian follicles that contained GFP-expressing oocytes.
>
> That suggested OSCs might indeed give rise to functional oocytes if transplanted into humans.
>
> Results were published in Nature Medicine [White et al., 2012]. The team also included researchers from Saitama Medical University. Tilly and colleagues are now optimizing the conditions for production of human oocytes *in vitro* and have started studies to assess whether OSCs in nonhuman primates can generate functional oocytes for IVF.

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Bukovsky, professor of reproductive biology associated with the Institute of Biotechnology of the Academy of Sciences of the Czech Republic, said he is most interested in seeing the work translated from ovarian tissue obtained from reproductive-aged women to women with ovarian failure who are unable to produce follicle cells or oocytes. 'Tilly's work supports the existing idea that purified germ cells transplanted into mouse ovaries will utilize existing immature follicle cells to produce new follicles and that the survival and function of oocytes *in vivo* requires interaction with these immature follicle cells,' he said. 'It will be very important that the team reproduce the experiments using ovarian tissue biopsies obtained from reproductive-aged women and from women with premature ovarian failure. I would very much like to see the mechanistic details.'

Questions aside, Oktay said the new study is 'a game changer because it increases the options available for obtaining viable eggs. When it comes to fertility restoration, it is all about options.'

Wu, professor of molecular reproduction and stem cell biology at the Bio-X Center of Shanghai Jiao Tong University, thinks there are still unaddressed technical challenges. 'It is very satisfying to see our protocol optimized and fine-tuned to obtain and differentiate human female germline stem cells into oocytes, but the team's culturing conditions will have to be optimized to avoid use of animal components, and mouse feeder cells will need to be replaced with either human feeder cells or a nonfeeder culturing system.' Wu told SciBX."

The article published in Nature Medicine (White et al., 2012) shows that primitive germ cells purified from the cortex of functional adult human ovaries form new ovarian follicles when injected into human ovarian cortical biopsies and xeno-transplanted into immunodeficient NOD-SCID mice. The article is an important confirmation of former observations on neo-oogenesis and follicular renewal in adult human ovaries by Bukovsky et al. (1995) and expanded thereafter (Bukovsky et al., 2004, 2005b, 2007, 2008c; Bukovsky, 2006a, 2007c, 2011c; Bukovsky and Virant-Klun, 2007c; Bukovsky and Caudle, 2012).

In addition, neo-oogenesis from secondary germ cells is already present in human fetal ovaries, during the second trimester of pregnancy (Bukovsky et al., 2005a; Bukovsky, 2011a). This is an observation not yet investigated by others.

In a recent commentary, Telfer and Albertini (2012, p. 354) indicated:

"Work of White et al. [2012] represents an advance that has the potential to change the nature of future infertility treatments, although many practical and conceptual obstacles remain before the clinical utility of their methods can be realized. Much effort will be required to improve the efficiency of isolation and transformation of OSCs into oocytes, as the number of OSCs that went on to form follicle-enclosed oocytes in the study by White et al. was small.

Even in the case of the mouse studies, very few GFP-positive oocytes were shown to fertilize and undergo even minimal embryogenesis, with many of these oocytes clearly arresting at the preblastocyst stage. Further, a more detailed characterization of genetic integrity (euploidy and the appropriate retention of epigenetic marks) and other hallmarks of oocyte quality (such as meiotic and developmental competence) are required before any clinical application of these techniques can be considered.

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> Given the response to earlier work from this laboratory, questions will undoubtedly linger as to whether these cells are only activated *in vitro* or whether they indeed contribute to de novo neo-oogenesis *in vivo*. Further research will be required before these issues can be fully resolved. Nonetheless, the findings of this study will change the tone of future discourse on the subject toward measured enthusiasm and, most importantly, will prompt speculation and tempered progress into what remains a major obstacle in the treatment of various forms of human infertility."

In an additional commentary Oatley and Hunt (2012, p. 196) indicated:

> "For OSCs, doubt will persist until clear evidence is provided that they give rise to genetically normal, developmentally competent eggs. In the meantime, skeptics are plagued by several nagging questions: What do these cells do in the ovary? Where do they come from? And, most importantly, if they can and do give rise to viable eggs in the adult ovary, why is female reproduction of such limited duration?"

Our data (reviewed recently by Bukovsky (2011a) and Bukovsky and Caudle (2012)) presented here appear to have already answered such questions.

Offspring from Oocytes Derived from Female Embryonic Stem Cells

By the end of 2012, Hayashi et al. reported that female (XX) embryonic stem cells were induced as pluripotent stem cells in mice and then induced into primordial germ cell-like cells (PGCLCs). When aggregated with female gonadal somatic cells as reconstituted ovaries, they underwent X-reactivation, imprint erasure, and cyst formation, and exhibit meiotic potential. Upon transplantation under mouse ovarian bursa, the PGCLCs in the reconstituted ovaries matured into germinal vesicle-stage oocytes, which then contributed to fertile offspring after *in vitro* maturation and fertilization.

To produce embryonic stem cells, the authors had first to utilize blastocysts flushed out from the uterus of fertile mice. From the autologous point of view, that is, to provide genetically related newborns, such an approach is not possible to use with infertile women lacking functional oocytes.

Summary on the Current Views

In conclusion, the prevailing belief is that the process of oogenesis occurs only in fetal gonads, and oogonia neither persist nor divide mitotically during sexual maturity with a few possible exceptions, such as in prosimian primates (Franchi et al., 1962; Zuckerman and Baker, 1977).

From our point of view, observations in human ovaries confirm that oogonia do not persist during sexual maturity, since during the PRP new female gametes and granulosa cells originate from bipotential OSC. Observations from ovaries of adult humans (Bukovsky et al., 1995a, 2004, 2005a,b, 2007) rats (Bukovsky et al., 2007) and mice (Allen, 1923; Johnson et al., 2004, 2005; Kerr et al., 2006; Zou et al., 2009; White et al., 2012) support a paradigm that the oocyte and follicular renewal in adult females during the PRP (Figure 4.1) exists throughout all animal species (see PRP theory). We are confident that in time most of reproductive biology scientists will confirm this for all higher vertebrates, including mammals.

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Why Does Menopause Occur?

As indicated above, Ji Wu and colleagues have shown that ovarian germ-line stem cell cultures derived from 5-day-old and adult mouse ovaries infected with GFP transgene produced functional oocytes when transplanted into ovaries of infertile mice. Such oocytes produced offspring expressing GFP of transplanted germ-line stem cells (Zou et al., 2009). The article confirms that OSC have the capacity to produce meiotically potent and functional oocytes in infertile mouse ovaries. This is also supported by human OSC culture studies, where some oocytes developing *in vitro* differentiate into parthenogenetic embryos (Bukovsky, 2008; Virant-Klun et al., 2008; Bukovsky et al., 2009a). This may indicate that the OSC differentiated *in vitro* represent functional oocytes.

A Physiological Role of OSC in Normal Ovaries

In a subsequent commentary (Tilly and Telfer, 2009) to the article of Ji Wu and colleagues (Zou et al., 2009), several important questions were raised regarding the *in vivo* biology for ovarian function and the relevance of this work to reproductive health in women. Is there a physiological role of OSC in normal ovaries? It has been suggested that although these stem cells originated from normal ovaries, their full germ-line potential may be the consequence of long-term culture and their oogenic activity normally suppressed *in vivo*.

Regarding the oogenic activity of human OSC in normal ovaries, it is suppressed unless two conditions are met (see Figure 4.5). First, there should be certain hormonal conditions accompanying the fetal midgestation and adult periovulatory periods, such as high circulating levels of estradiol and luteinizing hormone (LH)/human chorionic gonadotropin (hCG). Even under these conditions, however, only some OSC are converted into germ cells *in vivo* due to the requirement of local cellular signaling by immune-system-related cells (Bukovsky, 2006a, 2007c), (so-called immune physiology of the mammalian ovary (Bukovsky and Caudle, 2008) or immunoregulation of ovarian homeostasis (Bukovsky et al., 2008a)). Immunohistochemistry of the immune-system-related cells (T cells and MDCs) has shown that the emergence of germ cells from OSC *in vivo* requires primitive MDCs (CD14+) and activated (HLA-DR+) CD8+ T cells. The emergence of granulosa cells is accompanied by activated (HLA-DR+) MDCs (Bukovsky et al., 1995a, 2005a; Bukovsky and Caudle, 2008). Also, during adulthood, activated MDCs accompany migration of germ cells from TA to the ovarian cortex, where they enter ovarian vessels (Bukovsky et al., 1995a, 2004, 2005a; Bukovsky and Caudle, 2008 ; see Figure 4.2).

Transplantation of BM cells restores fertility in mice after chemotherapy-induced POF (Lee et al., 2007), possibly due to the addition of immune-system-related cells. It appears, however, that the immune-system-related cells are not required for oogenesis from OSC *in vitro*. Hence oogenic activity of OSC appears to be inhibited *in vivo*, unless appropriate hormonal and cellular signals occur. This may not be required *in vitro* (see below).

Availability of Granulosa Cells

Survival and function of oocytes *in vivo* requires their interaction with granulosa cells, and the number or activity of granulosa cells may restrict the function of germ cells (Tilly and Telfer, 2009). Regarding granulosa cells, marked differences exist between adult ovaries in different species. In small laboratory rodents, granulosa cells originate from solid epithelial cords (Evans and Swezy, 1931). The ovarian structure resembles

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human fetal ovaries with an abundance of OSC-derived granulosa cells (Bukovsky et al., 2005a, 2007). However, in adult human ovaries during the PRP the development of OSC-derived cortical nests of primitive granulosa cells occurs (Van Blerkom and Motta, 1979; Motta et al., 1980). Granulosa cell nests are transported through dense ovarian stroma to the lower cortex to assemble with newly formed oocytes provided by vascular transport or migration from the adjacent cortical OSC crypt (Bukovsky et al., 2004). Our observations show that the number of newly formed adult primordial follicles is determined by the availability of granulosa cell nests. Superfluous vascular oocytes degenerate in the medullary venules (Bukovsky et al., 1995a, 2004; 2008c).

After the PRP and in ovaries with POF, however, degenerating intravascular oocytes and ovarian cortical granulosa cell nests are virtually absent (Bukovsky, 2006a; Bukovsky and Virant-Klun, 2007). This indicates that the availability of transplanted germ cells in adult human ovaries lacking granulosa cell nests may not be sufficient for follicular renewal. Alternatively, transplantation of uncommitted autologous OSC may be a source of both cell types required for follicular renewal: the granulosa cell nests and germ cells. Furthermore, transplantation of autologous committed germ cells may be accompanied by uncommitted autologous OSC, if available. Cultured human OSC could be frozen for an extended storage and future use in order to produce both the granulosa cells in the absence of estrogens and the germ cells in the presence of estrogens (Bukovsky et al., 2005b, 2006a).

The restoration of ovarian function in ovaries with artificially depleted oocytes (Zou et al., 2009) was successful probably due to the preservation of the mouse OSC niche and the preserved granulosa cells. However, POF in human females is supposedly caused by the programmed premature termination of the OSC niche function (Bukovsky, 2011a). Many of such ovaries still carry OSC but fail to renew their missing primordial follicles. Mature oocytes can, however, be produced by OSC culture (Bukovsky et al., 2006b).

Why Do OSC Not Prevent Menopause?

Another important query raised is that even if the activity of OSC *in vivo* is shown to replenish the follicular pool and is accepted, why do they fail to maintain ovarian function with advancing age (Tilly and Telfer, 2009). Indeed, OSC from anovulatory and postmenopausal ovaries have the capacity to differentiate *in vitro* into oocytes (Bukovsky and Virant-Klun, 2007). However, it has to be taken into account that the immune system shows a significant functional decline between 35 and 40 years of age (Mathe, 1997) and concomitantly the ovarian follicular renewal ceases. Continuation of ovarian function until the menopause is based on utilization of aged primordial follicles previously formed (Bukovsky et al., 2004). The age-associated changes in the immune system may be responsible for the termination of neo-oogenesis and follicular renewal *in vivo* (Bukovsky et al., 1995a; Bukovsky, 2006b).

During development in human females, germ cells differentiate much earlier in the embryonic period, compared to granulosa cells and fetal primordial follicles, which appear during the second trimester of fetal intrauterine life (Peters and McNatty, 1980). There is a striking correlation between the period at which organ components are present during early ontogeny and that organ's functional longevity (Bukovsky, 2011b). It is likely that the lack of formation of granulosa cells required for the formation of new primordial follicles and the resulting cessation of ovarian ovulatory function is central to the occurrence of the menopause.

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Perimenopausal Disorders

During the perimenopausal period the risk of acute myocardial infarction rises sharply, as well as hypertension and increased lipids and body weight, vascular intima-media thickness, breast cancer risk, and aging brain disorders, especially dementia and Parkinson's disease. Differentiation of OSC is dependent on the TCS ensuring tissue homeostasis. The lack of OSC differentiation around 50 years of age (Table 4.2) suggests that homeostasis is particularly altered at that time in order to terminate any ovarian activity. Such perimenopausal alteration of homeostasis may also induce disorders of other tissues – see list above.

Table 4.2. Evaluation of menopausal status, OSC in ovaries, and presence of oogenesis and morulae in primary and secondary ovarian cultures.

				CK + OSC/ epithelial crypts		Oogenesis in primary and secondary cultures		
Case[1]	Age[2]	POF pre/post menopause	Diagnosis	Left ovary	Right ovary	Primary	Secondary	Morulae
1	30	POF	POF	Yes	Yes	Yes	N.A.	No
2	36	Pre	PFD	No	Yes	Yes	Yes	No
3	38	POF	POF	No	No	No	N.A.	No
4	39	Pre	Endometriosis	Yes	Yes	Yes	N.A.	No
5	40	POF	POF	Yes	Yes	Yes	N.A.	No
6	42	Pre	Fibroids	Yes	No	Yes	Yes	Yes
7	43A	Pre	Menorrhagia	Yes	Yes	Yes	Yes	No
8	43B	Pre	Fibroids	Yes	No	Yes	Yes	No
9	45	Pre	Endometriosis	Yes	Yes	Yes	Yes	Yes
10	48A	Pre	Menorrhagia	No	Yes	Yes	Yes	Yes
11	48A	Pre	Fibroids	No	Yes	Yes	Yes	No
12	49[3]	Pre	PFD	No	No	No	N.A.	No
13	50[3]	Pre	Fibroids	No	No	No	N.A.	No
14	52[3]	Post	Ovarian cyst	No	No	No	N.A.	No
15	53	Post	PFD	No	Yes	Yes	Yes	No
16	55	Post	Uterine bleeding	Yes	No	Yes	Yes	Yes
17	60	Post	PFD	No	Yes	Yes	Yes	Yes
18	67A	Post	PFD	Yes	Yes	Yes	N.A.	No
19	67B	Post	PFD	Yes	No	Yes	N.A.	No

[1]Arranged by age.
[2]Letters are added for patients exhibiting identical age.
[3]Perimenopausal women.
PFD, pelvic floor disorders (uterine/vaginal prolapse with or without urinary incontinence); POF, premature ovarian failure; pre, premenopause; post, postmenopause; N.A., not available.
(*Source:* Data adapted from Bukovsky and Virant-Klun, 2007 with permission, © Informa UK Ltd., and Bukovsky and Caudle, 2012 © Antonin Bukovsky.)

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Clinical Approaches for the Treatment of Ovarian Infertility

A Lack of Uncommitted Granulosa Cell Nests Causes a Degeneration of the Available Germ Cells

When such nests are not available, the remaining germ cells within several days increase to the 50 μm oocyte size, show profound cytoplasmic ZP expression, and degenerate, either within the ovarian medullary vessels (Figure 4.4I, and Figure 4.12A–D and F) or elsewhere (Figure 4.12E). Figure 4.12G shows the ZP expression limited to the oocyte surface in a normal secondary follicle.

The availability of transplanted germ cells in adult human ovaries lacking granulosa cell nests may not be sufficient for follicular renewal (Bukovsky, 2011c). Unlike mice, adult women with ovarian failure do not exhibit uncommitted granulosa cell nests (Bukovsky and Virant-Klun, 2007; Virant-Klun et al., 2011b) compared to women with follicular renewal (Bukovsky et al., 1995a, 2004). Hence, without available uncommitted granulosa cell nests, attempts to produce new primordial follicles by transplantation of autologous germ cells will not be successful (Bukovsky, 2011c).

Restoration of the OSC Niche after Chemotherapy

We maintain that ovarian infertility is caused by alteration of the OSC niche normally consisting of vascular pericytes, immune-system-related cells sensitive to chemotherapy (monocyte-derived cells and particularly CD8+ T cells), and autonomic innervation (Bukovsky, 2011a,b). In cancer patients, it should be possible to collect BM or circulating white blood cells prior to chemotherapy, and return them thereafter back to the patient to restore the OSC niche (Bukovsky, 2005a, 2011a,b).

Oogenesis *In Vitro*

The Bukovsky et al. (2005b) study for the first time demonstrated that cultured OSC exhibit neo-oogenesis *in vitro*. The neo-oogenesis was detected regardless of the advanced patient's age or existing premature ovarian failure (POF), except for the ovaries not exhibiting the presence of OSC *in vivo* (Bukovsky and Virant-Klun, 2007). The fertilization of mature oocytes developed *in vitro* requires intracytoplasmic sperm injection, since eggs developed *in vitro* do not express surface ZP proteins (see Figure 4.13A) – required for sperm affinity (van Duin et al., 1994). It has been shown that intracytoplasmic sperm injection into zona-free human oocytes results in normal fertilization and blastocyst development (Ding et al., 1999) and oocyte morphology does not affect the fertilization rate, embryo quality, and implantation rate after intracytoplasmic sperm injection (Balaban et al., 1979). Moreover, oocytes developing *in vitro* may utilize a phylogenetically ancient mechanism, known from *Drosophila* ovaries (Senger et al., 2011). They divide in order to produce several satellite (nurse) cells, which are exploited to provide additional organelles required for the oocyte growth, that are provided *in vivo* by granulosa cells injecting the ooplasm and forming a Balbiani body (Bukovsky et al., 2004).

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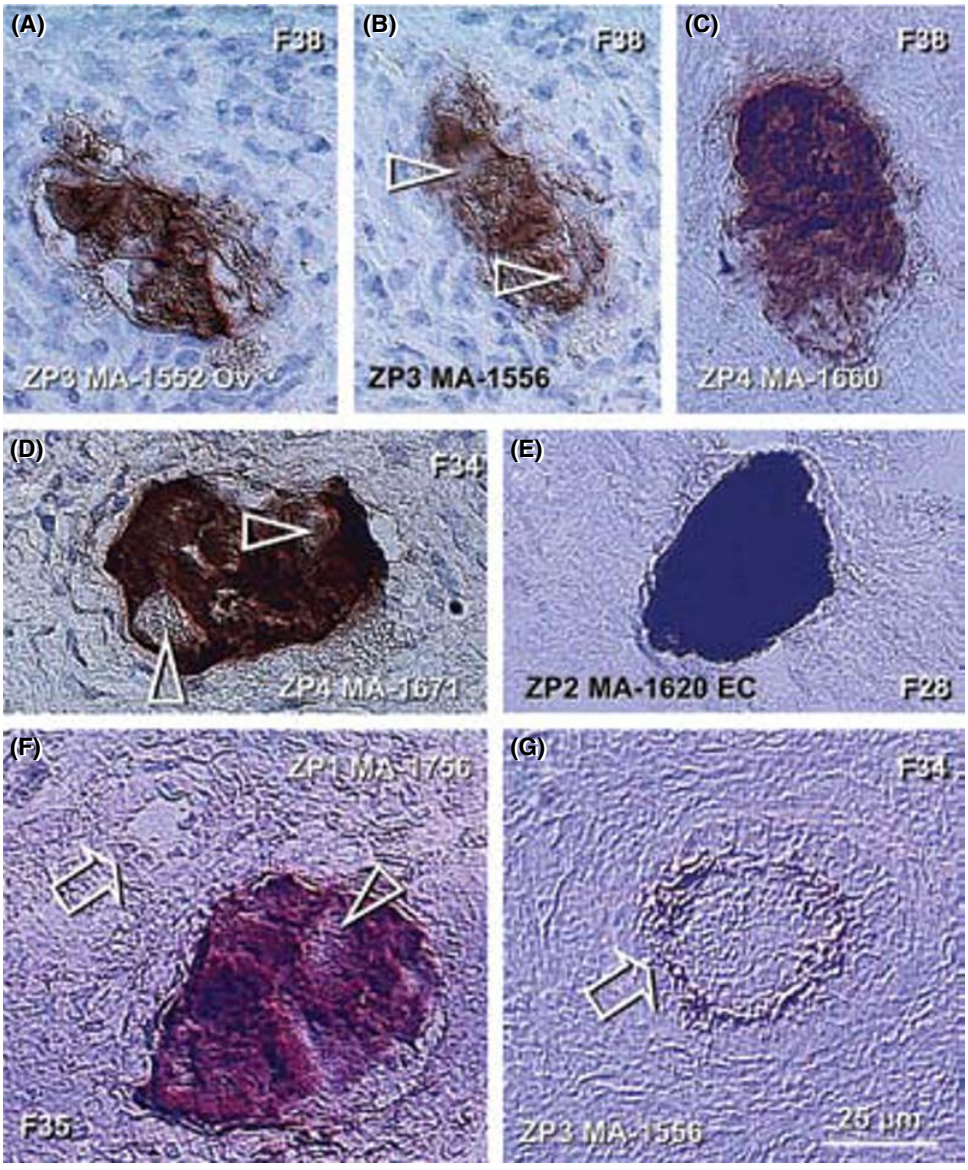


Figure 4.12. Intravascular degenerating human oocytes. Degenerating oocytes in venules of ovarian medulla (**A–D** and **F**) and uterine endocervical stroma **(E)** expressing ZP proteins identified by MAb clones against ZP3, ZP4, and ZP 2 (Bukovsky et al., 2008d) as indicated in panels (kindly provided by Dr Satish K Gupta). Arrowheads indicate unstained oocyte nuclei, arrow in **(F)** shows an arteriole, arrow in **(G)** shows ZP expression at the oocyte surface in a normal secondary follicle. F28–F38 indicates patient's age. ((A and C) Adapted from Bukovsky et al., 2008c with permission, © Elsevier, remainder reprinted from Bukovsky and Caudle, 2012, © Antonin Bukovsky.)

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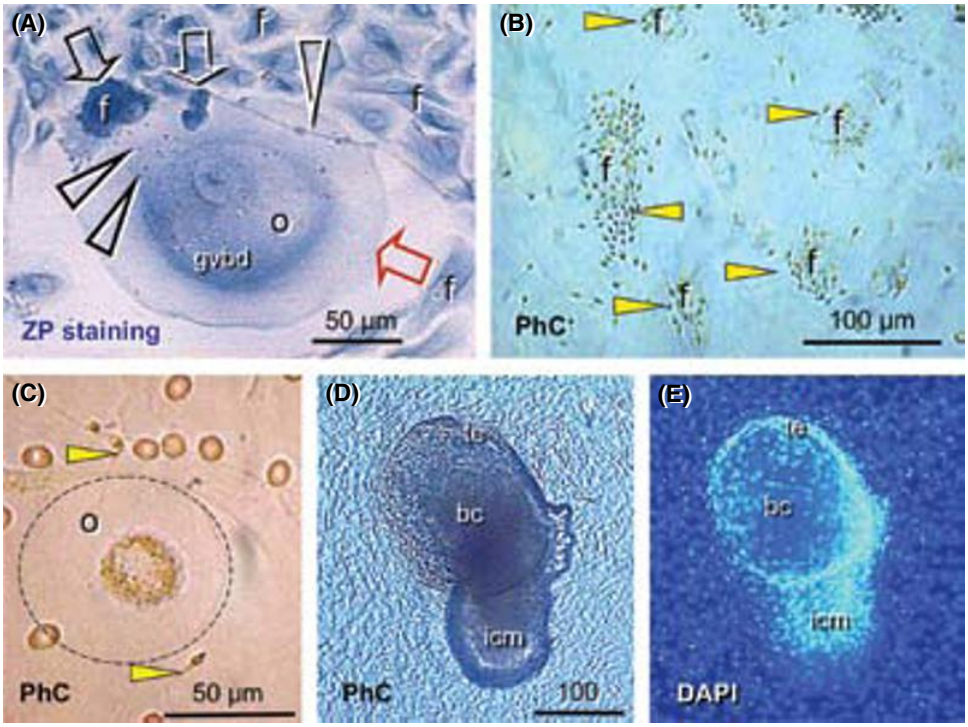


Figure 4.13. Oocyte and parthenote development *in vitro*. **(A)** The oocyte (o) development in OSC culture is accompanied by satellite (black arrow) and neuronal (white arrow) cells. Black arrowheads indicate organelles moving from the satellite cell into the oocyte and white arrowhead indicates neuronal extension. Note ZP staining of fibro-epithelial cells (f) but no expression of ZP proteins at the oocyte surface (red arrowhead). Sperm associated (arrowheads) with fibroepithelial cells **(B)** but not with the oocytes **(C)**. (**D** and **E**) The parthenote shows a trophectoderm (te), blastocoel (bc), and inner cell mass (icm). (A) Staining for ZP proteins (B, C and D) are live cultures in phase contrast (PhC); (E) DAPI staining of the fixed culture. (Adapted with permission from A. Bukovsky, "Ovarian Stem Cells and Mammalian Neo-Oogenesis," *Microsc. Microanal*. **14** (Suppl 2) (2008): 1474–1475 © Cambridge University Press, and A. Bukovsky and M. R. Caudle, "Immunoregulation of Follicular Renewal, Selection, POF, and Menopause *in Vivo*, Vs. Neo-Oogenesis *in Vitro*, POF and Ovarian Infertility Treatment, and a Clinical Trial," *Reprod. Biol. Endocrinol*. **10**, no. 1 (2012): 97. http://www.rbej.com/content/10/1/97 © Antonin Bukovsky.)

Developmental Potential of OSC In Vitro

The alteration of ovarian function and the onset of the menopause are associated with the changes in ovarian immunoregulation (Bukovsky, 2011a). Yet, in OSC cultures, such immunoregulation is absent and the neo-oogenesis is renewed, including cultures from POF and postmenopausal ovaries exhibiting a presence of OSC (Table 4.2). In addition, our observations also indicate that OSC show a strong binding of natural autoantibodies *in vivo* (see Figure 4.8E), which are absent in OSC cultures. This suggests that the OSC have the ability to differentiate into oocytes if *in vivo* immunoregulation is absent.

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Cell Types Developing from Omnipotent OSC

After observations in adult and fetal human ovaries, we asked ourselves if a similar potential for OSC could be demonstrated *in vitro*. Our observations show that oocytes, fibroblasts, and epithelial-type cells can develop spontaneously, as well as neuronal cells (Bukovsky et al., 2005b).

Culture Conditions and Techniques

The surface of intact ovaries is gently scraped in an aseptic laminar flow hood with a sterile stainless steel surgery knife blade No. 21 (Becton Dickinson, AcuteCare, Franklin Lakes, NJ). This procedure is selected with the intention to include OSC and some adjacent TA and ovarian stromal cells.

The cells are collected into sterile petri dishes containing tissue culture medium supplemented with heat-inactivated 20% fetal bovine serum (FBS; Gibco/BRL, Grand Island, NY) and antibiotics (50 µg/mL gentamycin, 100 U/mL penicillin, and 100 µg/mL streptomycin). The tissue culture media utilized is either Dulbecco's modified Eagle's medium (DMEM) containing 25 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES), 4500 mg/L glucose, and phenol red (DMEM-HG; with estrogenic stimuli) or DMEM/Ham's F12, phenol red free (DMEM/F12; without estrogenic stimuli). No other treatment is imposed during the culture.

The cells are spun down (1000 × *g*, 5 min, 24°C), diluted in 0.75–1.5 mL of supplemented media, seeded in either six or three wells of a 24-well plate (250–350 µL per well) (Fisher Scientific, Pittsburgh, PA), and cultured in a humidified atmosphere with 5% CO_2 at 37°C. The number of wells is chosen according to the size of the ovaries. Cells collected from larger ovaries are seeded into six wells, and those from small ovaries are seeded into three wells. All ovaries involved in the experiment were anovulatory, and no CL was detected. The culture medium was changed once after 24 h. This left only adherent (viable) cells in culture, and eliminated nonadherent cells and the majority of contaminating erythrocytes. The cell cultures were monitored daily by phase-contrast microscopy and live cells evaluated by immunohistochemistry after 5–6 days of the initial seeding. Viability of cells was apparent from their active movement, changes in shape, and movement of their nuclei. The number of adherent cells in a single well of a 24-well plate ranged between ~100 and 1000 during the late culture period (day 5 or 6).

Estrogens are Essential for the Neo-Oogenesis In Vitro

Ovarian stem cell cultures maintained in DMEM-HG with estrogenic stimuli (phenol red) showed large cells exhibiting the phenotype of oocytes on day 5. These cells reached 100–180 µm in diameter and showed a centrally located germinal vesicle break-down with nucleus and nucleolus (see Figure 4.13A).

In contrast, utilization of DMEM/F12 without estrogenic stimuli in another culture from the same patients produced no large cells exhibiting an oocyte phenotype on day 5. However, 12-day cultures of ovarian cells showed the presence of oocytes. It is thought that natural estrogenic stimulation is provided by ovarian stromal cells which accompany OSC in culture.

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It was found that stromal tissue from human ovaries produces progesterone, androgens, and estrogens *in vitro* (McNatty et al., 1979). In addition, ovarian stromal cells produce high levels of androgen *in vitro*, similar to that of the thecal cells (McNatty et al., 1980). These androgens can be converted into estrogens by fibroblasts (Nelson and Bulun, 2001) present in OSC cultures (Bukovsky et al., 2005b). The OSC are also capable of secreting hCG (Blaustein, 1982) and steroid hormones, including estrogens (Auersperg et al., 2001). Hence the hormonal conditions required for transformation of OSC into oocytes *in vivo* (Table 4.1) also can be achieved *in vitro*. The OSC cultures without estrogenic stimuli showed the presence of granulosa type cells on day 5 (Bukovsky et al., 2005b). It is useful to mix the 5-day OSC cultures with and without estrogenic stimuli from the same patient in order to accelerate the oocyte maturation. The granulosa cells from culture without estrogenic stimulation may provide the Balbiani body (Bukovsky et al., 2004) and contribute to the expression of ZP proteins by developing oocytes (Martinez et al., 1996). Since ZP proteins are sperm ligands (Prasad et al., 2000), their expression by developing oocytes *in vitro* may preclude the need for utilization of intracytoplasmic sperm injection (ICSI) for their fertilization (see below). *In vivo*, new granulosa cells develop in functional ovaries from OSC during the midfollicular phase, when the estrogenic stimuli and LH levels are low, and new oocytes are formed during the periovulatory period, when the estrogenic stimuli and LH levels are high (Bukovsky et al., 1995a, 2004). It requires twice as long for cultures initially lacking estrogen to reach a level of estrogens produced by ovarian stromal cells compared to media with a sufficient exogenous estrogen (phenol red) level from the beginning of culture.

Development of Oocytes and Parthenogenetic Embryos In Vitro

Primary OSC Cultures

The advanced development of oocytes (o, Figure 4.13A) *in vitro* with germinal vesicle breakdown (gvbd) is accompanied by fibro-epithelial (f) satellite cells (black arrow) substituting a granulose-cell-derived Balbiani body to provide additional organelles (black arrowheads) needed by the developing egg. To increase the volume of the oocyte, the human granulosa cell microvilli penetrate deep into the ooplasm of developing oocytes *in vivo* and supply a variety of organelles such as Golgi vesicles, endoplasmic reticulum membranes, and nascent forms of smooth endoplasmic reticulum (Motta et al., 1994).

In vitro, neural-type cells are also involved (white arrow, Figure 4.13A) with an extension (white arrowhead) over the developing oocyte. Note strong expression of ZP proteins in fibro-epithelial cells on the cell surface but a lack of ZP expression at the oocyte surface (red arrow). The presence of sperm causes their association (arrowheads, Figure 4.13B) with fibro-epithelial cells (f), but not with the oocytes (Figure 4.13C), due to the lack of surface ZP expression (red arrow, Figure 4.13A).

Eventually, some oocytes differentiate into parthenotes with a trophectoderm (te, Figure 4.13D and E), blastocoel (bc) and inner cell mass (icm) (Bukovsky, 2008), similar to some oocytes in *in vitro* fertilization (IVF) cultures (Santos et al., 2003). This indicates that the OSC-derived oocytes developed *in vitro* are functionally similar to the mature follicular oocytes. As noted, the lack of ZP expression at the surface of oocytes developed *in vitro*, will mandate utilization of ICSI for their fertilization.

Images from time-lapse photography show that early developing oocytes (o, Figure 4.14A) are low in optically dense cytoplasmic organelles (white open arrow).

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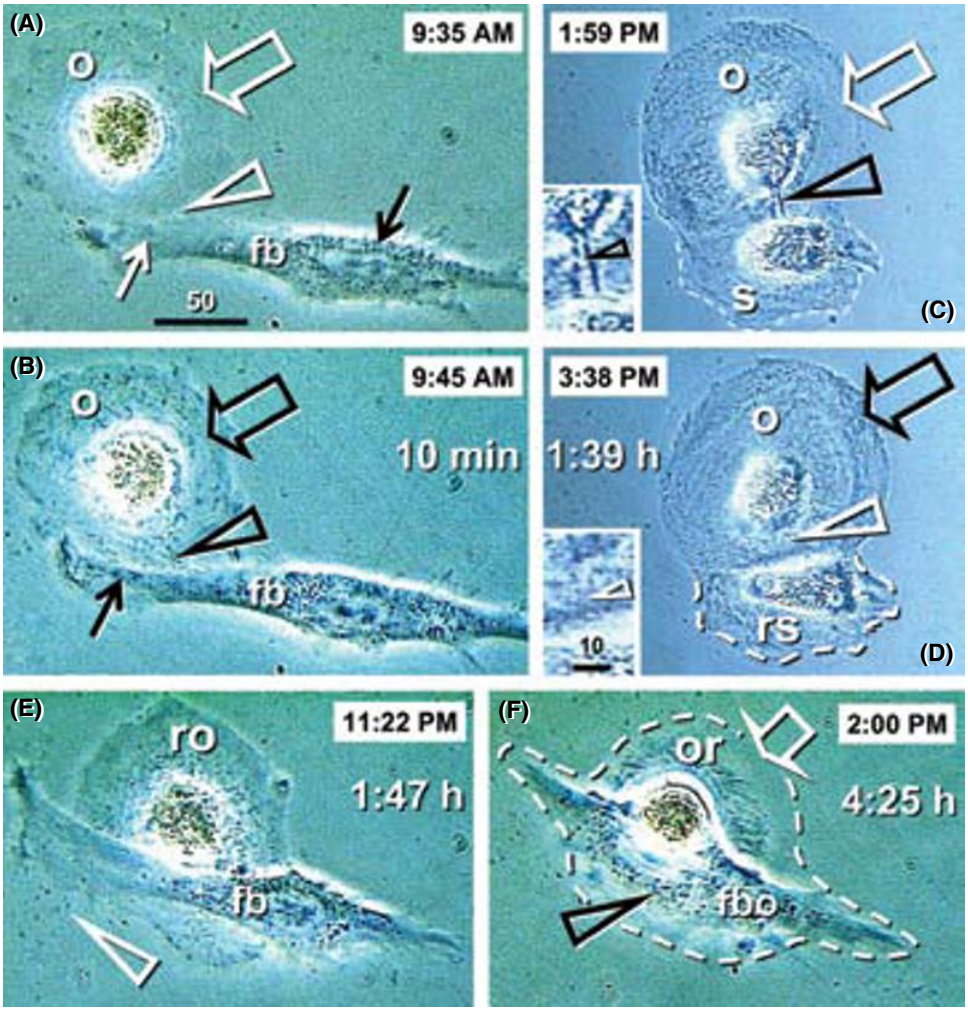


Figure 4.14. *In vitro* developing oocytes supplied with organelles from fibroblasts resulting in fibro-oocyte hybrids, or by satellite cells produced by the oocytes and exploited for the progression of the oocyte growth. Images from time-lapse photography (time in hours:minutes). **(A)** *In vitro* developing oocytes (o) deficient in organelles (white arrow) can be joined (arrowhead) by a fibroblast (fb and white arrowhead). **(B)** The optically dense organelles are supplied to the oocyte. **(C)** Alternatively, the oocyte is supplied by adjacent satellite cell (s) with an extended tube (black arrowhead; see detail in inset). **(D)** When completed, the tube disappears (inset) and the satellite is regressing (rs). **(E)** In contrast, the fibroblast moves above the oocyte and releases organelles out (white arrowhead) of the regressing oocyte (ro). **(F)** Subsequently, a fibro-oocyte (fbo) hybrid is formed exhibiting oocyte remnants (ro). Bar in (A) for (A–F). (Panels A–D adapted from A. Bukovsky, "Ovarian Stem Cell Niche and Follicular Renewal in Mammals," *Anat. Rec*. (Hoboken.) **294**, no. 8 (2011): 1284–1306. with permission, © Wiley-Liss, Inc, and complemented with E and F from A. Bukovsky and M. R. Caudle, "Immunoregulation of Follicular Renewal, Selection, POF, and Menopause *in Vivo*, Vs. Neo-Oogenesis *in Vitro*, POF and Ovarian Infertility Treatment, and a Clinical Trial," *Reprod. Biol. Endocrinol*. **10**, no. 1 (2012): 97. http://www.rbej.com/content/10/1/97. © Antonin Bukovsky.)

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They can be joined (arrowhead) by fibroblast-type cells (fb), which provide additional organelles. Such fibroblast-type cells initially show optically dense organelles (black solid arrow) close to the nucleus, but not in the arm extended toward the oocyte (white solid arrow). Within 10 min (Figure 4.14B), however, optically dense organelles are apparent in the extended arm (solid black arrow), within adjacent oocyte cytoplasm (black arrowhead), and in distant oocyte regions (open black arrow).

Alternatively, developing oocytes (o, Figure 4.14C) deficient in cytoplasmic organelles (white arrow) are supplied by satellite cells (s), that is, nurse cells, which are produced by the oocytes themselves (see below). The satellite cell shows an extended tube (black arrowhead; see also detail in the inset) providing the cytoplasm with organelles for the oocyte. Figure 4.14D (1 h, 39 min from Figure 4.14C) shows that when such a process is completed, the oocyte exhibits an enhanced content of the optically dense organelles (black arrow, Figure 4.14D) and the tube draining the satellite cell disappears (white arrowhead – see detail in the inset). The satellite cell size is reduced (dashed line) and the content is altered (compare with Figure 4.14C).

The fate of oocytes accompanied by fibroblasts may, however, be different. Figure 4.14E (1 h, 47 min from Figure 4.14A) shows that the fibroblast moves above the oocyte and releases the organelles out (white arrowhead) of the regressing oocyte (ro). Figure 4.14F (4 h, 25 min) shows fusion of the fibroblast (rich in organelles – black arrowhead) with a structure showing oocyte remnants (or) lacking organelles (white arrow), a so-called fibro-oocyte (fbo) hybrid.

Figure 4.15A shows an early stage after oocyte (o) division producing the satellite (s) cell. Note a similar content of cytoplasmic organelles; asterisks indicate former (regressing) satellites. Figure 4.15B indicates that oocyte growth is accompanied by a regressing satellite (rs) exhibiting nuclear alteration and vacuolization in the perinuclear space (arrowhead). Note also depletion of cytoplasmic organelles compared to the satellite in Figure 4.15A. Figure 4.15C shows progressive oocyte growth and its separation (arrowhead) from the satellite cell remnants (sr). A large isolated oocyte (Figure 4.15D) exhibits a germinal vesicle (gv) and a thick ZP cytoplasmic membrane (zp), which, however, lacks expression of ZP proteins (see Figure 4.13A).

Secondary OSC Cultures Derived from Frozen Primary Cultures

Primary OSC cultures can be trypsinized and stored frozen for later utilization. Figure 4.16A–C shows images from time-lapse cinematography of oocyte development in secondary OSC culture, beginning 4 h after seeding (F36, 36-year-old woman – for the OSC presence in the ovarian biopsy see Figure 4.18A). An early developing cell (Figure 4.16A, time 1 min, 15 s of cinematography) shows a cytoplasmic tail (arrowhead). At 18 min, 7 s multiple cytoplasmic eruptions (arrowheads) are evident (Figure 4.16B). At 31 min, 14 s the 40-μm oocyte-like cell developed (Figure 4.16C, yellow arrowheads indicate a cell surface, red arrowhead a polar body).

Figure 4.16D–F shows a secondary OSC culture from a 55-year-old postmenopausal women stained for ZP after 3-days of seeding (d3sc ZP). Oocyte precursors can produce, by several divisions, a "chain" of satellite cells. Such a chain is presented in Figure 4.16D. Note that cells in the chain are interconnected by intercellular bridges lacking division by cytoplasmic membranes (open white arrowheads). A membrane with ZP expression is apparent on their surface (open black arrowheads).

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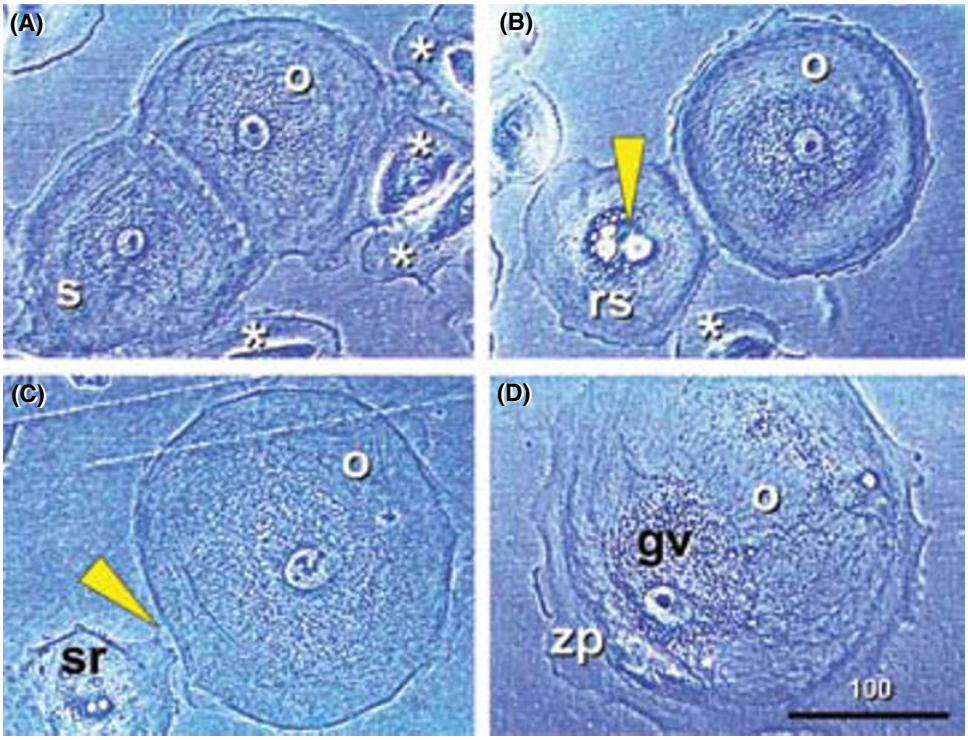


Figure 4.15. Oocyte satellites *in vitro*. **(A)** Early stage after oocyte (o) division producing the satellite (s) cell. Asterisks indicate additional small satellites. **(B)** Oocyte growth and regressing satellite (rs) with vacuolization in the perinuclear space (arrowhead). **(C)** Progressive oocyte growth and its separation (arrowhead) from the satellite remnants (sr). **(D)** Large isolated oocyte exhibits germinal vesicle (gv) and a thick zona pellucida (zp) cytoplasmic membrane. (Adapted from Bukovsky, 2011a with permission, © Wiley-Liss, Inc.)

The last cell in the chain represents a satellite remnant (sr). Regressing satellites (rs) follow, and a large satellite (s) is close to the developing oocyte (o) leading the chain. The developing oocyte shows formation of the cell surface in the bridge (solid yellow arrowhead). An asterisk indicates the leading oocyte nucleus and the red arrowhead a developing polar body. Figure 4.16E shows two "leading" oocyte type cells with surface ZP expression, each of which shows expulsion of two polar bodies (red arrowheads), retention of two pronuclei (asterisks), and surface ZP expression (open black arrowheads). Yellow arrowheads indicate a line of separation from a regressing satellite (rs) and satellite remnants (sr) lacking surface ZP. A large oocyte (120 μm) with a nucleus (asterisk), germinal vesicle (gv), and faint surface ZP expression (open black arrowhead) is shown in Figure 4.16F.

These observations resemble the formation and fate of *Drosophila melanogaster* ovarian cysts (egg chambers). In *Drosophila*, ovarian cysts are produced through a series of synchronous mitotic divisions during which cytokinesis is not completed. After completion of four mitotic divisions, all 16 cells enter the premeiotic S phase.

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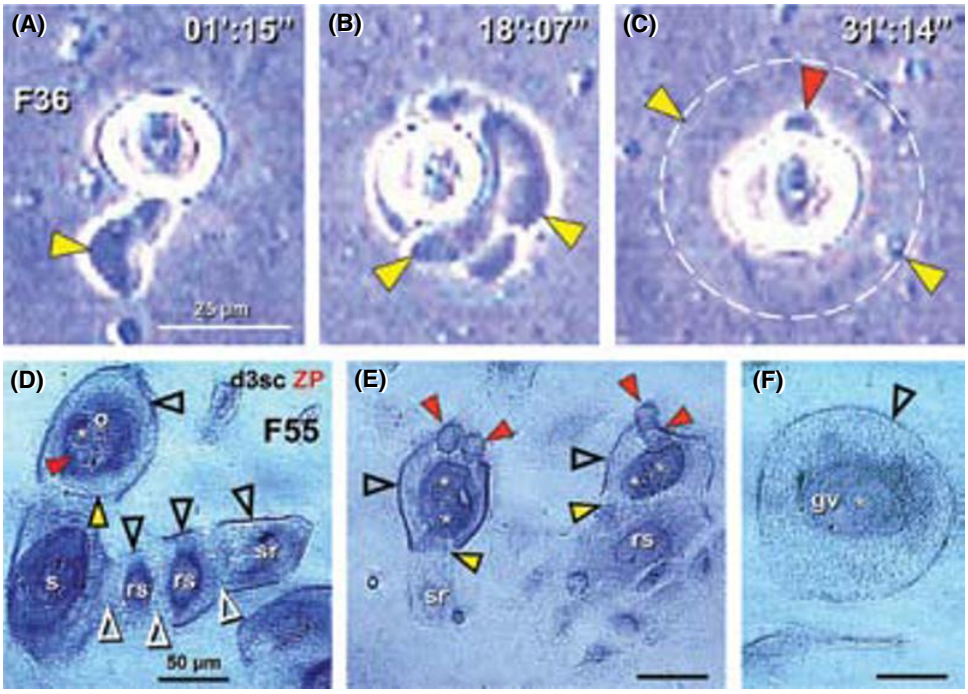


Figure 4.16. Differentiation of early oocytes and behavior of oocytes in secondary OSC cultures. (A–C) Time-lapse cinematography of oocyte development in secondary OSC culture. **(A)** Early developing cell with a cytoplasmic tail (arrowhead). **(B)** Multiple cytoplasmic eruptions (arrowheads). **(C)** Development of the 50 µm oocyte-like cell (yellow arrowheads indicate cell surface, red arrowhead a polar body). Time in minutes:seconds. (D–F) Day 3 secondary OSC culture stained for ZP (d3sc ZP) – 55-year-old postmenopausal women 3 days after seeding. **(D)** Oocyte (o) with a "chain" of satellite cell (s), regressing satellites (rs), and satellite remnants (sr) interconnected by intercellular bridges (open white arrowheads) and surface ZP expression (open black arrowheads). The "leading" oocyte shows formation of the cell surface in the bridge (solid yellow arrowhead). Asterisk indicates cell nucleus and red arrowhead developing polar body. **(E)** Two "leading" oocyte type cells with surface ZP expression, each of which shows expulsion of two polar bodies (red arrowheads) and retention of two pronuclei (asterisks). Yellow arrowheads indicate a line of separation from remnants of the "satellite" cells lacking surface ZP. **(F)** Large oocyte (140 µm) with surface ZP expression (arrowhead), nucleus (asterisk), and germinal vesicle (gv). (Reprinted from Bukovsky and Caudle, 2012 © Antonin Bukovsky.)

However, only the true oocyte at the center of the syncytium remains in meiosis and continues to develop. The remaining 15 cells lose their meiotic features and develop as polyploid nurse cells. In contrast to nurse cells, the oocyte remains in prophase of meiosis I until it proceeds to the first meiotic metaphase late in oogenesis. The oocyte fate is dependent on the unique presence of the *missing oocyte* (mio) gene, which is required for the maintenance of the meiotic cycle and oocyte identity. The *mio* gene associates with the conserved nucleoporin seh1, which influences the oocyte development. In *Drosophila* females lacking seh1 about 20% of egg chambers develop with 16 polyploid nurse cells and no oocyte (for data and review see McNatty et al.,

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1979). Oocyte syncytia (ovarian cysts) also develop in mouse and human fetal ovaries (Peters and McNatty, 1980; Pepling and A. C. Spradling, 1998, 2001).

Ovarian Stem Cell Cultures Versus In Vivo Oocyte and Follicular Development in Mammals

The behavior of developing oocytes in human OSC cultures resembles their behavior in ovarian cysts, from adult *Drosophila* to mouse and human fetuses. In other words, the oocyte development from human OSC *in vitro* may utilize the mio gene and nucleoporin seh1 mechanisms to produce their own nurse (satellite) cells, thereby preventing them from developing into additional oocytes. The oocytes developed *in vitro* are functionally competent since some continue to differentiate into parthenotic embryos (Figure 4.13D and E), like some cultured follicular oocytes.

In adult invertebrates and lower vertebrates (fish, amphibia), all periodically developed oocytes are ovulated. In higher vertebrates, however, each oocyte lies in the ovarian follicle, where its further development is dependent on the activity of granulosa cells providing additional organelles required by growing oocytes (Balbiani body). The activity of granulosa cells is regulated by the follicular TCS niche (Bukovsky, 2011a). In addition, the selection of dominant follicle(s) ensures the number of ovulations in a species-specific manner, which is optimal for the maintenance of the certain number of fetuses during pregnancy. Therefore, once granulosa cells are available during ontogeny, the development of oocytes into ovarian cysts with nurse cells is switched into follicular development. *In vitro*, the follicles with granulosa cells and the TCS niche are absent, so oocytes developing from OSC utilize the developmentally primitive mechanism of ovarian cysts formed by the oocyte and its nurse cells.

A Comparison of the Primary Versus Secondary OSC Cultures

Our observations indicate that the behaviors of primary and secondary OSC cultures are different. The primary OSC cultures derived from original OSC contain ovarian stromal cells, which contribute to oocyte growth and development. Such oocytes show the ability to develop into parthenotes, similar to some follicular oocytes during standard IVF techniques. This suggests that oocytes developed *in vitro* are in principle similar to the cultured follicular oocytes. The lack of expression of ZP proteins at the developed oocyte surface will, however, require ICSI for their fertilization, unless they are stimulated to express ZP by granulosa cells from primary OSC cultures without estrogenic stimulation. Secondary OSC cultures derived from frozen primary cultures utilize a developmentally ancient mechanism known from the *Drosophila* ovaries. They produce their own satellites for the oocyte maturation and mature oocytes express ZP proteins. This may be useful for standard oocyte fertilization. Yet, the targeted ICSI may still be found useful for the fertilization of the selected oocytes.

Development of Embryonic Stem Cells from Parthenotes Developed In Vitro

The DAZL protein, expressed in human oocytes, preimplantation embryos, and ESCs (Cauffman et al., 2005), is strongly expressed in early (4-cell stage) parthenotes (Figure 4.17A-C). Resulting morulae (Figure 4.17D and E, no immunohistochemistry)

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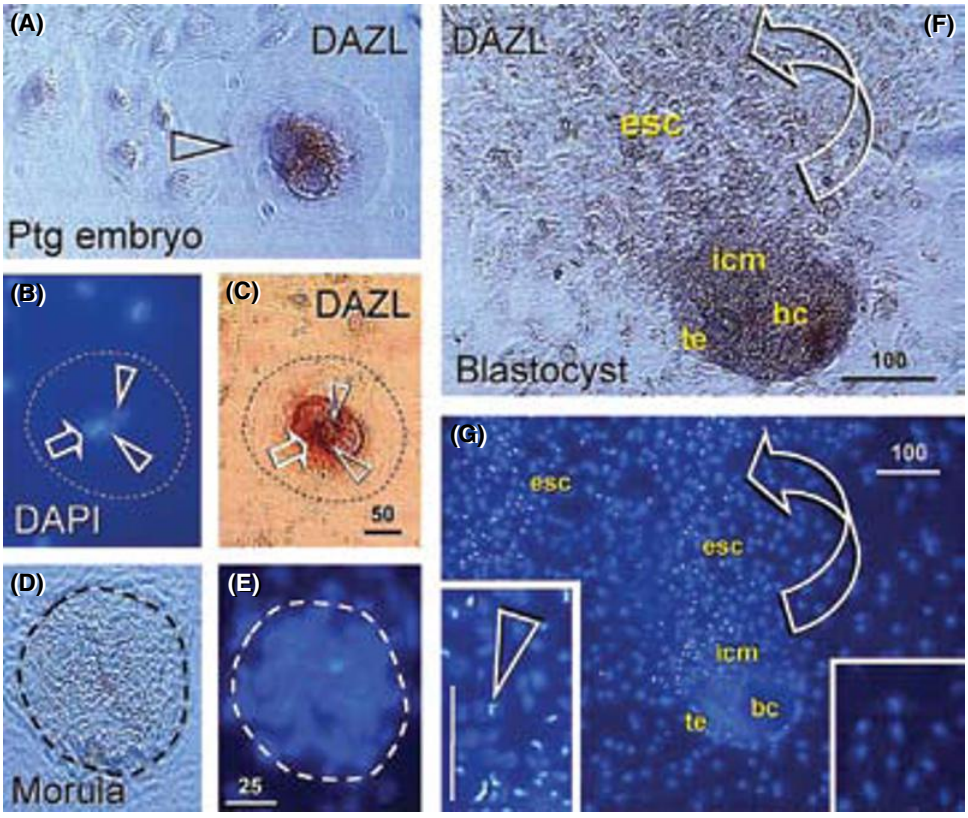


Figure 4.17. Parthenotes in OSC cultures. (**A–C**) Four-cell embryo. (**D** and **E**) Morula. (**F** and **G**) Blastocyst consisting of blastocoel (bc), trophectoderm (te), and inner cell mass (icm) releasing (arched arrow) DAZL + embryonic stem cells (esc). Scale in (C) for (A–C). (A, C and F) DAZL staining; (B, E and G) DAPI. (Adapted from A. Bukovsky, "Ovarian Stem Cells and Mammalian Neo-Oogenesis," *Microsc. Microanal.* **14**(Suppl 2) (2008): 1474–1475 with permission, © Cambridge University Press.)

may develop into blastocysts producing DAZL$^+$ ESCs from the embryonic inner cell mass into the culture (arched arrow and esc, Figure 4.17 F). The inner cell mass and the released ESCs are mitotically active compared to the other cells lacking DAZL expression and by pronounced 4′,6-diamidino-2-phenylindole (DAPI) staining (arrowhead in left vs. no mitoses in the right inset, Figure 4.17G) (Bukovsky, 2008).

These observations indicate that OSC cultures could be sources of eggs for the treatment of female ovarian infertility, and can also produce ESCs for autologous regenerative medicine.

Development of Oocytes from Postmenopausal and POF Ovaries

Ovarian cultures derived from atrophic postmenopausal ovaries are capable of producing new oocytes, some of which differentiate into parthenogenetic morulae,

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suggesting functional potential. Apparently, the OSC may differentiate and proliferate both during advanced postmenopause (Bukovsky, 2005b, 2006a) and in young women with POF (Bukovsky and Virant-Klun, 2007; Virant-Klun et al., 2008, 2009; Virant-Klun and Skutella, 2010).

Cultures from Ovaries Lacking OSC Fail to Produce Oocytes

The presence of OSC in ovaries is essential for the development of oocytes in ovarian cultures. Figure 4.18A shows CK + OSC (arrowhead) in the ovary of a 36-year-old woman (F36). However, perimenopausal ovaries (around 50 years of age, ages 49–52 in our observations – see below) often lacked OSC (Figure 4.18B), as well as some ovaries (one of three cases) of women with POF (Bukovsky and Virant-Klun, 2007). On the other hand, women in advanced menopause exhibited OSC regularly (Bukovsky,

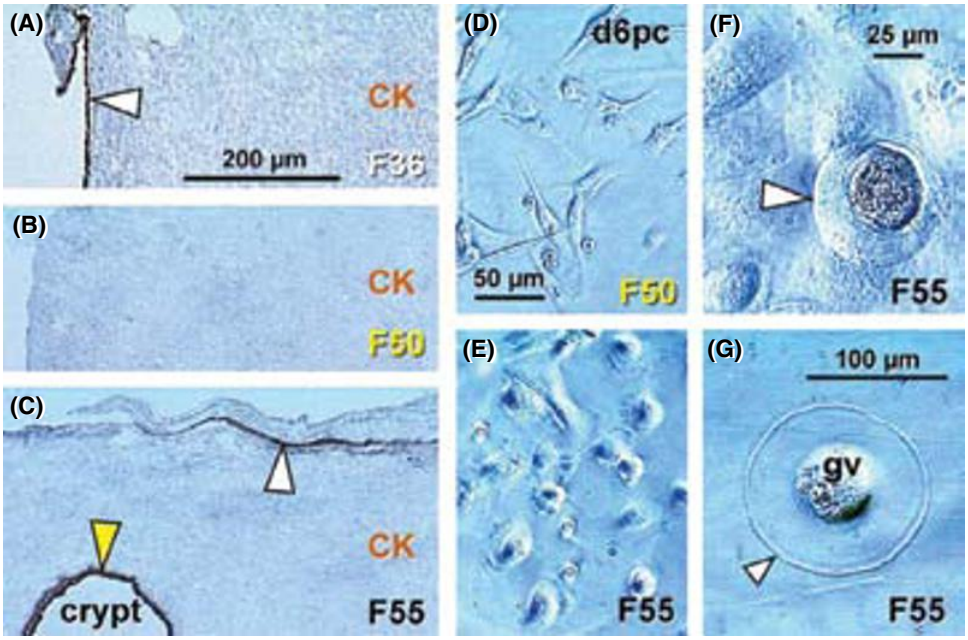


Figure 4.18. Influence of the presence versus absence of OSC in the ovary on ovarian cultures. Occurrence of OSC in pre- and postmenopausal ovaries (A–C): note a lack of adult primordial follicles in all samples. **(A)** Staining for CK with hematoxylin counterstain shows CK + OSC (arrowhead) in the ovary of 36-year-old woman (F36). **(B)** Ovary of 50-year-old woman without OSC. **(C)** Activity of the TA (white arrowhead) with OSC in 55-year-old ovary. The OSC is also present in the cortical crypt (yellow arrowhead) in the lower cortex. (D–G) Phase contrast from live day 6 primary ovarian cultures (d6pc). **(D)** Ovarian culture of a 50-year-old female shows only narrow fibroblasts (note lack of OSC in (B)). **(E)** Cluster of epithelial-type cells in culture from a 55-year-old female (note OSC in (C)). **(F)** Detail from another cluster of epithelial cells shows small (30 µm) round oocyte type cell (arrowhead) with a prominent nucleus. **(G)** Large (120 µm) round oocyte type cell with germinal vesicle (gv) and thickened plasma membrane (arrowhead). (Reprinted from Bukovsky, 2011a with permission, © Wiley-Liss, Inc.)

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2006a). Figure 4.18C shows the ovary of a 55-year-old women exhibiting CK + OSC on the ovarian surface (white arrowhead). Ovarian stem cells are also present in the cortical crypt in the lower cortex (yellow arrowhead). Ovarian culture of the 50-year old (Figure 4.18D) showed only narrow fibroblasts. A cluster of epithelial type cells was observed in culture from the 55-year-old postmenopausal female (Figure 4.18E). Detail from another cluster of epithelial cells showed a 30 μm round oocyte-type cell (arrowhead, Figure 4.18F) with a prominent nucleus. A large (120 μm) round oocyte-type cell with a germinal vesicle (gv) and thickened plasma membrane (arrowhead) was also detected (Figure 4.18G).

These observations show that ovaries lacking OSC are unable to produce oocytes in ovarian cultures. The presence of OSC is essential for *in vitro* oocyte development. The OSC usually persist in postmenopausal ovaries, serving as a source for development of epithelial cell clusters in ovarian cultures. Some of these OSC-derived epithelial cells appear to be precursors of gradually differentiating oocytes. Spontaneous development of parthenogenetic embryos in ovarian cultures, which can also be observed during oocyte handling in standard IVF (Santos et al., 2003), indicates the functional similarity of *in vitro* developed eggs with cultured follicular oocytes. Development of oocytes from OSC derived from ovaries with no naturally present follicles and oocytes (POF and postmenopausal women; Bukovsky and Virant-Klun, 2007) confirms the ability of OSC to produce new eggs in the absence of a OSC niche being present during neo-oogenesis *in vivo*.

The observations reported above and arranged according to the patient's age are summarized in Table 4.2. There were three POF cases, out of which two cases (1 and 3) exhibited OSC and exhibited oogenesis *in vitro*. No oocytes in culture were observed in case 2 lacking OSC in their ovaries. Three perimenopausal women (ages 49–52) lacked OSC in the ovaries and produced no oocytes *in vitro*. It is possible that during the perimenopausal period immune physiology causes an alteration of the OSC differentiation from TA precursors.

Neo-Oogenesis In Vitro Versus Conventional IVF

Previous *in vitro* observations of other investigators, including IVF practices, dealt with established follicular oocytes collected from mature ovarian follicles. Follicular oocytes show a uniform pattern, since they have already accumulated organelles supplied by granulosa cells (Balbiani body) and a thick ZP membrane. Cultures of follicular oocytes do not contain satellite cells, which appear to be involved in the stimulation of oocyte development in OSC cultures (Bukovsky et al., 2005b; Bukovsky, 2008). In contrast, oocytes evolving *de novo* in ovarian cultures show a different morphology during sequential stages of development which cannot be observed in follicular oocyte cultures.

Conventional IVF with autologous follicular oocytes is usually not used in women over 40 years of age, due to a high failure rate of implantation as well as the increasing incidence of fetal abnormalities. The live birth rates in IVF under age 31 are 66–74%, and decline thereafter, being 18–27% for ages 41 and 42, and 6–11% for age 43 or higher, reportedly due to the diminished ovarian reserve, since the rate is 60–80% for all recipient ages using donor eggs (Luke et al., 2012). In contrast, fresh oocytes develop *de novo* from OSC in culture regardless of the female age. Therefore, oocytes

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newly developed *in vitro* could be utilized for IVF in women exceeding 40 years of age with improved success rates and theoretically a low incidence of fetal abnormalities versus follicular oocytes collected from ovaries of aging females (see Figure 4.1).

More importantly, conventional IVF and assisted reproduction technique (ART) can never accomplish the provision of genetically related children in women lacking the ability to produce their own follicular oocytes, including young women rendered sterile after chemotherapy associated with oocyte loss and women with POF. Autologous oocytes from OSC in culture have been shown to produce blastocysts after fertilization (Bukovsky et al., 2006a), and therefore could be used in subsequent IVF and ART procedures.

Clinical Trial

This section is an adapted version of "Potential treatment of ovarian infertility" published as part of Bukovsky and Virant-Klun (2007).

Differentiation of Oocytes from OSC In Vitro

Early in 2005, we established primary OSC cultures and observed that OSC have a capacity to differentiate into distinct somatic cell types (epithelial cells, fibroblasts, granulosa, and neural-type cells) and also oocytes (Bukovsky et al., 2005b). The functional capacity of such OSC-derived oocytes is confirmed by their development into parthenogenetic embryos expressing DAZL protein – see Figure 4.17. Although OSC originate from fetal mesothelial cells covering peritoneal cavity, the adult peritoneal mesothelial cells do not have characteristics of stem cells as they persist unchanged in culture (Bukovsky and Virant-Klun, 2007). Therefore, OSC may represent a new type of adult stem cell with unique totipotent features.

Potential Treatment of Ovarian Infertility

The criteria for the utilization of OSC cultures in the treatment of the female ovarian infertility were elaborated (Bukovsky et al., 2006b) as follows.

Suitability of Patients for Clinical Trial

Patients with the diagnosis of premature ovarian failure (POF) may be included in the clinical trial. Optimally, these patients failed to conceive due to a lack of their own functional oocytes during previous standard IVF therapy, or such therapy was impossible due to the lack of oocytes within ovaries, and they are considering new options to have a genetically related child before using donated oocytes. Patients should provide a detailed medical history and available laboratory results for consideration in the trial. Ultrasound or magnetic resonance imaging (MRI) images of ovaries should be carried out, and patients advised to utilize certain hormonal therapies several weeks prior to the procedure.

Prospective patients and their partners should not carry any genetic alterations that can be transmitted to the child. Of particular importance is the exclusion of POF with

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the fragile X premutation (>200 CGG (cytosine-guanine-guanine) trinucleotide repeats of FMR1 gene), since the birth of a child in such women may result in mental retardation of the progeny (Corrigan et al., 2005). Genetic alterations are detected in a proportion of patients with POF, particularly those with primary amenorrhea (Rebar, 2000), and fragile X premutation was detected in 4.8% of patients with POF (Gersak et al., 2003). Therefore, evidence on the lack of a known genetic abnormality should be provided, or the patients tested.

If needed, additional laboratory investigation from blood and urine, as well as imaging procedures would be done after admission. All women considered should have a male partner with normal semen quality. Women with infertile partners (i.e. with azoospermia) should be excluded.

A specialist in gynecology and obstetrics who is familiar with this new technique should explain the therapy of ovarian infertility with cultured OSC to the patient. An interdisciplinary committee for *in vitro* fertilization should evaluate the medical documentation of each patient and her male partner, and approve inclusion into the trial. An institutional review board (IRB) should approve the clinical trial.

Collection of OSC and In Vitro Culture of Oocytes

Ovarian stem cells and small ovarian biopsies are collected during laparoscopy. The OSC and cells collected by scraping of tissue biopsies are cultured for 5–10 days to determine whether or not they can produce oocytes. If oocytes develop, they can be genetically analyzed. In the future, they may be fertilized by classic IVF, or by ICSI with the partner's semen after approval of the Medical Ethics Committee. Embryos, if developed, are cultured to the blastocyst stage, and before transfer into the uterus, evaluated by preimplantation genetic testing. Embryos may be cryopreserved. When a woman is hormonally prepared, at most two normal blastocysts can be transferred into the uterus and supernumerary blastocysts are cryopreserved for a potential later need of the patient. In case of a pregnancy, amniocentesis should be performed for genetic evaluation of the fetus.

Potential Pitfalls

During the clinical trial, the following complications of cultured cells could occur: oocytes could not develop, oocytes could not be appropriate for fertilization, oocytes could not be fertilized, fertilized oocytes could not develop into embryos, or embryos could not be transferred into the uterus because they were genetically abnormal.

Initiation of the First Clinical Trial

Criteria for initiation of a clinical trial were found appropriate and its initiation in the IVF Laboratory, Department of Obstetrics and Gynecology, University Medical Center Ljubljana, approved by the Slovenian Committee for the Medical Ethics. Early in 2006, Dr Antonin Bukovsky (AB) and Dr Irma Virant-Klun (IVK) met in the IVF Laboratory to initiate the trial. The objective was to evaluate whether there are OSC in infertile women with POF, whether they contain putative stem cells, and whether they can develop into oocytes capable of fertilization *in vitro*. After informed consent

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process, three patients aged 30, 38, and 40 years with POF and no naturally present oocytes in their ovaries and with normospermic partners were selected.

Collection of the OSC was performed by Dr Andrej Vogler of the Department of Obstetrics and Gynecology, in collaboration with AB and IVK. The cells were collected during diagnostic laparoscopy by scratching the ovarian surface with scissors (Figure 4.19A) and brush (Figure 4.19B), and ovarian biopsies (Figure 4.19 C and D) were collected from both ovaries. The cells were collected from the scissors and the brush into the culture medium. Before the end of laparoscopy, the ovaries were washed in reverse Trendelenburg position with 37 °C warm saline, and the liquid with cells was collected from the cul-de-sac space. The collected liquid was then spun down and cells in the pellet were dissolved in culture medium. From half of each biopsy and collected OSC, the cell cultures were set up in DMEM/F12 medium with phenol red (weak estrogenic action), supplemented with antibiotics and 20% comprehensively heat-inactivated serum (59 °C, 60 min) of the corresponding patient. Cultures were monitored daily.

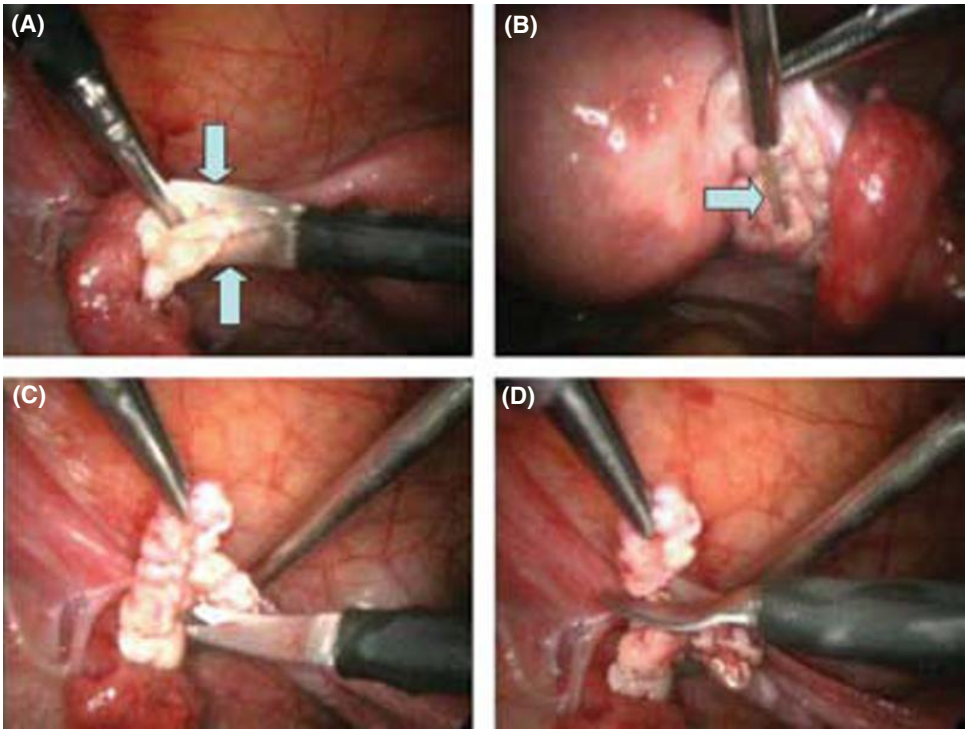


Figure 4.19. Snapshots from the collection of the OSC and ovarian biopsy from POF ovary by a laparoscopy. The OSC are collected by scratching the ovarian surface with scissors (arrows in **A**) and with a brush (arrow in **B**). Ovarian biopsies (**C** and **D**) are collected from each ovary. (Reprinted from A. Bukovsky and M. R. Caudle, "Immunoregulation of Follicular Renewal, Selection, POF, and Menopause *in Vivo*, Vs. Neo-Oogenesis *in Vitro*, POF and Ovarian Infertility Treatment, and a Clinical Trial," *Reprod. Biol. Endocrinol.* **10**, no. 1 (2012): 97. http://www.rbej.com/content/10/1/97, © Antonin Bukovsky.)

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Ovarian cells attached to the bottom of the dish began to differentiate into epithelial and fibroblast cell types, and some of them into oocytes. On day 3 of culture, the initial medium was replaced with *in vitro* maturation medium (Medicult IVM, Copenhagen, Denmark) supplemented with follicle-stimulating hormone (FSH) (75 mIU/mL), hCG (5 IU/mL) and 10% heat-inactivated patient's serum. Prepared male partner's sperm was added several hours later. Embryo-like structures developed in the OSC cultures of two POF women on the next day. They detached spontaneously and were transferred into the wells with standard medium for *in vitro* fertilization, where they developed progressively to the morula-, preblastocyst- and blastocyst-like structures. They were frozen to be later genetically analyzed and transferred into the uterus, if normal. Oocytes developed *in vitro* were analyzed genetically and were shown to express genes related to oocytes and pluripotent stem cells.

The remaining oocytes developed *in vitro* were analyzed genetically and were shown to express genes related to oocytes and pluripotent stem cells.

Material from biopsies was investigated by immunohistochemistry for the presence of OSC and granulosa cells of primordial follicles (cytokeratin expression). No primordial or other follicle types were found. Development of oocytes and embryo-like structures after *in vitro* insemination of cultures correlated with the presence of OSC in the biopsies. In one woman with no OSC in both biopsies no oocytes developed and embryo-like structures were absent after utilization of *in vitro* maturation and sperm (Bukovsky and Virant-Klun, 2007).

Results of this research confirm the presence of OSC in some infertile women with POF, which are capable of developing into oocytes and be fertilized *in vitro*. These observations indicate that adult human ovaries are capable of producing new oocytes for follicular renewal. This fails to occur *in vivo* in POF patients. Ovarian stem cultures offer a new chance for infertile women with POF to have genetically related offspring, and should be investigated further.

Conclusions

Our *in vivo* studies in 1995 and 2004 showed for the first time that the OSC in adult human ovaries are a bipotent source of new ovarian granulosa and germ cells. In 2005, we described similar properties of OSC in ovaries of midpregnancy human fetuses.

Our *in vitro* studies, starting in 2005, showed for the first time that in addition to neo-oogenesis and follicular renewal in human and rat females, human OSC are capable of differentiating into functional oocytes, indicated by their ability to produce parthenogenetic embryos, including OSC from POF and postmenopausal ovaries. Conventional IVF with autologous follicular oocytes is usually not utilized in women exceeding 40 years of age, due to the high failure rate and fetal abnormalities. This is due to the cessation of oocyte and follicular renewal at 38 ± 2 years of age. In contrast, however, functional oocytes develop *de novo* from OSC in culture regardless of age. Therefore, they can be used for IVF in women exceeding 40 years of age, possibly without a risk of high failure rates and high fetal abnormalities.

Most importantly, what conventional IVF and ART cannot accomplish is to provide genetically related children to women lacking the ability to produce their own follicular oocytes, including young women rendered sterile from chemotherapy associated with

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oocyte loss, and women with POF. This may be solved by clinical use of autologous oocytes from OSC in culture in the future.

Ovarian stem cells are present in premenopausal and postmenopausal women, with exception of the narrow perimenopausal period (ages 49–52). This may be caused by the overall alteration of tissue homeostasis, which also causes sharply increased risk of acute myocardial infarction, hypertension, increased body weight, vascular disorders, breast cancer, and brain disorders.

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Chapter 5

Oct4-EGFP Transgenic Pigs as a New Tool for Visualization of Pluripotent and Reprogrammed Cells

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Introduction

Pluripotent cells are unique because they can self-renew for long periods of time and possess the ability to differentiate into any cell of an organism. These properties render these cells promising candidates for cell therapies of degenerative diseases. Various cells, including cardiomyocytes, neurons or insulin-producing cells have been successfully differentiated *in vitro* from pluripotent cells. These cells can be used in cell replacement therapies and first clinical trials are already underway. These cells can also be modified genetically and used for the production of transgenic animals in biomedicine. However, prior to clinical application in humans, pluripotent cells need to be carefully evaluated in a suitable large animal model with respect to survival, functional integration, microbial safety, and tumorigenicity. To date, true germ-line competent pluripotent stem cells (PSCs) have been described only in the laboratory mouse and rat. However, the frequently used mouse and rat models have only limited value for preclinical testing of novel therapies due to profound differences to humans. The domestic pig is considered to be a promising preclinical model for novel cell therapies. Pigs have similar genetics anatomy and physiology to humans and have a long and successful record in biomedicine for the benefit of human patients. The availability of true porcine PSCs would thus be crucial for advancing regenerative medicine to clinical application. In spite of the fact that numerous attempts have been made, germ-line-capable porcine pluripotent cell lines have not yet been established. This suggests a high degree of species specificity with regard to regulation of pluripotency. Thus characterization and identification of porcine pluripotency specific markers and mechanisms underlying pluripotency are largely unknown. An accurate and sensitive tool for visualizing porcine pluripotent cells would be very useful for gaining a better understanding of the *in vivo* localization and behavior of porcine stem cells. This prompted us to produce transgenic pigs carrying the Oct4 gene fused to the enhanced green fluorescent protein (EGFP) cDNA to allow identification of pluripotent cells in the domestic pig. The transcription factor Oct4 is a stem-cell-specific marker gene,

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essential for the maintenance of pluripotency in the mouse and human. Green fluorescent protein and other fluorescent protein reporters are well suited for non-invasive imaging of living cells (Wiedenmann et al., 2009). Green fluorescent protein is expressed in whole tissues and even entire organisms and can be monitored without interfering with the physiology of the host (Hanson and Kohler, 2001). This chapter describes the production of Oct4-EGFP transgenic pigs that were used as a model for the study of porcine pluripotent cells.

Pluripotent Cells in Pigs

Pluripotent stem cells can be isolated from the inner cell mass (ICM) of blastocysts (embryonic stem cells – ESCs), the genital ridges of fetuses (embryonic germ cells – EGCs) or from germ-line tumours (embryonic carcinoma cells – ECCs). There is growing interest in the isolation of porcine PSCs, and the establishment of stable cell lines attributed to the growing significance of the domestic pig in biomedical research and development. These cells could improve the success and efficiency of genetic modification to create transgenic animals for modeling human diseases and could also serve for the derivation of differentiated cells for cell transplantation.

Embryonic Stem Cells

Embryonic stem cells are derived from the ICM of preimplantation mammalian embryos. True ESC lines cultured *in vitro* retain their pluripotent potential and can differentiate into cells representing derivatives of the three germ layers. The first successful isolation of stable ESC lines from the ICM of blastocysts was performed 42 years ago in the mouse (Evans and Kaufman, 1981; Martin, 1981). Since then, stem cell research has become one of the fastest growing research fields. Embryonic stem cell lines were established from Rhesus monkey and Marmoset blastocysts (Thomson et al., 1995, 1996) and in 1998 from humans (Thomson et al., 1998). More recently, ESCs have been isolated from blastocysts of the laboratory rat (Buehr et al., 2008; Li et al., 2008). However, up to date, only mouse and rat ESCs show all criteria for pluripotency, including *in vitro* differentiation into cells of the three germ layers, formation of teratomas upon injection of cells into immune-compromised mice, and chimeras with germ-line transmission after complementation with host blastocysts.

Numerous attempts have been made to isolate ESCs in farm animals, but true pluripotent ESC lines from farm animals have not yet been produced as none of the ESC lines was compatible with the production of germ-line chimeras. Thus pluripotent ESC lines isolated from farm animals were commonly termed as embryonic stem-like cells or putative ESCs (Brevini et al., 2010b; Nowak-Imialek et al., 2011a).

The establishment of ESC-like cell lines from porcine embryos was reported for the first time more than 20 years ago (Notarianni et al., 1990; Piedrahita et al., 1990). Attempts towards the establishment of porcine ESC lines entailed different sources of embryos, including *in vivo* collection (Talbot et al., 1993b) and blastocysts produced *in vitro* (Miyoshi et al., 2000), embryos after parthenogenetic activation (Brevini et al., 2010a), or somatic cell nuclear transfer (Kim et al., 2010). Methods for the derivation of ESCs and *in vitro* culture conditions are usually based on protocols successfully

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used in human, mouse or primates and have to be adapted for porcine cells. Several types of feeder layers in combination with growth factors were tested for their ability to support proliferation of porcine embryonic stem-like cells, albeit without success. Porcine *in vitro* cultures contained a population of cells that showed epithelial (Chen et al., 1999; Miyoshi et al., 2000), trophoblast, or fibroblast morphology (Strojek et al., 1990), which is distinctly different from the mouse. Rarely the derived porcine embryonic stem-like cell lines met some of the critical pluripotency criteria. These cells could be maintained in the *in vitro* culture for prolonged periods of time and formed embryoid bodies, but differentiated only in a limited number of cell types (Talbot et al., 1993b; Chen et al., 1999). Most of the porcine embryo stem-like cell lines were poorly characterized, primarily based on morphology and the expression of pluripotent markers.

Alkaline phosphatase (AP) staining has been widely used for identification of porcine embryo stem-like cell lines, which is, however, a weak marker of pluripotency (Talbot et al., 1993a; Chen et al., 1999; Shiue et al., 2006). Some studies have demonstrated expression of stem-cell markers previously used successfully in mouse or human, including Oct4, NANOG, transcription factor SRY-box 2 (SOX2) or stage-specific embryo antigen 1 (SSEA-1) (Wianny et al., 1997; Blomberg et al., 2008; Alberio et al., 2010). Unfortunately expression was inconsistent among the various cell lines. Moreover, the limited availability of porcine-specific antibodies frequently hampered unequivocal identification of porcine-specific pluripotent cells. Porcine embryo stem-like cells formed teratomas after injection into immunodeficient mice (Hochereau-de Reviers and Perreau, 1993; Anderson et al., 1994). In a few experiments somatic chimeras were produced after injection of putative ESCs into blastocysts (Chen et al., 1999; Vassiliev et al., 2010). Differences in morphology, gene expression, and pathway regulation of pluripotency were observed between mouse and human ESCs, pointing towards a relatively high degree of species specificity with regard to pluripotency (Ginis et al., 2004; Rao, 2004). The successful establishment of porcine ESCs requires the identification and characterization of a set of porcine-specific markers for pluripotent cells and pluripotency-maintaining factors (Munoz et al., 2008).

Primordial Germ Cells

Primordial germ cells (PGCs) are undifferentiated embryonic precursors of the gametes. Primordial germ cells differentiate during gastrulation from pluripotent epiblast cells and were first discovered in the extraembryonic region. They migrate through the allantois and hindgut and colonize the genital ridges (Godin et al., 1990). Primordial germ cell cultures were established for the first time in the mouse (Matsui et al., 1992; Resnick et al., 1992). Primordial germ cells can differentiate into sperm or eggs (Durcova-Hills et al., 2006). Mouse PGC cultures were isolated from the caudal region of 6.6. dpc embryos. These cells could be cultured *in vitro* for prolonged periods of time when they were grown on feeder cells in medium supplemented by growth factors such as stem cell factor (SCF), basic fibroblast growth factor (bFGF) and leukemia inhibitory factor (LIF). Under these culture conditions, unipotent PGCs gave rise to colonies of cells called EGCs, which resemble pluripotent ESCs (Matsui et al., 1992). The underlying mechanism for the conversion of the unipotent cells into

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pluripotent cells remains elusive. The reprogramming of PGCs in culture is a complex process that requires both dedifferentiation and acquisition of a PSC phenotype (Durcova-Hills et al., 2006). Mouse EGCs are similar to mouse ESCs with regard to morphology and pluripotency. These cells can be differentiated *in vitro* into cells of the three germ layers and can form teratocarcinomas. After injection into host blastocysts they formed germ-line chimeras (Labosky et al., 1994).

Attempts to derive EGCs have been reported in other species, including human (Shamblott et al., 1998), pig (Shim et al., 1997), cow (Choi and Anderson, 1998), buffalo (Huang et al., 2007), sheep (Ledda et al., 2010), rabbit (Kakegawa et al., 2008), rat (Leitch et al., 2010), and chicken (Park and Han, 2000). Whereas isolation, *in vitro* culture conditions, and characterization of mouse PGCs have been studied extensively, only little is known about these cells in farm animals. To date, only few laboratories have reported porcine cell lines that fulfilled important criteria indicative for pluripotency. Since porcine ESCs had not yet been established, the derivation of porcine germ cell cultures provided a promising alternative to pluripotent ESCs. Porcine PGCs could be maintained in culture for prolonged periods of time (Petkov and Anderson, 2008; Shim et al., 1997). Culture on feeder cells in medium with knockout serum supplemented with growth factors, including LIF, SCF, and bFGF, supported proliferation of porcine PGCs with a morphology very similar to mouse ESCs (Petkov and Anderson, 2008): these cells could be identified by markers typical for mouse PGCs, such as AP, SSEA-1, SSEA-4, and Oct4 (Shim et al., 1997; Lee et al., 2000; Petkov and Anderson, 2008). Expression of Oct4 decreased over time in cultured PGCs, probably due to changes occurring in these cells under cell-culture conditions (Petkov and Anderson, 2008; Petkov et al., 2011). These cells had the ability to differentiate *in vitro* into a wide range of cells representing the three germ layers (Piedrahita et al., 1998; Mueller et al., 1999). After injection into blastocysts, these cells contributed to somatic tissue in chimeric piglets but without germ-line transmission (Shim et al., 1997; Piedrahita et al., 1998; Mueller et al., 1999; Rui et al., 2004). Functional and molecular analysis indicated that porcine EGCs are multipotent rather than pluripotent (Petkov et al., 2011). To date, porcine PGC lines are poorly characterized and their pluripotent state has not yet been demonstrated. The availability of *in vitro* culture conditions supporting the proliferation of porcine cells and a better understanding of pluripotency would enable the successful establishment of true pluripotent porcine cell lines.

Pluripotent Cells Generated after Reprogramming

Differentiated somatic cells can be successfully reprogrammed to a pluripotent state by different strategies. These include: somatic cell nuclear transfer (SCNT), fusion of somatic cells with pluripotent cells or the generation of induced pluripotent cells (iPSCs) by overexpression of defined factors. During SCNT, the donor cell is introduced into an enucleated oocyte, the DNA of the transferred nucleus is reprogrammed by epigenetic mechanisms, a viable embryo is formed which eventually will generate cloned animals upon transfer to recipients. The generation of the first cloned animal "Dolly, the sheep" dramatically changed the view of scientists on pluripotency and differentiation. The successful delivery of a live cloned animal demonstrated that the epigenetic status of a terminally differentiated somatic cell can be reprogrammed into a pluripotent state which is compatible with the development of a new organism

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(Wilmut et al., 1997). The combination of nuclear transfer and ESC technology offers the possibility for cell replacement. Embryonic stem cells derived from cloned blastocysts are genetically identical to the somatic donor cell. This allows the production of autologous PSCs (Munsie et al., 2000). However, this raises significant ethical questions regarding the use of human oocytes for the generation of SCNT-derived cells. Functional ESCs could be derived successfully from cloned embryos in mouse (Kawase et al., 2000) and monkey (Byrne et al., 2007). The feasibility of isolating pluripotent cells with germ-line capacity from reconstructed farm animal embryos still needs to be demonstrated. Whereas SCNT protocols were significantly improved in the past decade, on average only 3–6% of the transferred cloned porcine embryos survived to term (Fulka and Fulka, 2007; Petersen et al., 2008).

The factors responsible for successful reprogramming are present in the ooplasm of enucleated oocytes. This epigenetic reprogramming entails the erasure of the gene expression program of the respective donor cell and the reestablishment of the well-orchestrated sequence of expression of the estimated 10,000–12,000 genes that regulate embryonic and fetal development (Kues et al., 2008). However, the basic mechanism of epigenetic reprogramming of the genomic program of a somatic cell into a pluripotent state is only poorly understood. Future studies are needed in order to identify factors that are critical for successful reprogramming.

The genome of a somatic cell can also be reprogrammed into the pluripotent state by fusion of the somatic cell with a pluripotent cell (Do and Scholer, 2004). In contrast to SCNT mediated reprogramming, reprogramming via cell fusion depends on factors present in the karyoplast of pluripotent cells and is independent of DNA replication and cell division (Do and Scholer, 2004). Several pluripotent cell lines, including ESCs (Do and Scholer, 2004), EGCs (Tada et al., 1997), and ECCs (Flasza et al., 2003) have been shown to be capable of reprogramming different somatic cells. The fused cells displayed several features of pluripotent cells, including morphology and differentiation into cell types indicative for the three germ layers and reactivation of pluripotency genes (Do and Scholer, 2004, 2005). The pluripotency of fused cells was confirmed in the mouse model by their ability to form teratomas that contained derivatives of the three germ layers (Vasilkova et al., 2007) and chimera formation with contribution to the placenta after blastocyst injection (Do et al., 2011).

Since true pluripotent cell lines have not yet been established from farm animals, reprogramming via cell fusion can be achieved only by using PSCs from species such as the laboratory mouse and rat. We have recently developed an interspecies cell-fusion model, using mouse ESCs as the pluripotent partner and porcine fibroblasts as the somatic partner (Nowak-Imialek et al., 2010). Porcine–mouse hybrids even showed similarities with mouse ESCs, including morphology and proliferation rate. The hybrids reactivated the porcine endogenous Oct4 gene, which is an essential PSC marker. However, the reprogrammed cells rapidly lost the porcine chromosomes. Presumably, incompatibilities between the porcine and mouse genome after interspecies cell fusion caused splicing errors and subsequently loss of the porcine chromosomes from hybrids (Nowak-Imialek et al., 2010). The fused cells are tetraploid, which limits their therapeutic application.

One of the most promising reprogramming methods is the generation of iPSCs from somatic cells. Differentiated cells both from mouse and human could be reprogrammed into a pluripotent state by overexpression of only four transcription factors,

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such as Oct4, SOX2, c-Myc and Klf4 (Takahashi et al., 2007) or by Oct4, SOX2, NANOG, and Lin28 (Yu et al., 2007). Induced pluripotent stem cells are nearly identical to ESCs with regard to morphology, self-renewal, proliferation, gene expression profile, and *in vitro* differentiation potential. Mouse iPSCs meet the important pluripotency criteria, including chimera formation with germ-line transmission (Zhao et al., 2009). The unlimited self-renewal and differentiation potential into any cell type renders iPSCs as promising for cell therapies. The establishment of iPSCs from any human somatic cells, regardless of the age, paves the way for patient-specific cell-based therapies (Hussein et al., 2013). Prior to clinical application, the therapeutic potential of iPSCs must be evaluated in an appropriate large animal model. Besides mouse and human, the successful reprogramming of somatic cells has been demonstrated in a variety of mammalian species, including pigs (Esteban et al., 2009; Ezashi et al., 2009, 2012; Wu et al., 2009; West et al., 2010). Porcine iPSCs shared characteristics of pluripotent cells, including morphology, rapid proliferation, and activation of endogenous PSC markers such as Oct4, NANOG, and SOX2 (Esteban et al., 2009; Ezashi et al., 2009; Wu et al., 2009; West et al., 2010). However, the exogenous reprogramming factors delivered from vectors were not silenced as regularly observed in iPSCs from mouse, human, or other species (Esteban et al., 2009; Montserrat et al., 2011). The cells differentiate *in vitro* into derivatives of the three germ layers, form teratomas with cells representing the three germ layers, and generate chimera animals without germ-line transmission (Esteban et al., 2009; Ezashi et al., 2009; Wu et al., 2009; Montserrat et al., 2011). Recently, germ-line transmission of porcine iPSCs with the birth of live transgenic piglets was demonstrated for the first time (West et al., 2011). However, the frequency of chimera generation with germ-line transmission of porcine iPSCs was low and only based on PCR evidence (West et al., 2011). The availability of true porcine iPSCs will provide pluripotent cells in those species, in which the derivation of ESCs from blastocysts has not yet been successful.

The Oct4-EGFP (OG2) Transgene as a Tool for Monitoring Pluripotency

Visualization of stem cells using fluorescent proteins in transgenic cell lines or animals allows monitoring of pluripotency. The GFP isolated from jellyfish can be used as a reporter of gene expression and protein localization within living cells, tissues, or embryos using conventional fluorescence and confocal microscopes (Chalfie et al., 1994; Hadjantonakis and Nagy, 2001). The major advantage of GFP is that the molecule fluoresces only in response to UV or blue light, unlike other fluorescent proteins that require cofactors or substrates for activity. Expression of GFP can be observed *in vivo* within individual cells, cell populations, or in whole organisms in real time (Lorang et al., 2001). Such GFP-expressing cells can be enriched from a heterogeneous cell population by fluorescence-activated cell sorting (FACS) (Hadjantonakis and Nagy, 2001). Additionally, GFP expression can be directly visualized by fluorescence microscopy in tissue after fixation in methanol or 4% paraformaldehyde/0.1% glutaraldehyde (Luby-Phelps et al., 2003).

Green fluorescent protein is commonly used as a reporter protein for monitoring of gene expression. Cells with stably integrated reporter genes can be monitored after

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transplantation for long-term studies (Mothe et al., 2005). The emergence of mouse stem cells can be monitored with the aid of the transgenic mouse model expressing GFP under control of the Oct4 promoter (Yoshimizu et al., 1999). The Oct4 gene is a member of the Pit-Oct-Unc (POU) transcription factor family that is critically involved in controlling self-renewal and maintenance of pluripotency in stem cells (Scholer et al., 1990; Boiani and Scholer, 2005). In the mouse, the Oct4-EGFP transgene mimics the expression profile of the endogenous Oct4 gene (Yoshimizu et al., 1999). The Oct4-EGFP transgene is silent in somatic cells, but active in the ICM of blastocysts and ESCs, primordial germ cells, and in ovary and testis (Yeom et al., 1996; Yoshimizu et al., 1999; Boiani et al., 2002; Ohmura et al., 2004; Youn et al., 2012). Monitoring the GFP, expression of the Oct4 gene could be determined successfully in live pre- and postimplantation mouse embryos (Yoshimizu et al., 1999). The transgene provides a useful tool to isolate germ cells at various developmental stages without additional staining (Yoshimizu et al., 1999). In addition, the mouse spermatogonial stem cell population expressing Oct4-EGFP can be enriched from testicular cells by FACS (Ko et al., 2010).

Human embryonic stem cell lines harboring the Oct4-EGFP transgene were used for monitoring pluripotency and optimizing passaging methods (Gerrard et al., 2005; Bajpai et al., 2008). As in the mouse, the expression profile of EGFP in these cell lines mimics the expression of the endogenous Oct4. Species-specific differences in Oct4 expression were shown after microinjection of a Oct4-EGFP transgene into zygotes of various species derived *in vitro* and *in vivo* (Kirchhof et al., 2000). The Oct4 protein was detected exclusively in the ICM of mouse blastocysts, whereas in porcine and bovine blastocysts it was found in both the ICM and the trophectoderm.

Reprogramming efficiency could be successfully monitored in mice by the emerging EGFP fluorescence after cell fusion (Do and Scholer, 2004; Tat et al., 2011), through ectopic expression of the transcription factors, including Oct4, SOX, c-*Myc*, and *Klf4* (Wernig et al., 2007), or after conversion of adult germ line stem cells into PSCs (Ko et al., 2009). The Oct4-EGFP transgene was also used in miniature pigs (Miyoshi et al., 2009) and cattle (Habermann et al., 2007; Wuensch et al., 2007) to demonstrate the reactivation of the Oct4 gene after SCNT, or after cell fusion (Ozawa et al., 2010). However, in these cases only Oct4-EGFP transgenic cells or embryos were generated and no Oct4-EGFP transgenic animals were reported.

Oct4-EGFP (OG2) Transgenic Pigs

True pluripotent porcine cell lines have not yet been derived (Chen et al., 1999). The *in vitro* culture conditions that are compatible with self-renewal of porcine pluripotent cells are still an enigma. In addition, little is known about marker genes in porcine stem cells. Using our well-established somatic cell nuclear transfer protocol (Petersen et al. 2008), we produced Oct4-EGFP (OG2) transgenic pigs expressing the murine 18 kb Oct4-EGFP (GOF-18/EGFP) construct exclusively in pluripotent cells (Nowak-Imialek et al., 2011b). The *Oct4*-EGFP pigs are equivalent to the existing OG2 mouse model and are the first large animal model for functional reprogramming. These pigs provide a useful tool for studying pluripotent and reprogramming cells in the domestic pig (Figure 5.1).

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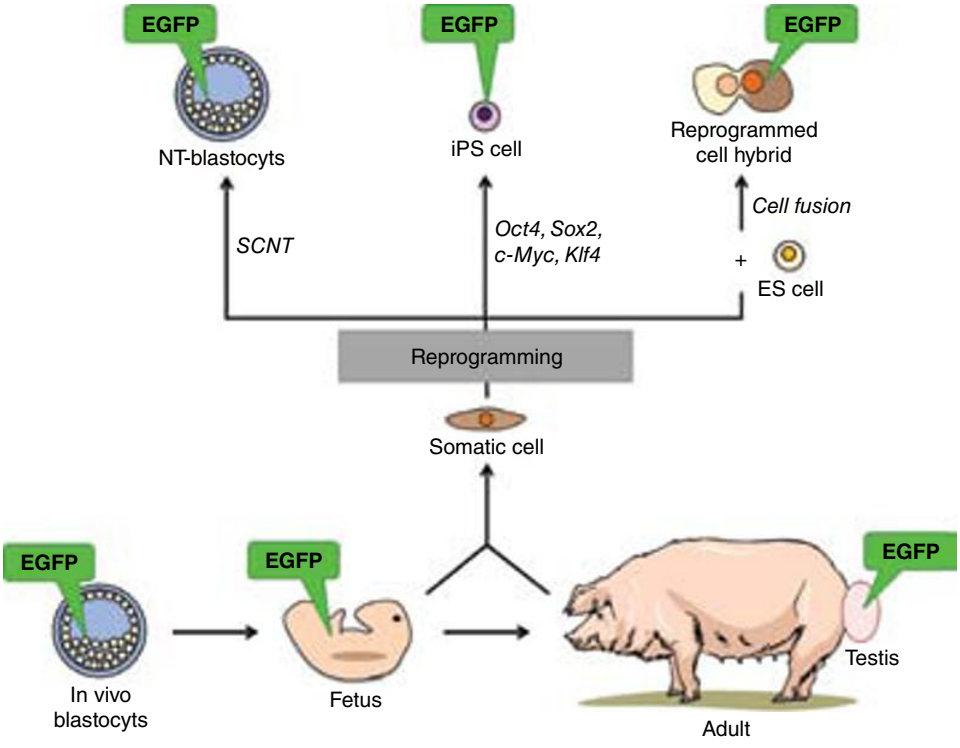


Figure 5.1. Non-invasive imaging of Oct4-EGFP-expressing cells in pigs. Oct4-EGFP (OG2) pigs proved a useful model for monitoring pluripotent cells expressing the Oct4 gene visualized by reactivation of the EGFP fluorescence. Expression of the EGFP reporter was confined to the ICM and trophectoderm of blastocyst after SCNT, or blastocysts produced *in vivo* or *in vitro*, in genital ridges isolated from an OG2 fetus and in testicular cells from testis of an adult OG2 boar. Reprogramming of somatic cells from OG2 animals after SCNT, or after fusion with mouse ESCs, or after introduction of reprogramming factors resulted in Oct4-EGFP reactivation.

Generation and Characterization of Oct4-EGFP (OG2) Transgenic Pigs

The production of germ line reporter transgenic pigs using a minigene construct based on the 3.9 kb human Oct4 promoter (hOct4) was not successful (Figure 5.2), although this construct drove Oct4 expression in human ESCs (Gerrard et al., 2005). Porcine fibroblasts transgenic with the human Oct4-EGFP construct were used as donor cells in SCNT. Neither *in vitro* cultured blastocysts nor genital ridges collected from transgenic fetuses showed reactivated EGFP fluorescence. We then used a mouse Oct4-EGFP (GOF-18/EGFP) construct containing the genomic clone of the entire Oct4 promoter (9 kb) fused to the EGFP cDNA and approximately 9 kb of the Oct4 exon/intron region (Figure 5.2). The EGFP was detected in the ICM and the trophectoderm of blastocysts produced by SCNT with OG2 porcine fibroblasts or collected 5 days after mating of a wild-type sow to an Oct4-EGFP transgenic boar (Figure 5.3). This indicates that regulation of pluripotency in preimplantation development differs between mouse and pig. Porcine blastocysts hatch 7 days after

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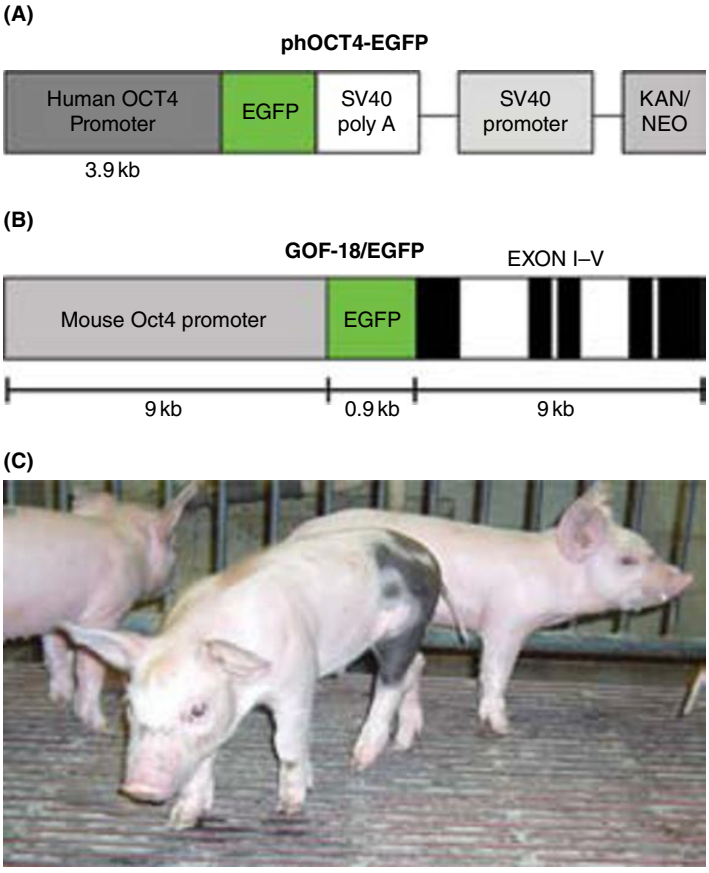


Figure 5.2. Schematic representation of gene constructs used for generation of Oct4-EGFP transgenic pigs. **(A)** The human phOct4-EGFP construct used in the first experiment contains the human Oct4 promoter (3.9 kb) followed by EGFP cDNA (0.9 kb). **(B)** Mouse Oct4-EGFP(GOF-18/EGFP) construct containing Oct4 exons I-V (black boxes) and inserted EGFP cDNA (green box). **(C)** Oct4-EGFP transgenic pigs. (Nowak-Imialek et al., 2011b.)

fertilization and the conceptuses do not attach to the endometrium prior to day 13–14 (Oestrup et al., 2009). This might explain why the expression of Oct4 is less tight in the domestic pig than in the laboratory mouse. Expression of the Oct4-EGFP transgene was restricted to germ cells isolated from genital ridges of porcine fetuses and testicular cells from adult animals; the transgene was not expressed in spermatozoa (Figure 5.3). The EGFP expression profile in adult testis was similar to the expression of the endogenous Oct4 gene in the transgenic pigs. Purification of EGFP positive cells is necessary to identify and to characterize Oct4 expressing cells in porcine testis. Our Oct4-EGFP pig model clearly demonstrates that the mouse OG2 transgene is suitable for visualization of PSCs in the domestic pig. Expression of EGFP in porcine blastocysts, germ cells and testis was visualized using an inverted fluorescence microscope containing a fluorescein isothiocyanate (FITC) filter. Results of our

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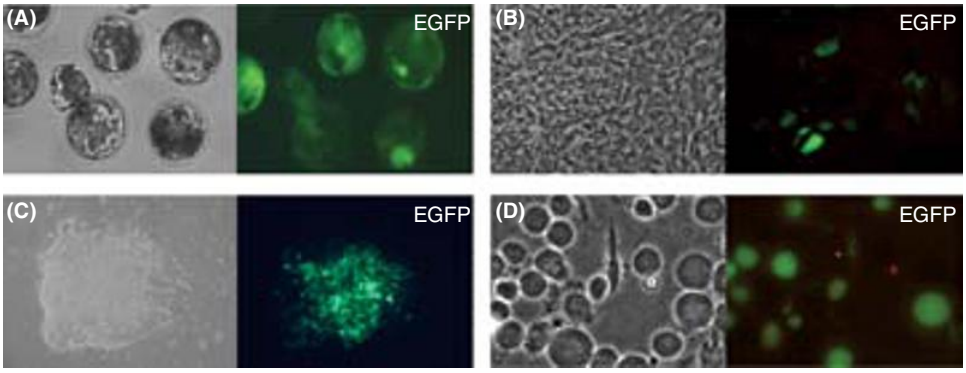


Figure 5.3. Reactivation of the Oct4-EGFP (OG2) transgene in pigs. **(A)** Porcine cloned and *in vitro* cultured blastocysts from days 5 or 6; bright-field image (left) and corresponding EGFP fluorescence image (right). **(B)** Porcine genital ridges isolated from Oct4-EGFP (OG2) transgenic fetuses on day 25 after squash preparation; bright-field image (left) and corresponding EGFP fluorescence image (right). **(C)** Mouse–pig hybrid colony; bright-field image (left) and corresponding EGFP fluorescence image (right). **(D)** Testis isolated from adult OG2 pigs after squash preparation; bright-field image (left) and corresponding EGFP fluorescence image (right). (Nowak-Imialek et al., 2009; Nowak-Imialek et al., 2011b.)

study show that the use of a genetic construct possessing all regulatory elements of the Oct4 gene obviously is mandatory for the successful generation of germ-line-specific Oct4-EGFP pigs.

Reprogramming Using Porcine Oct4-EGFP (OG2) Transgenic Cells

Cells from the Oct4-EGFP transgenic pigs can be successfully used for studying reprogramming and regaining of pluripotency. The usefulness of the transgene for monitoring reprogramming was first demonstrated by fusion of porcine Oct4-EGFP fibroblasts with pluripotent mouse ESCs (Nowak-Imialek et al., 2010). The resulting interspecies hybrids formed colonies with typical mouse ESC morphology, upregulated endogenous genes associated with pluripotency, and activated the EGFP fluorescence (Figure 5.3). The emergence of the EGFP fluorescence in fused cells indicated that the Oct4-EGFP transgene was reactivated by unknown factors from mouse ESCs. Unfortunately, mouse–pig hybrids were unstable and lost the EGFP fluorescence during *in vitro* culture. Probably incompatibilities between mouse and pig genome after cell fusion caused a loss of porcine chromosomes (Nowak-Imialek et al., 2010).

The introduction of the four reprogramming genes Oct4, SOX2, *Klf4*, *c-Myc* using viral vectors or transposons into Oct4-EGFP transgenic fibroblasts resulted in EGFP expressing iPSC colonies which displayed characteristics typical for pluripotent cells (Nowak-Imialek et al., 2009; Nowak-Imialek et al., 2011b; Kues et al., 2012). Porcine iPSCs showed long-term proliferation and expressed typical stem-cell markers. Transposon-mediated porcine iPSCs differentiated into derivatives of the three germ layers and formed teratomas after injection into immunodeficient mice. But the variable morphology in different passages and the low efficiency in the generation of Oct4-EGFP

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positive colonies indicated that optimal reprogramming and culture conditions were not yet obtained (Kues et al., 2013). The abundant availability of porcine Oct4-EGFP transgenic somatic cells provides a novel monitoring system for studying factors critical for maintaining long-term pluripotency of porcine cells. In addition, this system is promising for establishing a culture system for porcine reprogrammed cells.

Conclusions

Since the first establishment of ESCs *in vitro*, significant progress in stem cell research has been made. Nevertheless, true pluripotent cell lines from farm animals, including the domestic pig, have not yet been derived. This limits the use of the domestic pig as a useful preclinical model for cell therapies. The present culture conditions used for the derivation of pluripotent porcine cell lines are still inefficient and do not reliably allow proliferation of reprogrammed porcine cells. The fragmentary knowledge on the regulation of pluripotency in the domestic pig hampers long-term proliferation of PSCs. The recent generation of iPSCs from a variety of somatic cells provides a good alternative to ESCs and opens a new opportunity in stem cell research. The Oct4-EGFP transgenic pigs carrying the marker gene for stem cells are a valuable tool for monitoring pluripotent cells in the pig. Non-invasive imaging based on Oct4-EGFP expression can provide convenient means to monitor porcine stem-cell localization, survival and proliferation, as well as function. The fluorescent reporter Oct4 gene can be also used for isolation of stem cells after sorting according to the EGFP fluorescence. Moreover, the Oct4-EGFP fluorescent reporter gene facilitates visualization of stem cells using histological methods.

In summary, Oct4-EGFP transgenic pigs offer a useful non-invasive imaging system for the monitoring of porcine stem cells and will play a critical role in the study of stem cell biology and reprogramming in the pig.

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Chapter 6

Regulation of Adult Intestinal Stem Cells through Thyroid Hormone-Induced Tissue Interactions during Amphibian Metamorphosis

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Introduction

Amphibian metamorphosis mimics the evolutionary process to adapt from the aquatic to terrestrial vertebrate life over 350 million years ago. During metamorphosis, the amphibian larva-specific organs such as the tail and gills are completely involuted, whereas the adult-specific ones such as the limbs develop *de novo*. Numerous other organs dramatically remodel from the larval to adult form (Shi, 1999). Among them, the *Xenopus laevis* small intestine is one of the best-studied organs at the cellular level. While most of the primary (larval) epithelial cells undergo apoptosis during metamorphosis, a small number of undifferentiated cells appear. They actively proliferate, and differentiate into the secondary (adult) epithelium, by interacting with the surrounding connective tissue (Ishizuya-Oka and Shi, 2007). The adult epithelium acquires a cell-renewal system (McAvoy and Dixon, 1977; Ishizuya-Oka and Shimozawa, 1987a), which is similar to that of the adult mammalian intestine (Cheng and Bjerknes, 1985; Madara and Trier, 1994). This implies that intestinal stem cells analogous to the mammalian ones develop in amphibians during metamorphosis.

In the adult mammalian intestine, the epithelium undergoes rapid and perpetual cell renewal that is driven by the stem cells. It is well known that the microenvironment around the stem cells, called the "niche," plays an important role in the maintenance of the epithelial cell renewal and is thus clinically important for regenerative and cancer therapies in the intestine. A growing body of recent evidence reveals several key signals constituting the stem cell niche (Sato et al., 2011). However, it still remains mostly unknown how the stem cell niche is established during normal mammalian development. Since amphibian metamorphosis is completely controlled by the thyroid hormone (TH) and can be reproduced by simply adding TH to the tadpole-rearing water (Dodd and Dodd, 1976; Kikuyama et al., 1993), the amphibian intestine provides us with a valuable opportunity to clarify mechanisms underlying development of the stem cells and their

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niche. To further study this issue at the molecular level, a number of TH response genes have been isolated from the *X. laevis* intestine by various methods, including subtractive differential screening from 1990s (Shi and Brown, 1993; Amano and Yoshizato, 1998; Buchholz et al., 2007; Heimeier et al., 2010). The expression and functional analyses of these TH response genes by using organ culture and transgenic techniques have gradually revealed molecular aspects of the larval-to-adult intestinal remodeling. Here, we review recent progress in this field, focusing on TH-activated signaling pathways that mediate complicated epithelial–connective-tissue interactions essential for the stem cell development, and propose their evolutionary conservation roles in development and/or homeostasis of the adult mammalian intestine.

Thyroid Hormone Induces Intestinal Stem Cells during *Xenopus* Metamorphosis

The tadpole small intestine before metamorphosis is long and has only a single fold known as the "typhlosole," which runs longitudinally only in the anterior part of the intestine (Marshall and Dixon, 1978a; Figure 6.1). Histologically, the intestine mainly consists of simple columnar larval epithelium, immature connective tissue, which is very thin except in the typhlosole, and thin layers of inner circular and outer longitudinal muscles. The major epithelial cells are absorptive cells differentiated as larval type where the brush border is much longer than that of adults (Bonneville, 1963; Fox et al., 1972). It should be noted that, although the epithelial cells rarely proliferate at random, neither morphologically undifferentiated cells (Hourdry and Dauca, 1977; Marshall and Dixon, 1978a) nor cells positive for stem-cell markers can be identified before metamorphic climax (Ishizuya-Oka et al., 2001, 2003). Thus, a clearly definable stem cell population does not exist in the larval epithelium during this period.

At the early period of metamorphic climax (NF stage 60; Nieuwkoop and Faber, 1956), when the plasma level of TH abruptly increases (Leloup and Buscaglia, 1977), dramatic epithelial changes occur with a rapid shortening of the small intestine (Marshall and Dixon, 1978a; Ishizuya-Oka and Shimozawa, 1987a). Most of the larval epithelial cells (larval proper cells) begin to undergo apoptosis (Ishizuya-Oka and Ueda, 1996). At the same time, a small number of undifferentiated cells that are strongly stained red with methylgreen-pyronin Y (MG-PY) (adult progenitor cells) become detectable as small islets between the larval proper cells and the connective tissue in the entire small intestine (Figure 6.2A). They actively proliferate and invaginate into the connective tissue (Figure 6.2B and C), concomitantly with the increase in thickness and active cell proliferation of the connective tissue (Marshall and Dixon, 1978b; Ishizuya-Oka and Shimozawa, 1987a). Then, the adult progenitor cells gradually replace the larval proper cells and, as multiple intestinal folds develop, differentiate into the simple columnar adult epithelium (Figure 6.2E) (Hourdry and Dauca, 1977; McAvoy and Dixon, 1977). Thereafter, towards the end of metamorphosis (stage 66), the adult epithelium acquires the cell-renewal system along the trough–crest axis of the newly formed intestinal folds. That is, the adult epithelial cells proliferate in the trough of the folds and, as they migrate up, gradually differentiate into all cell types of mammalian intestinal epithelial cells except for Paneth cells; major absorptive cells possessing the short brush border and expressing intestinal fatty acid-binding protein (IFABP)

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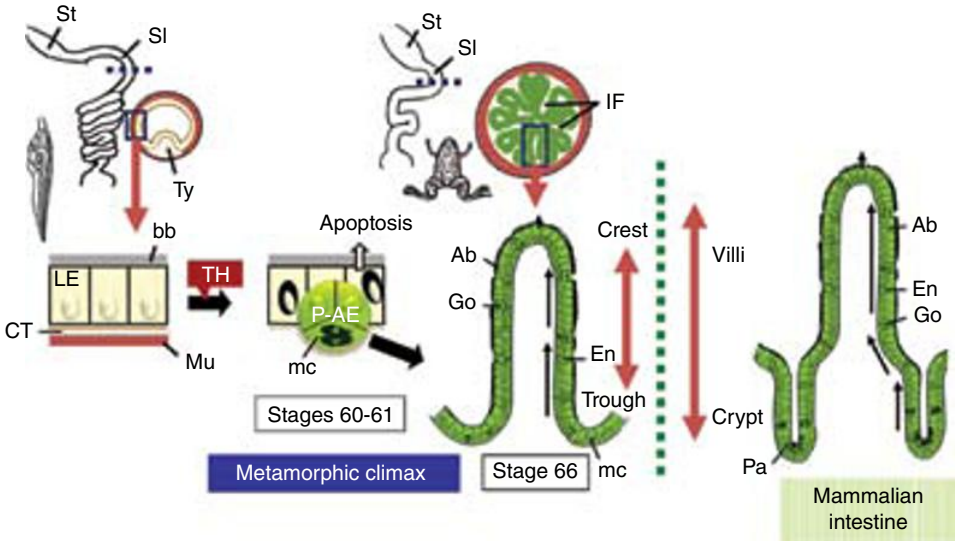


Figure 6.1. *Xenopus laevis* intestine remodels from larval to adult form, analogous to the mammalian adult intestine during metamorphosis. The small intestine (SI) before metamorphosis consists of the larval epithelium (LE) possessing the long brush border (bb), the immature connective tissue (CT), which is thin except in the typhlosole (Ty), and thin layers of inner and outer muscles (Mu). During early metamorphic climax (stages 60–61), when the plasma thyroid hormone (TH) levels become high, larval proper epithelial cells undergo apoptosis, whereas progenitor/stem cells of the adult epithelium (P-AE) appear as islets between the larval proper cells and the connective tissue. They generate the simple columnar adult epithelium (AE) with the progress of intestinal fold (IF) formation. At the end of metamorphosis (stage 66), the adult epithelium acquires a cell-renewal system along the trough–crest axis of the intestinal folds, similar to that along the crypt–villus axis in the adult mammalian intestine. Mitotic cells (mc) are localized in the trough. As the adult epithelial cells migrate up, they gradually differentiate into all cell-types of the mammalian ones except for Paneth cells (Pa), that is, major absorptive cells (Ab) possessing the brush border, goblet cells (Go), and enteroendocrine cells (En). Finally, they undergo apoptosis at the tip of the intestinal folds. St, stomach.

(Figure 6.2F), goblet cells, and enteroendocrine cells (Ishizuya-Oka et al., 1997; McAvoy and Dixon, 1978). Finally, they undergo apoptosis at the tip of the intestinal folds (Ishizuya-Oka and Ueda, 1996). These chronological observations implicate that the adult progenitor cells detectable at stage 60 include the stem cells analogous to those in the adult mammalian intestine. In fact, a growing body of immunohistochemical and *in situ* hybridization (ISH) data have shown that the adult progenitor cells express sonic hedgehog (Shh) (Ishizuya-Oka et al., 2001; Hasebe et al., 2008), Musashi-1 (Msi1) (Ishizuya-Oka et al., 2003), phosphorylated form of phosphatase and tensin homolog (P-PTEN), Akt (Ishizuya-Oka and Shi, 2007), and protein arginine methyltransferase 1 (PRMT1) (Matsuda and Shi, 2010; Shi et al., 2011), all of which are also expressed in the stem cells and their descendants of the adult mammalian intestine (van den Brink et al., 2001; de Santa Barbara et al., 2003; Kayahara et al., 2003; He et al., 2007). More importantly, the orphan leucine-rich repeat-containing G-protein-coupled receptor 5

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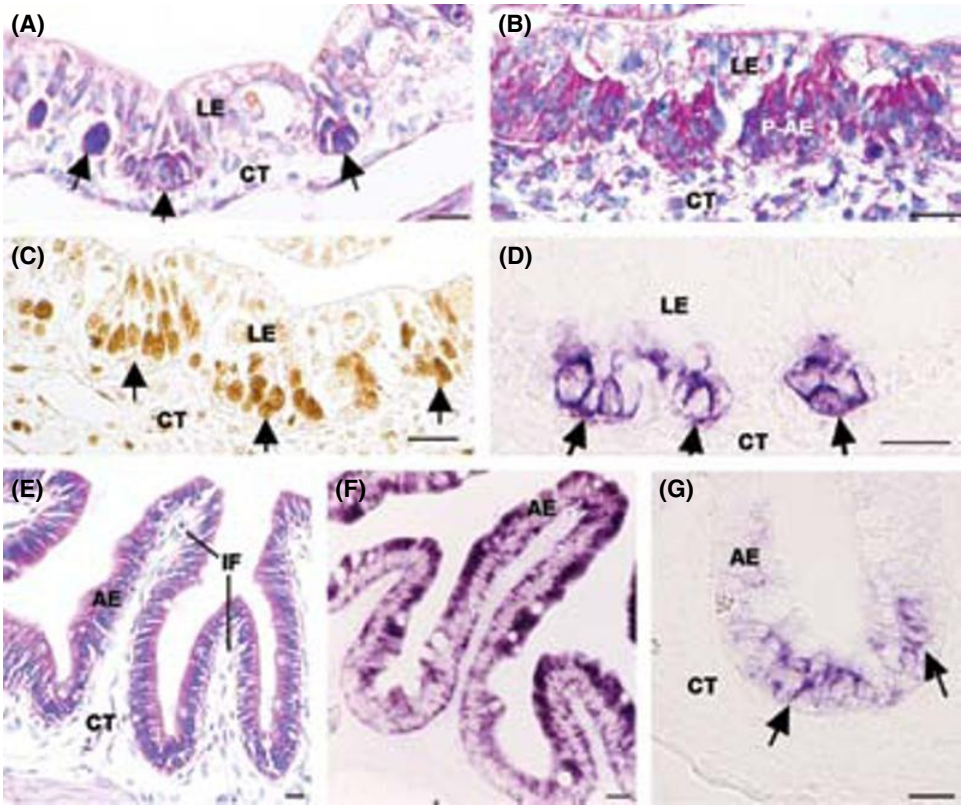


Figure 6.2. Development of adult progenitor/stem cells during *Xenopus laevis* metamorphosis. The cross-sections were stained with methylgreen-pyronin Y (MG-PY) **(A, B, E)**, immunostained with anti-BrdU antibody **(C)**, and hybridized with antisense Lgr5 **(D, G)** and IFABP **(F)** probes. **(A)** At stage 60: adult progenitor/stem cells strongly stained red with MG-PY (arrows) appear as islets between the larval epithelium (LE) and the connective tissue (CT). **(B–D)** During stages 61–62: adult progenitor/stem cells (P-AE) grow into the connective tissue by active proliferation (C, arrows) and express a stem-cell marker, Lgr5 (D, arrows). **(E–G)** At stage 66: high levels of IFABP mRNA **(F)** are expressed in the adult epithelium (AE) except in the trough of the newly formed intestinal folds (IF), where Lgr5 mRNA is localized (G, arrows). Bars, 20 μm.

(Lgr5), a well-characterized stem-cell marker in the adult mammalian intestine (Barker et al., 2007; Sato et al., 2009), is specifically expressed in the adult progenitor cells (Figure 6.2D). Thereafter, with the progress of intestinal fold formation, the Lgr5-positive cells become localized in the trough of the folds where the stem cells are assumed to reside (Figure 6.2G) (Sun et al., 2010). This conservation in the expression of stem-cell markers between the amphibian and the mammalian adult intestines enhances the usefulness of the *X. laevis* intestine as a model for the study of intestinal stem cell biology.

To experimentally investigate how the stem cells develop during amphibian metamorphosis, we first established an organ culture system for the small intestine isolated

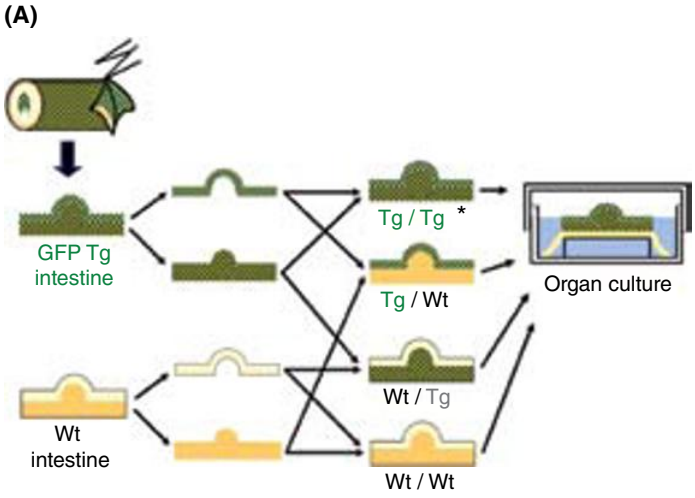
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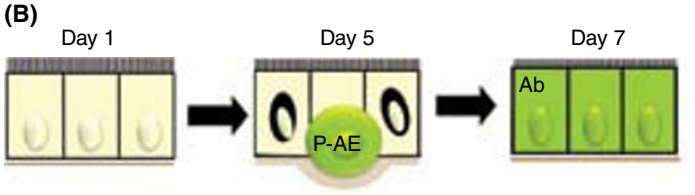
from of *X. laevis* tadpoles at stage 57 (before metamorphic climax), when metamorphic changes do not occur without TH treatment (Ishizuya-Oka and Shimozawa, 1992). Using this culture system, we have shown that TH can induce organ-autonomously the whole process of larval-to-adult remodeling in the anterior part of the small intestine (Ishizuya-Oka et al., 1997, 2003). Similar to natural metamorphosis, the adult progenitor/stem cells, which are stained red with MG-PY and positive for the stem-cell markers, became detectable after 5 days of TH treatment. They actively proliferated and then differentiated into the simple columnar adult epithelium expressing IFABP after 7 days. These results indicate that: (i) the stem cells originate from the tadpole intestine itself but not from the other organs; and that (ii) molecular mechanisms of the stem cell development can be clarified by assessing functions of TH response genes endogenously expressed in the *X. laevis* intestine. In addition, we found that TH failed to induce the adult epithelial development in the posterior part of the small intestine, in contrast to the anterior one where the larval connective tissue is mostly localized. More importantly, when the epithelium of the posterior small intestine was co-cultured with the connective tissue of the anterior one, adult progenitor/stem cells were now detected after 5 days of TH treatment (Ishizuya-Oka and Shimozawa 1992). This finding suggests important roles of the connective tissue in the adult stem cell development.

Larval Epithelial Cells Dedifferentiate into Stem Cells under the Control of the Niche

Given that the adult stem cells originate from the tadpole intestine through tissue interactions, what kind of larval intestinal cells are the origin of the stem cells? Although their origin has not yet been precisely clarified, our study using transgenic (Tg) *X. laevis* tadpoles for culture experiments *in vitro* provided the first experimental evidence for their epithelial origin (Ishizuya-Oka et al., 2009). We isolated the anterior part of the small intestine from wild-type (Wt) or Tg tadpoles that constitutively express green fluorescent protein (GFP) under the cytomegalovirus (CMV) promoter. Their epithelium was separated from the other non-epithelial tissues (non-E), mostly the connective tissue, and was recombined with homologous or heterologous Wt or Tg non-E (Figure 6.3A). In any recombinant intestine cultured in the TH-containing medium, just as in the intact Wt intestine, the adult progenitor/stem cells became detectable after 5 days and differentiated into the adult absorptive epithelium after 7 days. Importantly, whenever the epithelium was derived from GFP Tg intestine, both the adult progenitor/stem cells and the absorptive cells expressed GFP regardless of whether the epithelium was recombined with Wt or Tg non-E (Figure 6.3B). In contrast, whenever the epithelium was derived from Wt intestine, neither type of the adult cells expressed GFP. These results indicate that the adult stem cells originate from the larval epithelium but not from non-E. Since all of the larval epithelial cells in the small intestine before metamorphic climax are essentially differentiated as larval type, as mentioned above, it can be concluded that there are at least some differentiated larval epithelial cells that can dedifferentiate into the adult stem cells. In this regard, the larval epithelial cells are similar to the mammalian epithelial "transit amplifying cells,"

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Recombinant intestine	GFP expression: Adult progenitor/stem cell	GFP expression: Adult absorptive cell
Tg / Tg	+	+
Tg / Wt	+	+
Wt / Tg	–	–
Wt / Wt	–	–

Figure 6.3. Adult stem cells originate from the larval epithelium in the *Xenopus laevis* intestine. **(A)** Procedures for tissue recombination and organ culture. Tubular anterior small intestines were isolate from stage 57 wild-type (Wt) or transgenic (Tg) tadpoles expressing GFP. They were slit open and separated into the epithelium and non-epithelial tissues. Four kinds of recombinant intestines were made and cultured: *type of the epithelium/type of non-epithelial tissue. **(B)** The GFP expression of adult progenitor/stem cells (P-AE) on culture day 5 and adult absorptive cells (Ab) on day 7 in each kind of recombinant intestines. Expression was: −, undetectable; +, detectable.

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which can dedifferentiate into the stem cells during adult intestinal regeneration (Potten et al., 1997). In support of this, changes in the expression of nuclear lamins occur in the stem cells, concomitantly with their appearance, from differentiated cell-specific lamin A to embryo-specific lamin LIII (Hasebe et al., 2011a), similar to the changes in the expression of lamin during the mammalian somatic cell reprogramming (Mitalipov et al., 2007; Miyamoto et al., 2007). If so, the next question should be how the larval epithelial cells dedifferentiate into the stem cells by the inductive action of the TH?

Since previous studies suggested the involvement of the connective tissue in TH-induced adult stem cell development, we then investigated whether the TH directly acts on the larval epithelium or non-E, or both, to develop the stem cells, using Tg *X. laevis* tadpoles that express a dominant positive TH receptor (dpTR) under the control of a heat-shock-inducible promoter (Hasebe et al., 2011b). Metamorphic effects of TH are generally known to be mediated by nuclear TR, a transcriptional regulator (Shi, 2009). The dpTR functions as a constitutively liganded TR by specifically binding to the TH response elements (TRE) in the regulatory regions of direct TH response genes. Transgenic expression of dpTR activates transcription of these genes and leads to precocious metamorphosis even in the absence of TH (Buchholz et al., 2004, 2006). Thus, by using the dpTR Tg intestine for tissue recombinant culture experiments, direct TH-response genes could be tissue-specifically expressed at any time after heat shock in the medium deprived of TH. Whenever the epithelium was derived from dpTR Tg intestine, the epithelial expression of dpTR upregulated the expression of Shh, one of direct TH-response genes, after 5 days of heat-shock treatment (Stolow and Shi, 1995). More importantly, when the Tg epithelium was recombined with Tg non-E, these Shh-positive epithelial cells expressed other stem-cell markers and then differentiated into the absorptive epithelium after 7 days, just as they do during natural metamorphosis. In contrast, when the Tg epithelium was recombined with Wt non-E, the Shh-positive cells did not express other stem-cell markers nor differentiate into the adult absorptive epithelium after extended culturing. These results provide substantial evidence that the larval epithelial cells require not only TH signaling in the epithelium but also TH signaling in non-E to fully dedifferentiate into the stem cells (Figure 6.4). Thus, it seems likely that TH-response genes expressed in non-E, most likely the connective tissue, play some role in establishing the stem cell niche, which enables the epithelial dedifferentiation into the stem cells.

Thyroid-Hormone-Activated Signaling Pathways Control Stem Cells via Epithelial–Connective Tissue Interactions

Previously, a large number of genes have been identified as TH-response genes in the *X. laevis* intestine by using PCR-based subtractive differential screenings (Shi and Brown, 1993; Amano and Yoshizato, 1998) and cDNA microarrays (Buchholz et al., 2007; Heimeier et al., 2010). While some of them are directly regulated at the transcription level by the TH through the TR, the others are indirectly regulated downstream genes, suggesting the involvement of complex cell–cell and/or cell–extracellular-matrix (ECM) interactions in the intestinal remodeling (Shi and Ishizuya-Oka, 2001). The expression

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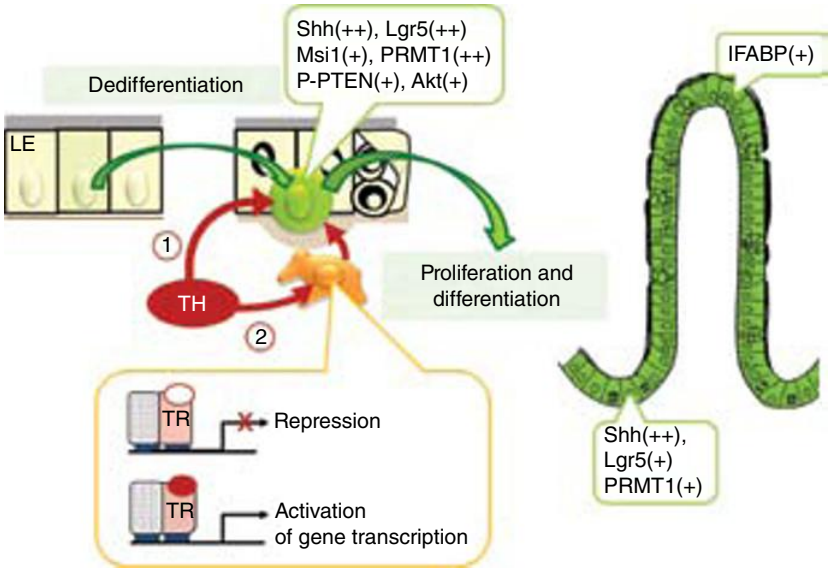


Figure 6.4. Schematic illustration showing thyroid hormone (TH)-induced adult stem cell development in the *Xenopus laevis* intestine. By the inductive actions of TH, some of the larval epithelial cells (LE) dedifferentiate into stem cells positive for markers such as Lgr5. They actively proliferate and differentiate into the adult epithelium where the stem cells are localized in the trough of intestinal folds. The entire processes of this adult epithelial development require not only TH signaling in the epithelium itself (1) but also that in non-epithelial tissues (2). In each nucleus of the tissue cells, TH binds to TH receptors (TR) and regulates the expression of direct TH response genes at the transcriptional level.

analysis of TH-response genes by ISH and immunohistochemistry have identified many genes with expression profiles that spatio-temporally correlate well with the larval-to-adult intestinal remodeling. Among them there are signaling molecules such as Shh, extracellular enzymes, and transcription factors. Although only fragmentary data are available for their function, recent studies using the culture system and transgenic technology have shed light on the key signaling pathways involved in development of the adult stem cells and their niche.

Juxtacrine Signaling

In relation to the epithelial–connective-tissue interactions, previous studies reported remarkable ultrastructural changes in their interface during the *X. laevis* intestinal remodeling. By electron microscopy, the basal lamina, which is a special extracellular matrix (ECM) separating the epithelium from the connective tissue, remains thin and continuous before metamorphosis (Figure 6.5A). During the early metamorphic climax, when the adult progenitor/stem cells become detectable, the basal lamina underlying the entire epithelium suddenly becomes thick by means of vigorous folding and then amorphous (Figure 6.5B and C) (Ishizuya-Oka and Shimozawa, 1987b). Through such modified basal lamina, subepithelial fibroblasts often make contacts

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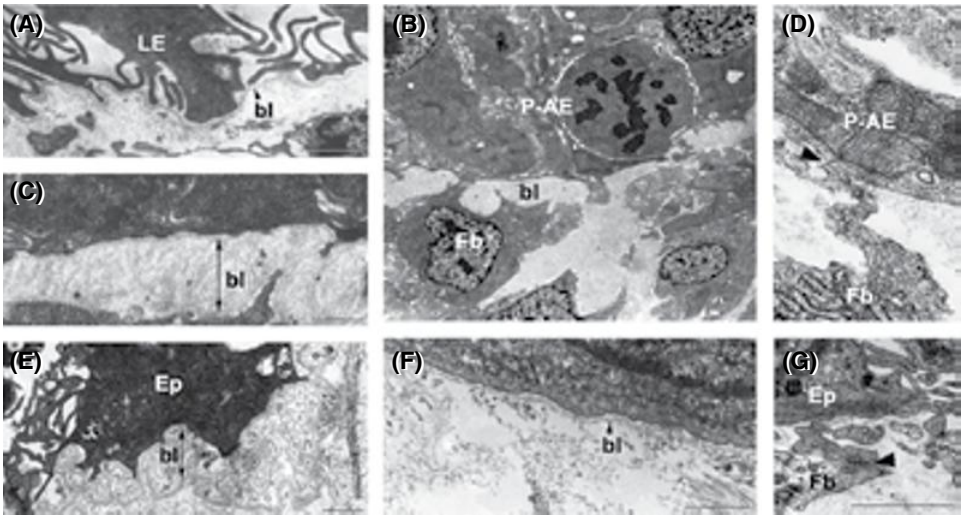


Figure 6.5. Electron micrographs of the epithelial–connective tissue interface in the *Xenopus laevis* intestine. **(A–D)** During natural metamorphosis: the basal lamina (bl) underlying the larval epithelium (LE) is thin and continuous until stage 59 **(A)**; at stages 60–61, when adult progenitor/stem cells (P-AE) appear as islets **(B)**, the basal lamina becomes thick by folding and amorphous **(C)**; through the modified basal lamina, subepithelial fibroblasts (Fb) often make contacts with the adult progenitor/stem cells (D, arrowhead). **(E, F)** Stage 57 intestines cultured *in vitro*: the basal lamina underlying the epithelilum (Ep) was modified after 3 days of thyroid hormone (TH) treatment **(E)**, whereas it remained thin when the anti-ST3 antibody was added to the TH-containing medium **(F)**. **(G)** Transgenic stage 54 intestine overexpressing ST3 for 4 days. Fibroblasts often made contacts (arrowhead) with epithelial cells through the modified basal lamina. Bars, 1 μm.

with the adult progenitor/stem cells (Figure 6.5D) but not with the larval proper cells undergoing apoptosis. Similarly, in the mammalian intestine, frequent cell contacts between the mesenchyme and the epithelium occur during development around birth (Mathan et al., 1972) when intestinal stem cells are considered to develop (Harper et al., 2011; Muncan et al., 2011). Thereafter, as the adult progenitor/stem cells differentiate into the simple columnar epithelium, the basal lamina underlying the adult epithelium becomes thin and flat again. At the same time, cell contacts between the two tissues become rare. This transient modification of the basal lamina followed by the cell contacts also occurred in the tadpole intestine cultured *in vitro* whenever the adult stem cells successfully developed (Ishizuya-Oka and Shimozawa, 1992). These observations strongly suggest that cell-to-cell contacts between the two tissues following the ECM remodeling play critical roles in the adult epithelial development but not in the larval epithelial apoptosis.

Matrix metalloproteinases (MMPs), a superfamily of Zn-dependent proteases known to degrade various ECM components (Birkedal-Hansen et al., 1993; McCawley and Matrisian, 2001), are noteworthy in connection with the basal lamina modification. Previously, many MMPs, including collagenases, gelatinases, stromelysin-3 (ST3; MMP11), and membrane type-1-MMP, have been identified as the TH-response genes

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in the *X. laevis* intestine (Shi et al., 2007). Although most of them are highly expressed during the intestinal remodeling, ST3 is the only MMP that has been experimentally demonstrated to be essential for the basal lamina modification.

Stromelysin-3, which was first identified as a gene associated with human breast cancer, is highly expressed in fibroblasts surrounding most types of invasive cancers, including colorectal (Boulay et al., 2001). In the *X. laevis* intestine, the expression of ST3 is also specific to fibroblasts and directly upregulated by the TH at the transcriptional level (Patterton et al., 1995; Fu et al., 2006). To investigate the functions of ST3, we first performed culture experiments *in vitro* by using an antibody against the catalytic domain of *X. laevis* ST3 (Ishizuya-Oka et al., 2000). Since ST3 protein is secreted in the enzymatically active form, the addition of the anti-ST3 antibody to the TH-containing culture medium should block the activity of ST3. This addition led to inhibition of TH-induced modification of the basal lamina in a dose-dependent manner (Figure 6.5E and F). Although a small number of the adult progenitor/stem cells were occasionally detected, they failed to invaginate into the connective tissue and never formed the adult absorptive epithelium. Next, the function of ST3 was examined *in vivo* by using Tg tadpoles that overexpress ST3 under the control of a heat-shock-inducible promoter (Fu et al., 2005). Overexpression of ST3 alone in the premetamorphic intestine did not induce the adult epithelial development but caused the basal lamina modification. Through such basal lamina that became amorphous, fibroblast–epithelial cell contacts frequently occurred, similar to those during natural metamorphosis (Figure 6.5G). These results strongly suggest that ST3 is one of the critical signals for the establishment of the stem cell niche, although other signals are also required for this process.

As a substrate of ST3, other than the ECM components, the 67-kd receptor for laminin (LR), a major ECM component of the basal lamina, has been identified. In addition, it has been shown that LR can be cleaved by the ST3 protein (Amano et al., 2005; Mathew et al., 2009). Thus, ST3 may alter interactions between the epithelial cells and the basal lamina, leading to the fibroblast–epithelial cell contacts. So far, the biological significance of cell contacts between the two tissues still remains unclear. Recently, in the adult mammalian intestine, the importance of cell biomechanics for the stem cell organization has been proposed (Buske et al., 2012). Thus, it is possible that mechanotransduction caused by the cell contacts may play some role in the stem cell development. To unravel it, future studies should be directed to identify mechanosensitive and/or juxtacrine signals that are specifically expressed in these contact sites. Their functional analysis could pave a way to clarifying unknown molecular aspects of the cell contacts.

As TH-response genes related to other juxtacrine signaling pathways, Notch1, hairy1, which is a transcriptional repressor activated by the Notch pathway (Buchholz et al., 2007), and Msi1, which is a positive regulator for the Notch pathway by suppressing the translational expression of Numb (Okano et al., 2005; Rezza et al., 2010), have been identified to date. Although the expression profiles of Notch1 and hairy1 have not yet been examined by ISH, Msi1 has been shown to be specifically expressed in the adult progenitor/stem cells from their first appearance (Ishizuya-Oka et al., 2003). These expression data suggest that the Notch pathway plays certain roles in the development of adult stem cells in the amphibian intestine. Also in the adult mammalian intestine, the stem cells express high levels of Notch1, suggesting a fundamental

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role of the Notch pathway in the maintenance of the stem cells (van den Brink et al., 2001; Fre et al., 2005; Pellegrinet et al., 2011). On the other hand, Paneth cells adjacent to the stem cells express Notch ligand Dll4 and function as one of the niche players (Sato et al., 2011; Vooijs et al., 2011). Since the amphibian intestine lacks Paneth cells throughout life, it is an interesting next task to identify what cells express Notch ligands and/or other Notch pathway components in the *X. laevis* intestine to understand how the Notch pathway is activated during the stem cell development.

Shh/BMP-4 Signaling Pathway

Sonic hedgehog is generally known to bind to a membrane-associated receptor, Patched (Ptc) (Marigo et al., 1996; Fuse et al., 1999), and relieve Ptc-mediated inhibition of the Smoothened (Smo) activity. This leads to the activation of the downstream transcription factors Glis 1–3, which finally regulate Shh target genes (Ruiz i Altaba, 1999; Sasaki et al., 1999). In the *X. laevis* intestine, Shh expression is directly upregulated by TH only in adult epithelial progenitor/stem cells, reaches its peak when they most actively proliferate, and is then downregulated toward the end of metamorphosis (Ishizuya-Oka et al., 2001; Hasebe et al., 2008). This expression profile strongly suggests a key role of Shh in the adult stem cell development. In contrast to the epithelium-specific expression of Shh, the expression of Ptc, Smo, and Glis 1–3 is in the connective tissue or muscles but not in the epithelium (Hasebe et al., 2012). In addition, their expression has been shown to be upregulated by Shh even in the absence of TH. These results indicate that Shh secreted by the adult progenitor/stem cells acts on the surrounding tissues in a paracrine manner with a positive feedback loop. More importantly, using the culture system, we have experimentally shown that Shh is involved in the *X. laevis* intestinal remodeling (Ishizuya-Oka et al., 2006). Addition of exogenous Shh protein to the TH-containing culture medium promoted cell proliferation of the connective tissue and muscles in a concentration-dependent manner after 5 days, when the adult progenitor/stem cells actively proliferated *in vitro*. At the same time, Shh upregulated the expression of bone morphogenetic protein 4 (BMP4) only in the connective tissue, just as Shh upregulates the expression of BMP4 only in the mesenchyme during gut organogenesis of higher vertebrates (Roberts et al., 1998; Sukegawa et al., 2000). For extended culturing, the added Shh caused developmental anomalies including the nonluminal epithelial structure, just as overexpression of Shh leads to abnormal differentiation in the *X. laevis* embryonic gut (Zhang et al., 2001). Thus, it seems likely that upregulation of Shh expression is essential for establishment of the adult stem cell niche, whereas subsequent downregulation of Shh expression is required for normal epithelial differentiation.

In agreement with the BMP4 expression upregulated by Shh *in vitro*, the expression of BMP4 in the connective tissue during the early metamorphic climax is higher the closer to the adult progenitor/stem cells which highly express Shh. This suggests that BMP-4 functions as a morphogen. Interestingly, the pan-hedgehog inhibitor, hedgehog interacting protein (Hip), is also transiently upregulated in the connective tissue during this period. Since the expression of Hip is contrary to that of BMP4 (Hasebe et al., 2008), it may locally suppress the activity of Shh. On the other hand, a major type I receptor for BMP4, BMPR1A, is expressed in both the connective tissue and the adult progenitor/stem cells (Ishizuya-Oka et al., 2006), suggesting direct effects of BMP4 on

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the both tissues. In fact, our culture study has shown that the addition of exogenous BMP4 protein to the TH-containing medium not only suppressed cell proliferation of the connective tissue, antagonizing the Shh function, but also caused precocious differentiation of the adult epithelium expressing IFABP (Ishizuya-Oka et al., 2006). Similarly, in the mammalian intestine, the expression of BMP4 is upregulated by Shh and promotes the epithelial differentiation (Scoville et al., 2008; Takashima and Hartenstein, 2012). In contrast, when excessive Chordin, an antagonist of BMP4 (Piccolo et al., 1996) that is also upregulated by TH in the *X. laevis* intestine, was added to the TH-containing culture medium, cell proliferation of the connective tissue was activated; that is, with the addition of Chordin, BMP4 deficiency led to the reduction of proliferation and the total number of adult progenitor/stem cells. This implies that a certain level of BMP4 is required to maintain the intestinal stem cells (Ishizuya-Oka et al., 2006), as reported in the case of mammalian embryonic stem cells (Qi et al., 2004; Ying et al., 2003) or primordial germ cells (Fujiwara et al., 2001). Furthermore, the *Xenopus* homolog of *Drosophila* Tolloid closely related to BMP1 (Tolloid/BMP1), which regulates the activity of BMP4 by degrading Chordin in the early *X. laevis* embryos (Wardle et al., 1999; Balemans and Van Hul, 2002), has also been identified as a direct TH-response gene. The expression of Tolloid/BMP1 is specific for the connective tissue and reaches its peak when the adult progenitor/stem cells actively proliferate (Shimizu et al., 2002). Taken together, it is highly possible that the BMP4 activity is spatio-temporally regulated by Tolloid/BMP1 through Chordin. Thus, the Shh/BMP4 pathway appears to be at least one of the key pathways that mediate epithelial–connective tissue interactions essential for the adult stem cell development in this amphibian model.

Wnt Signaling Pathways

In the adult mammalian intestine, it is well documented that the canonical Wnt signaling pathway, which is activated through translocation of β-catenin to the nucleus, plays a central role in the maintenance and proliferation of stem cells and their descendants (Sato et al., 2009; Medema and Vermeulen, 2011). Although its roles in the *X. laevis* intestinal remodeling have not yet been examined sufficiently, a number of genes related to this pathway have been identified as TH-response genes in the *X. laevis* intestine (Buchholz et al., 2007): β-catenin, a Wnt receptor Frizzled-2 (van de Wetering et al., 2002), Wnt targets CD44 (Moore and Lemischka, 2006) and Lgr5 (Sun et al., 2010), a Wnt agonist R-spondin-1 (Ootani et al., 2009), secreted Frizzled-related protein 2 (sFRP2) and Msi1, both of which are known as positive regulators for the Wnt pathway in the mammalian intestine (Bovolenta et al. , 2008; Kress et al., 2009; Rezza et al., 2010), and phosphorylated PTEN and Akt (Ishizuya-Oka and Shi, 2007), both of which are involved in the interplay between Wnt and BMP signals (He et al., 2004, 2007). Although their expression profiles remain mostly unclear, Lgr5 is specifically expressed in the adult progenitor/stem cells from their appearance, as mentioned above. Furthermore, Wnt5a and Ror2, components of the noncanonical Wnt pathway, have also been identified as TH-response genes (Buchholz et al., 2007), and their expression is transiently upregulated during the adult stem cell development (our unpulished oservations). These data lead us to consider that both canonical and noncanonical Wnt pathways are involved in not only the maintenance of stem cells, as

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reported in the adult mammalian intestine, but also their development, possibly through interaction with the Shh/BMP4 pathway. To fully understand the signaling network of the stem cell niche, further analyses of TH-response genges related to the Wnt pathways are necessary.

Conclusions

The amphibian metamorphosis from the evolutionary viewpoint resembles the mammalian postembryonic development around birth, since both phenomena are closely associated with the transition from aquatic to terrestrial vertebrate life. Indeed, amphibian intestinal remodeling shares common characteristics with mammalian intestinal maturation around birth; both phenomena occur concomitantly with the increase of plasma TH levels and lead to the establishment of the epithelial cell-renewal system fueled by the stem cells. In addition, recent studies have shown that some of epithelial cells dedifferentiate into stem cells during mammalian intestinal maturation (Harper et al., 2011; Muncan et al., 2011), just as during amphibian metamorphosis (Ishizuya-Oka et al., 2009). At the molecular level, a cDNA microarray study has shown that most of the genes that are upregulated during *X. laevis* intestinal remodeling, including ST3 and Shh, are also upregulated during the mammalian intestinal maturation, concomitantly with the increase of plasma TH levels (Kolterud et al., 2009; Heimeier et al., 2010). These similarities strongly suggest the existence of evolutionary conserved, TH-dependent gene regulation program leading to development of the adult stem cells and their niche (Ishizuya-Oka and Shi, 2011). Therefore, the amphibian intestine, where TH can trigger the whole process of stem cell development, serves as a valuable model to investigate its molecular mechanisms.

In mammals, in addition to the postembryonic development, homeostasis of adult organs is also known to be under the control of TH (Tata, 1993; Shi, 1999; Yoshizato, 2007). Although the action of TH on Shh remains unclear in the intestine, TH can upregulate Shh expression in the brain (Desouza et al., 2011). In the adult intestine, TRα1, one of TH nuclear receptors, has been shown to directly upregulate the expression of β-catenin and sFRP2 to promote proliferation of the stem cells and their ascendants in the crypt (Plateroti et al., 2006; Kress et al., 2009; Sirakov and Plateroti 2011). Considering that all of the adult intestinal epithelial cells are derived from the stem cells located in the crypt, it is tempting to speculate that TH may continue to act on the stem cells to maintain the epithelial cell-renewal throughout adulthood, by regulating Shh and/or Wnt pathways.

Recent expression and functional analyses of TH-response genes using this animal model have gradually elucidated the key signaling pathways that mediate the epithelial–connective tissue interactions essential for the adult stem cell development. So far, Shh secreted by adult progenitor/stem cells is one of the most important signals to start the adult stem cell development: Shh could be involved in establishment of the stem cell niche by acting on the surrounding tissues in a paracrine manner. One of Shh targets, BMP4, is secreted by fibroblasts and signals back to the adult progenitor/stem cells under the local control of Tolloid/BMP1/Chordin signaling. Bone morphogenetic protein 4 not only promotes adult epithelial differentiation but also is required to maintain the stem cells. On the other hand, ST3 secreted by fibroblasts is another early

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signal necessary for the establishment of the stem cell niche. Stromelysin-3 enables contacts between the adult progenitor/stem cells and subepithelial fibroblasts by altering cell–ECM interactions, which is possibly required for the adult epithelial development (Figure 6.6). Interestingly, during mammalian intestinal maturation, Shh expression is highly upregulated only in the intervillus pockets where the stem cells develop (Kolterud et al., 2009). Thereafter, Shh expression becomes localized in the

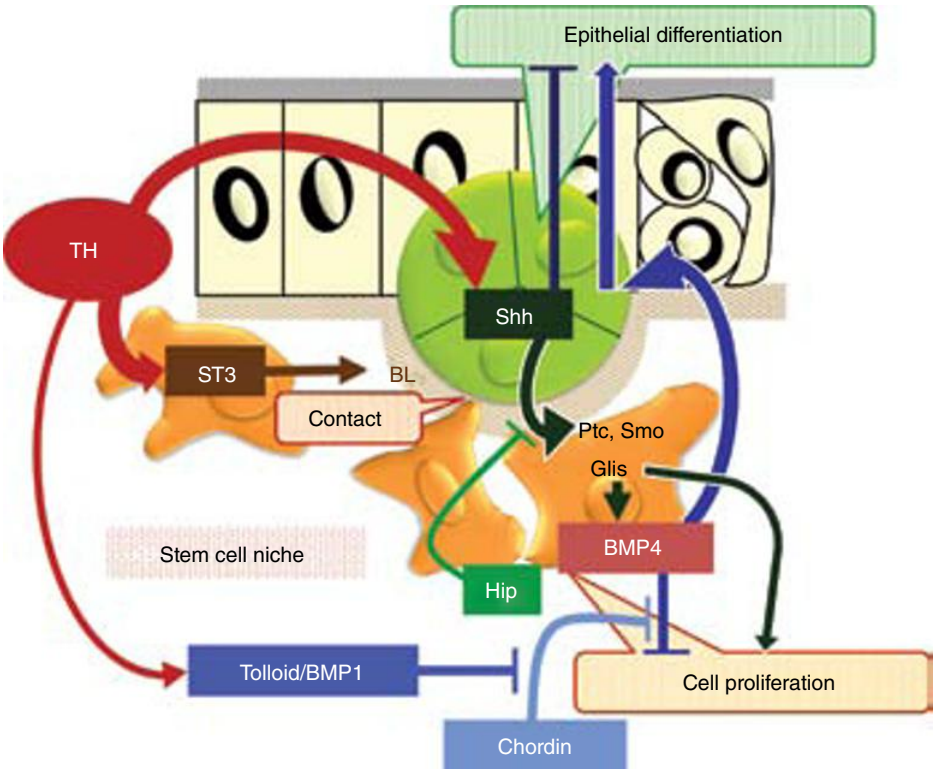


Figure 6.6. Proposed model of thyroid hormone (TH)-activated signal pathways involved in development of adult stem cells and their niche during *Xenopus laevis* metamorphosis. The TH directly upregulates Shh expression only in adult progenitor/stem cells. The Shh secreted by them acts on the connective tissue or muscles in a paracrine manner through Ptc, Smo, and Glis. The Shh, in which activity is inhibited by Hip, activates cell proliferation of the connective tissue and induces fibroblasts to express BMP4, which in turn suppresses cell proliferation of the connective tissue and promotes differentiation of the adult epithelium and/or maintains the adult stem cells under the control of Tolloid/BMP1 through degrading Chordin. On the other hand, the TH directly upregulates ST3 expression only in the connective tissue. The secreted ST3 modifies the basal lamina (BL), leading to frequent contacts between the adult epithelial progenitor/stem cells and fibroblasts. These Shh/BMP4 and ST3 signaling pathways are integrated, together with Wnt or Notch pathways activated in the adult progenitor/stem cells, into a signal network essential for adult stem cell development. Arrows and T-shaped bars indicate activation and repression, respectively.

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crypt where the stem cells reside (Crosnier et al., 2006; Varnat et al., 2010). In addition, when the stem cells actively proliferate during mammalian intestinal regeneration or carcinogenesis, the level of Shh expression becomes high (Berman et al., 2003; Nielsen et al., 2004). These results support our proposal that Shh functions as an early key player to establish the stem cell niche common to the amphibian and mammalian intestines. Possibly, the TH-activated Shh signaling pathway should be integrated, together with ST3 signaling and other pathways (such as Wnt and Notch), into a signal network to establish the stem cell niche. By using recent Cre/loxP (Rankin et al., 2009, 2011) and gene knockout technologies for frogs (Young et al., 2011; Lei et al., 2012), it is thus a challenging issue to clarify the entire signal network involved in stem cell development and/or maintenance, clarification of which is urgently needed for regenerative and cancer therapies, and to know how far the stem cell network is dependent on TH.

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Chapter 7

Stem Cell Therapy for Veterinary Orthopedic Lesions

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Introduction

Musculoskeletal conditions are major causes of injury in most countries. In the face of a poor response to conventional therapy, some biological therapeutics have been tested to increase the quality of repairing tissue and shorten the time required for healing. These therapeutics include stem cells, growth factors, natural and synthetic biomaterials, bank tissue, and engineered tissues, and they have become interesting issues for the scientific community and for animal and human health care.

Clinical applications of these biological therapies constitute a promising field. For the past few decades, scientists have studied stem cell pluripotency, differentiation, and immunomodulation using different *in vitro* and *in vivo* protocols. Much work has focused on understanding regenerative medicine, although there remains only fragmented knowledge regarding the cascade that regulates cell migration, its paracrine effects, and the tridimensional integration to different damaged tissues.

Although numerous papers have been published about stem cell therapy, its applications and results are polemic. Some professionals are very enthusiastic about its therapeutic effects and even offer the technique to clients. Others, however, believe in its potential but state that many more studies are needed before the technique should be used commercially. Finally, there are skeptics who criticize the high expectations for stem cell therapy.

A Complex Cascade

The goal of using stem cells is to build new tissue using cell. This goal can be achieved either via a direct contribution through differentiation into tissue-specific cell phenotypes or indirectly by trophic effects through the production of bioactive proteins, such as growth factors, antiapoptotic factors, and chemotactic agents. This information is mostly important regarding lesions or diseases with poor responses to conventional therapy (Smith et al., 2003; Richardson et al., 2007).

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Different applications have been explored, but the exact mechanism applied by seeded cells is not completely known. Progenitor cells are believed to differentiate at the specific tissue site of implantation, produce an appropriate extracellular matrix (ECM), and synthesize bioactive proteins such as growth factors and cytokines. These substances recruit endogenous stem cells and anabolically stimulate both stem and mature cells (Alves et al., 2013). All of these actions most likely occur together as a complex cascade.

Clinical Applications

Regenerative medicine has been tested on several clinical diseases and injuries. Initially, it was used only on blood cancers such as leukemia (Weissman, 2000). Then, experimental and clinical applications were applied to cardiac problems such as acute myocardial infarction and Chagas disease, which is a major cause of cardiomyopathy in Latin America (Perin et al., 2003; Jasmin et al., 2012). Regenerative medicine can also be used to treat diabetes type I (Zalzman et al., 2003), lung diseases (Neuringer and Randell, 2004), epithelial reconstruction (Li et al., 2004), and degenerative and traumatic neurologic diseases (Liu et al., 2012).

However, studies showing the results and safety of stem cell therapy on orthopedic lesions are more frequent. Experiments have mostly explored large bone defects (Muschler et al., 2003; Zamprogno, 2008), muscular dysfunctions (Chen and Goldhamer, 2003, Kerkis et al., 2008), cartilage lesions (Yamada, 2011), tendinitis (Young et al., 1998; Barreira et al., 2008), and desmitis (Fortier and Smith, 2008).

Orthopedic injuries have particular importance for horses and dogs. Rossdale et al. (1985) reported that orthopedic lesions are the most important reason for removing horses from work, even exceeding respiratory problems. In addition, a similar scenario occurs in dogs. With the extension of the lives of cats and dogs, degenerative diseases have an increased impact on joint, tendon, and ligament injuries.

Questions about Sources

Some cells have a common origin and similar functions. For example, connective tissue cells specialize in secreting extracellular matrices rich in collagen and are responsible for body structure. Connective tissue cells include osteocytes, myocytes, chondrocytes, adipocytes, and fibroblasts, which is the most undifferentiated cell type in this group. They have the capacity to differentiate into any mesodermal lineage cell in response to protein signaling and the tridimensional arrangement of the matrix (Alberts et al., 2010).

Embryonic and adult stem cells have been studied, and both types demonstrate promising results; however, the use of adult stem cells does not involve an ethical polemic, which facilitates studies and increases their applicability. Recently, Özen et al. (2012) reported that adult stem cells are found in a perivascular niche, where they maintain multilineage potential and self-renewal capacity as they interact with the microenvironment. Adult stem cells have surface markers that resemble pericytes and mesenchymal stem cells (MSCs). Moon et al. (2011) showed that somatic cells can be reprogrammed into induced pluripotent stem cells (iPSCs) by the transcription factors

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Oct4, SOX2, and Klf4 in combination with c-Myc, which has expanded the possibilities for stem cell therapy.

The source of the donor also varies. For example, an implant or transplant of cells between individuals from different species is called heterologous, but transplant within an individual is termed autologous, and transplants within the same species but with different individuals are called allogeneic (Tapp et al., 2009). Most studies use autologous transplantation to prevent immune reactions. However, if stem cells are truly immunomodulatory, then allogeneic transplants are possible and would not trigger rejection. This source may result in increasing the supply and in implementing stem cell therapy earlier in the course of the disease because it is not necessary to collect and culture these types of cells. Allogenic transplants are also more cost-effective than autologous ones, because there is no need to expand the patient cell population; however, additional costs involving quality assurance and cell storage would need to be added (Fortier and Travis, 2011).

For orthopedic injuries, MSCs are the primary focus of study. Independent of the donor, several sites of harvesting exist, including bone marrow (BM), adipose tissue (AT), peripheral blood (PB), and umbilical cord blood (UCB) (Alves et al., 2013). Bone marrow was the first source reported to contain MSCs; however, human ATs are readily available because they are discarded as liposuction surgery waste. Although liposuction is not performed on animals, harvesting these cells is much easier than harvesting BM. Additionally, Burk et al. (2012) affirmed that AT has a greater number of viable MSCs with a high rate of migration and cell differentiation than BM. However, only 30% of mesenchymal cells obtained from AT or BM are multipotent. According to Gorodetsky and Schäfer (2011), most of the cells have restricted potential to differentiate, which generates poor results regarding tissue regeneration. According to Kern et al. (2006), aging is also correlated with a decline in the number and differentiation potential of MSCs.

Umbilical cord blood harvesting is the least invasive technique; however, an animal reproduction program to access this source is necessary. Kern et al. (2006) compared some MSCs sources and verified differences in the success rates of isolating MSCs, which was 100% for BM and AT but only 63% for UCB. However, UCB-MSCs were able to be cultured the longest and showed the highest proliferation capacity, whereas BM-MSCs possessed the shortest culture period and the lowest proliferation capacity. The paper highlights UCB and AT as attractive alternatives in isolating MSCs, in which AT had the highest concentration of MSCs and MSCs isolated from UCB were expanded to higher numbers.

In our opinion BM is an advantageous source for treating tendon, ligament, and bone injuries because of its heterogeneous population composed of MSCs and hematopoietic stem cells, which are useful to replace both ECMs and blood vessels. However, BM harvest requires trained professionals, which limits its use. This source has minimal donor site morbidity and allows autologous implantation, which carries fewer regulatory and safety issues. Furthermore, in comparative experiments assessing multipotency, BM-derived MSCs frequently outperformed MSCs that were isolated from other sources (Vidal et al., 2007; Toupadakis et al., 2010).

At the same time, ATs have gained importance because they are easily accessible in large quantities and can be a good option as a source of avascular tissues such as cartilage. Even though their MSCs have similar marker profiles as BM-derived MSCs,

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they are less capable of differentiating into specific cell lineages, which can compromise results (Im et al., 2005; Toupadakis et al., 2010).

Adipose-derived nucleated cells (ADNCs) have been used for injection into tendon lesions either after extraction (Nixon et al., 2008) or expansion in cell culture, which is similar to the technique used for BM cells (de Mattos Carvalho et al., 2011). It is important to recognize the difference between these two approaches. The nucleated cell fraction released from the AT, referred to as the stromal vascular fraction (SVF), is a mixed population of cells that includes endothelial cells, preadipocytes, and a relatively low number of MSCs. However, because the SVFs are not expanded in culture, they are termed minimally manipulated and are therefore subject to fewer regulatory issues in human medicine. Although AD-MSCs are expanded in the laboratory and involve a greater cost and increased labor, they have the potential advantage to produce a greater number of cells and a more homogeneous MSC population (Alves et al., 2011).

Other Biologic Therapeutic Products

As previously mentioned, other autologous biological products have been used in human and animal therapy with evident clinical efficacy, minimally invasive collection, and easy and low-cost processing (Textor and Tablin, 2012). The clinical use of platelet-rich plasma (PRP) has been described in the literature (Vendramin et al., 2009; Milano et al., 2010). When activated, the PRP releases more than 800 proteins (Senzel et al., 2009) that have paracrine effects on mesenchymal cells (Dohan et al., 2010), fibroblasts, tenocytes (de Mattos Carvalho et al., 2011), chondrocytes (Yamada et al., 2012), and osteoblasts (Graziani et al., 2005).

Platelet-rich plasma is a chemotactic solution that contains many mitogenic factors, such as platelet-derived growth factor (PDGF), TGF-ß, epidermal growth factor (EGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), and interleukins, which promote fibrin glue formation (Browne and Branch, 2000). Clinical studies in different species and diseases have been published in recent years in horses (de Mattos Carvalho et al., 2011; Yamada et al., 2012), dogs (Ferraz et al., 2007), pigs (Lippross et al., 2011), goats (Jakse et al., 2003), rabbits (Kasten et al., 2008), and rats (Messora et al., 2008). Human studies have demonstrated the safety of the treatment (Kon et al., 2010), and to date, there are no reports of adverse effects associated with autologous implants.

Many tests were done to determining the best quality of the platelet-rich plasma and to check the method's reproducibility. We varied the strength and timing of centrifugation, the amount of the reduction plasma and the waiting time. To be considered platelet-rich plasma, the platelet concentration must be two- to eightfold higher than in plasma, and leukocytes present in the sample should appear to influence platelet behavior (Fortier and Travis, 2011).

Extracellular Matrix Influence

The ECM also plays an important role in understanding stem cell behavior at the implantation site. It provides fully functional space organization for tissues and their systemic integration. These molecules interact with cellular epitopes to activate or

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produce different substances (Smith and Webbon, 1996). The addition of substances such as Oct4 induces human cells to become iPSCs from blood cells or reprograms Sertoli cells into induced neural stem cells (Dangsheng, 2012).

Based on this information and the lack of further details, *in vitro* and *in vivo* studies are necessary to define all aspects of MSC behavior in different tissues. The success of stem cell therapy depends on using the appropriate cell type and particular method of stimulation for each tissue. This chapter aims to promote a revision of the orthopedic applications of stem cells in animals, mostly in horses, based on recent literature and on the experience of this group of authors.

Stem Cell Therapy for Tendinitis and Desmitis

Tendons are frequently injured by different mechanisms, and lesions are expressed as tissue degenerative phenomena with inflammatory reactions of differing degrees. Despite limited knowledge of the basic biology of tendons and ligaments, papers during the past decade promoted a greater understanding of tendon physiology and etiopathogenesis of lesions, which permits progress in treatment. Recent studies primarily focus on MSC therapy, adipose- and BM-derived, and detail the different aspects of their mechanism of action.

Alves et al. (2011) hypothesized that in tendon lesions, regardless of the source, the implantation of autologous MSCs in far greater numbers than are normally present within this tissue will improve the repair structurally, as shown by optimizing mechanical properties, organization, and composition, and functionally, as measured by reduced re-injury rates.

Normal tendons have great tensile strength due to the high proportion of type I collagen (>90% of total collagen), which is arranged in a hierarchical way (Hofmann and Gross, 2007). After injury, the thinner type III collagen (usually < 1%) is found in a higher amount and rapidly forms cross-links to stabilize the injury. Tendon tissues are poorly vascularized and predominantly utilize anaerobic energy systems, thus resulting in poor healing potential after an acute or overused injury.

Tendonitis is an important injury that occurs in horses with a high rate of recurrence, which may decrease athletic performance or result in premature retirement from sports (Kasashima et al., 2004). It usually requires a long rest period, which takes from 6 months to over 1 year to allow adequate tissue repair. Conventional treatments for tendonitis are primarily based on clinical and surgical approaches. However, recovered animals are rarely sufficiently healed to allow their full return to competitions at the same level as before injury (Marfe et al., 2012).

Mesenchymal stem cells can regenerate connective tissues, but increasing evidence shows that the mechanism of action may not be due to direct engraftment or differentiation. Mesenchymal stem cells secrete a variety of soluble autocrine and paracrine growth factors, which recruit MSCs, promote cell survival, and enhance endogenous connective-tissue cell proliferation. These growth factors stimulate mitosis in tissue progenitors, induce angiogenesis, and reduce apoptosis (Hoffmann and Gross, 2007; de Mattos Carvalho et al., 2011).

The use of MSCs in the treatment of equine tendonitis is promising. In our previous studies, histological improvement in tendon tissue in response to BM mononuclear cells therapy was observed (Barreira, 2005; Oliveira et al., 2011), and adipose-derived MSC therapy in equine tendinitis also improved repair (de Mattos Carvalho et al., 2011).

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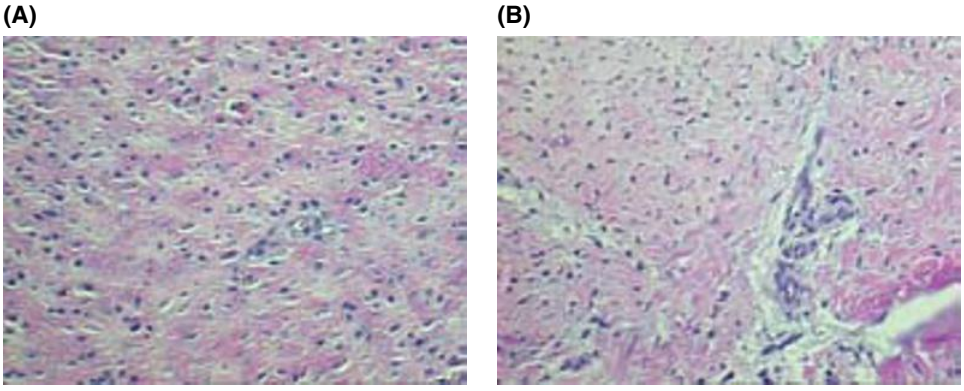


Figure 7.1. Results from Barreira et al. (2008). Histopathology of induced tendon lesions was carried out on horses at 48 days after induction. The treatment group was seeded with intralesional BM mononuclear fraction cells. **(A)** Observation of treatment group that presented an increase in inflammatory cell infiltration, increase of extracellular matrix synthesis and reduction of necrotic area, when compared to **(B)** the control group. (200×)

In a previous paper (Barreira et al., 2008), the effect of autologous BM cells on tendonitis was analyzed through biopsies at 48 days after lesion induction. High inflammatory cell infiltration, ECM synthesis, a reduced amount of necrotic areas (Figure 7.1), a small increase in cellular proliferation (KI-67/MIB-1), and low immunoreactivity to transforming growth factor beta 1 (TGF-β1) were observed. These observations suggested that the acceleration of tendon repair occurred in the treated group.

Using a similar protocol, Oliveira et al. (2011) increased vascularization in the treatment group at 120 days and showed an improvement in tendon healing in treated limbs by the characterization of collagen fibers of types I and III in the new tissue. Therapy using autologous implants of the mononuclear fraction improved tissue organization and its quality, resulting in the significantly higher expression of collagen type I (Figure 7.2).

The same protocol was applied to racing thoroughbreds with superficial digital flexor tendon (STDF) and suspensory ligament injuries as part of our research. In tendinitis, BM-derived stem cell therapy appears to shorten rehabilitation time, except at the insertion site (enthesopathies). Cell implantation was not difficult, it must be guided by ultrasound using an established protocol under standing sedation and after regional analgesia.

Reports describing the beneficial effects of using BM suspended in its supernatant suggest that it improves therapeutic effects (Smith, 2008). However, BM also can be suspended in autologous serum (Pacini, 2007), plasma (Smith et al., 2003) and platelet-rich plasma (PrP; Del Bue et al., 2008).

Due to the ease of AT harvesting and the higher percentage of MSCs in this source, our group later studied adipose stem cell treatment on induced equine tendinitis (de Mattos Carvalho et al., 2011). After 150 days of treatment, higher expression of collagen type I was observed (Figure 7.3) and was correlated with a higher quality of repair tissue. In subsequent studies, we added PC to ADMSC using a similar protocol to verify PC influence (de Mattos Carvalho et al., 2011). Although no difference was observed in the gene expression of collagen type III and factor VIII in the groups with

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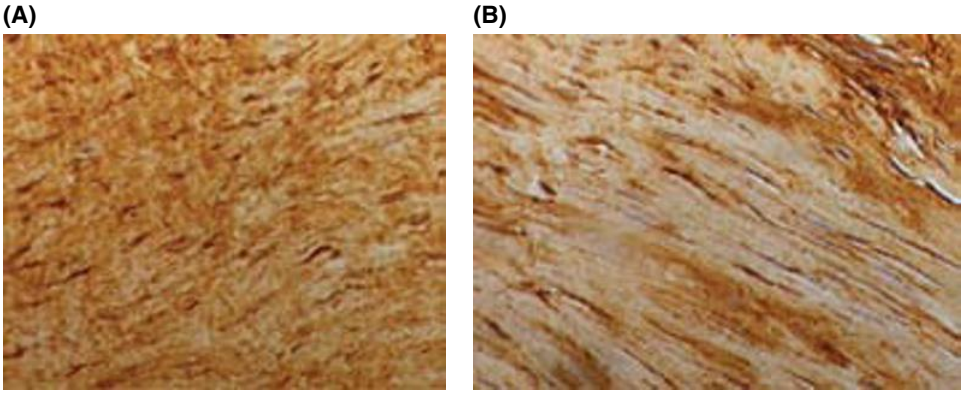


Figure 7.2. Results from Oliveira et al. (2008). Study of tendon lesions treated with autologous implants of the mononuclear fraction. The pictures show immunohistochemistry of collagen type I from equine tendon at 120 days after lesion induction. Observation of higher expression of collagen type I in **(A)** the treatment group (46.9%), when compared to **(B)** the control group (36.5%) (200×).

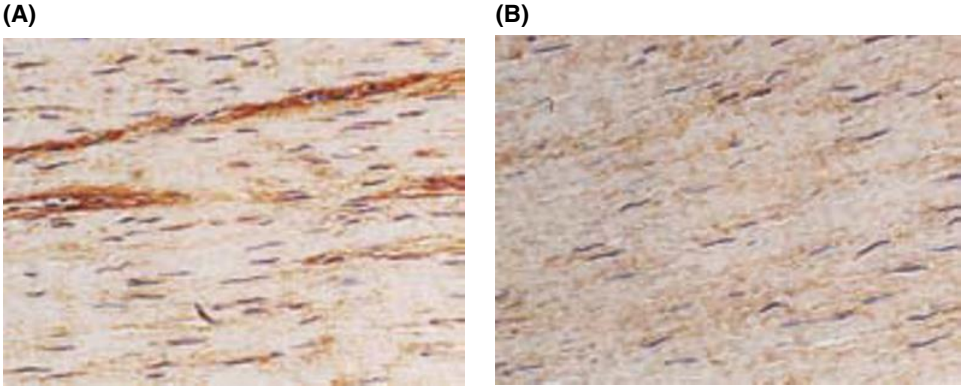


Figure 7.3. Results from de Mattos Carvalho et al. (2011). Study of equine tendinitis treated with adipose-derived mesenchymal stem cells. Higher expression of collagen type I was seen in **(A)** the treatment group, when compared to **(B)** the control group by immunohistochemistry analysis at 150 days (400×).

and without PC treatment after 16 weeks, MSCs in addition to PC limited the progress of induced lesions, which can be explained by the anti-inflammatory and immunomodulatory action of the therapy (Bendinelli et al., 2010). Several mechanisms involve the anti-inflammatory effects of MSCs that benefit tendon repair, including an increase in chemokines, the suppression of cytokine secretion from dendritic cells and a reduction of T lymphocytes and natural killer effects. The use of MSCs in addition to PC or PRP has been described previously and was correlated with improvement via a follow-up ultrasound (Argüelles et al., 2008). Furthermore, increased local blood cell flow and metabolic activity were also observed, which results in tissue repair maturation (Bosch et al., 2010).

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Further studies need to be conducted to define the ideal protocol for equine and dog tendonitis and desmitis therapy. The best time to seed cells needs to be confirmed. Currently, stem cell therapy is used in horses from 7 to 45 days after the onset of the lesion. It remains necessary to determine whether it is possible to prevent further injury when such therapy is applied to a injury under development. Furthermore, the repeated implantation protocol needs to be studied further. Seeding cells two to three times in a repair process appears to have a positive effect because the injury maintains a higher concentration of growth factors, which accelerate cellular metabolism. Studies also need to determine the best source of cells. Until now, BM and fat have been the most popular, but recent studies on satellite cells in cartilage and muscle have shown promising results, which can be tested in tendons. It is also necessary to elucidate all the cascades of metabolic events of stem cells in tissue repair. Based on these ideas, these studies must have strong biomechanical analyses to verify whether a significant reduction in the healing time is associated with an increase in new tissue strength.

Stem Cell Therapy for Cartilage Defects

Articular disease is common in humans and domestic animals, mostly horses and dogs. According to Frisbie et al. (2005), 51% of racing horses have orthopedic problems, and cartilage lesions are the most frequent occurrence. Among these diseases, osteoarthritis (OA) causes the greatest impact because of its debilitating, chronic, and progressive profile. In dogs, hip dysplasia is one of the most frequent joint diseases. Rettenmaier et al. (2002) published a study of 2885 dogs where the prevalence of hip dysplasia was 17.7–19.7% with radiologic signs of OA.

Cartilage is a specialized tissue for absorbing impact, which acts as an elastic sponge that absorbs and expels water during weight bearing. Cartilage does not contain blood vessels or innervations and is composed of chondrocyte columns. Chondrocytes synthesize the surrounding ECM, which promotes its nutrition using synovial fluid and blood from subchondral bone. Cartilage ECM contains primarily collagen type II soaked in proteoglycan (aggrecan), glycoproteins, and hyaluronic acid (HA). The interaction between hyaluronan and aggrecan generates hydrophilic molecules, which allows water to flow easily inward and outward (Richardson, 1992).

Osteoarthritis is a multifactorial disease that affects structures in the joint environment (Felson and Neogi, 2004). It can be caused by systemic (e.g., genetics, obesity), local (e.g., synovitis, capsulitis), and biomechanical (e.g., muscle weakness, subchondral bone overload) factors. Repeated trauma and joint instability are also involved and can be considered primary causes of OA in sport horses (Richardson, 1992; Abramson and Krasnokutsky, 2006; Semevolos and Nixon, 2007, Carmona and Prades, 2009). The cartilage becomes yellowish, opaque, soft, wrinkly, and cracked or fractured with decreased impact absorption and exposed subchondral bone, which results in stiffer joints (Felson and Neogi, 2004; Schwarz and Saunders, 2011). Cytokines, particularly interleukin 1, are released and activate enzymes as matrix metalloproteinases (MMPs) that degrade cartilage and change synovial fluid (Schwarz and Saunders, 2011).

Osteoarthritis is diagnosed by imaging techniques, by arthroscopy (Denoix, 2010) and recently by biomarkers in synovial fluid, serum, and urine (Berg-Johansson, 2009). Radiology is extremely useful in serial analysis because it enables studying the disease

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progression and treatment responses, which vary among individuals. Some patients present a quiescent profile, but others have progressive cartilage loss and a decrease in joint space (Raynaud et al., 2004). With progression, subchondral bone sclerosis and osteophyte formation occur. However, radiology is not able to detect early changes, thus requiring association with ultrasonography or magnetic resonance imaging (MRI). Ultrasonography allows an earlier diagnosis than radiology by detecting thickening of the capsule, thinner and rougher cartilage, and enthesopathy. Magnetic resonance imaging has higher sensitivity in detecting bone and soft-tissue injuries, but its availability is not frequent in some countries. Although computed tomography may contribute to the diagnosis, it is not a technique of choice (Rasera et al., 2007; Denoix, 2010; Schwarz and Saunders, 2011). Therefore, complete joint evaluation needs a technique combination.

Many of the detected lesions are asymptomatic and, therefore, the exact incidence is unknown. It is likely that symptoms and joint degeneration are dependent on lesion size, location, and patient characteristics (Bos, 2010).

Regardless of the etiology of OA, the response to conventional treatments is not satisfactory because no modification of the long-term disease occurs (Frisbie et al., 2005; Carmona and Prades, 2009). The goals of cartilage defect repair should always be a combination of symptom relief and prevention of early joint degeneration. Pharmacological treatment consists of: (i) anti-inflammatory steroids or nonsteroids, which provide pain relief and decrease the effusion; (ii) glycosaminoglycans, mostly in chondroitin sulfate, which decreases prostaglandin and increases proteoglycan levels; and (iii) HA, which improves synovial fluid viscosity. Additionally, arthroscopy allows lesion debridement, treatment, cell implantation, and even creates microfractures, which cause the release of growth factors that stimulate stem cell migration (De Biasi et al., 2004; Frisbie et al., 2007; Fortier et al., 2010).

The inability to prevent the development of fibrocartilage in tissue repair and the progression of the disease with conventional therapy results in a need to search for new possibilities. In this regard, regenerative medicine suggests the use of biological alternatives in horses, such as the use of precursor cells, IRAP (protein receptor antagonist of interleukin-1) and PC or PRP (Weissman, 2000; Fortier and Travis, 2011).

Studies on cell therapy in animal chondral lesions seek to understand the biology of synovial joint diseases, allowing analysis of the safety and effectiveness of the techniques. However, many variables affect the behavior of progenitor cells *in vivo* because ECM interactions result in different tissue and immunomodulating tropism. Cartilage stem cell therapy is the only currently approved modality by the Food and Drug Administration (FDA), ensuring the therapeutic safety; however, the success rate is still extremely variable. These results can be explained by the aging of individuals, which decreases the quality and number of chondrocytes, and by the protocol. Questions about the source of precursor cells, the use of scaffolds, and cartilage growth factors still persist (Gorodetsky and Schäfer, 2011).

Many studies have explored different sources of precursor cells for therapy of joint damage and the most frequent are BM and AT. Mesenchymal stem cells are the main cell type involved to chondral defects stem-cell therapy. Improvement in tissue repair and an increase in ECM production have been achieved using stem cells from BM aspirates through intra-articular injections and intralesional applications with biocompatible scaffolds (Fortier et al., 2010).

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Fortier and Smith (1998) pointed to the use of biological agents and tested the chondrogenic potential of equine BM MSCs *in vitro*. The culture demonstrated rounded cells that produced type II collagen and proteoglycans. Our group (Yamada et al., 2012) tested different associations with MSCs from AT as a treatment of induced chondral lesions in horses. The best result regarding the morphological aspects of joint damage occurred with MSC treatment in addition to PC.

Embryonic and induced pluripotent cells (iPCs) are also a focus of study (Kern et al., 2006; Gorodetsky and Schäfer, 2011). However, only 30% of MSCs obtained from AT or BM are multipotent. Most of these cells have restricted differentiation potential, which generates poor results in the regeneration of damaged cartilage. However, samples from these sources, after differentiation into chondrocytes, tend to be hypertrophied and mineralize the newly formed matrix (Gorodetsky and Schäfer, 2011). Therefore, the use of chondral precursors originating from mature chondrocytes has been explored. These cells show innate chondrogenic and osteogenic but not adipogenic differentiation capacity, suggesting an enhanced direction for the chondrogenesis of mesenchymal cells. Even in the absence of growth factors, chondrogenic cells should synthesize sulfated glycosaminoglycans and more collagen type II than type I *in vitro* (Amaral et al., 2011).

Studies involving stem cells in the treatment of cartilage defects indicated that the cells present the formation of smooth white repair tissue that is apparently fibrocartilaginous and well attached and completely fills the chondral defect (Muschler et al., 2003; Yamada et al., 2012). Chondral lesions that were not submitted to treatment with MSCs presented a failure to fill the lesion and persistent articular cartilage degradation. Studies reported that lesions treated with stem cells present, as a consequence, a lower rate of occurrence of erosions and fibrillations surrounding the lesion, lower rates of osteophytes, and less articular cartilage degeneration. However, none of the treatments minimized joint inflammation, which was measured by PGE2 levels in the synovial fluid (Yamada et al., 2012).

When considering the use of cell therapy in dogs, Black et al. (2007, 2008) observed an improvement in lameness, range of joint motion, and pain scores in cases of chronic osteoarthritis of the hip and elbow joint with intra-articular injection of adipose-derived MSCs. Although the outcomes were subjectively evaluated, these results represent important progress because osteoarthritis induced by hip dysplasia is a major cause of lameness in dogs.

Other approaches in small animals include intra-articular injection after partial meniscectomy in the stifle, medial coronoidectomy in elbows with dysplasia, and Achilles tendonitis. Research in dogs showed the improvement of angiogenesis, chondrogenesis, greater immune cell infiltration, and proliferation of the fibroblasts in induced meniscal tears compared to untreated joints. After stem cell injection, the dog owners perceived pain (visual analog scale (VAS)) to be significantly improved at both 6 weeks and 6 months after joint injections (Britt, 2012).

Several studies have compared the efficacy of MSCs with chondrogenic potential from BM, AT, and even nasal cartilage (Tapp et al., 2009; Amaral et al., 2011); however, controversy exists regarding the best source. The therapeutic action of different sources requires prior knowledge of the gene expression of surface markers at different stages of differentiation (Tapp et al., 2009) and its interactions in the tridimensional environment (Yamada, 2011).

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Doubts also exist about the use of scaffolds for cell therapy in cartilage lesions; however, some authors report their need and recommend the use of hydrogel, fibrin glue (Tapp et al., 2009), or micromass (Gorodetsky and Schäfer, 2011), while others claim that scaffolds are not necessary (Frisbie and Stewart, 2011; Watts and Nixon, 2011). If there is no need of a scaffold, the transplantation technique does not require arthroscopy; therefore, it becomes easier, cheaper, and less risky.

The use of PRP has become popular in recent studies and even in veterinary services because it stimulates the local metabolism (Vendramin et al., 2009; Milano et al., 2010).

Platelet-rich plasma can be injected into the joint with or without arthroscopy, into the lesion, or into the synovial fluid – activated or nonactivated. When activated, PRP presents a gel form and appears to have better results in cartilage repair, regardless of the protocol (Milano et al., 2010; Fortier and Travis, 2011; Gorodetsky and Schäfer, 2011;). Ito et al. (2006) demonstrated that fibrin is useful in stem cell therapy because it supports cell growth in a three-dimensional scaffold. Fibrin glue offers a stable surface for chondral growth and has osteoinductive potential that allows the migration of chondrocytes to the injury site.

Fortier et al., 2010 usually induces cloth formation on the lesion site by subcondral bone perforation to promote cell adhesion. The cloth acts as an intrinsic PRP, but the technique still needs arthroscopy, which raises its risk and cost.

The use of autologous PRP in articular cartilage defects in horses was also studied by our group. Cartilage lesions were induced on medial femoral trochlea, followed by intralesional PrP injection in the treatment group and saline solution in the control group (Figure 7.4). Synovial fluid analysis was carried out every 15 days, until day 150, when biopsy was performed. According to Yamada et al. (2012), the use of PRP resulted in better quality tissue, although it did not minimize joint inflammation, as measured by PGE2 levels in the synovial fluid. The treatment group presented white, smooth fibrous tissue well attached to the lesion site, while in the control group a lack of defect filling was noted (Figure 7.4).

In our opinion, a combination of biological factors such as MSCs and PRP, promotes synergistic therapeutic effects, minimizes articular cartilage inflammation, and produces better quality tissue. Indeed, ideal protocols have to be defined in order to optimize results.

Stem Cell Therapy for Bone Problems

Despite its solid appearance, bone is a dynamic organ with constituent cells that communicate constantly to maintain both structural and metabolic functions and to respond to external stimuli such as mechanical loading, inflammation, and hormones.

Under chondral ossification, osteoblasts (OBs) arrive from vessels and replace chondrocytes, while osteoclasts (OCs) also arrive and create a bone cavity. As bones grow, OBs produce an organic matrix that is mineralized by the deposition of calcium and phosphate in the form of hydroxyapatite. Some OBs become surrounded by bone and differentiate into osteocytes, which maintain cellular communication via tiny cannaliculi to respond to mechanical forces. Osteoclasts play a critical role in remodeling bones as they grow because these cells are capable of restoring both organic and inorganic

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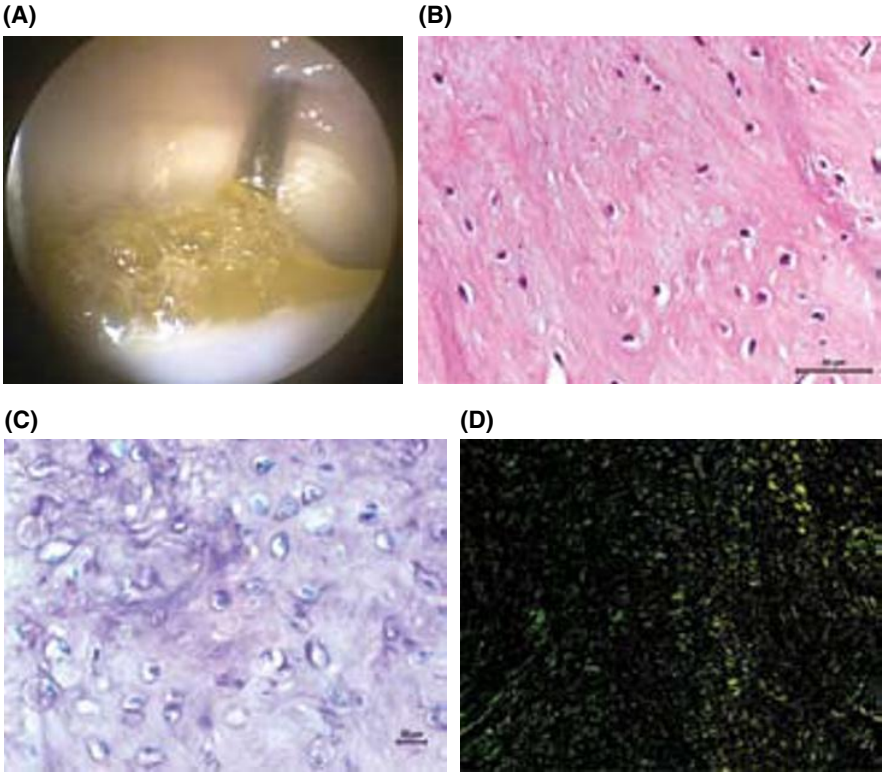


Figure 7.4. Results from Yamada et al. (2012). Study of equine cartilage defects treated with adipose-derived mesenchymal stem cells presenting better quality of tissue repair. **(A)** Autologous PrP injected in articular cartilage defects in horses. **(B)** Photomicroscopy under natural light of treated group stained by hematoxylin and eosin, showing formation of fibrocartilaginous tissue, with a predominance of chondrocytes (400×), and **(C)** stained by toluidine blue, showing glycosaminoglycan presence (400×). **(D)** Photomicroscopy under polarized light stained by Picrosirius Red, in which G1 presents thinner fibers and predominance of green related to type II collagen formation (200×).

components. In mature bones, chondrocytes remain only at the joint surfaces, where they maintain joint cartilage. Osteoblasts and OCs are also present and are active in mature bone, where they interact in bone remodeling units. Throughout life, OCs remove bone and OBs replace it during normal homeostasis; however, their activities are balanced, leading to a complete replacement of the skeleton every 10 years. Communication between these cell types is critical to bone health, and disturbances in this relationship are found in many diseases or lesions (Novak, 2011).

Fatigue injury of the third metacarpal bone ("Bucked shins") is a self-limited disease that afflicts approximately 70% of thoroughbred yearlings horses in their first year of training. Affected animals usually have poor performance and training intermissions. Conventional therapy relies on the type and severity of lesion and usually requires a

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rest period of 2–3 months, with a high risk of recurrence. In a study by our group, the effect of autologous BM mononuclear fraction in equine fatigue injury of the third metacarpal bone was examined. After therapy, the animals were submitted to a progressive training program and returned to full training in week 3. Clinical improvements were observed in 66.7% of the animals, and recurrence occurred in 33.3%. Although treated horses were capable of returning to exercises more quickly, the re-injury rate was similar to that observed in conventional therapy (Rebelo et al., 2010).

Regenerative medicine approaches to bone injuries primarily focus on atrophic nonunion, cysts, and the replacement of large loss of bone tissue. Large bone defects are usually caused by trauma, infection, or tumors, and atrophic nonunion is usually caused by insufficient blood supply, interposition of soft tissue, and consequence of infection. The treatment of large bone defects remains a challenge for orthopedic reconstructive surgeons. The generation of bone grafts with osteoconductive, osteoinductive, and osteogenic properties via tissue engineering strategies may resolve this problem (Khan et al., 2009).

Many *in vitro* and pre-clinical studies were performed to investigate the applicability of different stem cell types for bone regeneration. Some promising results using adult MSCs focused on the differentiation of bone cells, which enhanced bone healing and vascularization. Experiments based on critical size defects have been conducted in rabbit femurs and showed a progression in the healing rate treated with BM-derived MSCs. Better healing of the bone gap at the osteotomy site was obtained, while lyophilized bone chips alone did not achieve complete osteointegration in some cases (Dallari et al, 2007).

Nonetheless, regenerative medicine used for bone healing has reached the patient in the form of cell-therapy approaches to treat localized bone defects or systemic diseases of the skeleton. Autologous MSCs were successfully seeded in a number of patients to enhance fracture/osteotomy healing, fill bone defects, treat pseudarthrosis, bone cysts, osteonecrosis, or enhance spinal fusion with relevant clinical applications (Schmitt et al., 2012).

Therefore, it appears that the combined use of homologous bone, gels of platelets, and autologous MSCs is an ideal biological stimulus for bone regeneration.

Stem Cell Therapy for Muscular Diseases

Skeletal muscle growth and repair are mediated by a resident population of myogenic precursors called satellite cells (Ferrari et al., 1998). They reside around muscle fibers, exhibit robust regenerative capacity *in vivo*, and can be isolated easily, but they lose their stemness and survival *in vitro*. Therefore, Gilbert et al. (2010) tested the influence of hydrogel in a muscle stem cell (MuSC) culture to determine changes in their behavior. The authors postulated that hydrogel mimics the elasticity of brain, cartilage, and muscle and reported better results compared to using plastic. According to the authors, the use of MuSCs is superior to using induced pluripotent or embryonic stem cells, in which differentiation must be directed because they are native and have a natural identity to the microenvironment. Once the limitations in culturing are resolved, these cells may be useful in muscular-disease therapy.

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Independent of the source, stem cell therapy in muscle lesions is not as popular as in tendons, cartilage, or bone because usually myositis responds well to conventional therapy and has a minor impact on performance and quality of life. However, stem cell therapy of large muscle ruptures (Brown et al., 2012) or intractable degenerative disorders (Rudnicki, 2003) are described. Brown et al. (2012) reported cases of acute semitendinosus muscle tears of working German shepherd dogs treated with stem cells. The cells were obtained from a stromal vascular fraction of a falciform ligament and were seeded into the muscle (intra- and paralesional) as well as given intravenously. Complete recovery was observed after 10 weeks, and the dogs returned to full work.

In 2003, Rudnicki suggested that hematopoietic stem cells contribute to muscle regeneration, but the findings were most promising regarding neuromuscular degenerative disorders such as Duchenne muscular dystrophy (DMD). According to Kerkys et al. (2008), golden retriever dogs are the best animal model for therapeutic trials for DMD because they naturally present this disease. In the study, they transplanted human dental pulp stem cells (hIDPSC) either by arterial or muscular injections into four puppies aged 28–40 days with natural DMD. Two noninjected dogs were kept as controls. The seeded human cells presented significant engraftment in dog muscle, and although human dystrophin expression was modest, chimeric muscle was observed. No signs of immune rejection were observed, and these results suggested that hIDPSC cell transplantation may be performed without immunosuppression. Better clinical conditions were observed in dogs that received monthly arterial injections, and they were clinically stable at 25 months of age. These data suggest that systemic multiple deliveries are more effective than local injections for muscular diseases, which contribute to ideal protocol definitions for stem cell therapy for chronic conditions.

Although many stem cell clinics offer the therapy for animals with muscle lesions or diseases, little scientific literature regarding this topic has been published.

Other Applications

Other interesting cases exist in which stem cells may be helpful. Horses hoof avulsion is an example of a rare condition in horses that can severely limit their performance and even become a life-threatening condition. Only a few cases of total hoof loss are reported in the literature, and their poor prognosis is the primary message. When recovery occurs, it takes approximately 2 years for the animal to return to its normal activities. Our group reported a case of total hoof avulsion treated by a combination of conventional and stem cell therapy. When the injury was no longer life threatening, an autologous BM mononuclear fraction was injected into different areas at the distal portion of the affected limb. During the following days, marked clinical improvement was noted, and the presence of exuberant and healthy granulation tissue was observed (Figure 7.5). Continuous improvement occurred, and the horse was able to gallop 4 months after the accident, although with grade 3 lameness. One year after the accident, the animal was almost sound. Based on the literature, a faster recovery occurred. We conclude that stem cell treatment accelerated the healing process and improved the quality of life of the patient (Barreira et al., 2010).

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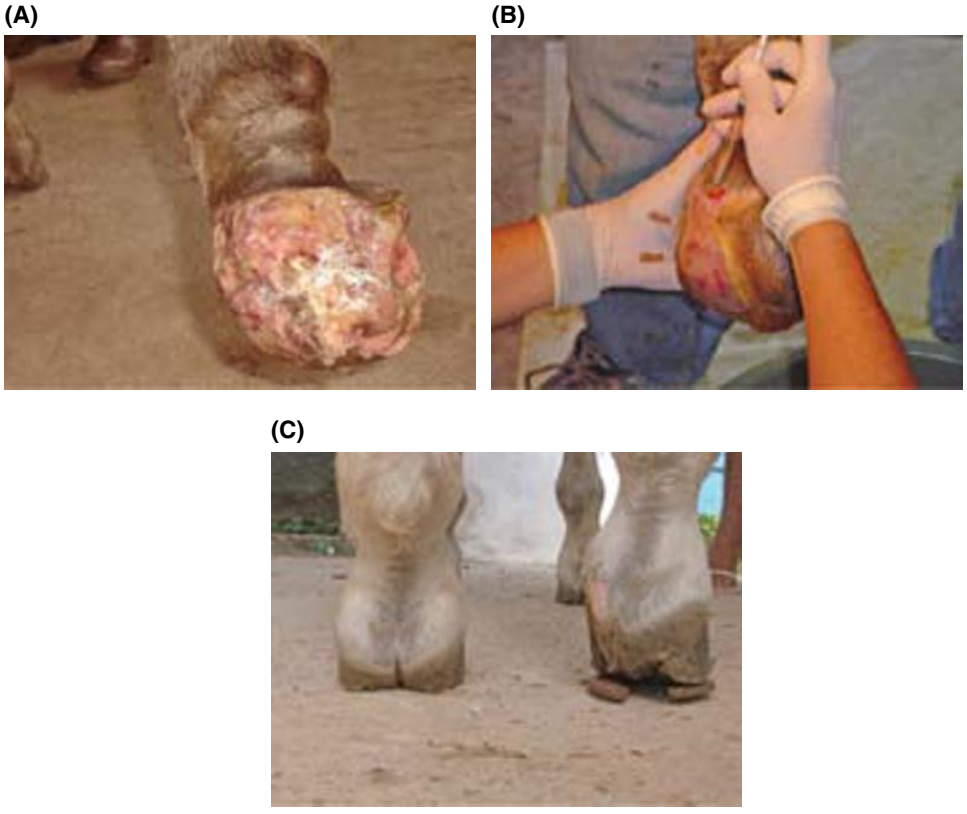


Figure 7.5. Results from Barreira et al. (2010). **(A)** A case of traumatic hoof avulsion in a 2-year-old horse; **(B)** treated with autologous BM mononuclear fraction; **(C)** observation of hoof conditions at 18 months after treatment, when the horse was able to gallop normally.

Conclusions

Nowadays, regardless of tissue source, the allogeneic transplant of MSCs represents a promising protocol for the attendance of patients because of the quick attendance of requirements of orthopedic injuries and diseases, even though, it still has to define ideal protocols that are still required. Although much has been discovered about this new type of therapy, it is important to consider that this technology is relatively new and further studies on basic stem cells and tissue biology are necessary. To realize the full therapeutic potential of stem cells, a number of open questions have to be answered. Such studies should include more complex experiments with longer time periods of study and should involve the treatment of orthopedic lesions to certify the safety and improved efficacy of the therapeutic use. Apart from all the particularities involving MSCs, the regenerated tissue must also provide the appropriate tridimensional structure, including the production of an extracellular matrix and a functional tissue-specific biomechanical behavior. To achieve clinical applications of MSCs, an interdisciplinary approach with biologists, bioengineers, and clinicians will be essential.

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Chapter 8

Sex Steroid Combinations in Regenerative Medicine for Brain and Heart Diseases: The Vascular Stem Cell Niche and a Clinical Proposal

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Current Possibilities in Regenerative Medicine

The perivascular tissue-specific stem cell niche is essential for the preservation and differentiation of endogenous stem cells (Bukovsky, 2011a). The composition/effectors, function, tissue-specific differences, and alterations with age of stem cell niches are still poorly understood. The goal of regenerative medicine is to replace altered or lost functional cells in certain tissue with exogenous stem cells, such as embryonic stem cells (ESCs) or adult-derived induced pluripotent stem cells (iPSCs) capable of differentiation into tissue-specific functional cells (Barker, 2012). Human iPSCs can be generated from tissue cells (e.g. skin and lung fibroblasts, endometrium) and from menstrual blood (Nishino et al., 2011). Neural progenitor cells have been generated from cells in human urine (Wang et al., 2012). The replacement of lost functional cells with exogenous stem cells has, however, a number of obstacles, such as ethical concerns, immune responses after administration, the potential of teratoma formation, the inability of stem cells to regenerate, cell cycle arrest, differentiation into functional tissues, and apoptosis (Choumerianou et al., 2008; Rogers, 2009; Tesche and Gerber, 2010). In addition, most attempts to establish ESCs from large mammals have not been successful (Li et al., 2011). Human iPSCs exhibit teratoma formations when tested in immuno-compromised animals (Suhr et al., 2009). Recent study also indicates that iPSCs retain a distinct genomic methylation memory of their past, which can reduce their therapeutic utility (Lister et al., 2011). Long-term reprogramming of iPSCs was attained by their continuous passaging, however, resulting in possible therapeutic application (Nishino et al., 2011).

An alternative approach would be to induce a patient's own pluripotent stem cells to differentiate *in situ*. This could be successful in acute/traumatic disorders where the appropriate perivascular tissue-specific stem cell niche required for the maintenance of

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stem cells and their differentiation into the functional state is preserved. There is also the possibility of using a process transforming nonstem cells into different types of cells called autologous transdifferentiation. For instance, differentiated exocrine pancreatic cells can be converted into hepatocytes by treatment with a dexamethasone (Shen et al., 2000). Pancreatic β-cells can differentiate from hepatocytes with gene transfer by recombinant adenoviruses (Ber et al., 2003) or from adult exocrine pancreatic cells with epidermal growth factor (EGF) and leukaemia inhibitory factor (Baeyens et al., 2005). Differentiated vascular smooth muscle cells (SMCs) can be converted into neuronal cells by sex steroid combinations (Bukovsky, 2009) (see below).

Another possibility is to alter the differentiation path of committed intrinsic stem cells from their established differentiation course. For example muscle progenitor cells committed to the myogenic pathway transdifferentiate to neurons, following their inoculation into the developing brain of newborn mice (Sarig et al., 2010). This indicates that a distinct stem cell niche can convert (reprogram) muscle-committed stem cells into a different path of differentiation.

Such approaches eliminate most of the roadblocks listed above but do not address concerns about the efficiency of such stem cell differentiation in enhancing the return of normal tissue function (Rogers, 2009). Long-lasting disorders may be associated with an altered perivascular tissue-specific stem cell niche causing persisting tissue dysfunction. Degenerative diseases may be caused by an altered stem cell niche stimulating apoptotic degeneration of functional tissue cells. Thus improper tissue function may be maintained by an improper tissue-specific stem cell niche, and stimulation of stem cells toward differentiation could thereby cause their dysfunction or degeneration.

Attempts to improve altered tissue function by stem cell differentiation into functional cells may not be successful unless we are able to manage stem cell niche repair (Bukovsky, 2011a). Animal experiments dealing with disorders of the pancreas, heart, liver, and nervous system have been more successful than human clinical trials (Tesche and Gerber, 2010). This may be due to preservation of the proper perivascular tissue-specific stem cell niche shortly after induction of a disorder in the animals.

Vascular Pericytes and Morphostasis: Pluripotency of Stem Cells

Mesenchymal stem cells (MSCs) from various tissues reside in a perivascular location. These can be identified as pericytes functioning as mural cells in the microvessels (Feng et al., 2007). Pericytes secrete large quantities of bioactive trophic factors stimulating tissue intrinsic progenitor cells (Caplan, 2009). Beside their trophic function in the perivascular tissue-specific stem cell niche, some pericytes also exhibit MSC properties and are capable of nuclear reprogramming into distinct cell types such as testicular Leydig cells (Davidoff et al., 2009), cardiomyocytes (Dohmann et al., 2005), cartilage and bone (Augello et al., 2010; Crisan et al., 2012), and neural and neuronal cells (Bukovsky, 2009). Hence pericytes display a dual role in the perivascular tissue-specific stem cell niche: exhibiting a trophic function for intrinsic progenitor cells and reprogramming into tissue-intrinsic progenitor cells when such cells are required. Pericytes show a gene expression similar to neural cells, and both pericytes and

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neuronal cells originate from the neural crest (Sato et al., 2003). The activity of the perivascular tissue-specific stem cell niche is modulated by autonomic innervation (Chow et al., 2011), which regulates pericyte numbers and their function (Wu et al., 2003; Wiley et al., 2005).

Other essential components of the perivascular stem cell niche are monocyte-derived cells (MDCs) associated with the microvasculature (Bukovsky, 2011a). It has been shown that depletion of MDCs in one testis of adult rats selectively abolishes the differentiation of Leydig cells from mesenchymal precursors (Gaytan et al., 1994) such as vascular pericytes (Davidoff et al., 2009). Recent findings indicate that CD14+ primitive MDCs (pMDCs) in the circulation are involved in a variety of physiologic functions in addition to their innate and acquired immune responses, such as repair and regeneration of tissues (Seta and Kuwana, 2010). CD14+ pMDCs also stimulate the proliferation of tissue-intrinsic progenitor cells, and MDCs regulate their preservation in the tissue-specific functional state (Bukovsky, 2011a). Therefore, MDCs have multiple roles in the perivascular tissue-specific stem cell niche. They stimulate division of intrinsic progenitor cells, participate in the stimulation of their differentiation, and preserve differentiated cells in a tissue-specific functional state.

It appears that the perivascular tissue-specific stem cell niche is a relatively straightforward structure, consisting of pericytes regulated by autonomic innervation and perivascular CD14+ pMDCs. It is physically associated with the microvasculature throughout the body, influencing tissue intrinsic progenitor cells required for tissue regeneration and repair. This basic "tissue control unit" (TCU) requires incorporation into a more complex system called the tissue control system (TCS), which exhibits marked diverse involvement among the various tissues. Tissues vary in their extent of cellular differentiation from stem cells to apoptosis (Bukovsky, 2011a).

In some tissues functional cells remain relatively young (brain, pancreas, skeletal muscle). Others reach a moderate stage of differentiation enabled by intraepithelial T cells (gut epithelium), and some tissues differentiate into cornified (apoptotic) superficial cells by binding immunoglobulins (stratified epithelium of the epidermis and uterine ectocervix). Interruption of cell differentiation before reaching a functional state in early ontogeny causes persistent tissue dysfunction due to the immaturity; differentiation of tissue cells during early ontogeny more than functionally required may cause subsequent autoimmune and degenerative diseases. It is important that the tissue attains its functional stage of differentiation as soon as possible during early ontogeny. Tissues differentiating very early into a functional stage, such as the heart during the embryonic period, can function for a 100 years in humans. On the other hand the normal ovary, which differentiates during the fetal period, can function for about only 50 years. Delayed functional differentiation of certain tissue during early ontogeny causes early alteration of its function during adulthood (see Bukovsky, 2011a for details).

The Role of Sex Steroids in the Neural Stem Cell Niche

Neural stem cells (NSCs) accompany the NSC niche residing in adult brain subventricular zones of the lateral ventricles. In these zones, accompanied by hippocampal neurogenesis for short-term and long-term memory (Zou et al., 2012), new functional

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neurons are generated. Beside NSCs, the niche contains astrocytes (the most abundant cell of the human brain), ependymal cells, and vascular endothelial cells (Lee et al., 2012). Involvement of immune cells in cross-talk with NSCs plays an important role in the stem cell niche behavior (Kokaia et al., 2012). Microglia and other immune cells produce a myriad of cytokines influencing self-renewal, proliferation, glial differentiation, oligodendrogenesis, neurogenesis, migration, survival quiescence, and neuronal differentiation in the subventricular and subgranular (hippocampal) zones of the central nervous system (CNS) (Gonzalez-Perez et al., 2012).

Sex steroids may play a role in the organization of structural connections in the brain. Estradiol and progesterone may enhance cortico-cortical and subcortico-cortical functional connectivity, and testosterone may increase functional connectivity between subcortical brain areas (Peper et al., 2011). The age-related loss of androgens in man is associated with symptoms that include depression and impaired cognitive function. Lower free testosterone (TS) levels can be detected 5–10 years prior to the diagnosis of Alzheimer's disease (AD). Animal experiments suggest that both estrogens and androgens may play a role in preventing such neurodegeneration. In clinical trials, the most extensively studied steroids have been androgens. Androgen therapy, however, has no effect on cognitive performance, as well as verbal and nonverbal memory in elderly patients. In addition to the gonads, some steroids are also synthesized in adrenals and in the brain; the latter are sometimes termed neurosteroids (reviewed by Fuller et al., 2007).

Sex steroids have the potential to stimulate the proliferation and differentiation of existing NSCs. They easily pass through the blood–brain barrier and bind to the abundant sex steroid receptors in the brain, particularly areas important for the regulation of emotions, cognition, and behavior (Westberg and Eriksson, 2008). It remains to be determined whether utilization of a single sex steroid alone has a role in prevention or treatment of neurodegenerative diseases and traumatic neurologic injuries.

Cellular Reprogramming: A Small Molecule Perspective for a Chemical Approach

Chemical approaches for stem-cell-based therapy have followed two strategies. Initially, the cells in culture were manipulated to obtain homogeneous stem cell types specific for particular tissues. A more promising approach is the utilization of chemicals to stimulate existing endogenous stem cells to proliferate and regenerate. Such a chemical approach can act on target stem cells or their niches *in vivo*, without requiring stem cell implantation, to promote cell survival, proliferation, differentiation, reprogramming, and homing (Yu et al., 2008).

Various combinations of transcription factors have been used to create iPSCs, but cellular reprogramming to iPSCs is an inefficient and slow process involving stepwise stochastic events. Activation of endogenous transcription factors by small molecules (cell-lineage-specific growth factors and cytokines) may be more efficient than providing exogenously reprogrammed iPSCs (Nie et al., 2012).

Another possibility is the use of natural small molecules passing blood barriers in combinations causing direct transformation of one adult cell type into progenitors of a distinct cell type (see below).

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Sex Steroid Combinations Cause Nuclear Reprogramming of Ovarian Stem Cells into Neural- and Neuronal-Type Cells *In Vitro*

Our initial observations of cultured ovarian stem cells (OSCs) indicated that they are capable of differentiating spontaneously into distinct cell types, including neuronal-type cells (inset, Figure 8.1A; Bukovsky et al., 2005). Our subsequent experiments tested different sex steroid combinations in ovarian cell cultures (Bukovsky et al., 2008).

Cultured ovarian epithelial stem cells primarily differentiate into large epithelial cells lacking the expression of stage-specific embryonic antigen 1 (SSEA-1) and neural cell adhesion molecule (NCAM). A few epithelial cells in untreated cultures show weak staining for Thy-1 differentiation protein (Figure 8.1A) abundant in neuronal cells (Cohen et al., 1981), and similar expression of SSEA-4. The addition of individual gonadotropins (FSH and/or hCG), EGF, estradiol (E2), progesterone (PG), and TS alone (at 60 μm for each steroid), or E2 + PG and E2 + TS combinations showed no change in either cell morphology or immunohistochemical staining. No changes were observed in control cultures, including those with the sex steroid vehicle (Bukovsky et al., 2008).

On the other hand, utilization of TS mixed with PG 1 day after E2 pretreatment produced a marked effect 1 h after the treatment. There was transdifferentiation of epithelial cells into small cells, some strongly expressing SSEA-1 (arrowhead, Figure 8.1B), a glycoconjugate of NSCs and precursor cells (Pruszak et al., 2007). An asymmetric division resulting in SSEA-1+ and SSEA-1− daughter cells is shown in Figure 8.1C. Note the stained early extensions (arrow) associated with the SSEA-1+ cell. Three hours after treatment, neuronal-type cells developed and strongly expressed Thy-1 antigen (white arrowhead, Figure 8.1D), a glycosylphosphatidylinositol (GPI)-anchored protein expressed by neurons (Rosen et al., 1992). Many cells developed Thy-1+ extending processes (black arrowhead), characteristic of neuronal differentiation.

Stage-specific embryonic antigen 4 is commonly used as a cell surface marker of pluripotent human ESCs and SSEA-4 cells enriched in the neural stem/progenitor cell fraction (Barraud et al., 2007). Stage-specific embryonic antigen 4 is strongly expressed in neuronal-type cell bodies (black arrowhead, Figure 8.1E) but not extensions (white arrowhead). Control immunohistochemistry produced no staining in neuronal or other cell types. In phase-contrast observations, large numbers of putative stem cells were found detached from the chamber bottoms floating in the center of wells (Figure 8.1F). These cells show bubble-like "anchoring" extensions (arrowhead, Figure 8.1G), which apparently facilitate the attachment of trypsin-detached and seeded non-neuronal cells. Since such putative NSCs did not reattach, these cells can be transported where needed.

Similar transdifferentiation was observed in both porcine and human granulosa cell cultures. *In vivo*, pluripotent ovarian ESCs give rise to two distinct cell types, ovarian germ cells and granulosa cells (Bukovsky et al., 1995, 2004). Granulosa cells characteristically differentiate *in vitro* into fibroepithelial cells (Bukovsky et al., 2008).

The treatment of long-lasting porcine granulosa cell cultures on day 0 with EGF, hCG, and E2 alone, or with a mixture of PG + TS had no effect on the occurrence of neural-/neuronal-type cells, but a combination of E2 + PG + TS produced a small

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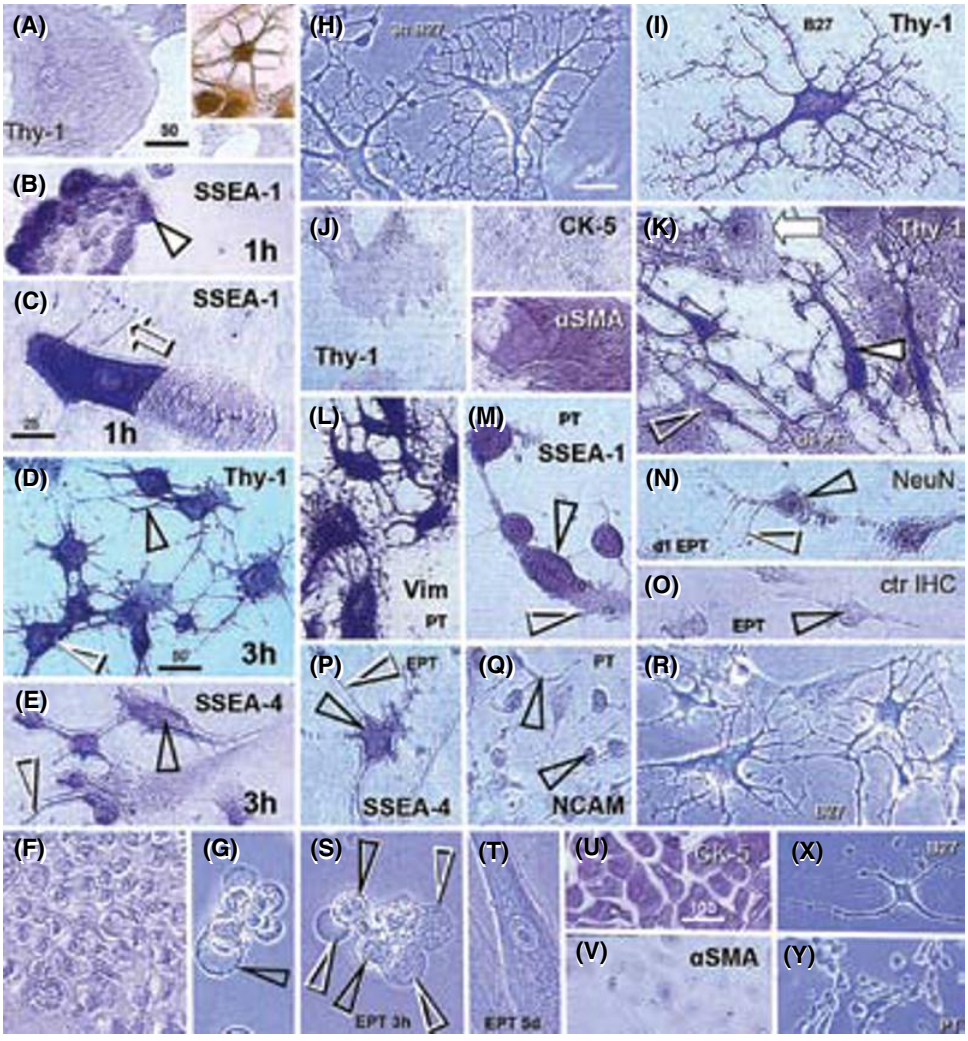


Figure 8.1. Ovarian, smooth muscle cell, and amniotic cultures after sex steroid combinations. Transdifferentiation of ovarian epithelial cells **(**A–I), vascular SMCs (J–T), and amniotic cells into neural- and neuronal-type cells (U–Y). **(A)** Control ovarian epithelial cells show low Thy-1 expression by epithelial cells. Inset shows spontaneous differentiation of neuronal-type cell in ovarian culture. One day after E2 pretreatment and 1 h after PG mixed with TS the ovarian cells dedifferentiated into small cells strongly expressing SSEA-1 – arrowhead **(B)**. **(C)** Asymmetric division resulting in SSEA-1+ and SSEA-1- daughter cells. The arrow indicates stained early extensions. **(D)** Three hours after treatment, the cells exhibited a neuronal morphology with strong expression of Thy-1 neuronal glycoconjugate in extensions (black arrowhead) and bodies (white arrowhead). **(E)** Such cells also expressed SSEA-4 in their bodies (black arrowhead) but not extensions (white arrowhead). **(F)** Putative stem cells were found to detach from the chamber bottoms floating in the center of the wells. **(G)** These cells show bubble-like "anchoring" extensions (arrowhead). Ovarian epithelial cells 3 h after culturing in B27 medium showed direct

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number the subsequent day. No changes were observed in the vehicle-treated cells. Additional steroid treatment on day 1 consisted of PG + TS. There was a marked conversion of fibroepithelial into neural-/neuronal cell types in cultures pretreated the first day with E2 + PG + TS or PG + TS. The neural-/neuronal-type cells expressed SSEA-4 in the porcine neuronal cell bodies. Strong NCAM expression was detected in small stem-like cells. Stage-specific embryonic antigen-1 staining was seen in neuronal cell types but not in fibroepithelial cells (Bukovsky et al., 2008).

The presence of NSCs with "anchors" after the combined steroid treatment in human OSC cultures suggests that such stem cells could be used in autologous regenerative treatment of neuronal disorders in women. Once developed, numerous NSCs are released in the culture medium and are easily collected without trypsinization. Human ovarian stem cultures can be readily obtained regardless of the patient's age. They could be frozen, recultivated, transdifferentiated into neural-/neuronal-type cells by sex steroid combination, and used for autologous stem cell therapy.

Finally, sex steroid mixtures can stimulate proliferation and differentiation of NSCs in the CNS. They can also transdifferentiate potential sources of NSCs such as adult bone marrow and peripheral blood. These may be a natural source of NSC precursors, just as they are a source of hematologic and other nonhematologic stem cell lineages (Song and Sanchez-Ramos, 2002; Kuci et al., 2008). New NSC can differentiate from peripheral blood precursors *in vitro* (Kuci et al., 2008), possibly under the influence of sex steroids *in vivo*. Circulating NSCs can home within the CNS (Corti et al., 2005), and could be stimulated to differentiate further by sequential steroid treatment, that is, E2 followed by PG + TS (Bukovsky et al., 2008).

Figure 8.1. (*Continued*) transdifferentiation into neuronal-type cells (H) strongly expressing Thy-1 **(I)**. Control vascular SMCs show weak Thy 1 **(J)**, no cytokeratin (CK-5, upper inset), and strong αSMA (lower inset). **(K)** One day after PG + TS (PT) the cells exhibited enhanced Thy-1 expression and transition into neuronal-type cells (open and solid arrowheads). **(L)** Some cultures consisted of neuronal-type cells only. **(M)** Staining for SSEA-1 revealed asymmetric division (black vs. white arrowheads). **(N)** Expression of neuronal nuclear antigen (NeuN) in cell bodies 1 day after E2 + PG + TS (EPT) treatment. **(P)** Staining for SSEA-4 was similar to ovarian cultures (see panel E). **(Q)** The NCAM expression in cell bodies and extensions (arrowheads). **(R)** Vascular SMCs after 3 h in the B27 medium. **(S)** Detached cells (black arrowheads) showing bubble-like "anchoring" extensions (white arrowhead). **(T)** Reestablishment of the vascular SMC type within 5 days. **(U)** Culture of amniotic epithelial cells show KC-5 staining and no αSMA **(V)**. **(X)** Amniotic epithelial cells show a transdifferentiation in B27 medium but responded poorly to PT treatment (Y vs. K and L). Numbers above bars indicate microns. (A–G adapted from A. Bukovsky, M. R. Caudle, and M. Svetlikova, "Steroid-Mediated Differentiation of Neural/Neuronal Cells From Epithelial Ovarian Precursors in Vitro," *Cell Cycle* **7**, no. 22 (2008): 3577–3583. © Landes Bioscience. H–V adapted from with permission from A. Bukovsky, "Sex Steroid-Mediated Reprogramming of Vascular Smooth Muscle Cells to Stem Cells and Neurons: Possible Utilization of Sex Steroid Combinations for Regenerative Treatment Without Utilization of in Vitro Developed Stem Cells," *Cell Cycle* **8**, no. 24 (2009): 4079–4084. © Landes Bioscience. X and Y reprinted with permission from A. Bukovsky, "Immune Maintenance of Self in Morphostasis of Distinct Tissues, Tumor Growth, and Regenerative Medicine," *Scand. J. Immunol.* **73**, no. 3 (2011a): 159–189, © Oxford: Wiley-Blackwell Scientific Publications.)

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Vascular SMCs Show *In Vitro* Nuclear Reprogramming into Neural- and Neuronal-Type Cells and Vascular Smooth Muscle Stem Cells

We tested the effect of Neurobasal/B27 neuron culture medium with 2 mm Glutamax (B27 medium) on cultured OSCs (Bukovsky, 2009). (B27 medium is used to preserve hippocampal neuronal cells *in vitro* since it substitutes in the role of astrocytes (Brewer et al., 1993).) Within 3 h there was transdifferentiation of epithelial cells into interconnected neuronal-type cells (Figure 8.1H; phase contrast of living culture). Such cells strongly expressed Thy-1 neuronal glycoconjugate (Figure 8.1I), similar to cultures exposed to sex steroid combinations. These observations show that sex steroid combinations are as effective as B27 medium.

Subsequently, we cultured human vascular SMCs, non-epithelial mesenchymal cells known to express sex steroid receptors (Hodges et al., 1999, 2000; Ma et al., 2005), to test them as a control cell type due to their morphology and expression of NSCs and neuronal markers. Unexpectedly, vascular SMCs subjected to sex steroid combinations also transdifferentiated into neural- and differentiating neuronal-type cells (Bukovsky, 2009). These cultured vascular SMCs showed weak expression of Thy-1differentiation protein (Figure 8.1 J), characteristic of human vascular pericytes (Bukovsky et al., 2001). They did not express cytokeratin 5 (CK-5, upper inset), but strongly expressed α-smooth-muscle actin (αSMA; lower inset). The addition of individual sex steroids did not change SMC morphology (except PT – see below) or Thy-1 expression. However, 1 day after treatment with PG + TS (d1 PT, Figure 8.1K), transdifferentiation of SMCs into neuronal-type cells occurred, with strong Thy-1 expression (white arrowhead). Note also the enhanced Thy-1 expression in the remaining SMCs (arrow; compare with Figure 8.1 J). The open arrowhead indicates an SMC that is in the process of transdifferentiation into a neuronal cell.

The phenomenon which we labeled the "brain *in vitro*" feature (Bukovsky et al., 2008) is shown in Figure 8.1L, where all SMCs transdifferentiate into interconnected neuronal-type cells (vimentin stain). The emerging neural-/neuronal-type cells show strong SSEA-1 expression. Figure 8.1M shows asymmetric division resulting in SSEA-1+ (black arrowhead) and SSEA-1− (white arrowhead) daughters such as seen in transdifferentiated ovarian surface epithelium (OSE) cultures (see Figure 8.1C).

Neural nuclei protein (NeuN) expression in cultures after 1 day of E2 + PG + TS (EPT, Figure 8.1 N) treatment is present in neuronal cell bodies (black arrowhead) but not extensions (white arrowhead). Control immunohistochemistry produced no staining of neuronal (arrowhead, Figure 8.1O) or other cell types. Neuronal-type cells show SSEA-4 (Figure 8.1P) in their bodies (black arrowhead) but not extensions (white arrowhead), like OSC cultures (Figure 8.1E). Neuronal cell bodies and extensions express NCAM (arrowheads, Figure 8.1Q) characteristic of later stages of neuronal differentiation (Pruszak et al., 2007). The NCAM is used for the isolation of human embryonic stem-cell-derived neurons (Pruszak et al., 2007).

Utilization of B27 medium without steroids caused a rapid (within 3 h) transdifferentiation of SMCs into neuronal-type cells (Figure 8.1R; phase contrast of living culture) similar to ovarian epithelial cell cultures (see Figure 8.1H). These data clearly demonstrate that both the SMCs and ovarian epithelial cells have the potential to

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transdifferentiate into neuronal-type cells in media stimulating the neurons and neuronal differentiation of ESCs (Brewer et al., 1993; Shin et al., 2006).

Treatment with E2 + PG + TS caused a rapid (within 3 h) conversion of SMCs into stem-type cells (black arrowheads, Figure 8.1S) exhibiting bubble-like "anchors" (white arrowheads) signaling the capability of attachment. Within several days these cultures reestablished the vascular SMC type (Figure 8.1T). This demonstrates that sex steroid combinations can cause transdifferentiation of SMCs into neuronal and vascular smooth muscle stem cells.

For positive control for CK-5 staining we used a culture of amniotic epithelial cells (Figure 8.1U) which do not express αSMA (negative control, Figure 8.1V) (Bukovsky, 2009). Amniotic cultures responded well by transdifferentiation into neuronal-type cells after B27 treatment (Figure 8.1X), but not after sex steroid treatment (Figure 8.1Y) – compare with Figure 8.1K (Bukovsky, 2011a).

Estradiol-17β in Vascular SMC Cultures after Testosterone, Progesterone, and Testosterone + Progesterone Treatment

Our earlier study (Bukovsky et al., 2008) indicated that pretreatment with E2 is essential for the transdifferentiation of OSCs and granulosa cells into NSCs and neuronal-type cells after PG + PT treatment. Some cultures were capable of transdifferentiating after PG + PT alone, possibly due to the conversion of TS (and PG) by aromatase. We tested whether PG, TS, or both were being converted into E2 in vascular SMC cultures. The medium with 10% fetal bovine serum (FBS) and spent control culture medium showed low E2 levels (E2 pg/mL 1050 ± 150 and 1477 ± 742 structural equation modeling (SEM), respectively); cultures after TS treatment showed significantly higher production of E2 (22,086 ± 4650 SEM); PG pretreatment was even more effective (71,920 ± 9090 SEM); but the highest E2 levels were obtained by PG + TS pretreatment (198,202 ± 40,088 SEM) (Bukovsky, 2009).

These data confirm that E2 is produced in cultures pretreated with PG and TS. This explains the source of E2 from PG and TS conversion by vascular SMCs. *In vivo*, advancing age is associated with lower levels of circulating estrogens. However, in postmenopausal women and aging men E2 continues to be produced in a number of extragonadal sites, including vascular SMCs and numerous sites in the brain (Simpson, 2002). Vascular SMCs express aromatase and 17β-hydroxysteroid dehydrogenase type I, which converts estrone, the major product of aromatase, to the more potent estrogen E2 (Murakami et al., 2001). Therefore, the PG and TS treatment *in vivo* may induce the transdifferentiation of vascular SMCs into vascular stem cells and neural-/neuronal-type cells in sites where this is needed.

Combination of Progesterone and Testosterone Might be Sufficient for Brain Regenerative Treatment

High circulating levels of a PG + TS combination are unlikely to occur naturally in either human females or males. The E2 + PG or E2 + TS combinations are not capable of transdifferenting ovarian epithelial cells or vascular SMCs into stem and neuronal-type

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cells. However, E2 + PG or E2 + TS mixtures may arise locally, in the brain for example, as a combination of neurosteroids (Fuller et al., 2007). Our observations (Bukovsky et al., 2008; Bukovsky, 2009) suggest that either E2 + PG + TS treatment or PG + TS treatment and E2 produced by targeted cells expressing aromatase + 17β-hydroxysteroid dehydrogenase type I are effective.

Studies suggest that large doses of PG for up to several days after injury can limit CNS damage, reduce loss of neural tissue, and improve functional recovery (reviewed by Wang et al., 2008). This is probably due to the combined effect of PG and locally produced E2. However, treatment with PG + TS may enhance E2 production and induce transdifferentiation of vascular SMCs into neural-/neuronal-type cells (PT, Figure 8.1K–M and Q). This may be even more effective in repairing CNS injuries.

Vascular SMC Cells

The ability of vascular SMC to transdifferentiate into neural- and neuronal-type cells is especially important because (unlike OSCs) vascular SMCs accompany all vessels as pericytes, including the CNS microvasculature in both females and males. The combination of all three sex steroids causes a rapid conversion of differentiated vascular SMCs into stem-type cells. These cells subsequently differentiate into mature vascular SMCs. Vascular SMCs, also known as pericytes, regulate endothelial cell properties and contribute to the stability and maintenance of blood vessels. They are pluripotential, serving as precursors for a variety of other cell types (Thomas, 1999). Hence vascular SMCs accompany capillaries and post-capillary venules (Shepro and Morel, 1993) and function as MSCs in various tissues, including bone marrow stromal stem cells (Doherty et al., 1998; Bianco et al., 2001; Shi and Gronthos, 2003; Farrington-Rock et al., 2004).

Pericytes represent a perivascular niche of MSCs residing in virtually all organs and tissues. They are multipotent progenitor cells, giving rise to mesenchymal and epithelial cells *in vitro* and *in vivo*. Their differentiation potential is related to their tissue of origin. All MSC populations derived from specific tissues, including bone marrow, spleen, muscle, aorta, vena cava, kidney, lung, liver, brain, and thymus, show expression of αSMA suggesting a relationship to pericytes (da Silva et al., 2006).

Altogether, either systemic or local treatment with sex steroid combinations could stimulate transdifferentiation of resident vascular SMCs to treat neurodegenerative, traumatic and ischemic neurological disorders, and vascular diseases without requiring stem cells developed *in vitro*.

The morphostatic maintenance of tissues declines with age, probably due to degenerative changes in the immune system. This is accompanied by an increased incidence of tissue dysfunctions such as type 2 diabetes mellitus associated with pericyte pathology (Hayden et al., 2010) and neurodegenerative diseases caused by apoptosis of neuronal-type cells (Nishimura, 2010). Regenerative approaches are more likely successful in acute/traumatic injury treatment than in chronic tissue disorders (Bukovsky et al., 2009), unless we are able to manage the attenuated or lost "stop effect" of MDCs in specific tissues associated with aging (Bukovsky, 2011a).

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Cellular Therapy Without Cells

Within the field of regenerative medicine of neurodegenerative and traumatic neurologic disorders, there is considerable interest in cellular therapy such as engraftment of NSCs in the CNS to induce neuronal renewal and repair. Neural stem cells can be isolated from the neonatal or adult CNS and propagated *in vitro* in the presence of mitogenic growth factors (Daadi, 2002; Shihabuddin, 2002). Alternative sources of NSCs include ESCs, umbilical cord blood, amniotic epithelial cells, bone marrow stem cells, and mobilized peripheral blood CD133+ cells (O'Shea, 2002; Song and J. Sanchez-Ramos, 2002; Miki and Strom, 2006; Kuci et al., 2008). After several passages, these cells can be transdifferentiated into NSCs by fibroblast growth factor-1, 12-*O*-tetradecanoylphorbol-13-acetate (protein kinase C activator), isobutylmethylxanthine (a nonspecific inhibitor of phosphodiesterases), and forskolin (protein kinase A activator), or all-trans-retinoic acid and 2-merkaptoethanol (Scintu et al., 2006). Due to toxicity, these substances are not suitable for treatment *in vivo*, however.

An alternative to the use of organ-/tissue-specific stem cells for functional engraftment at particular sites (topic therapy) is the idea of "systemic regenerative therapy" or "*in situ* regenerative treatment" by common low-molecular-weight substances capable of crossing blood–tissue barriers, such as the blood–brain barrier.

Our study has shown that sex steroid combinations (but not individual sex steroids) can directly transdifferentiate cultures of vascular SMC known to posses sex steroid receptors (Hodges et al., 1999, 2000; Ma et al., 2005) into neural- and neuronal-type cells (Bukovsky, 2009). As we have noted, this is important since, unlike OSCs, the vascular SMCs accompany all vessels, including the CNS microvasculature, as pericytes in both genders.

We have also reported that sex steroid combinations produce vascular smooth muscle stem cells which differentiate within a few days back into mature SMCs (Bukovsky, 2009). These combinations can therefore induce the renewal (regeneration) of vascular pericytes known to play an essential role in vascular physiology. Pericytes regulate endothelial cell properties and contribute to the stability and maintenance of blood vessels, They may serve as precursors for other cell types (Thomas, 1999), such as cardiomyocytes (Dohmann et al., 2005). Regenerative treatment could therefore treat vascular disorders, such as type 2 diabetes, which affects microvasculature pericytes throughout the body (Richards et al., 2010), vascular atherosclerosis, and cardiomyocyte regeneration after myocardial infarction. Thus systemic regenerative therapy and *in situ* regenerative treatment with sex steroid combinations are new approaches to cell therapy without the need of extraneous stem cells.

The Potential of Sex Steroid Combinations for Systemic Regenerative Treatment Trials

We conducted *in vitro* studies to determine the doses of sex steroids required to induce transdifferentiation of vascular SMCs. Control cultures (Figure 8.2A) and media with vehicle alone (Figure 8.2B) showed no spontaneous transdifferentiation. Similar results were found with 60 μm E2 (Figure 8.2C) and 60 μm TS (Figure 8.2D) 1 day

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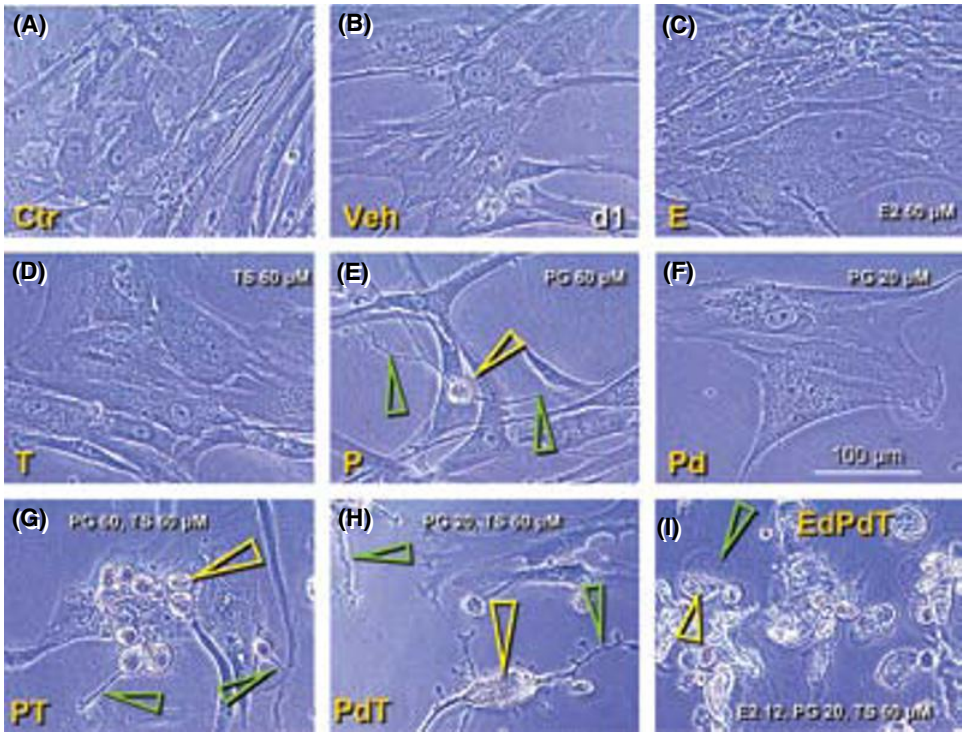


Figure 8.2. Sex steroid doses and combinations in vascular SMC cultures: **(A)** control, **(B)** vehicle, **(C)** high estrogen (E2 60 µm), and **(D)** high testosterone (TS 60 µm) treated cultures of vascular SMCs showed no morphological changes. **(E)** High progesterone (PG 60 µm) treatment was, however, associated with occasional neuronal-type cells. **(F)** This was not observed in cultures with diluted PG (20 µm). **(G)** Cultures with high PG and high TS showed numerous neural-type cells. **(H)** Cultures with diluted PG and high TS showed direct nuclear transdifferentiation into neuronal-type cells with branching extensions. **(I)** Diluted estradiol and PG and high TS caused formation of undifferentiated stem-type cells. Open yellow arrowheads indicate neural-/neuronal-type cell bodies; green arrowheads indicate extensions; solid arrowhead in panel I indicates bubble-like anchor; Ctr, control; E2, estradiol; Ed, estradiol diluted; P, progesterone; Pd, progesterone diluted; PT, progesterone + testosterone; Veh, vehicle. (Reprinted with permission from A. Bukovsky, "The Role of Resident Monocytes and Vascular Pericytes in the Stem Cell Niche and Regenerative Medicine," *Stem Cell Stud.* **1**, no. e20 (2011b): 126–147, © PagePress Publications.)

after the treatment. However, 60 µm PG caused the occasional appearance of neuronal-type cells (yellow arrowhead, Figure 8.2E) with their characteristic elongated early extensions (green arrowheads). This dose of PG corresponds to the concentration of 16 mg/kg found to reduce cell death, gliosis, and functional deficits after traumatic brain injury in rats (Djebaili et al., 2005). Progesterone may have some *in vivo* neuroprotective effect. The lower PG dose (20 µm) had no *in vitro* effect on the development of neuronal-type cells, however (Figure 8.2 F).

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When the 60 µm PG was combined with 60 µm TS, numerous small NSCs (yellow arrowhead, Figure 8.2G) with short extensions (green arrowheads) appeared. This shows that numerous new NSCs can be generated by addition of high-dose TS to high-dose PG treatment.

When a lower concentration of PG (20 µm) was used in cultures along with 60 µm TS, the vascular SMCs showed direct nuclear transdifferentiation into differentiating neuronal-type cells (yellow arrowhead, Figure 8.2H) with branching extensions (green arrowheads). It is important to note that the *in vitro* dose of diluted PG corresponds to the dose of PG suppositories (200 mg, used twice daily) utilized in the prevention of miscarriage and preterm labor (Check et al., 2005; Borna and Sahabi, 2008). The dose of TS is similar to intramuscular weekly injections of androgens (600 mg) tested in human males (Gray et al., 2005; Coviello et al., 2008). Hence, it should be safe to use daily doses of 400 mg progesterone combined with weekly injections of 600 mg testosterone in clinical trials to treat CNS dysfunctions.

When a low dose of E2 (12 µm) was added to 20 µm progesterone and 60 µm TS, numerous stem cells (yellow arrowhead, Figure 8.2I) with bubble-like anchors (green arrowhead) developed in vascular SMC cultures. Since such cells differentiate back into mature SMCs (see Figure 8.1S and T) within several days, the treatment of vascular disorders with such a therapy is plausible in order to regenerate vessels. A 12 µm *in vitro* dose of E2 corresponds 120 mg in humans. Transdermal 17β-estradiol 50 mg twice weekly has also been used for hormone-replacement therapy (Schram et al., 1995).

Pericytes regulate endothelial cell properties and contribute to the stability and maintenance of blood vessels. As we have noted, they are thought to be pluripotential, serving as precursors for a variety of other cell types (Thomas, 1999), including cardiomyocytes (Dohmann et al., 2005). A combined treatment with all three sex steroids (400 mg of PG daily + 600 mg of TS weekly + 50 mg of E2 twice a week) for a period of 2–4 weeks could aid vascular regeneration after a stroke and regeneration of cardiomyocytes after myocardial infarction.

Taken together, in aging individuals or patients at risk of CNS or vascular and heart disorders, occasional treatment (once a year) with combined PG + TS of E2 + PG + TS for a period of 2–4 weeks may have a preventive or regenerative effect. Since E2 can be locally derived from PG and TS, several doses of PG + TS combination (e.g., 400 mg of PG daily + 600 mg of TS weekly for 2–4 weeks) may be sufficient for the CNS, vascular and heart regeneration. This could also be applicable in acute injury such as spinal cord trauma, stroke, or myocardial infarction.

The Pros and Cons of Regenerative Medicine

The aim of what we term regenerative medicine is to replace altered tissue-specific cells with new cells originating either from stem cells or reprogrammed differentiated cells from one lineage into another.

As indicated above, therapies using transdifferentiation of autologous progenitor cells *in situ* by drugs or hormones may be practical approaches to treat neurological and vascular disorders. This may be more successful in acute traumatic and ischemic disorders with preserved perivascular organ-specific stem cell niches with TCS-mediated

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regulation of stem cell differentiation and preservation of a functional state. In chronic or degenerative diseases, however, TCS-mediated function may be altered. As a result, attempts to regenerate organ function may not be successful due to the lack of preservation of tissue-specific cells in a state required for proper tissue function.

It has been shown recently that aging is associated with DNA hypermethylation of MDC (Rakyan et al., 2010). This may be improved by therapeutic levels of 5-aza-2′-deoxycytidine (5-AZA-dC), a potent inhibitor of DNA methylation (Laurenzana et al., 2009). Systemic "transcriptional therapy" could return the MDC into a normal state within specific tissues, perhaps reversing the aging process. It may be more practical to influence the MDC within an affected tissue by *in situ* application of a DNA methylation inhibitor influencing resident tissue-specific self-renewing MDC in the basic TCU and cryptopatches.

In diseases caused by a high MDC "stop effect" such as diabetes, cirrhosis, livers, or autoimmune and degenerative diseases, it may be possible to improve tissue function. Tissue affected by lower than required "stop effect" of MDC, such as muscular dystrophy, may require the opposite approach, that is, stimulation of DNA methylation in resident MDC.

If we achieve the ability to manage the function of the tissue-specific stem cell niche and TCS-mediated functional preservation, we may be able to treat early postnatal tissue disorders, improve regenerative medicine, and delay physical and mental aging.

Conclusions

Available evidence shows that morphostasis is a complex event requiring: (i) renewal from stem cells; (ii) preservation of tissue-specific cells in the proper differentiated state; and (iii) regulation of tissue quantity. This can be executed by the TCS consisting of immune-system-related components, vascular pericytes, and autonomic innervation. Morphostasis is established epigenetically, during morphogenetic developmental immune adaptation, that is, during the critical developmental period. Subsequently, tissues are maintained in the state of differentiation reached during adaptation by a "stop effect" of MDC. Alteration of tissue differentiation during the critical developmental period causes persistent alteration of tissue function. Morphostasis is altered with aging, due to degenerative changes of the immune system. This results in aging of individuals and an increased incidence of degenerative diseases.

A promising aspect in regenerative medicine is a cellular chemical approach, such as the combination of sex steroids to stimulate endogenous stem cells for treatment of neuronal, vascular, or heart disorders. In aging individuals or patients at risk, occasional treatment with combined sex steroids for a period of 2–4 weeks may have a regenerative or preventive effect.

Regenerative medicine in affected patients, however, may be more successful in acute/traumatic disorders with an intact morphostatic stem cell niche compared to those with chronic degenerative diseases with an altered stem cell niche. Chronic degenerative diseases may be less responsive to regenerative medicine due to defects in the TCS-mediated morphostasis, such as an alteration of the "stop effect" of MDC.

The ability to manage the "stop effect" of MDC by transcriptional therapy may prove effective in treating early postnatal tissue disorders, such as muscular dystrophy

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and type 1 diabetes. It may delay physical, hormonal, and mental aging of an individual, including the delay of menopause in women, sexual dysfunction in aging men, age-associated high blood pressure, type 2 diabetes, and autoimmune diseases. Degenerative diseases may be associated with tissue cell apoptosis. If we are able to alter morphostasis, we may disrupt the cellular apoptotic process and improve the chances of treating chronic degenerative diseases.

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Chapter 9
Hair Follicle Stem Cells

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Introduction

Skin is one of the largest organs in the body, representing around 16% of body weight (Fawcett and Bloom, 1994). Skin seals our body, protecting us from infection, injury, and dehydration. It contains multiple sensory receptors (tactile) that receive stimuli from our external environment and it has an important role in thermoregulation and water balance.

The upper layer of skin, the epidermis, is a multilayered epithelium consisting of undifferentiated basal cells and more differentiated suprabasal cells. In development, the epidermis also gives rise to specialized appendages including hair follicles, sebaceous glands (SBs), nails, and sweat glands. The basal cells actively proliferate and differentiate into the upper layers, which are eventually sloughed off from the surface. The turnover is fast: humans completely renew their epidermis in about 2 weeks.

To accomplish the formidable task of self-renewal and maintenance of appendages, skin uses reservoirs of stem cells, located in specialized environments or niches. They are present in the epidermis, hair follicle, and sweat glands (Fuchs, 2007; Fuchs and Nowak, 2008; Blanpain and Fuchs, 2009; Pasolli, 2011; Legué, 2012). Epidermal stem cells have long been used for therapy in burn patients (Green, 1991) but insights into how adult stem cells contribute to epidermal homeostasis and repair have only recently been attained (Mascre et al., 2012). This chapter will focus on the isolation, characterization, and function of stem cells in their niches in hair follicles.

Hair Follicle Development and Structure

The embryonic epidermis is derived from the ectoderm as a single layer of multipotent cells (Hardy, 1992). Specific signaling pathways act to trigger hair follicle morphogenesis in concert with the population of skin mesenchymal cells. At about embryonic day 14.5 of mouse development, the interaction between embryonic epidermis and the underlying mesenchymal cells induces the formation of small invaginations of the epidermis,

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the hair placodes. Mesenchymal cells are then instructed to organize in condensates under the placodes, forming the dermal papilla (DP) (Figure 9.1). As a result of the cross-talk between placodes and DP, placodes grow progressively downward to form a hair germ, hair peg, and finally a mature hair follicle (Figures 9.1 and 9.2).

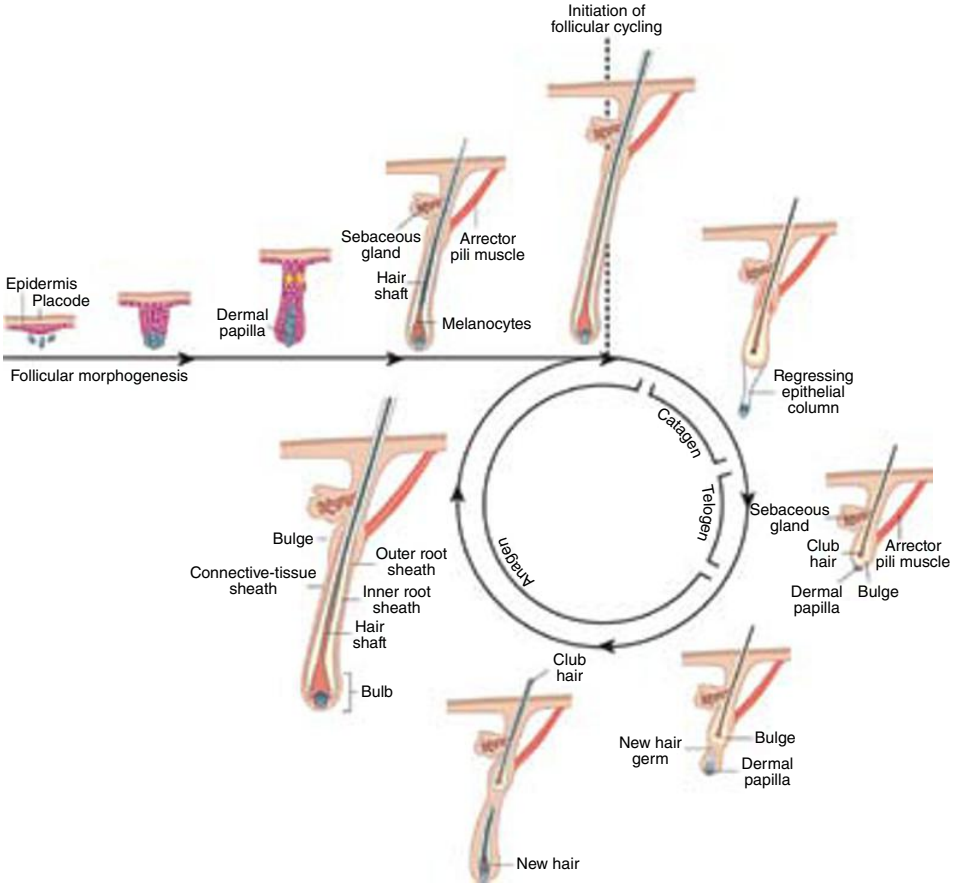


Figure 9.1. Embryonic hair follicle morphogenesis and the adult hair cycle. Hair follicle morphogenesis starts when small invaginations termed placodes appear in the basal layer of the epidermis, accompanied by aggregations of dermal cells. Placodes will grow downward to form a hair germ and later a hair peg, in which matrix cells start encapsulating the dermal papilla (DP), hence giving rise to the hair bulb. In mice, hair follicle morphogenesis is completed at about P9, with the presence of a fully differentiated hair shaft and sebaceous glands. The hair follicle enters the anagen phase of the hair cycle, when the hair shaft is growing and protruding through the skin surface. Follicles then progress to the catagen phase, where cells in the lower two-thirds of the follicle undergo massive apoptosis. The DP is connected to the follicle by an epithelial strand, which regresses and brings it to rest under the bulge, the stem cell compartment. After the resting period of telogen, stem cells become activated again and a new hair is formed. (Reprinted by permission from Macmillan Publishers Ltd: *Nature* (Vol **445**, no. 7130, page 834), Copyright 2007.)

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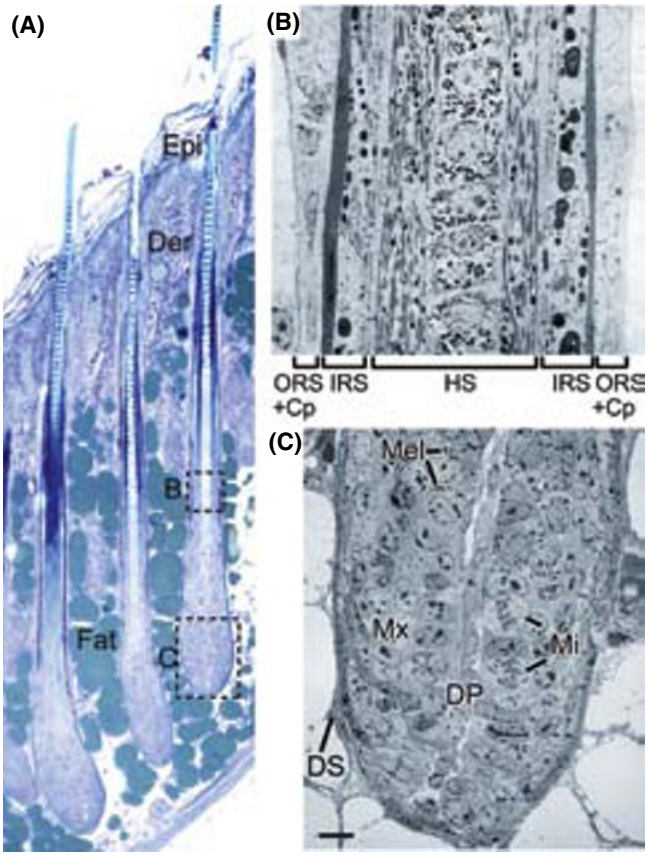


Figure 9.2. (**A**) Hair follicles in mouse anagen backskin. Toluidine-blue stained 1 μm section shows epidermis (Epi), dermis (De) and hair follicles. Hair shafts emerge from the surface of the epidermis. Subcutaneous fat appears in green. Boxed areas B and C are shown in the corresponding electron micrographs. (**B**) Ultrathin section through the mid-segment of an anagen hair follicle. The hair shaft (HS) is composed of three layers. The hair shaft is surrounded by the inner root sheath (IRS), which is composed of three layers. The IRS is encased by the companion layer (Cp) and the outer root sheath (ORS). (**C**) Ultrathin section of a hair bulb. The central column of cells is the dermal papilla (DP). Matrix cells are arranged in an onion-skin-like fashion. Mitoses (Mi) are frequent in this transit amplifying cell population. Melanocytes and keratinocytes containing melanin granules (Mel) are located in the upper area of the hair bulb. Bar = 10 μm for B and C and 80 μm for A. (Hilda Pasolli, The Hair Follicle Bulge: A Niche for Adult Stem Cells. *Microscopy and Microanalysis*, vol. **17**, issue 4. Reprinted with permission. Copyright © 2011, Cambridge University Press.)

The leading front of the hair follicle, the matrix, is composed of highly proliferative cells that maintain close contact with the DP, forming the hair bulb (Figure 9.2A, C). Matrix cells are a transit amplifying cell population: they arise from stem cells and undergo a limited number of cell divisions before initiating terminal differentiation. The most external layer is the outer root sheath (ORS), which maintains contact with

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the basement membrane and is continuous with the epidermis, while the inner layers differentiate into the inner root sheath (IRS) and the hair shaft. The IRS is composed of three concentric layers: the cuticle, Huxley, and Henle layers (Figure 9.2B). The IRS acts as a channel for the protruding hair, guiding it to the surface of the epidermis. The hair shaft is the part of the hair follicle that emerges in the epidermis, and it is also composed of three different concentric layers: the medulla, cortex, and cuticle. At birth, SG precursors appear in the upper part of the hair follicle and the SG forms shortly thereafter. For most hair follicles in mouse backskin, maturation completes toward the end of the first postnatal week, when the terminally differentiated hair shafts break through the skin surface.

In addition to these epithelial cells (also called keratinocytes), hair follicles are populated by cells of different embryonic origin, melanocytes. Through the production of the pigment melanin, melanocytes protect the skin from the deleterious effects of ultraviolet (UV) radiation. Melanocyte precursors derive from the neural crest. They migrate, proliferate, and differentiate en route to their final destination in epidermis and hair follicles. In the case of murine hair follicles, mature melanocytes are located in the upper hair bulb, in close contact with the DP (Figure 9.2C).

Hair Follicles in Homeostasis: the Hair Cycle

Hair follicles undergo cyclic bouts of growth, destruction, and regeneration throughout life, producing a new hair with each cycle. Matrix cells, the transit amplifying population, multiply actively during the growth phase, called anagen (Figure 9.1). At one point proliferation and differentiation into hair shaft and IRS slow down and the hair follicle enters a destructive phase known as catagen. During this phase, massive apoptosis reduces the follicle to an epithelial strand that connects the noncycling part of the follicle with the DP. Once completed, hair follicles lie dormant in a resting phase, telogen (Figure 9.1). With the onset of the new anagen phase, the hair follicle starts a new round of hair growth and it is able to regenerate itself. To accomplish this daunting task, the hair follicle uses a reservoir of stem cells to give rise to the multiple cell lineages of the hair follicle.

One of the most distinguishing features of stem cells is their slow-cycling nature (Bickenbach, 1981; Wright and Alison, 1984). This property allows them to retain their proliferative potential and to avoid DNA errors that could occur during replication. The first procedures developed to image hair follicle stem cells (HFSCs) were based in their slow cycling properties. When a label is introduced into the DNA of stem cells, it will be retained for an extended period of time, allowing for the identification of stem cells as label-retaining cells (LRCs). Using repeated administration of tritiated thymidine for a prolonged period, Cotsarelis et al. (1990), identified a discrete population of mouse hair follicle cells. For many years it was thought that HFSCs reside in the matrix area of the bulb. Surprisingly, the population of LRCs found by this group was located in an unexpected area of the outer root sheath below the SG, at the site of attachment of the arrector pili muscle. Historically, this area of the hair follicle has been called the "Wulst" or bulge (Leydig, 1859) (Stohr, 1904) (Unna, 1876). The bulge marks the end of the "permanent" or noncycling portion of the hair follicle. All keratinocytes below it undergo apoptosis during catagen.

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The identification of LRCs in the bulge posed a challenging question to scientists: are these LRCs indeed stem cells? Are LRCs able to self-renew and to produce all skin epithelial cell lineages? It was essential to develop a methodology that allowed the purification and characterization of bulge cells to demonstrate that they function as stem cells. In the long run, such a procedure could be used for preparing stem cells for prospective stem-cell-based therapies. In 2004, Elaine Fuchs and George Cotsarelis' research groups accomplished this task with strategies that combined generation of transgenic mice with fluorescence-activated cell sorting (FACS). Transgenic mice were engineered to carry specific labels in their bulge cells, which were subsequently purified using FACS. The Fuchs laboratory approach was to induce for a limited time the expression of a transgene encoding a long-lived histone coupled to green fluorescent protein (GFP), driven by the keratin 14 (K14) promoter (Tumbar et al., 2004). All cycling K14-expressing cells became positive, including basal epidermal cells, ORS cells, and, therefore, bulge cells. By administration of a drug to the mice, the GFP label can be switched off and the label is "diluted" after several months. They found that cells retaining the GFP label were situated in the bulge and could be isolated and characterized by standard FACS of cells expressing a high level of GFP (Figure 9.3A).

The Cotsarelis group based their approach on the expression of the intermediate filament protein keratin 15 (K15) (Morris et al., 2004). They have previously shown that the K15 promoter has specific activity in bulge cells (Liu et al., 2003). They engineered mice in which the K15 promoter drives the expression of GFP, thus also allowing FACS of bulge cells.

Purification of bulge stem cells has since been accomplished in our laboratory by FACS based on the expression of bulge surface markers such as α6 integrin and CD34 coupled with K14-GFP expression (Blanpain et al., 2004).

Purification of bulge cells was a crucial step on the way to formally demonstrating that they were indeed functional stem cells. The contribution of bulge cells to skin epithelial cell lineages was analyzed by skin reconstitution assays, in which dispersed epidermal or hair follicle cells are mixed with dermal cells in chambers and implanted onto the back of syngenic or immunocompromised mice. Another essential method for analyzing *in vivo* contribution is lineage tracing, a technique that marks all the progeny of a single cell.

Grafting and lineage tracing experiments have shown that bulge cells are stem cells and that offspring of a single bulge stem cell can form all epithelial cell layers of a new hair follicle in homeostasis (Blanpain et al., 2004; Morris et al., 2004; Claudinot et al., 2005). Bulge stem cells are also able to contribute to interfollicular epidermis during wound repair, and to the SG when its own resident stem cells are defective (Morris et al., 2004; Tumbar et al., 2004; Ito et al., 2005; Horsley et al., 2006).

To elucidate the special qualities of bulge cells, investigators have performed gene expression profiling of purified bulge cells. Nearly 150 genes are preferentially expressed in the bulge relative to the proliferating basal cells of the epidermis (Blanpain et al., 2004; Morris et al., 2004; Tumbar et al., 2004). Microarray data have revealed new markers that contribute significantly to the understanding of HFSCs' biology. Among the upregulated bulge genes are transcription factors such as Lhx2, Sox9, Tcf3, Nfatc1, and Lgr5 (a transmembrane receptor) (Figure 9.3B) (Trempus et al., 2003; Blanpain et al., 2004; Morris et al., 2004; Tumbar et al., 2004; Nguyen et al., 2006; Rhee et al., 2006; Horsley et al., 2008; Jaks et al., 2008).

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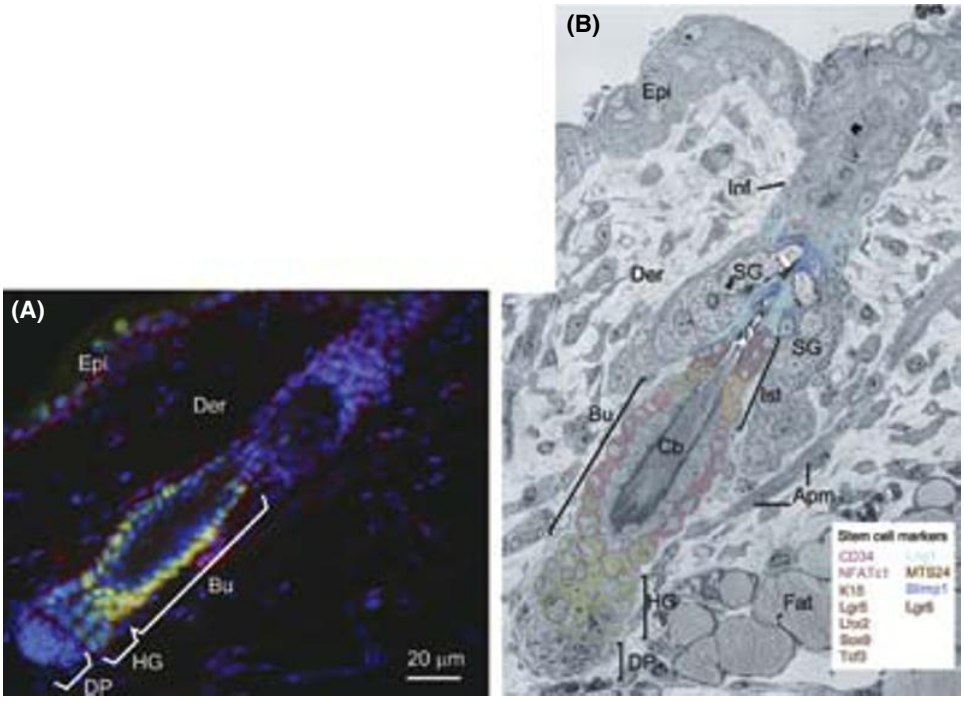


Figure 9.3. Stem cell populations in the hair follicle. (**A**) Hair follicle in telogen phase. Slow-cycling cells (marked by histone H2-GFP) are located in the outer layer of the bulge (Bu). Immunofluorescence labeling for β4 integrin (in red) demarcates the basement membrane. (**B**) Electron micrograph of a telogen follicle, pseudocolored to show the localization of the different pools of stem cells. The outer layer of the bulge (red) is home to CD34+ and NFATc1+ cells. K15, Lhx2, Sox9, and Tcf3 are expressed in both the outer and inner layer of the bulge and in the hair germ (green). Lgr5 shows a more restricted pattern of expression, being found in the outer bulge cells in contact with the hair germ (green cells delineated in red) and in the hair germ itself. Lgr6 (yellow), MTS24 (brown) and Lrig1 (blue) are expressed in overlapping populations in the isthmus and infundibulum. Blimp1, marker of sebocyte progenitors, is located close to the sebaceous glands. Fat denotes the subcutaneous adipose tissue. Apm, arrector pili muscle; Bu, bulge; Cb, club hair; Der, dermis; Epi, epidermis; HG, hair germ; Inf, infundibulum; Ist, isthmus, SG, sebaceous gland. Bar = 20 μm for A and B. (Hilda Pasolli, The Hair Follicle Bulge: A Niche for Adult Stem Cells. *Microscopy and Microanalysis*, vol. **17**, issue 4. Reprinted with permission. Copyright © 2011, Cambridge University Press.)

Heterogeneity Within the HFSC Population

The bulge, home to HFSCs, is located below the SGs at the base of the noncycling portion of follicles. Recent research has shown that two additional areas of the hair follicles are important for their contribution to skin homeostasis: the isthmus and the infundibulum (Watt and Jensen, 2009). The isthmus is the region of the hair follicle that extends from the bulge to the SG and the infundibulum is the portion of the hair follicle above the SG and continuous with the interfollicular epidermis.

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Slow-cycling HFSCs are located in the outermost layer of the bulge (Figure 9.3A) and are marked by high levels of integrins (α6β4 and α3β1), keratins (keratin 5 (K5), K14 and K15) and cell surface markers (CD34 and Leu-rich repeat-containing G protein-coupled receptor 5 (LGR5)) (Blanpain et al., 2004; Morris et al., 2004; Tumbar et al., 2004). They also express transcription factors, including SOX9, LIM homeobox 2 (LHX2), nuclear factor of activated T cells, cytoplasmic 1 (NFATC1), T cell factor 3 (TCF3), and TCF4 (Figure 9.3B). Loss-of-function studies show that all of these factors help to maintain features of "stemness" (Vidal et al., 2005; Nguyen et al., 2006, 2009; Rhee et al., 2006; Horsley et al., 2008; Nowak et al., 2008).

The inner layer of bulge cells forms at the end of the hair cycle (see below). These cells do not express integrins or CD34 but they do express some HFSC markers, including NFATC1, SOX9, LHX2, and TCF3. They also exhibit features not seen in HFSCs, including unusual intercellular adhesions to the hair shaft and expression of K6 (Hsu et al., 2011).

The bulge base features a small cluster of cells, known as the hair germ (Figure 9.3A and B). Transcriptional profiling shows that hair germ cells are more similar to HFSCs than to their transient amplifying progeny, that is, to matrix cells (see below) (Greco et al., 2009). Except for NFATC1 and CD34, the hair germ expresses the HFSC markers mentioned above. They can be distinguished from the bulge HFSCs by their high levels of placental cadherin (P-cadherin).

Several markers of the isthmus and infundibulum have been identified. Marker Lrig1 is expressed in the junctional zone, which comprises the lower part of the infundibulum and the upper part of the isthmus (Jensen et al., 2009). The upper part of the isthmus specifically expresses the marker MTS24 (Figure 9.3B; Nijhof et al., 2006). In the uppermost part of the isthmus, at the opening of the SG canal, there is an additional population of cells expressing Blimp1(Horsley et al., 2006). In the central isthmus there is a population of cells expressing Lgr6, which partially overlaps with the MTS24 and Lrig1 populations (Figure 9.3B).

Are all of these different subpopulations stem cells, and how do they contribute to hair follicles and epidermis? Many of these cell populations are able to reconstitute all skin lineages in chamber grafts and lineage tracing experiments. The cell populations located in the isthmus/infundibulum area, although able to reconstitute all cell types in reconstitution assays, show a more restricted contribution when subjected to fate-mapping. For example Lrig1 cells contribute to the junctional zone, SGs, infundibulum, and the interfollicular epidermis, but not to the hair follicle (Jensen et al., 2009). Blimp1 cells, on the other hand, are unipotent SG progenitors and form only SGs in reconstitution assays (Horsley et al., 2006).

The Hair Follicle Stem Cell Niche

It is not random that HFSCs are located in the bulge area. Stem cells are usually found in well-protected, highly vascularized, and innervated areas. The location of the bulge away from the skin surface offers a unique microenvironment to protect stem cells against mechanical trauma and UV radiation and to regulate their function. In addition, it provides HFSCs with the possibility of interaction with a diverse arrangement of cellular components, including nerve terminals, blood vessels, and diverse

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dermal cell types. Scientists have just started to unveil the role of the niche components in stem cell function. It is not unlikely that additional niche components and their role in stem cell regulation will be revealed in the near future.

Components of the HFSC Niche

Melanocyte Stem Cells

Hair follicle stem cells share the bulge niche with melanocyte stem cells (MSCs; Nishimura et al., 2002). They are close neighbors that can be found adhering to each other in the bulge and hair germ area. In a coordinated process, both stem cell types are simultaneously activated during anagen, when MSCs differentiate into melanocytes that populate the hair bulb (Rabbani et al., 2011; Tanimura et al., 2011). The pigment is transferred to the differentiating matrix cells and subsequently to the forming hair shaft. In addition to intrinsic mechanisms regulating their maintenance in the niche, MSCs are dependent on HFSCs for their survival. Different factors secreted by HFSCs regulate the behavior of MSCs. Whether MSCs regulate HFSC behavior remains to be examined. The bulge niche has also been implicated as a residence for smooth muscle progenitors responsible for generating the arrector pili muscle, which is located just above the bulge (Fujiwara et al., 2011).

$K6^+$ Layer

Although the cells in the inner layer of the bulge express many HFSC markers, they do not function as stem cells. Recent research has clarified their origin and properties (Hsu et al., 2011). The $K6^+$ layer originates from cells of the lower ORS that survived catagen and then home back to the bulge. Despite their failure to regain proliferative potential, they perform essential functions in the bulge. They function to anchor the club hair to the bulge, through distinctive attachment structures thus preventing cyclical alopecia in the animals. And most remarkable, K6 cells function as a signaling center in the bulge, maintaining HFSC quiescence through FGF18 and BMP6 signaling.

Dermal Papilla

Located just beneath the hair germ, the DP is an essential activation center. Signaling from the DP is indispensable for hair follicle growth and integrity. For a review see Sennett and Rendl, (2012).

Nerve Terminals

Four types of different sensory neurons converge on the upper bulge. Ultrastructural studies show a close contact between the upper bulge cells and these endings. It has been recently shown that some of these neurons are a source of Shh (sonic hedgehog) and signal to upper bulge cells (Brownell et al., 2011).

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Adipose Tissue

Two longer range signaling inputs from the adipose tissue participate in regulating HFSCs behavior. Subcutaneous adipocytes send out waves of alternating inhibitory and activating factors that help synchronize HFSC niches within the skin (Plikus et al., 2008). Most recently, adipocyte precursors were implicated in transmitting regulatory factors that probably signal to receptors on the surface of the dermal papilla and/or dermal sheath (Festa et al., 2011).

Imaging Hair Follicle Stem Cells in their Niche

The original methods to identify HFSCs were based in their quiescence (LRCs). Subsequently, different genes were found to be expressed with certain degree of specificity in HFSCs, and their products were then used as molecular markers. Once it was possible to irreversibly label HFSCs, lineage-tracing methods were devised to analyze the fate of stem cells *in vivo*. Lineage tracing provided snapshots of stem cells and their progeny over time that revealed retrospectively the fate of HFSCs.

The next challenge was to develop an approach to study stem cell behavior in its niche by live imaging. Intravital two-photon microscopy has been successfully adapted to visualize the hematopoietic stem cell in the bone marrow (Lo Celso et al., 2009; Xie et al., 2009). These are invasive methods that do not allow monitoring stem cells for long periods. Skin, on the contrary, is a much accessible tissue for live imaging (Ra et al., 2011; Uchugonova et al., 2011). More recently, this technique has been applied to investigate the HFSC niche (Rompolas et al., 2012). They monitored the behavior of stem cells and their progeny during hair regeneration by imaging stem cells expressing a histone coupled to GFP. A useful tool of two-photon microscopy is the option to eliminate specific cells by laser-induced ablation. They targeted dermal papilla cells with a reporter expressing red fluorescent protein (RFP) and proceeded to ablate dermal papilla cells. Hair follicles with ablated mesenchyme remained quiescent, hence demonstrating the requirement of the DP for hair regeneration. Laser ablation offers the exciting possibility of eliminating other components of the stem cell niche such as the dermal sheath, nerves, and arrector pili muscle, provided that reporter fluorescent lines are available for these components.

The Potential of Skin Stem Cells for Therapeutic Use

There has been significant progress in our understanding of the basic biology of skin stem cells. We have progressed in our understanding of the role of bulge and sweat gland stem cells in homeostasis and wound healing. However, there is still much to investigate for the potential of skin stem cells in regenerative medicine. Being a source of abundant, easily accessible stem cells, it is attractive to envision a future where skin stem cells can be used for treatment of genetic skin disorders, chronic ulcers, and even baldness.

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Chapter 10

The Potential of Using Induced Pluripotent Stem Cells in Skin Diseases‡

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Introduction

The skin and its appendages are a multifunctional organ indispensable for the survival of an animal, which provide physical and immunological barriers against external insults, transmit tactile and temperature sense and enable thermoregulation, and, in some cases, social communication. This polyfunctionality is secured by the well-orchestrated interactions between the main body of the skin consisting of epidermis, dermis, subcutaneous fat, vasculature, nerve, and lymphatics and individually specialized miniorgans, including hair follicles (HF), nails, sebaceous glands, and sweat glands. One of the most characteristic features of this complex system is that it continuously self-renews. Considering the diversity of differentiation status of cell populations comprising the organ, it is reasonable to speculate that multiple tissue stem cell populations exist and provide progenies to respective cell lineages, including keratinocytes and melanocytes, two major cellular subsets of the epidermis and HF.

Over the past decades, radioisotopic, chemical, or transgenic cell labeling and fate-tracking techniques in combination with cell-biological assays and bioinformatics analyses have successfully identified several tissue stem cell populations (interfollicular epidermal, HF, melanocyte, and sebocyte stem cells) and their biomarkers. Importantly, the characterization of those stem cell subsets facilitated better understanding of their

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roles and involvements in the pathogenesis and pathophysiology of dermatological disorders: destruction of hair follicle stem cells (HFSCs) results in permanent hair loss in scarring alopecia, loss of melanocyte stem cells causes hair graying, mutated HFSCs give rise to basal cell carcinoma, etc. With their high proliferative capacity and plasticity, skin stem cells hold great promise as cell sources enabling regenerative medicine therapies for intractable skin disorders, such as severe burn, chronic ulcers, and vitiligo. In addition, stem cell gene correction in combination with tissue engineering can be applied to treat genodermatoses, including epidermolysis bullosa or xeroderma pigmentosum. However, further investigation and application of human stem cells is currently hampered by the lack of efficient isolation and propagation methodologies.

In the seminal study by Takahashi and Yamanaka (2006), skin-derived fibroblasts were successfully reprogrammed into induced pluripotent stem cells (iPSCs), similar to embryonic stem cells (ESCs). Later studies reported successful generation of keratinocytes and melanocytes from human iPSCs, clearly indicating that human iPSCs (hiPSCs) should provide valuable materials to further dissect the roles of skin stem cells and their progenies in the maintenance of skin homeostasis and the development of skin diseases and tumors. For instance, the concept of cancer stem cells may be tested, once hiPSCs were successfully induced into immortal melanoma cells. In particular, hiPSCs are favorable material for bioengineering of the skin, which may facilitate the management of currently intractable skin diseases and the development of new therapies and drug discovery (Figure 10.1).

This chapter summarizes recent advances in iPSC technology, especially focusing on successful generation of different cell lineages in the skin from hiPSCs, and discusses the advantages and promise of using hiPSCs for the investigation and treatment of skin diseases.

A New Era in Regenerative Medicine: iPSC Technology

In 2006 Takahashi and Yamanaka reported the landmark discovery that mice embryonic dermal fibroblasts can be directly reprogrammed to a pluripotent state, similar to ESCs, with defined transcription factors (Klf4, Oct4, Sox2, c-Myc). Since, many different cell types such as lymphocytes (Hanna et al., 2008), stomach and liver cells (Aoi et al., 2008), neural progenitors (Kim et al., 2009), pancreatic β cells (Stadtfeld et al., 2008), keratinocytes (Aasen et al., 2008), and melanocytes (Utikal et al., 2009) from a number of other species including human (Takahashi et al., 2007; Yu et al., 2007), rat (Li et al., 2009), pig (Wu et al., 2009) and rhesus monkey (Liu et al., 2008) have been successfully reprogrammed to a pluripotent state using the original schema reported by Takahashi and Yamanaka (2006; Figure 10.2.). These iPSCs, like ESCs, possess unlimited proliferative capacity and are able to differentiate into all three embryonic germ layers, as demonstrated by teratoma forming assay. In this assay the differentiated tumor contains tissues from all three embryonic germ layers; for mice iPSCs, when injected into murine blastocysts they contributed to all tissues including germ line tissues; and for some iPSC lines when injected into tetraploid blastocysts they generate mice. Induced pluripotent stem cells hold great promise as an ethical tool for investigating basic questions of cellular plasticity and pluripotency, and importantly, for cell-based therapies for incurable

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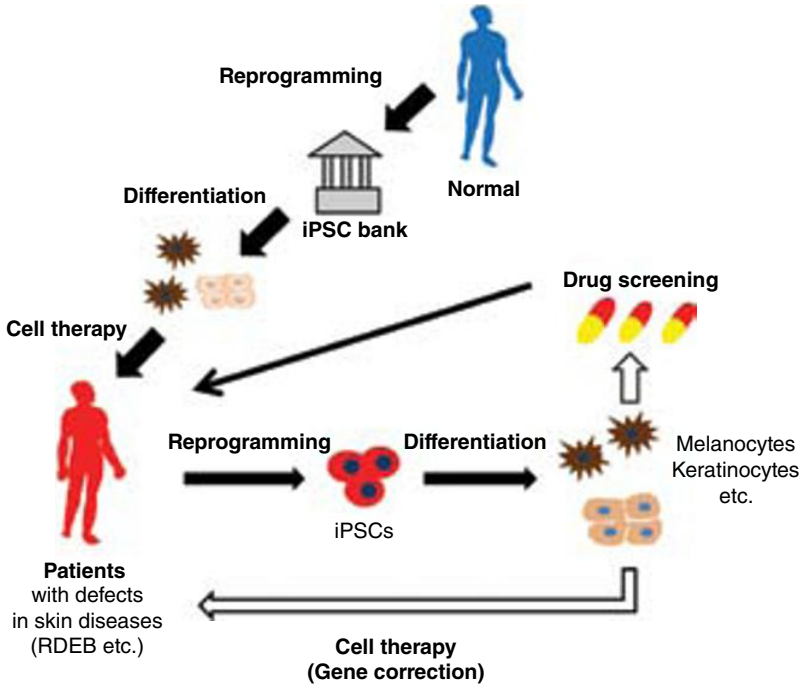


Figure 10.1. The clinical potential of induced pluripotent stem cells (iPSCs). Since the discovery of iPSCs, potential clinical applications of the technology have been postulated. Induced pluripotent stem cells generated from normal individuals may be stored in 'iPSC banks'. Induced pluripotent stem cell lines may then be differentiated into a variety of cell types and subsequently utilized for drug screening or cellular therapy. For patients with genetic diseases, patient-specific iPSC lines provide chances to correct individual mutations.

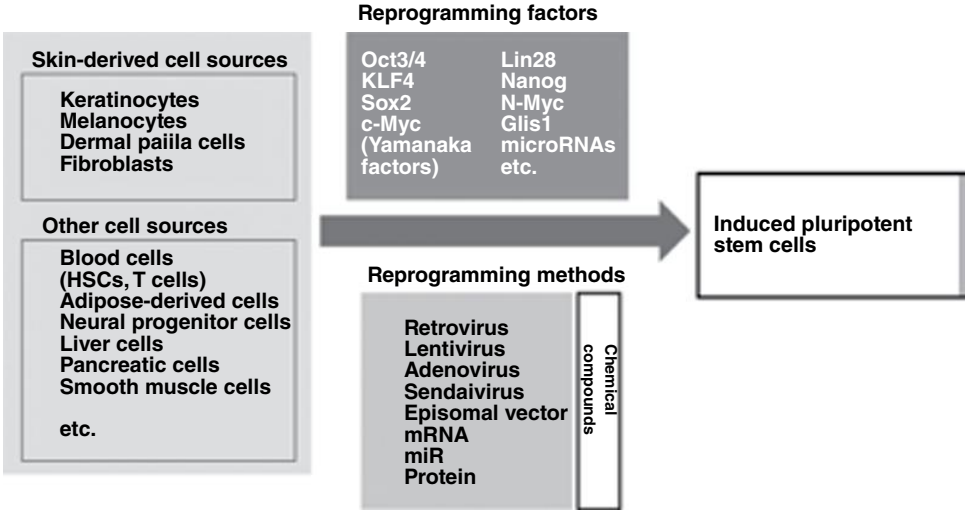


Figure 10.2. Cellular sources and methods for iPSC generation. A variety of somatic cells, including somatic cells derived from the skin (fibroblasts, keratinocytes, dermal papilla cells and melanocytes), can be reprogammed into iPSCs. To supply clinically applicable iPSCs, nonintegration methods of iPSCs are being investigated.

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diseases such as spinal cord injury and genetic disorders (Daley, 2012; Yamanaka, 2012; Nakamura and Okano, 2013).

Originally, the reprogramming discovered by Takahashi and Yamanaka (2006) adapted a retroviral delivery system of the four transcription factors (Oct4, Sox2, Klf4, and c-Myc – known as Yamanaka factors). How such a small subset of transcription factors can destabilize the somatic program and lead to the establishment of a pluripotent transcriptional network has not yet been fully uncovered. However, this method has been validated in multiple laboratories across the world, with multiple cell types with an efficiency of approximately < 1%. Later attempts to improve efficacy has been successful by using lentiviral delivery systems. Retroviruses only infect dividing cells, so by using a lentiviral delivery system both dividing and nondividing cells can be infected (Yu et al., 2007). Potential problems with both the retroviral and lentiviral methods is that the proto-oncogene reprogramming factors Klf4 and c-Myc may be integrated into the infected cell's genome, which could increase the risk of tumorigenicity hindering the translational potential of this method for cell-based therapies. Therefore, to improve safety, attempts have been made to further improve reprogramming methods. Strategies to remove integrated sequences have been developed using lentiviral vectors with loxP sites that serve as substrates for Cre-mediated excision of most of the integrated sequences (Chang et al., 2009; Soldner et al., 2009; Somers et al., 2010). However, minor remnant exogenous transgene sequences would be a concern if this technology were to be used in clinical settings. Therefore, alternative nonintegration reprogramming methods are being developed, such as infection with adenovirus or Sendai virus, transfection with episomal or minicircle vectors, transfection with synthetic mRNA or miRNA, and transposition with piggyback transposon (Ho et al., 2011; Hussein and Nagy, 2012). Considering efficiency and safety, currently the episomal and Sendai virus methods would be favorable methods to generate iPSCs for clinical-grade therapies (Rao and Malik, 2012).

Pluripotent stem cells hold great potential for regenerative medicine. Similarities between iPSCs and ESCs exist, in particular their morphology, global gene expression, self-renewal capacity, pluripotency and their downstream use for *in vitro* disease model, disease specific pharmacological testing, and potential for cell therapy applications to treat currently incurable diseases (Puri and Nagy, 2012). However, differences between iPSCs and ESCs have given rise to the multifaceted debate as to which cell type is preferential. Since the discovery of human ESCs in 1998 (Thomson et al., 1998), there has been an ongoing ethical objection to using human ESCs in research and for clinical applications. Use of iPSCs derived from adult somatic cells is free from such ethical opposition. Despite this, much investigation has been undertaken into the nature of ESCs, their differentiation ability, and their derivatives being used as functional replacements in disease. Indeed, clinical trials using products derived from human ESCs have started. The much publicized first trial of human ESC-derived products, sponsored by Geron Corporation, delivered oligodendrocyte progenitors to four spinal cord injury patients before halting the trial without publication of initial phase 1 results in order to focus on alternative corporate priorities (Baker, 2011). Furthermore, another trial to treat age-related macular degeneration, sponsored by the company Advanced Cell Technologies, have reported initial findings. Only one of the two patients showed evidence of persistent cells, but both were reported to show some restoration of visual perception (Schwartz et al., 2012). Although it is difficult to draw conclusions from

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these early trials, it is evident that clearing the high regulatory hurdles, extensive preclinical cell characterization, and quality control can be met by stem cell researchers before exposing patients to the risks of ESC-derived products (Daley, 2012).

However, obstacles to clinical applications using ESC and iPSCs remain. Both ESC and iPSC acquire abnormal karyotypes during expansion and prolonged passage (Taapken et al., 2011). In addition, elimination of residual pluripotent stem cells should be required prior to any transplantation. Furthermore, the differentiation capacity of iPSCs and functionality of their differentiated derivatives vary, so this may prove a challenge in the future (Veraitch et al., 2011). Partial explanation of this phenomena may be due to iPSCs retaining residual methylation signatures (or "epigenetic memory") characteristic of their somatic origin, favoring differentiation to lineages related to the donor cell, leading to implications for downstream disease modeling or treatment. To overcome this observation, this differentiation propensity can be reset using chromatin-modifying compounds (Kim et al., 2010). However, the increasing ability to direct iPSCs and ESCs to any cell type for therapeutic potential holds enormous potential for these cell types in regenerative medicine. The debate on iPSCs versus ESCs is ongoing, but, ESCs are considered the gold standard of pluripotency, while iPSCs offer patient-specific cells from adult individuals, giving the possibility of curing diseases using cells or tissue grafts with perfect histocompatibillity match.

The skin is a highly complex organ comprising a variety of different somatic cell types that can be isolated and reprogrammed into a pluripotent state. Main components of the skin comprise three layers: the epidermis (stratified epithelial tissue consisting of keratinocytes), dermis (collagenous tissue from which fibroblasts and melanocytes can be isolated), and subcutaneous fatty tissue. Skin appendages, including pilocebaceous units and sweat glands, and blood/lymphatic vessels and nerves are buried within this main structure. Especially, the HF, a complex mini-organ consisting of epithelial (keratinocytes in the outer root sheath) and specialized hair inductive mesenchymal cells (the dermal papilla (DP) at the base of the HF and the dermal sheath wrapping the HF structure) attracts great interests in terms of regenerative medicine because its stem cell populations have been studied intensively. The skin is a particularly attractive reservoir of material for iPSCs as samples from this easily accessible organ can be taken by skin biopsies or from redundant tissue following face-lift surgeries. Furthermore, the cellular components from these skin samples can be isolated easily, either enzymatically or by microdissection. Indeed, even plucked HFs have provided a convenient source of iPSCs (Aasen et al., 2008).

Classically, the four Yamanaka transcription factors Oct4, Sox2, Klf4 and c-Myc can reprogram somatic cells into pluripotent stem cells. However, as mentioned above, the use of oncogenic reprograming factors, Klf4 and c-Myc, remains a challenge in iPSC generation. This has lead to recent attempts to reprogram somatic cells using less transcription factors. Recently, mouse neural stem cells that endogenously express Sox2, Klf4 and c-Myc, were successfully reprogrammed by two (Oct4 and Klf4) or one factor (Oct4) (Kim et al., 2009). This led to the speculation that cells expressing endogenous Yamanaka factors may be susceptible to reprogramming without forced expression of the endogenous factor. This is a major step towards finding cells that can be manipulated into iPSCs more easily, however, isolating equivalent neural stem cells from humans is technically and ethically demanding.

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In addition to fibroblasts, other populations within the skin can be utilized to generate iPSCs, such as keratinocytes, DP cells, and melanocytes (Aasen et al., 2008; Utikal et al., 2009; Tsai et al., 2010, 2011; Higgins et al., 2012). Where keratinocytes from juvenile human foreskin and plucked HFs have provided material for iPSC generation, four-factor transduction was reported to be at least 100-fold more efficient and twofold faster compared with reprograming of human fibroblasts (Aasen et al., 2008). The molecular signature of murine DP cells includes c-Myc, Klf4, and Sox2, which may confer a more reprogrammable state. Indeed, mice DP cells have recently been successfully reprogrammed using two (Oct4 and Klf4) and one (Oct4) factors (Tsai et al., 2010, 2011). Upregulation of Sox2 was also observed in human DP cells (Ohyama et al., 2012). Human DP cells can be converted to iPSCs, however, reprogramming with less than four Yamanaka factors was not possible (Higgins et al., 2012). Melanocytes, like neural stem cells, are neuroectodermally derived and express endogenous Sox2. Therefore, investigations to reduce the manipulation required to reprogram mice and human melanocytes to iPSCs revealed that Sox2 is dispensable for reprograming (Utikal et al., 2009). These findings are summarized in Table 10.1. As stem cell populations in the skin are further characterized, additional cellular sources for iPSCs generation may evolve.

A Brief Overview of Stem Cells and Their Function in the Skin

The skin and its appendages constitute an intriguing organ, particularly due to their capacity to continually self-renew throughout life: both in physiological homeostasis and in response to injury. These renewing and repairing capabilities imply the presence of stem cells within the skin to ensure these functions. Classic label-retaining experiments revealed a slowly dividing population of stem cells that retain nucleotide analogs identifying stem cell populations in the basal layer of the interfollicular epidermis (Mackenzie and Bickenbach, 1985; Morris et al., 1985; Ghazizadeh and Taichman, 2001) and the bulge region of HFs (Cotsarelis et al., 1990; Lyle et al, 1998).

Despite intensive characterization of basal layer cells, isolation of pure populations of human interfollicular epidermal stem cells has not been achieved and, therefore, it has been difficult to identify a hierarchy of progenitors. Past morphological studies supported the epidermal proliferative unit (EPU) hypothesis where a column structure comprising a central stem cell and surrounding stem-cell-derived transient amplifying cells sustains overlying stratified keratinocyte layers in mice. However, recent transgenic studies have challenged this dogma and proposed a new concept that the epidermis is maintained by a single functionally equal progenitor population (Clayton et al., 2007; Poulson and Lechler, 2010; Doupe and Jones, 2012). Whether these observations are applicable to human epidermis remains a future question. Although several promising candidates have been proposed, cell surface markers to define *bona fide* interfollicular epithelial stem cells, especially that of human, have not been identified. Further investigation is necessary to fully elucidate the stem cell system within the human epidermis.

Compared to interfollicular epidermal stem cells, relatively more is known about HFSCs, which were previously believed to be located in the bulbar area of the HFs.

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Table 10.1. Skin as a source of iPSCs.

	Mouse			Human		
	Endogenous expression of reprogramming factors	Reprograming factors to induce iPSC	Efficiency of iPSC induction (%)	Endogenous expression of reprogramming factors	Reprograming factors to induce iPSC	Efficiency of iPSC induction (%)
Fibroblasts		OSKM	0.056 (Utikal *et al.*, 2009) (Takahashi *et al.*, 2006)		OSKM	<0.01 (Takahashi *et al.*, 2007) (Aasen *et al.*, 2008)
Keratinocytes (foreskin)					OSKM	1 (Aasen *et al.*, 2008) 0.00001 (Carey *et al.*, 2009)
					OSK	1 (Santamaria *et al.*, 2010)
Keratinocytes (plucked hair)					OSKM	1 (Aasen *et al.*, 2008)
Dermal papilla	SKM	OSKM OK O	1.38 0.024 (Tsai *et al.*, 2010) 0.088 (Tsai *et al.*, 2011)	SN	OSKM	0.02 (Higgins *et al.*, 2012)
Melanocytes	S	OSKM OSK OKM	0.19 0.02 0.03 (Utikal *et al.*, 2009)	S	OSKM OSK OKM	0.05 0.01 0.01 (Utikal *et al.*, 2009)

O, Oct4; S, Sox2; K, Klf4; M, c-Myc; N, NANOG

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However, early studies demonstrated that a whole HF could be regenerated despite the hair bulb being surgically removed (Oliver, 1966). Over two decades later, label-retaining experiments showed that the HFSCs reside in the HF epithelial bulge, an insertion point of arrector pili muscle within the outer root sheath, both in mice and human (Cotsarelis et al., 1990; Lyle et al, 1998). Establishment of isolation methods for bulge HFSCs has greatly accelerated our understanding of bulge-cell biology (Trempus et al., 2003; Morris et al., 2004; Tumbar et al., 2004; Ohyama et al., 2006). Lineage tracking experiments and hair reconstitution assays indicated that bulge stem cells possess multipotency to repopulate HFs, sebaceous glands, and the epidermis. An intriguing question is whether interfollicular epidermal stem cells and bulge stem cells are interchangeable. Interestingly, Ito et al. (2007) reported that epidermal stem cells contribute to hair follicle neogenesis and repopulate the bulge stem cell niche after wounding.

Recently, deeper insights into stem cell-niche interactions in the HF have been highlighted. Unexpectedly, cross-cell signaling between HF stem cells and other cell populations, including melanocytes, adipocytes and muscles or nerves has been suggested to contribute to HF maintenance. For instance, melanocyte stem cell activation during HF regeneration is influenced by neighboring epithelial stem cells (Rabbani et al., 2011; Tanimura et al., 2011), the arrector pili muscle attaches to bulge cells which promotes muscle differentiation (Fujiwara et al., 2011), signals from peripheral nerves alter the properties of upper bulge stem cells (Brownell et al., 2011), and fat cell precursors induce HF cycling (Festa et al., 2011).

Additional putative stem cell populations in the skin, such as melanocytes, and sebaceous and sweat gland stem cells have been identified from elegant genetic tracking experiments (Horsley et al., 2006; Lu et al., 2012; Nishimura et al., 2002). Melanocyte stem cells were identified in the sub-bulge area, which are maintained in a quiescent state by TGF-beta signaling (Nishimura et al., 2005; Lu et al., 2012; Tanimura et al., 2011) during HF morphogenesis. During anagen, they are activated with HFSCs to generate mature bulb melanocytes (Nishimura et al., 2002) that produce and transfer pigment to differentiating hair matrix cells, resulting in pigmented hair fibers. Sebocytes within the sebaceous gland secrete lipids into the infundibular opening of the associated HF, enabling hair shaft elongation (Stenn, 2001). Blimp1+ progenitors in the isthmus of the HF were shown to give rise to terminally differentiated sebocytes. However, unlike the bulge HFSCs, they do not contribute towards interfollicular epidermis in normal homeostasis or wounding (Horsley et al., 2006). The sweat gland has a relatively simple architecture. The glandular part of the sweat gland consisting of basal myoepithelial and suprabasal luminal cells is connected to the skin surface by a duct. Recently, it was demonstrated that in ventral paw skin the glandular luminal cells are maintained by suprabasal unipotent progenitors that are independent of the myoepithelial cells (Lu et al., 2012). Additional studies are likely to further define these stem cell populations, and their interactions within the skin.

Therefore, distinct skin stem cell populations and their complex interplay are only just being defined. Considering variety and their respective roles in the skin, attempts to achieve full reconstruction of the human skin with cycling HFs using human iPSCs is likely to require induction of individual stem cell populations from iPSCs.

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Generation of Melanocytes and Keratinocytes from iPSCs

Melanocyte Generation

Melanocytes originate from the neural crest and, once matured, reside in the basal layer of the epidermis and the matrix of HFs. They contribute to skin and hair coloring by generating melanin pigment, which is packed in melanosomes, and finally transported to keratinocytes. In addition, melanocytes play an important physiological role in providing protection from harmful ultraviolet rays (Yamaguchi et al., 2007). Either defects in or a lack of melanocytes can lead to pigmentary disorders, including vitiligo and dyschromatosis (Dessinioti et al., 2009). To date, cultured melanocytes may represent a potential future therapy for such disorders (Abu et al., 2010). However, in contrast to neonatal foreskin melanocytes, expansion of adult epidermal melanocytes is challenging, because the expandable capacity of adult melanocyte culture from human biopsy is limited. Thus, development of methods to generate large numbers of autologous melanocytes is required, considering the therapeutic implications. To address this issue, melanocyte generation from ESCs has previously been reported in mice and human (Yamane et al., 1999; Fang et al., 2006).

Genetic disorders of hypopigmentation including Waarrdenburg syndrome, piebaldism, albinism, Chedaki-Higashi syndrome, and Griscelli syndrome, are caused by either melanoblast or melanocyte functional defects (Tomita and Suzuki, 2004). Patient-specific iPSC-derived melanocytes may faithfully recapitulate the disease phenotype and contribute significantly to revealing disease mechanisms and drug discovery by using high-throughput screening systems. In addition, gene mutations have recently been corrected in iPSCs, as described below. Grafting of melanocytes derived from these gene-corrected iPSCs may potentially be used for cell therapy. During embryonic development, nonpigmented melanoblasts, the precursors of melanocytes, are derived from the neural crest cells and migrate to their destination stimulated by differentiation signals. A number of studies have shown that factors such as Wnt, stem cell factor (SCF) known as c-kit ligand, and endothelian-3 (ET3) are important for development (White and Zon, 2008; Ernfors, 2010). Based on this knowledge, generations of melanocytes from iPSCs have been reported in mouse and humans using Wnt3a, SCF, and ET3 through the embryoid body (EB) formation procedure (Ohta et al., 2011; Yang et al., 2011). Interestingly, during the melanocyte induction process, the appearance of neural crest stem-like cell population was observed (Ohta et al., 2011). Thus, the melanocyte generation process from iPSCs seems to mimic the *in vivo* developmental process. In addition, Wnt3a and 12-tetradecanoylphorbol-13-acetate (TPA) were shown to be dispensable for the melanocyte generation from mouse iPSCs (Yang et al., 2011), suggesting that there remain possibilities to improve the contents of the melanocyte induction cocktail to increase the productivity of melanocytes from iPSCs. Nissan et al. (2011) reported melanocyte generation from human iPSC using a moderate concentration of bone morphogenetic protein (BMP) without EB formation (Figure 10.3). In addition, melanin transfer from melanocytes to keratinocytes in a three-dimensional culture system was also shown in the study. Currently, more efficient induction of neural crest stem cells from iPSCs may be a key point to increase the productivity of melanocytes from iPSCs. Efficient methods to generate neural crest stem cells from iPSCs using BMP and Wnt inhibitors with have been reported (Lee et al.,

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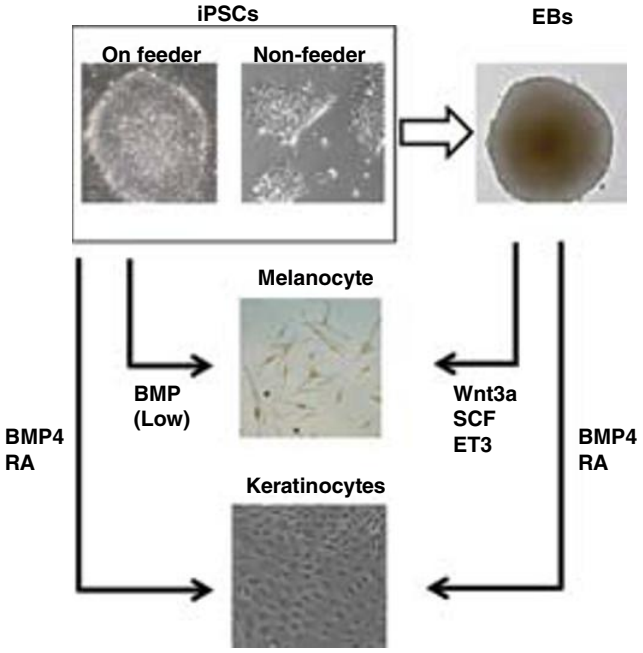


Figure 10.3. Methods for generating iPSC-derived melanocytes and keratinocytes. Human iPSCs have been successfully differentiated into both melanocyte and keratinocyte lineages by exposing them to key signaling molecules and mediators, including WNTs and BMPs.

2010; Menendez et al., 2013), which will contribute to a new protocol for melanocyte generation from iPSCs. Thus, this induction system may allow further elucidation of human melanocyte properties, including currently unidentified specific markers.

Based on the epigenetic memory theory that iPSCs can retain their epigenetic memory of original cell sources (Kim et al., 2010), keratinocytes, easily accessible and a cell population of the same ectodermal origin, may be a suitable cell source for the generation of iPSC-derived melanocytes. In addition, higher reprogramming efficiency of more than 100-fold in iPSC generation from keratinocytes compared to fibroblasts was also reported (Aasen et al., 2008). However, it should be considered that keratinocytes obtained from sun-exposed areas might possess ultraviolet-ray-induced DNA damage (Galach and Utikal, 2011). Therefore, selecting the location of skin biopsy for the generation of iPSCs is probably important. In conclusion, the generation of melanocytes from iPSCs may be useful for understanding of human melanogenesis biology, analyses of the mechanism of pigmentary disorders, drug discovery, and potential development of cell therapy for pigmentary disorders.

Keratinocyte Generation

Established therapies using autologous and allogeneic keratinocytes, including cadaver-derived cells, have been used for cell therapy in patients with large burns and ulcers (Lemaitre et al., 2011). Allogeneic keratinocytes have a drawback regarding

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immunoreaction and risk of virus contamination. Thus, iPSC-derived keratinocytes from either autologous samples or iPSC banks may be an ideal source for cell therapy, considering the unlimited proliferative potential, immunogenicity, and ethical advantages compared to ESCs.

The generation of keratinocytes from iPSCs has been achieved based on knowledge from ESC studies (Uitto, 2011). Previous studies reported the generation of keratinocytes and/or keratinocyte-lineage cells from human and mouse ESCs (Iuchi et al., 2006; Ji et al., 2006; Haase et al., 2007). Recently, Guenou et al. (2009) generated human keratinocytes using BMP4 and ascorbic acid by bypassing formation of EBs, and the yield of keratinocytes was 50–60% from human ESCs after 6 weeks. The study is quite intriguing in that both melanocytes and keratinocytes could be generated only by BMP4, depending on the different concentration of BMP4 without formation of EBs (Figure 10.3). In addition, in the human ESC study, the generation of keratinocytes from human ESCs was reported by stage-specific addition of activin, which can inhibit neural differentiation similar to BMP4 (Kidwai et al., 2012). Thus, activin may be applied for keratinocyte differentiation from human iPSCs instead of BMP4. In mouse studies, a collagen-IV-coated dish was used to enrich keratinocyte stem cells during the course of induction of keratinocyte lineage cells from mouse iPSCs (Bilousova et al., 2011), suggesting an advantage of using collagen IV for keratinocyte induction from human iPSCs. In future studies, there still remains room to improve the keratinocyte induction methods from human ESCs and iPSCs.

The success of generation of keratinocytes from human iPSCs offers many opportunities to investigate skin diseases. In human studies, keratinocytes were generated from recessive dystrophic epidermolysia bullosa (RDEB) (Itoh et al., 2011; Tolar et al., 2011) and junctional EB patient-derived iPSCs (Tolar et al., 2013) using retinoic acid (RA) and BMP4, based on the findings that RA promotes ectodermal fate and BMP4 blocks neural fate. Both normal and RDEB iPSC-derived keratinocytes generated three-dimensional skin equivalents, showing their functional cell differentiation (Itoh et al., 2011). In addition, it was informative that iPSCs derived from RDEB, which has a COL7A1 mutation, were able to differentiate into both hematopoietic and nonhematopoietic lineages (Tolar et al., 2011). Thus, the generation of disease-specific iPSC-derived keratinocytes may deepen our understanding of disease mechanisms and potentially lead to drug discovery. It also may be possible to correct gene mutations in RDEB-derived keratinocytes using new technologies, which is discussed later.

Induced pluripotent stem cells derived from epithelial and mesenchymal components may be advantageous materials for hair regeneration. Current attempts to regenerate HFs center on combining receptive epithelial and trichogenic dermal mesenchymal components and grafting them into an *in vivo* environment (for reviews see Ohyama et al., 2010; Yang and Cotsarelis, 2010). Investigations into different epithelial components for such assays have suggested that neonatal cell sources may be more efficient in forming HF-like structures than adult human keratinocyte cells at the same passage (Ehama et al., 2007). However, supply and ethical restraints may limit the use of such neonatal keratinocyte cells in routine HF regeneration. Therefore, it may be advantageous to generate epithelial and mesenchymal precursors from human iPSCs for the purpose of HF bioengineering. To support this hypothesis, it has been reported that *de novo* HF generation in immune-deficient mice was possible by grafting human iPSC-derived ectodermal precursors, which may include keratinocyte progenitors,

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mixed with mouse mesechymal dermal cells, suggesting that iPSC-derived epithelial lineages may possess the ability to contribute to *in vivo* HF morphogenesis (Veraitch et al., 2013).

Use of iPSCs for the Investigation of Skin Cancers and Melanomas

Melanomas account for an astounding 75% of all skin cancer deaths (http://www.cancernetwork.com). Overall, melanoma incidence increased at 3.1% ($P < 0.001$) per year in the United States (Linos et al., 2009), and advanced melanoma patients have a poor prognosis with a median survival between 3 and 11 months. However, effective new treatments have recently been developed, including molecular targeted therapies such as BRAF/MEK inhibitors (Bollag et al., 2012), and immunotherapies such as anti-CTLA-4 mAb, anti-PD-1 mAb, and adoptive cellular therapy (Bluestone and Small, 2012; Restifo et al., 2012; Ott et al., 2013). The heterogeneity of cancer is thought to be one of the obstacles for development of effective therapy (Marusyk et al., 2012; Fisher et al., 2013). The cancer stem cell (CSC) hypothesis supports the generation of heterogeneous cancers and has been suggested for leukemia, brain tumors, and breast cancers (Dalerba et al., 2007; Clevers, 2011; Nguyen et al., 2012). Cancer stem cells are defined as a cell population that has self-renewal potential and an ability to generate heterogeneous cell lineages. In the majority of solid tumors, CSCs exert resistance to chemotherapy and radiotherapy, leading to cancer relapse. Accordingly, investigation of CSC biology is indispensable for the development of effective cancer treatment. Enrichment of CSC populations may be achieved with the combination of cell surface makers, the ability of spheroid formation, side population based on the exclusion ability of Hoechst 33342 dye, enzymatic activity such as aldehyde dehydrogenase, and tumorigenic ability of transplanted CSCs in immunodeficient mice (Visvader and Lindeman, 2008). Attempts to explain tumor heterogeneity were first proposed with the stochastic (clonal evolution) model, and more recently the CSC model. However, it is still controversial whether a single model can explain tumor heterogeneity (Magee et al., 2012). In addition, the dynamic stemness model, where CSCs and/or CSC-derived cells and differentiated cancer cells may convert to one another, has been proposed in recent studies (Hatina, 2012). Indeed, conversion of nonstem-like cells to stem-like cells was reported in human mammary epithelial cells (Schwitalla et al., 2013). In addition, it has been reported that epithelial–mesenchymal transition induced non-CSC to become CSC-like cells (Mani et al., 2008). Thus, the study of the CSC plasticity is also an important issue, especially *in vivo,* when considering the design of cancer therapy.

In melanoma, CD20, CD133, ABCB5-positive cells, and side population cells have been reported as possible CSC populations (Grichnik, 2008; Zabierowski and Herlyn, 2008). However, Quintana et al. (2010) have recently shown the difficulty of enrichment of tumor-initiating cells in nonobese diabetic/severe combined immunodeficient interleukin-2 receptor-γ chain null (NSG) mouse xenograft model, which lacks T, B, and NK cells, by using proposed melanoma CSC markers. They also reported that primary melanoma tissues contain a large number of tumor-initiating cells, which were more frequent than estimated from the CSC hypothesis. The discrepancy between

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this study and the previous may be explained by the difference of experimental conditions, including enzymatic digestion of the cancer tissues, use of matrigel, and the presence of NK cells. This study clarified that tumor-initiating assay of human cancer cells in xenografting models may suffer from some problems, including lack of the appropriate niche and presence of unusual immune responses such as mouse NK cells, and different hypoxia conditions, leading to a microenvironment different from that surrounding actual human tumors. It has been reported that stem cell niche supports the stemness of CSCs depending on cell adherent molecules, growth factors, and cytokines. Thus, it is important to develop new assays mimicking the *in vivo* tumor microenvironment in order to evaluate human tumor-initiating ability *in vivo*. Quintana et al.'s (2008) study showed the difficulty of enriching melanoma stem cells by simply using cell surface markers. If melanoma stem cells exhibit plasticity *in vitro*, they can hardly be distinguished by cell surface marker expression. In fact, H3 lysine-4 demethylase JARID1B-positive melanoma cells were reported to have high tumorigenic ability compared to the negative cells (Roesch et al., 2010). At the same time, they observed bidirectional change between the JARID1B-positive and -negative melanoma cells, suggesting a possibility that CSCs have a reversible phenotype termed "dynamic stemness." This study showed that CSCs might change their phenotype reversibly by epigenetic regulation. Thus, the CSCs plasticity should be considered in the characterization of melanoma stem cells.

Currently, the origin of tumor-initiating melanoma stem cells remains unknown. Melanoma stem cells may be developed from melanocyte stem cells or melanocytes via dedifferentiation and oncogenic mutations. The generation of cells possessing stem cell properties from either differentiated cells or progenitor cells has been reported in intestinal epithelial cells, B cell progenitors, glial progenitors, and neurons (Liu et al., 2011; Friedmann-Morvinski et al., 2012; Schwitalla et al., 2013; Sugihara and Saya, 2013), suggesting the possibility that quiescent adult epidermal melanocytes may also be a cell source of CSCs of melanoma. In some cases, NKIbetab positive putative memelanocyte stem cells, which reside in the lower permanent portion (LPP) of the HF (Nishimura, 2011), may contribute to the generation of melanoma stem cells and/or melanoma. Additional populations of melanocyte stem cells may exist. For instance, neural-crest-derived progenitors residing in the dermis may be an additional candidate for melanocyte stem cells. Indeed, p75-positive cells derived from human foreskin have been reported to have potential to differentiate into melanocytes (Li et al., 2010). However, due to lack of definitive markers, it is difficult to convincingly show the presence of dermal melanocyte stem cells.

Reprogramming technology including iPSC is very useful to study CSCs. Similarities between iPSCs and cancer cells have been implicated by global gene expression studies and epigenetic status analyses. Of note, a new concept of induced cancer stem cells (iCSCs) is quite intriguing (Wong et al., 2008; Sugihara and Saya, 2013). Human CSC-like cells, which express a stage-specific lineage marker SSEA-1, and had both self-renewal and differentiation ability with tumorigenicity, were generated from fibroblasts using hTERT, H-RasV12, and SV40LT and ST antigens (Scaffidi and Misteli, 2011). Successful generation of iCSCs has also been reported in ostosarcoma, brain tumors, ovarian tumors, and leukemia lymphoma in mice (Sugihara and Saya, 2013). The advantages of iCSCs include: (i) unlimited proliferative capacity and easy accessibility; (ii) relatively easy evaluation of the roles of cancer-related molecules by

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gain/loss of function studies; (iii) ideal cell sources for drug discovery screening. It is impossible to analyze mechanisms of tumorigenesis retrospectively in patients. This system could allow analyzing transformed processes chronologically. Based on the knowledge from iPSC studies, the ability of tumorigenesis may be greatly affected by reprogramming status. Insufficiently reprogrammed, immature cell populations were considered to be more tumorigenic.

Human melanoma stem cells may be generated from fibroblasts using combinations of transcription factors, potentially leading to the identification of melanoma stem cell markers. In addition, melanoma stem cells may be generated from iPSC-derived melanocyte stem cells by the introduction of oncogenes such as BRAF, β-catenin, N-ras, MITF, hTERT, and shRNA for tumor suppressor genes such as p16, p53, and PTEN. We are currently attempting to enrich human iPSCs-derived neural crest stem cells to generate melanoma stem cells. Other groups have been trying to reprogram cancer cells to iPCCs (induced pluripotent cancer cells) and/or CSCs, including chronic myeloid cells, gastrointestinal cells, and melanoma cells (Utikal et al., 2009; Miyoshi et al., 2010; Kumano et al., 2012, Ramos-Mejia et al, 2012). Intriguingly, mouse melanoma cells were reprogrammed into iPCCs and chimeric mice carrying these iPCCs were free of obvious tumors (Utikal et al., 2009), suggesting the possibility that pharmacological reagents with reprogramming potential may reset tumor-specific epigenetic memory and cancel their tumorigenicity in cancer cells. In many cancers, upregulation of stemness marker genes has been reported (Semi et al., 2013). It may be possible to consider that melanoma stem cells could be generated from melanocytes accompanied by a reprogramming procedure to erase the original epigenetic memory and oncogenic mutations, leading to the dysregulation of cell proliferation control and disturbance of the reprogramming induced senescence (RIS) (Banito and Gil, 2010). Once the underlying mechanism of epigenetic regulation enabling reprogramming is fully elucidated, novel diagnostic approaches to evaluate the risk of tumorigenesis may be developed. In addition, as shown in leukemia, a drug that differentiates immature cancer cells (e.g. CSCs) to differentiated cells may reduce the number of CSCs. Promoting the cell proliferation of quiescent state of CSCs *in vivo* and interrupting the connection between CSCs and the niche environment, including chemokine blocking and the homogenization of heterogeneous cancer cells, represent new cancer treatment strategies taking advantages of iPSCs and regenerative medicine technologies (see Figure 10.4).

In skin cancers, basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) are the major nonmelanoma skin cancers (Colmont et al., 2012). The identification of cancer origins of both BCC and SCC have been attempted using mouse models adopting wound injury and chemical mutagenesis by 7,12- dimethylbenz(a)anthracene (DMBA)-TPA or genetically engineered mice with dysfunction of HH signals and p53, and overexpression of Ras oncogenes. Cell-lineage-tracking analyses elucidated that various epidermal compartments, including interfollicular epidermis, infundibulum, isthmus, and bulge regions, gave rise to BCCs depending on the types of oncogenic mutations (Blanpain, 2013). In addition, CD34, CD133-postitive cells, and integrin $\alpha6\beta1^{high}$ SCC cells were shown to have tumor-initiating cell ability. Although some skin tumors, especially BCCs, have been considered to orig*i*nate from bulge stem cells (Thieu et al., 2012; Blanpain, 2013), further studies are needed to identify *bona fide* origins and establish their reliable markers. Recently, Youssef et al. (2012) showed that basal cells could be reprogrammed to embryonic HF progenitor-like cells by SmoM2 induction for BCC

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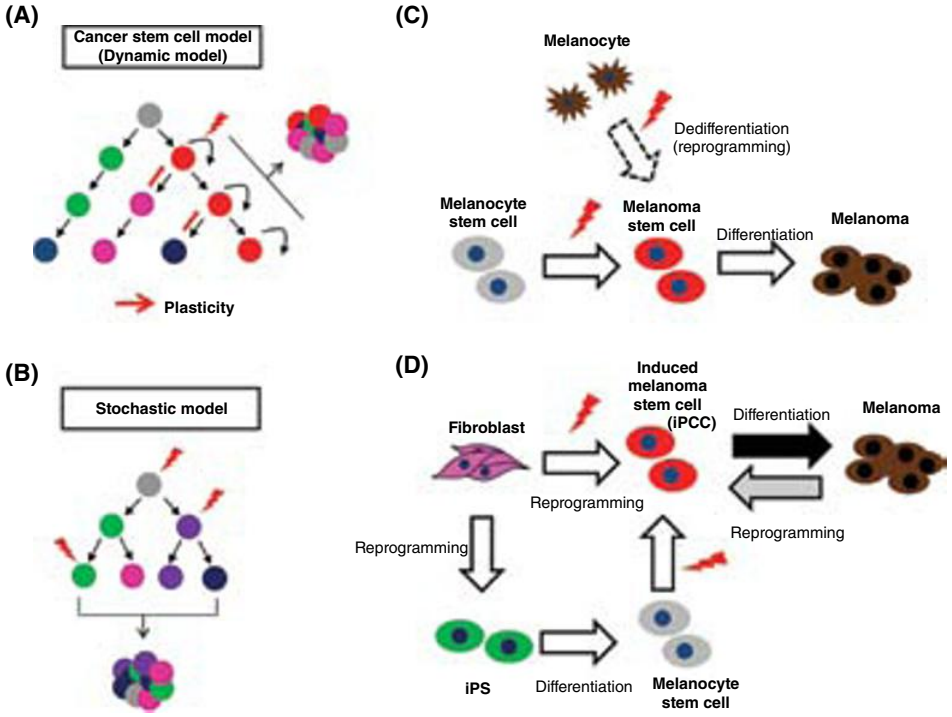


Figure 10.4. The cancer stem cell (CSC) model, concepts of plasticity of CSCs, and induced CSCs (iCSCs). **(A, B)** The CSCs model for explanation of tumor heterogeneity. In the CSC model, some cell populations maintain CSC potential, which involves self-renewal, differentiation, and tumorigenicity. Recent studies have shown that non-CSCs possess plasticity to reacquire CSC properties (dynamic model). Red arrows: dedifferentiation **(A)**. In the stochastic model, most cancer cells have the ability to generate tumors triggered by oncogenic insults **(B)**. **(C)** The model of melanoma stem cell and melanoma development. Melanoma stem cells may originate from normal melanocyte stem cells after exposure to oncogenic insults. Alternatively, melanocytes may directly dedifferentiate into melanoma stem cells to form melanoma through a reprogramming process driven by oncogeneic insults. **(D)** The concept of iCSCs and reprogramming of cancer cells (adopting melanoma as an example). Based on reprogramming technology, fibroblasts can be transformed into iCSCs using oncogenes and reprogramming factors directly or via somatic tissue stem cells. The established iCSCs may give rise to tumor tissue under defined medium conditions. The reprogramming of cancer cells may generate iPCCs and/or CSC-like cells depending on reprogramming status. In all figures, red wavy arrows shows oncogenic insults.

tumor formation, suggesting the necessity of chronological *in vivo* tracing of cell fate to identify the tumor origins without relying on specific cell-fate markers. Moreover, the existence of heterogeneity of CSC populations (e.g., migrating CSCs and stationary CSCs) was recently reported in SCC (Biddle et al., 2011), pancreatic cancer, and breast cancers (Hermann et al., 2007; Brabletz, 2012). Thus, the heterogeneity in CSCs needs to be appropriately assessed in the investigation of tumor-initiating cell populations. Ideally, CSCs should also be studied at single cell level. Rapid high throughput screening

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by the next generation sequencer may enable deeper understanding of melanocyte and melanoma stem cell biology and lead to the development of new types of diagnostic and therapeutic strategies for various skin cancers.

The Potential of iPSCs Technologies in Dermatology

Induced pluripotent stem cells represent an accessible and ethical source of pluripotent cells that have been shown to possess the ability to differentiate into multiple cell lineages, including melanocytes and keratinocytes. Skin tissues may also provide an ideal cell source for the generation of iPSCs. Recently, Guha et al. (2013) have reported an additional advantage that iPSC-derived differentiated cells in *in vivo* mouse models have a low risk of immune rejection. Thus, iPSC technology holds a great promise of contributing to the development of new cell-based treatments for intractable dermatological disorders.

Developments are in progress, including the establishment of an iPSC bank, that will enable clinical application of iPSC technology. Well-defined guidelines for clinical-grade iPSC generation are required (Carpenter et al., 2009; Goldring et al., 2011). Among currently available reprogramming methods, nonintegration episomal vector systems (Yu et al., 2007; Okita et al., 2011) may be preferable. Once fully established, nonintegration methods, in combination with pharmacological agents, may be more appropriate for safely achieving reprogramming. In addition, xenofree and nontoxic iPSC induction conditions are indispensable for good manufacturing practice.

Tumorigenecity represents potential problems that need to be solved for establishing iPSC-based cell therapy. Generation of pure iPSC-derived populations devoid of incompletely differentiated cells is one possible approach to reduce tumorigenicity (Ben-David and Benvenisty, 2011; Cunningham et al., 2012). Promising approaches, including an antibody depletion system using stage-specific embryo antigen 5 (SSEA-5) antibody and introduction of inducible suicide genes into iPSCs, have been proposed (Knoepfler, 2009; Tang et al., 2011). Identification of lineage-specific human cell surface markers would aid enrichment of target iPSC-derived cells. Alternatively, direct lineage conversion (Vierbuchen and Wernig, 2011) may be adopted. This technology allows the generation of desired differentiated cells without the induction of an intermediary pluripotent state. This approach may reduce tumorigenicity of generated cells, as the pluripotent state is bypassed. Successful direct lineage conversion from skin to both neuron and cardiomyocytes has been reported (Vierbuchen and Wernig, 2012). This technology may facilitate efficient generation of melanocytes and keratinocytes from dermal fibroblasts in the future, although additional combination/optimization of transcription factors and induction medium compositions are necessary. In addition, as each iPSC clone exhibits different biological behaviors, the most preferential clones need to be selected by further analyzing intrinsic properties, such as gene and surface marker expression, immune antigen expression including MHC, karyotype normality, gene mutations, epigenetic factors, and functional potential of differentiated cells.

In addition to tissue regeneration using iPSC-derived cells, iPSCs can be used for the treatment of genodermatoses. Feasibility of genetic correction in patient-specific iPSC lines is currently being investigated as well. The rapid progress of gene-editing technologies using zinc finger nuclease (ZFN) and, more recently, transcription activator-like effector nuclease (TALEN) and clustered regularly interspaced short

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palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems may make it possible to repair mutated genes in skin diseases (Hockemeyer et al., 2009; Ding et al., 2013; Mali et al., 2013). In particular, successful correction of gene mutation in patient specific iPSCs has been reported in RDEB (Tolar et al., 2011). However, these gene-correction technologies are under development and there remains some possibility of miss-editing unexpected loci in the systems (Fu et al., 2013). Thus, it is very important to undertake whole genome sequence prior to clinical application.

Conclusions

Assessment of the feasibility of using iPSC technology in clinics is in the early stages, and much remains to be investigated. Because of easy accessibility and observability of the skin, dermatology is one of most attractive fields for testing and applying this novel approach. Considering the complexity of stem cell systems supporting homeostasis of the skin, further accumulation of knowledge on individual skin stem cell subsets, including their molecular signature and biological behavior, is indispensable to make full use of iPSC technology. Evidently, intensive investigation in this regard would be rewarding, as iPSCs should provide valuable materials for regenerative medicine, deeper insights into stem cell and cancer biology, and new remedies for currently intractable skin diseases, including genodermatoses.

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Chapter 11

Mitochondrial Differentiation in Early Embryo Cells and Pluripotent Stem Cells

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Introduction

Generally known for their fundamental role as energy-producing organelles in eukaryotic cells, mitochondria are multifunctional organelles and serve critical functions in calcium homeostasis (Jacobson and Duchen, 2004), cell signaling, apoptosis (Danial and Korsmeyer, 2004), as well as playing a role in numerous other important functions (Dyall et al., 2004). To accommodate the different needs of different cells and tissues mitochondria have an enormous ability to modulate their functions, dynamic behavior, and metabolic activities, thereby also controlling cellular fates and cellular differentiation. These semiautonomous organelles contain their own genome of ca. 16.6 kb circular mitochondrial DNA (mtDNA), encoding for 13 essential protein subunits of complexes I, III, IV, and V of the respiratory chain of the mitochondrial oxidative phosphorylation (OXPHOS) complexes as well as 22 tRNAs used in mitochondrial protein synthesis and 2 rRNAs (12s and 16s) that are necessary for the translation of mitochondrial subunits (Anderson et al., 1981). About 1500 mitochondrial-related genes reside in the nuclear genome thereby functionally connecting the nuclear genome with the mitochondrial genome. Transcripts for most of the mitochondrial polypeptides are translated in the cytoplasm and imported into the mitochondria. An RNA primer generated by mitochondrial transcription factor A (TFAM) (Larsson et al., 1998) is required for mtDNA replication, which is coordinated by catalytic DNA polymerase gamma (POLG) and accessory POLYG2 subunits (Gray and Wong, 1992). Mitochondria are dynamic organelles that form a complex network and undergo fusion and fission to accommodate the various requirements of specific cell types and they are translocated along microtubules to their functional destinations using the microtubule motor proteins dynein and kinesin for effective translocations in most cell systems (Katayama et al., 2006). Most studies on mitochondria have been performed in differentiated cells in which mitochondria display an elongated morphology containing complex cristae and a dense mitochondrial matrix.

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In contrast to differentiated cells, pluripotent stem cells and early embryo cells contain mitochondria that display a round morphology and only few cristae, generally referred to as functionally immature mitochondria that are typical for undifferentiated cells. In these cells mitochondria mainly undergo glycolysis compared to more differentiated cells that use OXPHOS as the major source for energy generation. Significant interest has recently been generated to explore the intriguing mitochondrial structure–function relationships and determine how structure and function are correlated on molecular, biochemical, and metabolic levels, which would also allow pursuing therapeutic intervention in adversely affected mitochondria that are implicated in various diseases and disorders such as neurological disorders, heart disease, diabetes, cancer, and several others in which mitochondrial dysfunctions play a role.

This chapter is focused on the dynamic changes and similarities of mitochondrial morphology and metabolic activities in developing embryo cells and in pluripotent stem cells during differentiation.

Changes in Mitochondrial Morphology and Dynamics During Differentiation in Developing Embryos and Stem Cells

As mentioned in the introduction, like pluripotent stem cells, mitochondria in oocytes and in the very early preimplantation embryo are immature with round morphological features and no, or only few, cristae. These cells undergo glycolysis rather than OXPHOS metabolic activities (Varum et al., 2011; Krisher and Prather, 2012; Redel et al., 2012) and develop morphological and metabolic profiles as they differentiate into adult somatic cell mitochondria with complex morphology, including well-developed cristae, denser matrix, and an elongated or branched appearance that is correlated with changes in their metabolic state. Throughout development and differentiation mitochondrial maturation takes place, which includes a bioenergetic transition from mainly glycolytic to aerobic OXPHOS metabolism. The respiratory chain-complex density and adenosine triphosphate (ATP) production become increased while mitochondria become elongated and cristae-rich. One example of tricarboxylic acid (TCA) cycle activation occurs as the level of uncoupling protein 2 (UCP2) decreases. Uncoupling protein 2 is a member of the mitochondrial UCP family and plays a significant role in mitochondrial metabolism; the TCA cycle is suppressed when UCP2 levels are high and the TCA cycle is activated when UCP2 levels are low (reviewed by Xu et al., 2013).

While we are beginning to understand the importance of mitochondrial morphology and dynamics to cellular physiology we do not yet clearly understand the physiology of mitochondria in early embryos and in stem cells and the differentiation process from the round cristae-poor glycolysis state to the elongated cristae-rich OXPHOS state in mitochondria of differentiated cells. Studies have shown the clear correlation between mitochondrial morphology and shift in differentiation, as increasing the abundance of a single protein can drive differentiation of stem cells (Bach et al., 2003).

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It has clearly been shown that undifferentiated stem cells and early embryos both metabolize glucose with many similarities to the Warburg Effect (Varum et al., 2011; Krisher and Prather, 2012; Redel et al., 2012) that was first described by Otto Warburg for cancer cells (Warburg, 1956): the similarities between early embryos, stem cells, cancer cells, and yeast have been reviewed in detail by Krisher and Prather (2012).

Mitochondria are maternally inherited in mammalian embryos, and the unfertilized oocyte contains all mitochondria for subsequent development while the sperm's mitochondria are destroyed in the fertilization process (Luo et al., 2013). The oocyte's immature mitochondria are distributed equally to the dividing daughter cells during subsequent cell divisions and utilize glycolysis during these early cell-division stages. In mouse embryos, new oocyte transcription takes place during the late 4- and 8-cell stage; an increase in the rate of protein synthesis is observed during the morula–blastocyst transition, leading to the formation of the inner cell mass (ICM) and trophectoderm (TE) at the blastocyst stage. Preimplantation embryonic development has been described in various mammalian species and been presented in detail for porcine embryos (Martin et al., 2007; Hall et al., 2010), including stages of blastocoel formation and cellular differentiation into TE and ICM cells. This stage is critical for mitochondrial differentiation (Martin et al., 2007; Hall et al., 2010; Krisher and Prather, 2012; Redel et al., 2012). As cellular differentiation takes place in specific cells during these preimplantation development stages (reviewed by Schatten and Sun, 2014) mitochondria elongate and develop numerous cristae, which is first observed in TE cells during differentiation into an epithelial layer (Sun et al., 2001; Schatten et al., 2005; Martin et al., 2007). Different to TE cells those of the ICM retain spherical mitochondria that do not contain cristae. This is interesting, as cells of the ICM are pluripotent and can be isolated for culture of embryonic stem cells that do retain spherical mitochondrial shapes until differentiation is induced. Such pluripotent stem cells have been described by several investigators to contain functionally immature mitochondria with a globular shape and minimally developed cristae, indicative of a less active mitochondrial state (St John et al., 2005; Facucho-Oliveira and St John, 2009; Chung et al., 2010; Prigione et al., 2010; Suhr et al., 2010).

We do not yet have clear knowledge about the number of mitochondria in early pig embryos, but it is known that the copy number of the genome increases during oocyte maturation (Mao et al., 2012) and no additional replication takes place until the blastocyst stage. Specific data on mitochondrial differentiation from early- to late-stage embryos are not yet available, although this information would be important given that mitochondrial dysfunctions may be manifested during this stage of embryo development and result in implantation failures (reviewed by Krisher and Prather, 2012; Redel et al., 2012).

The expression of TFAM and POLG was determined in porcine embryos from the 2-cell stage to the expanded blastocyst stage using brilliant cresyl blue staining and real-time polymerase chain reaction (PCR). Real-time PCR was also used to measure mtDNA copy number (Spikings et al., 2007). These studies determined that increased POLG expression was not detectable in cells of the ICM until the expanded blastocyst stage: a small increase in TFAM gene transcription was observed from the morula to the expanded blastocyst stage (Antelman et al., 2008).

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Remodeling of mitochondrial dynamics and the metabolic system is important for differentiation, as increased energy is needed. The mtDNA copy number increases and the mitochondrial morphology becomes complex as has been described for differentiated cells. It is also worth noting that during later stages of preimplantation development cells do not undergo the rapid cell cycles with DNA replication that is important for the early developmental stages. Glycolysis generates less reactive oxygen species (ROS) compared to the OXPHOS pathway and therefore the early DNA replication stages are less exposed to detrimental ROS and potential DNA damage. The differentiation stages are associated with increased oxygen consumption and ROS levels and downregulation of glycolytic enzymes (Chung et al., 2007; Armstrong et al., 2010; Prigione et al., 2010; Tormos et al., 2011).

Mitochondrial dynamics and motility are other important aspects for mitochondrial functions. Maintenance of pluripotency depends on mitochondrial dynamics and proper mitochondrial network integrity (Todd et al., 2010). As mentioned in the introduction, mitochondria are translocated along microtubules to their functional destinations. In early embryo cells and in pluripotent stem cells mitochondria accumulate in the perinuclear region, which has led to a number of hypotheses to perhaps explain functions and close interactions with the nuclear genome (Katayama et al., 2006; Lonergan et al., 2007), but it is also possible that mitochondria are accumulating to this area by centrosomes, microtubule organizing centers that are closely associated with the nucleus and critical for cell division, thereby ensuring adequate distribution of mitochondria to the dividing daughter cells (Schatten et al., 2005). It has been shown that unequal distribution of mitochondria to the blastomeres leads to asymmetrical and disproportional patterns of mitochondrial inheritance (Van Blerkom et al., 2000) in 2- and 4-cell-stage human embryos, resulting in lysis of the blastomere that is deficient in mitochondria. Such asymmetrical inheritance of mitochondria can result in imbalanced apoptosis patterns during preimplantation development, resulting in an imbalance of ICM and TE cells and potential embryo loss (Zhong et al., 2008).

Figure 11.1 shows a simplified schematic diagram of metabolic activities correlated with mitochondrial morphologies. Figure 11.2 displays transmission electron microscopy (TEM) images of immature mitochondria in oocytes that are round and largely devoid of cristae. In this specific figure the images are taken from somatic cell nuclear transfer (SCNT) oocytes, referring to a procedure during which the oocyte's nuclear genome is removed and replaced by the somatic cell's (donor) genome from a differentiated fibroblast donor cell that is entirely fused with the "enucleated" oocyte. Figure 11.3 illustrates the difference in mitochondria morphology in the somatic donor cell that contains mature mitochondria displaying the typical elongated shape with well-developed cristae.

While TEM reveals the presence of cristae, it does not reveal the three-dimensional organization when only sections of cells are analyzed. Scanning electron microscopy (SEM) on the other hand can be used to analyze mitochondrial organizations in three dimensions, as shown in Figure 11.4 taken from Naguro et al. (2013), in which high-resolution field emission scanning electron microscopy (HRFESEM) reveals a well-preserved mitochondrial network in an olfactory-bulb granule-cell body. The three-dimensional organization of well-preserved cristae (yellow) is clearly shown. Naguro et al. (2013) described various types of mitochondrial networks that may be specific for different functions within cells and tissues.

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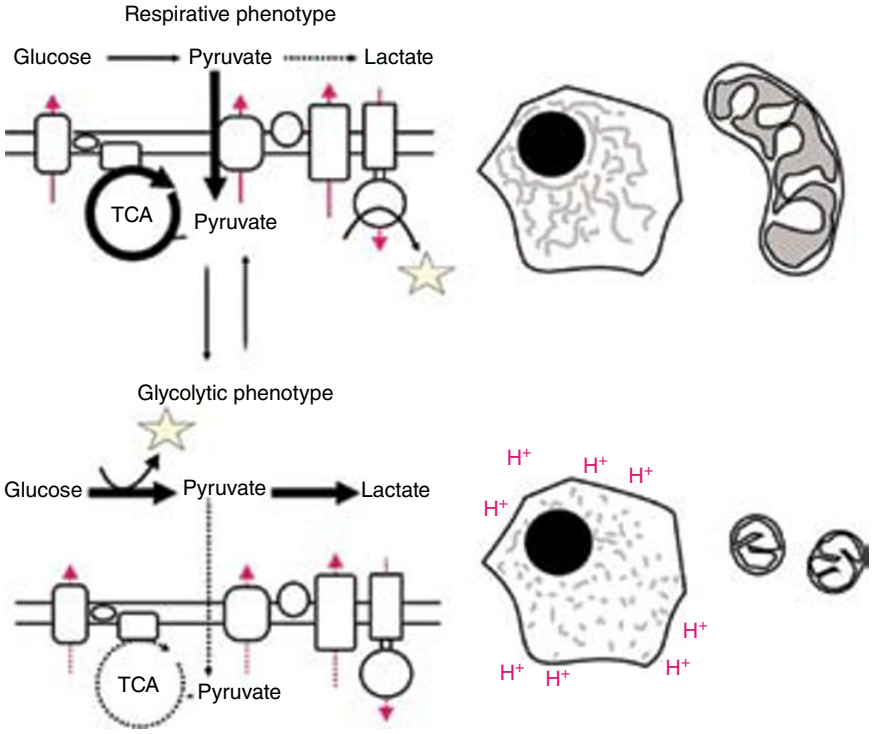


Figure 11.1. Simplified schematic representation of mitochondrial shape changes correlated with metabolic strategies and mitochondrial morphology. In the respirative phenotype, glucose is converted into pyruvate and further oxidized by mitochondrial respiration in elongated mitochondria with elaborate cristae. In the glycolytic phenotype, the majority of cytosolic glucose is converted into lactate, associated with acidification of the extracellular environment. Mitochondria appear round with no or only few cristae. (Modified from Alirol and Martinou, 2006.)

Biochemical and Metabolic Changes During Mitochondrial Differentiation

As mentioned above, early preimplantation-stage embryos and stem cells employ similar mechanisms to satisfy their cellular metabolism. The interest in stem cell differentiation led to an increased interest in mitochondrial differentiation when it was recognized that mitochondria play a significant role in stem cell differentiation and, importantly, that immature mitochondria may be experimentally manipulated into specific states of differentiation (reviewed by Krisher and Prather, 2012; Redel et al., 2012). As mentioned above, in embryo cells as well as in pluripotent stem cells metabolic transition from glycolysis to oxidative phosphorylation takes place and this transition is associated with changes in mitochondrial morphology and dynamics that are likely required for cellular differentiation. In addition, mitochondrial metabolism

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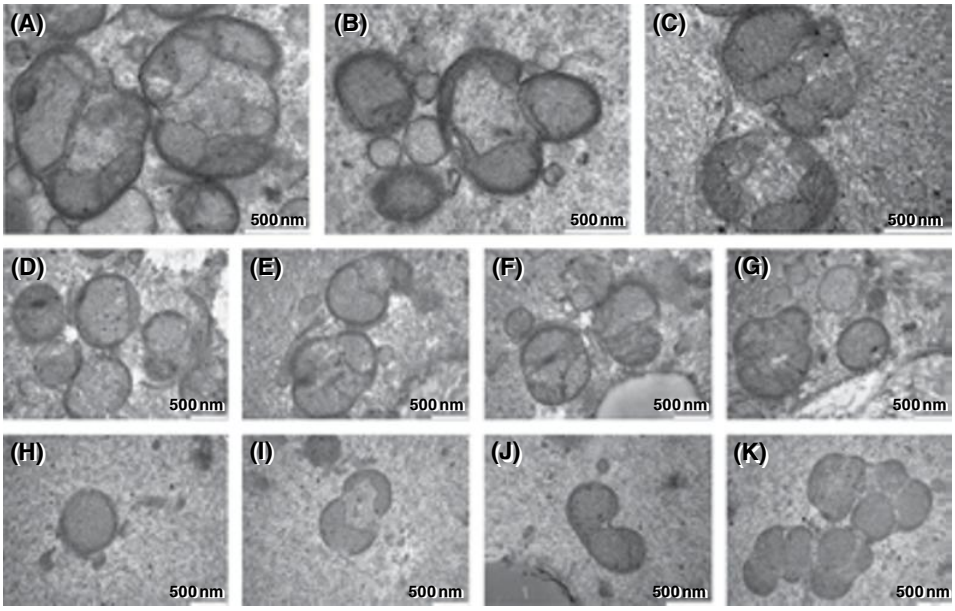


Figure 11.2. Transmission electron microscopy images depicting details of ultrastructural features in various mitochondria from SCNT eggs from 1-, 6-, and 20-h time points. (**A**, **B**) Mitochondrial clusters (1-h nuclear transfer time point). The clusters appear to be fragmented mitochondrial aggregations. (**C**–**G**) Clusters with complex substructures of lighter and darker osmiophilic areas, as well as complex associations with endoplasmic reticulum (ER) (6-h time point). (**D**, **E**) Endoplasmic reticulum is seen in association with mitochondria. (**H**–**K**) Single (**H**), double (**I**, **J**), or multiplexed pleomorphic (**K**) forms of mitochondria (20-h nuclear transfer time point). (*Microsc. Microanal.* **14**, 418–432. Copyright © 2008, Cambridge University Press.)

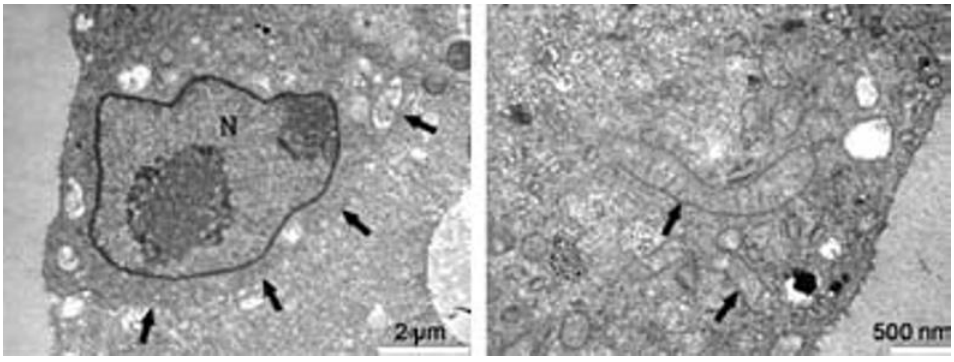


Figure 11.3. (Left) Transmission electron microscopy (TEM) of reconstructed oocyte at 30 min after fusion. The donor cell is still clearly separated from the oocyte cytoplasm (arrows); and typical somatic cell mitochondria are seen within the donor cell surrounding the donor cell nucleus (N). (Right) Higher magnification TEM image of somatic donor cell mitochondrial morphology with clear cristae (arrows) at 30 min after reconstruction. (*Microsc. Microanal.* **14**, 418–432. Copyright © 2008, Cambridge University Press.)

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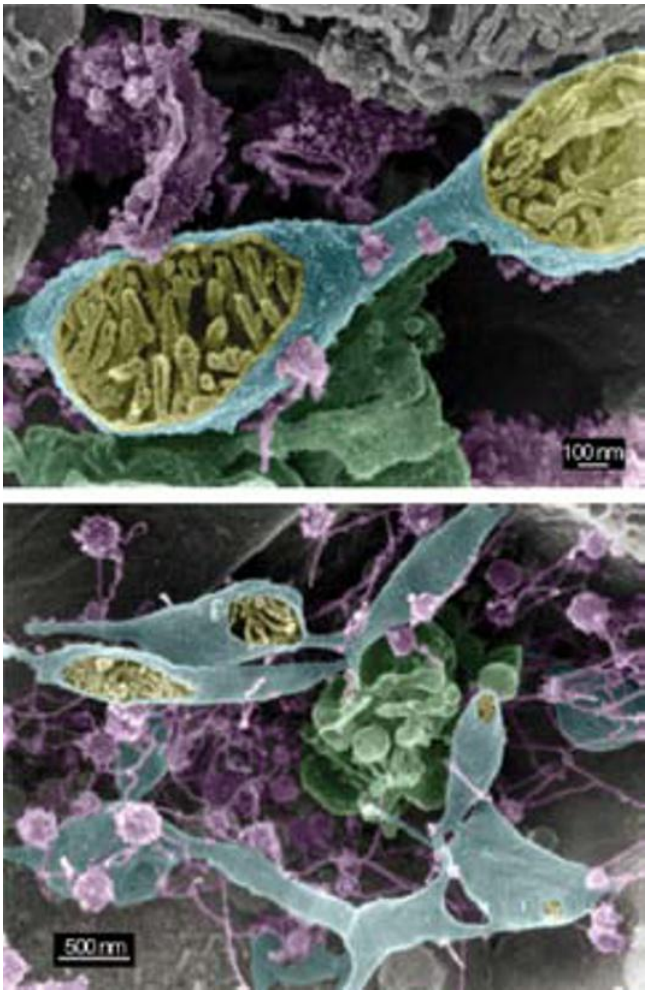


Figure 11.4. High-resolution field emission scanning electron microscopy (HRFESEM) displaying well-preserved mitochondrial network in an olfactory-bulb granule-cell body. Mitochondrial cristae (yellow) are well preserved. Golgi apparatus (green), endoplasmic reticulum (magenta), mitochondrial outer membrane (blue). (Schatten, Heide. Scanning Electron Microscopy for the Life Sciences. Copyright © 2013, Cambridge University Press.)

in embryonic cells and in stem cells has significantly been implicated in cell fate determination and development (Folmes et al., 2012; Rafalski et al., 2012).

While pluripotent stem cells favor glycolysis over OXPHOS, functional respiratory complexes are still present in mitochondria of pluripotent stem cells. We currently do not yet fully understand the regulation of metabolic preference for glycolysis and OXPHOS but data are emerging to understand the process of decoupling glycolysis from OXPHOS, which may involve moving pyruvate out of the mitochondria by mitochondrial UCP2

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(J. Zhang et al., 2011), and by higher levels of the glycolytic enzyme hexokinase II and lower levels of pyruvate dehydrogenase (PDH), which has been shown for pluripotent stem cells (Varum et al., 2011). Inhibition of key glycolytic enzymes promotes differentiation; on the other hand, inhibition of mitochondrial function with respiratory inhibitors promotes pluripotent gene expression and blocks pluripotent stem cell differentiation (Chung et al., 2007; Mandal et al., 2011). As mentioned above, UCP2 plays a critical role in coupling glycolysis to OXPHOS during pluripotent stem cell differentiation and changes in its levels are associated with the transition from glycolysis to increased mitochondrial glucose oxidation. The UCP2-mediated suppression of OXPHOS is important for the maintenance of pluripotency.

Changes in the mitochondrial permeability transition pore (mPTP) are important for mitochondrial maturation, which has been shown in differentiating cardiomyocytes (Hom et al., 2011). These studies showed that closing of the mPTP occurred during normal cardiomyocyte maturation; and experimentally induced closing of the mPTP resulted in mitochondrial maturation and cardiomyocyte differentiation. Other investigators have shown that while gene knockout of factors that are important for mitochondria biogenesis, such as TFAM, POLG, POLG2, and nuclear respiratory factor 1 (NRF1), or cofactors such as peroxisome proliferator-activated receptor gamma coactivator-related protein 1 (PPRC1), did not affect embryo implantation it caused embryonic lethality at a later stage; which is interpreted to mean that mtDNA depletion and insufficient supply of energy as well as metabolites are necessary for cell differentiation (Larsson et al., 1998; Huo and Scarpulla, 2001; Hance et al., 2005; Humble et al., 2013).

Several other methods are available to characterize immature and mature mitochondria, including various membrane potential probes. Mitochondrial membrane potential (Ψm) has been used as a measure for mitochondrial maturation using the mitochondrial membrane potential indicator 5,5′,6,6′-tetrachloro-1,1′3,3′-tetraethylbenzamidazol-carboncyanine (JC-1) (Zhang et al., 2008). Indicator JC-1 is a cationic dye for which the mitochondrial uptake is directly related to the level of Ψm. Greater mitochondrial uptake results in greater concentration of JC-1 aggregate, displaying a red fluorescent emission signal, while the JC-1 monomer displays green fluorescence. A higher red:green ratio indicates a more polarized, or more negative and hyperpolarized mitochondrial inner membrane.

The indicator JC-1 has been used to analyze mitochondria differentiation during embryo development and stem cell differentiation and it has been shown that stem cells from younger human fetal neurospheres displayed higher membrane potential when compared to neuronal stem cells isolated from older, larger neurospheres (Plotnikov et al., 2006).

Experimental Manipulations and Therapeutic Implications

The importance of understanding mitochondrial metabolism during embryo development and in pluripotent stem cells is highlighted by the fact that many developmental disorders and diseases are associated with dysfunctional mitochondria. Because mitochondria are versatile and can be manipulated experimentally it is possible to restore mitochondrial functions once we know the specific requirements for

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tissue-specific states. In aging oocytes containing dysfunctional mitochondria and in oocytes of women with metabolic diseases, transfer of mitochondria-containing cytoplasm from donated fresh oocytes has increased oocyte viability. Such oocyte manipulations have raised new possibilities for therapies but also have generated new discussions concerning ethical concerns and they are currently being evaluated for practical applications (Reinhardt et al., 2013).

Mitochondrial dysfunctions are associated with numerous diseases, including a number of neurological diseases (Rugarli and Langer, 2012; Cooper et al., 2012), diabetes (Fujikura et al., 2012; Yu et al., 2013, and references therein), cancer (Alirol and Martinou, 2006), the immune system (Campello and Scorrano, 2010), and aging (George et al., 2011), as well as other diseases and disorders that affect a large percent of the population worldwide. Reprogramming of mitochondria in disease states will allow new approaches for therapeutic intervention and may include gene-targeting technologies (Liu et al., 2011, 2012; Pan et al., 2011; Zhang et al., 2012).

Numerous approaches have been proposed to reverse pathophysiological pathways in mitochondria, including the glycolytic mitochondrial metabolism in cancer cells, through targeting known pathways or factors as well as food ingredients (Samudio et al., 2009; Keijer et al., 2011). However, much research is still needed to determine the specific pathways and steps in these pathways leading to mitochondrial dysfunction in order to therapeutically intervene to either prevent or reverse abnormal mitochondrial metabolism in disease states.

Conclusions

The renewed interest in mitochondrial metabolism has generated a significant amount of new data that are beginning to find applications in the biomedical field. We now know that the healthy mitochondrial metabolism is not only affected in cancer but also in numerous other diseases, such as neurological diseases, diabetes, diseases of the immune system, developmental disorders, and others. One goal of curing these disease states is to alter abnormal mitochondrial metabolism towards a healthy condition. Knowing and understanding the metabolic conditions and how they change in different environments leading to pathophysiological conditions is an important step toward this goal. The early embryo is an excellent system to study the mitochondrial metabolic changes from glycolysis to OXPHOS, and stem cells provide excellent material for directing mitochondrial metabolism towards cellular differentiation into specific tissues. On the other hand, reprogramming of somatic cells into stem cells will allow the generation of tissue-specific stem cells for personalized medicine. By studying these systems it will be possible to determine reprogramming steps from a glycolytic mitochondrial metabolism to the OXPHOS metabolism and vice versa in molecular detail and therefore provide specific points for therapeutic intervention. It will open up multiple possibilities to reverse faulty mitochondrial metabolism and open new directions toward reversal of metabolic reprogramming. More research is needed to study the driving mechanisms and signaling pathways that play a role in mitochondrial remodeling and lead to pathophysiological conditions in order to determine specific strategies for targeted treatments.

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Chapter 12

The Role of Centrosomes in Cancer Stem Cell Functions

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Introduction

Cancer stem cells (CSCs) comprise a small subpopulation of cells within a solid tumor that are also called cancer-initiating cells because of their enhanced ability to initiate tumor growth. This ability has been well demonstrated *in vitro* by isolating and injecting CSCs into immuno-compromised mice resulting in tumor initiation, which is not the case for more differentiated cells that do not have such tumor initiating ability (Todaro et al., 2007). Cancer stem cells, like other stem cells, have the ability to self-renew, giving rise to another malignant stem cell or to cancer cells (Al-Hajj et al., 2004; Sell, 2004; Houghton et al., 2006; Wicha et al., 2006). While we know that CSCs are different from the rest of the cancer cell population, they are still underexplored for their specific characteristics within specific tumors; however, it is known that CSCs respond to cancer therapy differently than the rest of the heterogeneous cancer cell population within a solid tumor and they can repopulate the tumor following seemingly effective tumor therapies (Houghton et al., 2006). Cancer stem cells were first discovered in leukemia and myeloma (Bruce and Van Der Gaag, 1963; Wodinsky and Kensler, 1966; Bergsagel and Valeriote, 1968; Park et al., 1971), and subsequently CSCs or CSC phenotype cells have been detected in many other cancers (Lapidot et al., 1994; Bhatia et al., 1997; Blair et al., 1997; Bonnet and Dick, 1997): including breast (Al-Hajj et al., 2003) and brain (Singh et al., 2003, 2004a; Galli et al., 2004) cancers, as well as bone (Gibbs et al., 2005), lung (Kim et al., 2005), melanoma (Fang et al., 2005), prostate (Collins et al., 2005), and colorectal (Cammareri et al., 2010) cancers, and others displaying specific surface markers (biomarkers such as $CD133^{+}$, $CD44^{+}$, $CD24^{low}$, and $CD166^{+}$) that indicate their CSC characteristics. As of now it is not certain how CSCs arise and how they interact with their microenvironment to maintain their stem-like characteristics (Perez-Losada and Balmain, 2003) and it is not known how and why their cell cycle becomes reinitiated to pursue cell proliferation and repopulation of tumor tissue. It has been recognized that the microenvironment in which CSCs are located plays a significant role in this process, as do several signaling pathways that are involved in cell cycle progression and cell division (reviewed by Tysnes and Bjerkvig, 2007).

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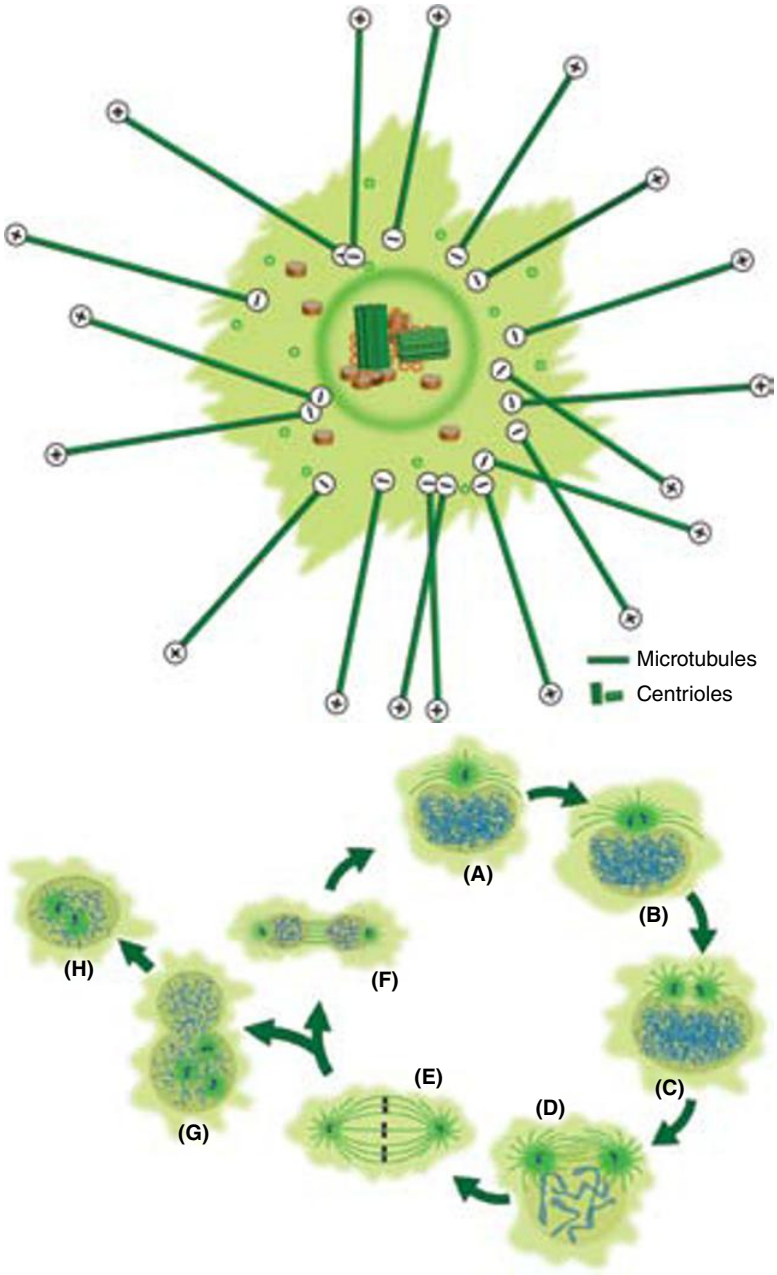


Figure 12.1. **(A)** A typical mammalian somatic cell centrosome contains two perpendicularly oriented centrioles (termed mother and daughter centriole) surrounded by centrosomal material, also referred to as pericentriolar material (PCM). The centrosomal material consists of a meshwork of proteins and includes gamma-tubulin and the gamma-tubulin ring complex for microtubule nucleation and other proteins for anchoring microtubules to the complex. The

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Centrosomes (microtubule organizing centers, MTOCs; Figure 12.1A) are central to cell cycle progression and cell division and they are critically implicated in abnormal cancer cell proliferation (e.g., Lingle and Salisbury, 2000; Schatten et al., 2000; Schatten, 2008; Figure 12.1B). Centrosomes organize microtubules to aid in a variety of cellular functions and they are essential for accurate nucleation and organization of microtubules that form the mitotic apparatus to separate chromosomes equally to the dividing daughter cells. Centrosome dysfunctions are implicated in abnormal microtubule organization and in abnormal chromosome segregation, leading to aneuploidy with consequences for genomic instability, which is a well-known hallmark characteristic for cancer cells. While the causes for centrosome dysfunctions are still being debated, it is clear that centrosome abnormalities are involved in cancer cell cycles and that dysfunctions can have different origins in different abnormally proliferating cells (Figure 12.2). Centrosome dysfunctions may cause aneuploidies directly, or indirectly by faulty signal transduction that affects centrosome regulation and microtubule organization.

Since centrosomes serve as central communication centers for signal transduction pathways and centrosomes are themselves precisely regulated by signal transduction molecules (reviewed by Schatten, 2008) a faulty cell cycle without return to resting stages may already be initiated by faulty signaling from the CSC niche, leading to the first steps of abnormalities that cannot be repaired by internal cell cycle surveillance mechanisms. DNA and centrosome cycles are well synchronized in a regular cell cycle but can become desynchronized under adverse conditions, resulting in abnormal chromosome segregation (reviewed by Schatten, 2008, 2013). In normal cell cycles, centrosomes are well regulated by several kinases but abnormalities in this regulation can lead to centrosomal abnormalities that are implicated in aberrant cancer cell division. As will be discussed below, aurora kinase A (AURKA) is one of the prime regulators of centrosome functions and is overexpressed in cancer cells, leading to centrosome amplification and chromosome mis-segregation (reviewed by Kais and Parvin, 2012). Studies in colorectal CSCs in which AURKA is overexpressed (Cammareri et al. 2010) have shown that inhibition of AURKA compromised the ability to form tumor xenografts in immuno-compromised mice, and it further reduced

Figure 12.1. (*continued*) minus ends of the polarized microtubules are associated with the centrosome complex. **(B)** A regular somatic cell cycle **(A–F)** displaying dynamics of the centriole–centrosome complex. **(A)** The interphase centrosome complex containing a pair of centrioles is connected to the nucleus and nucleates an array of interphase microtubules. **(B)** Duplication of the centriole–centrosome complex occurs during the S phase in synchrony with DNA duplication. **(C)** The duplicated centriole–centrosome complex separates and moves to the opposite spindle poles during early prophase. **(D)** Following nuclear envelope breakdown the centriole–centrosome complex matures into a division-competent centrosome complex, which includes cascades of phosphorylation as indicated in the text; it is localized at the two spindle poles during bipolar mitotic spindle formation. **(E)** The centrosome complex becomes highly compacted in metaphase and organizes the metaphase spindle with microtubules attached to the kinetochores. **(F)** Centrosomal material becomes decompacted during telophase and the centriole–centrosome complex relocates to the daughter nuclei after cell division. **(G–H)** Abnormalities of the centriole–centrosome complex can result in abnormal mitosis and in cells displaying aneuploidy, which is the case in cancer cells in which components of the centriole–centrosome complex are amplified or overreplicated.

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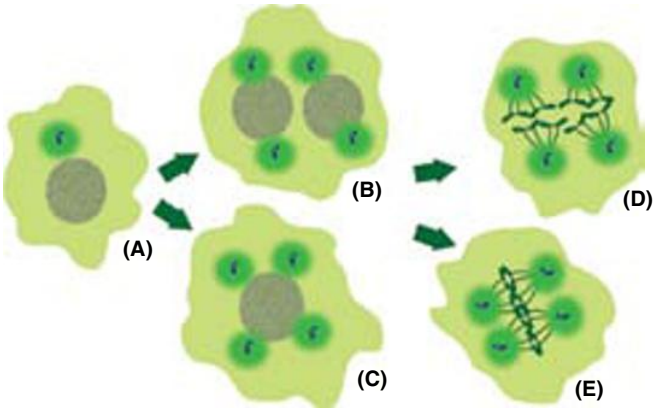


Figure 12.2. In noncancer cells the centriole–centrosome complex is closely associated with the nucleus **(A)** and its duplication is well synchronized with DNA duplication to form the mitotic apparatus that separates chromosomes equally to the dividing daughter cells (shown in Figure 12.1). Multipolar mitoses can have several origins and can be the result of cell fusion **(C)**, abnormal mitoses **(B)** resulting from centriole and/or centrosome abnormalities with consequences for abnormal cell division. Such cells with centriole–centrosome abnormalities can give rise to multipolar cells as displayed in **(D)** and **(E)** or in cells with a bipolar spindle that may appear normal but contain multiple centriole–centrosome components as a result of overexpression of specific centrosome proteins, abnormal centriole duplication, or other centrosome defects as discussed in the text.

migration of colorectal CSCs. Based on these data and others it has been proposed that targeting AURKA may be effective in inhibiting CSC proliferation and the ability to initiate tumor cell regeneration. These data also indicate that centrosome regulation plays a critical role in CSC proliferation.

We do not yet know for certain if aberrant signal transduction from the CSC microenvironment may play a role in aberrant initiation of centrosome functions and aberrant cell cycle progression, but it is likely, based on information that is available from various other cell systems, as will be discussed below. In embryonic stem cells it has been shown that primary cilia containing centrosomal material at their basal bodies are central for the initiation of signal transduction pathways and for communicating signaling molecules to the cell body, thereby initiating and influencing subsequent cell-cycle decisions. Several pathways are critical for cell proliferation and abnormalities in these pathways have been shown to play a role in cancer cell proliferation, including the Wnt, Notch, and Hedgehog embryonic signaling pathways. Inhibition of these pathways has been proposed as a therapeutic approach for targeting CSCs (reviewed by Takebe et al., 2011). In addition, as mentioned above, among the kinases that are overexpressed in cancer cells and provide a critical link between primary cilia, centrosomes, and CSC proliferation, AURKA drives centrosome abnormalities and abnormal cancer cell proliferation (Cammareri et al., 2010; Kais and Parvin, 2012; Chou et al., 2012; Schatten, 2013). Determining specific abnormalities will be important for the design of effective combination therapies to target CSC proliferation. These topics will be addressed

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in the present chapter focused on: (i) cancer stem cells and their microenvironments; (ii) centrosome regulation, dynamics, and amplification in cancer cells with implications for CSCs; (iii) centrosomes in multipolarity and aneuploid cell divisions; and (iv) the role of primary cilia signaling in centrosome functions and dysfunctions.

Cancer Stem Cells and their Microenvironments

In recent years, interest in CSCs has increased significantly for a number of different reasons, and it further increased when it was recognized that this small population of cells could be the cause for enhanced drug resistance and for recurrence of tumors that had been in remission. This new information generated new ideas for new tumor treatment strategies (Steg et al., 2011; reviewed by Al-Ejeh et al., 2011; LaBarge, 2010; Chow, 2013; Cammareri et al., 2010; Takebi et al., 2011), and new research has been initiated to explore the causes for CSC initiation and progression (Cammareri et al., 2010). As of now it is not clear how CSCs can (re)-initiate tumor growth but several hypotheses have been proposed that are currently being tested and pursued in a number of different laboratories. It has been recognized that the initiation and progression of CSCs can have various origins (Liu et al., 2011) and often involve, among other factors, three important classes of genes (proto-oncogenes, tumor suppressor genes, genes involved in DNA repair mechanisms) that play a role in cell cycle progression (reviewed by Tysnes and Bjerkvig, 2007). Uncoupling of biological processes through mutations, deletions, or amplifications of these genes may account for some of the underlying causes leading to cell-cycle initiation and progression of CSCs. In cancer cells, uncoupling of centrosome and DNA cell cycles have been well documented as a significant cause for cell-cycle deregulation (reviewed by Korzeniewski and Duensing, 2012; Saladino et al., 2012; Olivero, 2012; Lingle et al., 2002; Münger and Duensing, 2004; Duensing et al., 2000; Ko et al., 2006) and it may also play a role in CSC cycle deregulation leading to CSC progression and abnormalities that will result in aneuploidies and tissue heterogeneity, well-known hallmarks for cancer tissue. The role of centrosomes in aneuploidy and genomic instability will be discussed below.

The idea that one single abnormal cell might initiate tumor growth had already been proposed by Theodor Boveri (1914; translated into English 2008) over 100 years ago and it has further been explored by Grander (1998) who laid out evidence that tumors can arise from one single cell containing frequent genetic mutations resulting in malignant transformation. Several reports are available providing evidence that tumor can be initiated by CSCs or CSC-like cells (Reya et al., 2001; Marx, 2003; Singh et al., 2003, 2004a,b; Galli et al., 2004) giving rise to another malignant stem cell as well as a cell that will result in a phenotypically diverse cancer cell with the ability to repopulate a tumor.

For CSCs to be stimulated to reenter the cell cycle, the CSC microenvironment plays a critically important role and involves the CSC niche (Figure 12.3), which refers to stem cells typically being associated with a specialized cell or region (niche) providing the specific microenvironment (Ohlstein et al. 2004; Xie and Li 2007); the niche may include specialized cells that are in direct contact with the stem cells and may send out

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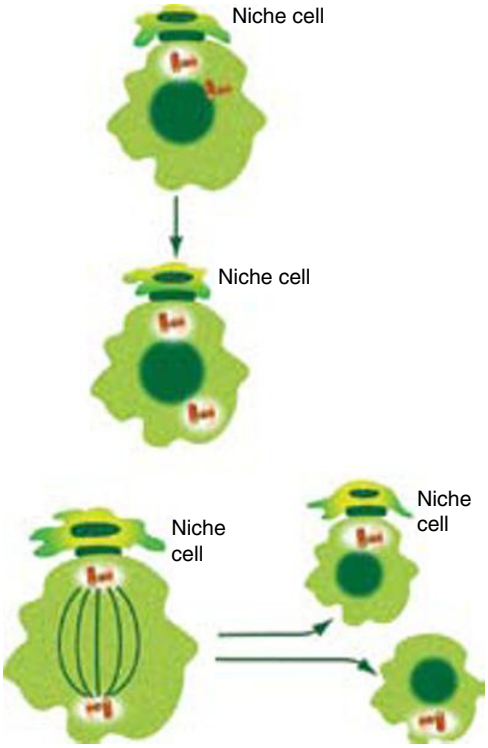


Figure 12.3. Schematic representation of stem cell niche interactions with stem cells. For stem cells to be stimulated to reenter the cell cycle the microenvironment plays a critically important role and involves the stem cell niche, which in this figure is represented by niche cells which may include specialized cells that are in direct contact with the stem cells and send out signals to initiate the stem cell cycle. In *Drosophila* male germ-line stem cells it has been shown that the centriole–centrosome complex plays determining roles in directing stem cell maintenance and differentiation, which may also hold true for cancer stem cells, as this complex is central to signal transduction molecules that influence cell cycle decisions. The interactions with the niche are critical for stem cell maintenance and differentiation.

signals to cause initiation of stem cell cycles. For adult stem cells, the niche provides molecular cues from neighboring connective tissue cells such as mesenchymal (fibroblast-like) cells and vascular cells (Watt and Hogan, 2000; Alison and Islam, 2009; Alison et al., 2010).

The characteristics of stem cells and their niches have been well explored in the *Drosophila* model and to a much lesser extent in mammalian systems that have mainly been limited to the mouse, in which hematopoietic stem cells, satellite muscle cells, central nervous stem cells, intestinal epithelium, hair follicle bulge, interfollicular epidermis, and spermatogonial stem cells have been explored. In the bulge of hair follicles, multiple stem cells are clustered, while in other tissue types CSCs may be interspersed among other cells (reviewed in detail by Morrison and Spradling, 2008). Stem cell niches are defined as local tissue microenvironments that are

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capable of maintaining and regulating one or more stem cells. However, this definition is broad and in several cases the stem cell niches cannot be defined precisely (reviewed by Morrison and Spradling, 2008). Excellent data are available for stem cells in *Drosophila* female and male gonads, for which niches and their influence on stem cells have been well documented and have been reviewed in several previous papers (Morrison and Spradling, 2008; Yamashita 2009a,b; Schatten and Sun, 2010, 2011, 2012; Roth et al., 2012). These studies have shown that after the initial signals are received by the stem cell a typical stem cell response results in asymmetric cell division in which one cell maintains stem cell characteristics while the other cell can undergo cell differentiation. The studies in *Drosophila* present an excellent example of asymmetric cell division in which centrosomes play determining roles in directing stem cell maintenance and differentiation (Yamashita et al., 2007); in the *Drosophila* male germ-line stem cells (GSCs) during mitosis the mother centriole–centrosome complex is maintained in the stem cell closely positioned to the niche, while the daughter centriole–centrosome complex migrates to the opposite stem cell pole to be inherited by the differentiating daughter cell. In this case the *Drosophila* system provided a suitable experimental model in which molecular methods were combined with ultrastructural approaches. Genetically engineered flies were experimentally manipulated to produce the centrosomal protein pericentrin/AKAP450 centrosomal targeting (PACT) tagged with fluorescent protein that then was induced to be expressed at different times and analyzed by a pulse–chase approach, allowing selective labeling of either mother or daughter centriole–centrosome complexes. The data showed that the mother centriole–centrosome complex was located near the hub, thereby ensuring inheritance by the stem cell that remains in the niche after division, while the daughter centriole–centrosome complex became located in the differentiating cell after division. It is known that the older (mother) centriole in the centrosome complex can accumulate more centrosomal material compared to the younger (daughter) centriole, which in this case allows increased microtubule nucleation and adequate anchorage of the stem cell to the hub. These experiments on *Drosophila* stem cells give insights in how the centriole–centrosome complex is involved in stem cell maintenance and stem cell differentiation. It is not known whether CSCs use similar mechanisms for stem cell maintenance and differentiation and anchorage to the stem cell niche; such investigations are still needed to determine more fully the capabilities and division characteristics of CSCs and the role of centrosomes in CSC proliferation into tumor tissue.

The niche provides the microenvironment that allows stem cells to either be quiescent or undergo self-renewal, which includes cell cycle initiation, progression, and cell division. In mammalian systems, symmetric and/or asymmetric cell divisions can follow initial stem cell stimulation by the niche (reviewed by Morrison and Kimble, 2006). Nevertheless, even in morphologically appearing symmetric cells molecular asymmetry may play a role in differentiation of cells that may not be distinguishable by morphological criteria alone; this may especially be the case for centrosomes, as centrosomal proteins within the centrosome complex can differ on qualitative and quantitative levels, resulting in different microtubule organizations with consequences for transport of molecules such as cell-fate determinants along microtubules to the dividing daughter cells (reviewed by Schatten, 2008, 2013; Schatten and Sun, 2011).

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This may also include posttranslational modifications of microtubules to allow differential transport of cellular components (Schatten and Sun, 2014).

The interactions with the niche are critical for stem cell maintenance and differentiation and result in differences of signals received by the stem cell and the differentiating cells. β1 integrins are important for stem cell maintenance and positioning of the niche in *Drosophila* (Tanentzapf et al., 2007) and β1 integrin has also been shown to be required for functional stem cell population maintenance and the establishment of asymmetric cell division in the mammary system (Taddei et al., 2008). The importance of niche cells for stem cell proliferation has been documented and it has been shown that different signals generated by the niche activate and initiate pathways that control the stem cell fate, which includes the Janus kinase/signal transducers and activators of transcription (JAK/STAT), bone morphogenetic protein/transforming growth factor beta (BMP/TGFβ), Hedgehog and P-element induced wimpy testis (Piwi) pathways for *Drosophila* (Kiger et al., 2001; Tulina and Matunis, 2001) to induce asymmetrical division. The differentiating cell is left without these signals from the hub (Fuller and Spradling, 2007). The specific signaling pathways differ in different stem cell systems and include the Hedgehog, Wnt, Notch and BMP/TGFβ1 for hematopoietic stem cells and Wnt and BMP/TGFβ1 signaling for the skin stem cell niche (Li and Xie, 2005). While it is clear that signals from the niche microenvironment are important for stem cell division the specific signaling pathways leading to asymmetric stem cell divisions have not yet been fully determined for most mammalian stem cell systems. Our current knowledge on asymmetric stem cell division is mostly derived from the *Drosophila* and *Caenorhabditis elegans* systems (reviewed by Knoblich, 2010) in which molecular components that establish asymmetry have been determined. *Drosophila* and *C. elegans* are easy models for basic studies but it is not yet clear if these data also hold true for mammalian systems.

We do not yet know whether abnormalities in niche stem cell signaling may play a role in abnormal CSC differentiation. In *Drosophila*, overexpression of JAK/STAT increases stem cell division and may lead to GSC tumors (Decotto and Spradling, 2005) and overexpression of components of the Wnt signaling pathway may lead to cancer in different tissues (Fodde and Brabletz, 2007). In prostate CSCs focal adhesions, JAK/STAT, and NFκB are associated with the prostate CSC phenotype (Birnie et al., 2008). New data from a number of different laboratories indicate that key cell signaling pathways are overexpressed in the CSC population (reviewed by Lang et al., 2009). As of now it is not yet clear whether pathways leading to centrosome amplification are involved in stem cell niche–CSC signaling resulting in abnormal cell-cycle progression and aneuploidy. The following section discusses pathways leading to centrosome amplification that may play a role in CSC cycle initiation and progression and may include AURKA amplification. The section following that addresses the potential role of primary cilia in this pathway and CSC initiation and progression.

Signal transduction, molecular interactions with their niches, and adherence to the stem cell niche decreases with age, resulting in a decrease in the self-renewal capabilities of stem cells. This has been shown for the *Drosophila* system in which E-cadherin and BMP declines with age, but a certain degree of recovery is possible as has been shown by Pan et al. (2007), who were able to genetically restore high levels of expression to increase function and lifetime of older stem cells.

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Centrosome Regulation, Dynamics, and Amplification in Cancer Cells with Implications for CSCs

The significance and importance of centrosomes for cell-cycle progression has been reviewed previously (e.g., Fry and Hames, 2004; Schatten, 2008; Schatten and Sun, 2010, 2011) and will not be addressed in detail here where the focus will be on specific centrosome regulation and dynamics related to stem cell biology and to centrosomal deregulation leading to centrosome dysfunctions in cancer. The role of centrosomes in CSCs will be discussed and implications for CSC cycle initiation and proliferation will be addressed.

Controlled and well-regulated signaling between CSC centrosomes and the microenvironment is important, as several of the critical mitotic cell-cycle regulators are concentrated at the centrosome and include Polo and AURKA (Barr and Gergely, 2007), and cdc2/cyclin B kinase (Jackman et al., 2003), which play major roles in directing and orchestrating centrosome and cell cycle functions. Misregulation of centrosomes leads to centrosome amplification, with consequences for genomic instability that may result in loss of tumor suppressor genes in some cells and tumor promoter genes in others. New research has focused on determining the mechanisms underlying centrosome amplification, with the goal to inhibit signaling pathways that allow centrosome amplification, some of which are tissue-specific.

As indicated above, phosphorylation of centrosome proteins plays a major role in cell-cycle-specific centrosome functions. Aurora kinase A is important for centrosome maturation that drives cell-cycle progression and cell proliferation, and it may be a critical link between centrosomes and CSC proliferation. Aurora kinase A plays central roles in the mitotic process and cell division, and its overexpression has been implicated in centrosome amplification in several cancers, including breast cancer (reviewed in Kais and Parvin, 2012). Aurora kinase A is a centrosome-associated serine/threonine kinase, and specific overexpression of AURKA causes multipolar mitotic spindles, as has been shown in animal models (Goepfert et al., 2002; Wang et al., 2006) in which early development of mammary tumors was reported and caused by AURKA overexpression. As will be discussed in in the following section, it is also associated with the basal body-centrosome area in primary cilia, which may be involved in CSC initiation. While it is not known how AURKA may drive centrosome amplification in CSC signaling through the primary cilium to the CSC centrosome, factors in the microenvironment may play a role in signaling cell-cycle initiation, thereby triggering cascades of subsequent signal transductions in which the centrosome, through its microtubule organizing capabilities, plays consequential roles in the process of cell proliferation.

The notion that centrosomes are central to CSC cycle initiation and progression is supported by the data derived from the *Drosophila* system, as discussed above. As mentioned above, AURKA signaling has long been implicated in the centrosome cycle (Carmena and Earnshaw, 2003; Meraldi et al., 2004) and it has further been shown that overexpression of AURKA plays a role in centrosome amplification and in driving abnormal signaling pathways. It has also been shown that overexpression of AURKA promotes chromosomal instability (CIN; the inability to maintain correct numbers of chromosomes after mitosis), cell-cycle progression, and therapeutic

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resistance (Gautschi et al., 2008; Cammareri et al., 2010). Furthermore, AURKA phosphorylates p53 leading to ubiquitination and degradation of p53 with consequences for resistance to apoptosis.

Chromosomal instability is considered a hallmark of cancer, but it is not clear how it originates in CSCs and how it becomes manifested in malignant progression. Several abnormal signaling pathways have been proposed to originate from the CSC microenvironment and BMI1/AURKA signaling has been particularly studied to drive abnormal CSC progression. Bone morphogenetic protein 1 is a member of the Polycomb group of chromatin-modifier proteins that is critical for stem cell self-renewal and it has been shown that BMI1 overexpression drives epithelial–mesenchymal transition (EMT) in head and neck cancer tissue (Chou et al., 2012), establishing a link between CSCs, EMT, and CIN. The studies by Chou et al. (2012) further showed that BMI1 is involved in upregulation of the mitotic AURKA through BMI1-induced *AURKA* gene expression, which led the authors to suggest that the *BMI1–AURKA* axis may be explored for therapeutic use in head and neck cancers. Such targeted therapies are important and may be part of personalized medicine to target patient-specific cancer tissue, as tissue heterogeneity and genomic instability are among the complex abnormalities associated with cancer that require complex and multiple approaches and strategies for effective treatment. Targeting multiple signaling pathways that inhibit abnormal centrosome functions are an attractive avenue to pursue and include specific targeting of AURKA as well as other pathways of the centrosome phosphorylation cascade.

One other target involves nucleophosmin, a nucleolar protein that has been implicated in cancer pathogenesis (reviewed by Tysnes and Bjerkvig, 2007). Nucleophosmin is mutated and rearranged in a number of hematological disorders (Grisendi et al., 2005). The nucleophosmin 1 gene is a transcriptional target of the proto-oncogene MYC (Boon et al., 2001; Zeller et al., 2001) that plays a role in the Wnt and Hedgehog pathways. Nucleophosmin protein modulates centrosome duplication, and it is itself phosphorylated by cyclin-dependent kinase 2 (CDK2)/cyclin E (Okuda et al., 2000), one of the major centrosome regulators that plays critical roles in centrosome cycle initiation and duplication. Cyclin-dependent kinase 2/cyclin E plays a role in nucleophosmin dissociation from centrosomes and allows subsequent centrosome cycle progression (Okuda et al., 2000).

Targeting CSC centrosomes may be achieved by targeting abnormally expressed centrosome proteins directly or by targeting signal transduction molecules that play a role in abnormal centrosome formation. Such approaches may include targeting specific overexpressed centrosome proteins or targeting abnormal phosphorylation of centrosome proteins. Targeting abnormal phosphorylation of cancer cell centrosomes will be important, as cancer cell centrosomes are consistently phosphorylated in all cell cycle stages (Lingle et al., 1998; reviewed by Schatten, 2008) and are therefore consistently division-competent, while they undergo precise cell cycle regulation in normal cell cycles. In non-cancerous cells, centrosomes become phosphorylated at the entry into mitosis, while they are dephosphorylated in interphase (reviewed by Schatten, 2008, 2013). Interruption of the centrosome phosphorylation cascade will result in centrosomes that are not consistently division-competent, thereby interrupting the aberrant cell proliferation cycles.

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Taken together, the microenvironment surrounding CSCs may trigger centrosome phosphorylation and initiate entry into the cell cycle followed by signal transduction cascades that may result in abnormal centrosome phosphorylation.

Centrosomes in Multipolarity and Aneuploid Cell Divisions

Aneuploid CSC populations have been reported (Singh et al., 2003; Galli et al., 2004) and the origin of such CSC abnormalities is actively being investigated. Tripolar, tetrapolar, and multipolar cells are hallmark features of cancer tissue and there are multiple explanations for causes on how multipolarity can arise; it is now clear that multipolar cells can arise by a variety of mechanisms (Figure 12.2), including mitotic aberrations, cytokinesis failure, and viral-induced cell fusion among other cell fusions that will be discussed below. Centrosome abnormalities have long been implicated in abnormal cancer cell division (Boveri, 1914, translated into English 2008; reviewed by Schatten, 2008; 2013) and the role of centrosome abnormalities leading to multipolarity and abnormal cell divisions has been studied in detail on cell, molecular, and genetic levels (Ganem et al., 2009; Korzeniewski et al., 2010; Fukasawa, 2012; Kais and Parvin, 2012; Korzeniewski and Duensing, 2012; Olivero, 2012; Prosser and Fry, 2012; Saladino et al., 2012; Yan and Chng, 2012).

The invertebrate sea urchin egg has allowed us to first understand the formation of tri- and multipolar centrosomes (Boveri, 1901; Schatten et al., 1986; reviewed by Schatten, 2008) caused by multiple sperm incorporations, each contributing one centriole during fertilization. In normal fertilization, the sperm centriole duplicates during the first embryonic cell cycle, resulting in the formation of the bipolar mitotic apparatus, while multiple centrioles contributed by multiple sperm incorporations form multiple poles during mitosis. These classic studies formed our understanding of abnormal cell divisions as a result of increased numbers of centrioles resulting from polyspermy (reviewed by Schatten, 2008). These observations are also important for understanding multipolar mitoses in cancer tissue that includes increased numbers of centrioles, overexpression of specific centrosome proteins (discussed earlier), various defects in cell-cycle regulation, and others (reviewed by Schatten, 2008, 2013); as mentioned above and shown in Figure 12.2 it may also arise from cell fusions (Weimann et al., 2003; Pomerantz and Blau, 2004; reviewed by Bjerkvig et al., 2005) that can result in centriole–centrosome abnormalities (Zhong et al., 2007) and may have implications for CSC proliferation.

Cell cycle abnormalities leading to multipolar mitoses have been discussed in previous reviews and include centrosome deregulation and centrosome protein amplification, centriole overduplication, epigenetic factors, and failure of centrosome clustering (Tysnes and Bjerkvig, 2007; Kwon et al., 2008; Ganem et al., 2009; Leber et al., 2010; Krämer et al., 2012). Centrosomes being modified by viral fusions that directly cause centrosome abnormalities have been documented and reviewed excellently by Duensing and his group (Korzeniewski and Duensing, 2012; and references therein). These studies showed that virus-induced centrosome alterations leading to malignancies are caused by the viral oncoproteins E6 and E7 that are consistently overexpressed in human papilloma virus (HPV)-associated cancers, such as cancers of the uterine cervix. These studies showed that both centrosome overduplications as

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well as centrosome accumulations are two different mechanisms leading to cancer cell centrosome aberrations and both types can be found within the same tumor tissue. Centrosome overduplication and centrosome amplifications can result from cell-cycle deregulations, often resulting in overexpression of specific centrosomal proteins (reviewed in Fukasawa, 2012) that frequently result in division errors. While multipolar mitoses are clearly detectable in cancer tissue, centrosome aberrations in bipolar spindles are less noticeable in histological preparations; these centrosome aberrations in bipolar spindles can be the result of amplified centrosomes that represent abnormally clustered centrosomal material, resulting in division errors and subsequent aneuploidies (reviewed by Krämer et al., 2012; Figure 12.2). Centrosome accumulations can also result from cell fusions in which two or perhaps even more fusigenic cells combine their DNA and associated centrosomal material, resulting in nuclear and centrosome amplifications.

Cell fusions between leukocytes and somatic cells have been studied for over a century (Aichel, 1911) and have been implicated in cancer progression; these early studies took place during the time when centrosomes were discovered and explored (Van Beneden, 1876; Boveri, 1887a,b, 1901) and were subsequently implicated in abnormal cell divisions when increased in numbers (Boveri, 1914; translated into English 2008). Both areas of research have been pursued on cell, molecular, and genetic levels, and subsequent investigations on cell fusions revealed phenotypic and genotypic diversity in tumors (Mekler, 1971; Warner, 1975; Larizza et al., 1984a,b; Rachkovsky et al., 1998; Duelli and Lazebnik, 2003). However, investigations on centrosomes in fused cells have not yet been pursued vigorously and this area of research is still only at the beginning; only few studies on centrosome abnormalities are available for somatic cell nuclear transfer (SCNT) in which two different cell types are fused (Zhong et al., 2007; reviewed by Sun and Schatten, 2007; Schatten, 2008; Schatten and Sun, 2011, 2012). Examples of fused cells have been described (reviewed by Tysnes and Bjerkvig, 2007) and cell fusions as a possible mechanism underlying cellular centrosome abnormalities with consequences for abnormal CSC and cancer cell proliferation are likely.

Spontaneous fusions have been reported and *in vitro* studies documented fusion of neural stem cells with embryonic stem cells, which resulted in tissue-specific properties of the fused hybrid cells (Ying et al., 2002). These fused hybrid cells may contain supernumerary centrosomes as a result of both cells contributing their centrosomal components, which will result in centrosome amplifications with consequences for aberrant cell divisions, as discussed earlier. This notion is supported by the fact that many fused cells are aneuploid. These abnormalities can be further enhanced when a stem cell is fused to a somatic cell with mutations. Such fusions may give rise to the significant heterogeneity that is observed in cancer tissue.

Bloodborne stem cells that are found in tissues may fuse with cells to aid in specialized functions such as tissue repair (reviewed by Tysnes and Bjerkvig, 2007). Other examples come from bone-marrow-derived cells that can home and repopulate the gastric mucosa in response to chronic *Helicobacter* infection, eventually contributing to metaplasia, dysplasia, and cancer (Houghton et al., 2004). It has further been shown that bone marrow cells can adopt the phenotype of other cells by spontaneous fusion, which has been studied in mouse bone-marrow cells that could fuse spontaneously with other cells and adopt the phenotype of the recipient cells (Terada et al., 2002;

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Ying et al., 2002; Rodic et al., 2004). These fused cells will have extra centrosomes that can result in aneuploidy during subsequent cell divisions. Our own studies on SCNT in which a somatic cell is fused with an enucleated oocyte (Zhong et al., 2007) have shown that centrosomal abnormalities play a significant role in defective cell divisions displaying multipolarity in about 40% of analyzed cells (Zhong et al., 2007; reviewed by Schatten and Sun, 2012). It may be possible that the CSC microenvironment provides signals that result in cell fusions and abnormal centrosome biology with supernumerary centriole–centrosome complexes.

Additional centriole–centrosome abnormalities may also be introduced into the cell by aberrant primary cilia, in which the basal body becomes dislodged to serve as additional nucleation center for abnormal microtubule formations resulting in multipolarity and subsequent abnormal chromosome segregation, as has been described for advanced stages of breast and prostate cancer (Lingle et al., 1998, 2002; Schatten et al., 2000; Schatten, 2013).

The Role of Primary Cilia Signaling in Centrosome Functions and Dysfunctions

The primary cilium (Figure 12.4) plays essential roles in signal initiation and signal transduction in almost all mammalian cells, including human embryonic stem cells and cells in developing and adult tissues (reviewed by Satir and Christensen, 2008; Schatten, 2008; D'Angelo and Franco, 2009; Veland et al., 2009; Schatten and Sun, 2011). It may play a role in signals received from the stem cell niche, although this specific area of research has not yet been pursued closely. Because of the newly discovered important signaling functions of primary cilia, the past decade has seen enormous progress in primary cilia research and the role of primary cilia in cellular

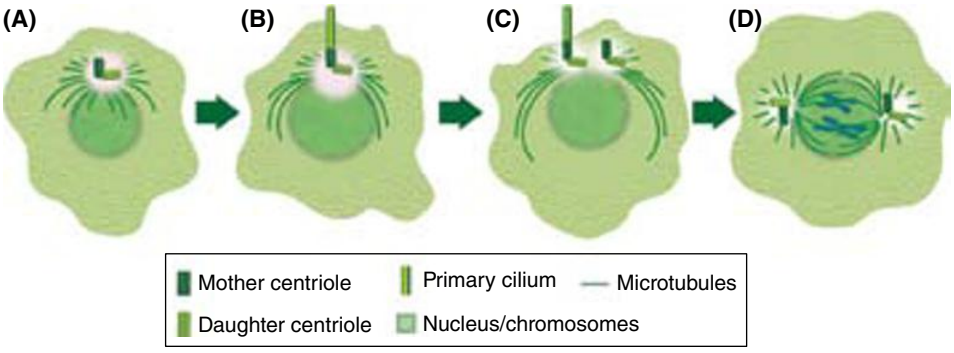


Figure 12.4. Schematic representation of primary cilium originating from the mother centriole of the centriole pair. This diagram shows the close cell-cycle relationships of the primary cilium with the mitotic poles. In G1 **(A)** the mother centriole associates with membrane components to build the axoneme of the primary cilium. The primary cilium grows during G2 **(B)**, duplicates during the S phase **(C)**, and becomes disassembled during mitosis **(D)**, when centrioles become associated again with the mitotic spindle poles.

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functions, including signaling of environmental factors through specific signaling pathways. This specialized nonmotile single cilium protruding from almost all cells in our body (Wheatley et al., 1996; D'Angelo and Franco, 2009; Veland et al., 2009) directly influences critical cell-cycle functions (reviewed by Schatten and Sun, 2010). Precise coordination between primary cilia and cellular centrosome functions is an important aspect for regulated cell-cycle progression (reviewed by Satir and Christensen, 2008; Berbari et al., 2009; Schatten and Sun, 2011; Schatten, 2013), and dysfunctions in the coordinated regulation have strongly been implicated in cellular dysfunctions that are associated with diseases including cancer (reviewed by Quarmby and Parker, 2005; Davenport and Yoder, 2005; Hildebrandt and Otto, 2005; Michaud and Yoder, 2006; Satir and Christensen, 2008; Schatten, 2008; D'Angelo and Franco, 2009; Veland et al., 2009; Schatten and Sun, 2010).

Closely related proteins located in the basal body of primary cilia and centrosomes are important for primary cilia–centrosome communication and coordination. This coordination is especially apparent and important considering that the primary cilium assembly and disassembly cycle is well synchronized with the cell's centriole–centrosome complex. A typical primary cilium assembly cycle (Figure 12.4) starts when the distal end of the cellular centrosome's mother (older) centriole becomes associated with a membrane vesicle (reviewed by Pan and Snell, 2007; Schatten and Sun, 2011) during the G1 stage, and the primary cilium's axoneme then directly assembles onto the microtubules of the mother centriole. Primary cilia are disassembled as the cell enters mitosis and centrioles become located to the mitotic poles. Primary cilia are then reassembled during exit from mitosis with the mother centriole always serving as the primary assembly and microtubule nucleation site (Sorokin, 1962).

Several important pathways require signaling through primary cilia and include the Wnt, hedgehog and platelet derived growth factor (PDGF) pathways (Sharma et al., 2008; Berbari et al., 2009); in addition, MAPK signaling through primary cilia is important for centrosome functions and cell-cycle progression. Downstream signaling cascades include phosphorylation and activation of the Akt and Mek1/2–Erk1/2 pathways (Schneider et al., 2005). As mentioned earlier, the oncogenic AURKA is localized to the basal body of primary cilia and may play a role in CSC initiation and progression. It may be possible that AURKA pathways originating at primary cilia may initiate abnormal signaling in CSCs, ascribing a possible role of AURKA in primary-cilia–cell-cycle dysfunctions. While primary cilia have not yet been determined in CSCs it is known that embryonic stem cells do have primary cilia and can differentiate differently depending on the signals they receive (Kiprilov et al., 2008; reviewed by Schatten and Sun, 2011). The regulation of the PDGF signaling pathway by primary cilia has been documented by Schneider et al. (2005), who determined that the PDGF receptor α (PDGFRα) localizes to primary cilia in murine embryonic fibroblast cells. These studies also showed that activation of Akt and ERK1/2 pathways by PDGF ligand requires primary cilia. Expression of PDGFRα is a poor prognostic indicator of breast cancer (Jechlinger et al., 2006; Carvalho et al., 2005) and links PDGF signaling through primary cilia to cancer (reviewed by Schatten, 2013). The role of primary cilia in hedgehog signaling has been well documented and the transmembrane protein, Smoothened (Smo), in the primary cilium plays a role in the activation of the hedgehog pathway: hedgehog-dependent transcription is mediated by the three transcription factors, Gli1, Gli2, and Gli3

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(Kasper et al., 2006; Haycraft et al., 2005). Hedgehog signaling and primary cilia are required for the formation of adult neural stem cells (Han et al., 2008). Hedgehog signaling through primary cilia has been implicated in cancer progression (Hassounah et al., 2012) and the presence or absence of primary cilia may play a role in cancer cell and CSC signaling, with implications for CSC initiation and progression (reviewed by Schatten, 2013).

Conclusions

Cancer stem cells, like other stem cells, have the ability to self-renew giving rise to another malignant stem cell or to cancer cells. Cancer stem cells are different from the rest of the cancer cell population, and it is known that they respond differently to cancer therapy than the rest of the heterogeneous cancer cell population within a solid tumor and that they can repopulate the tumor following seemingly effective tumor therapies. These findings call for new treatments that also eradicate CSCs, although our knowledge of CSC characteristics is still limited. To understand CSCs and their interactions with their niches, studies on centrosomes and centrosome regulation will be important, as centrosomes are vital for cell division and provide most suitable targets to inhibit cancer cell division.

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