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Mouse Cell Culture

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Edited by

Andrew Ward
David Tosh

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Preface

Techniques for the isolation, maintenance and growth of tissues normally found in a multicellular organism can be traced back for over 100 years. The pioneering developmental biologist Wilhelm Roux is generally attributed with the first reported tissue culture experiment after maintaining an explant of chick medullary plate for several days in a warm saline solution. This experiment recognised the need to provide cultured cells with conditions that resemble their normal environment, including an optimal temperature and an isotonic medium, and could be considered the founding principle of tissue culture. Tissue culture has become more sophisticated since then mainly through the ability to increasingly recognise the conditions needed to support specific cell types and, indeed, to instruct their behaviour in vitro. Tissue culture experiments have been used to address many different biological questions. Most obviously, cultured cells have been used to study the properties of the tissues from which they are derived. The accessibility of cultured cells, combined with the ability to expand a homogeneous cell population from a relatively limited source, opens up a wealth of possibilities for researchers. Cultured cells have been used to manufacture protein products, and, as a test bed for new drugs, they have been developed to model features of human disorders and, conversely, as a source of tissue replacement in human disease, providing new possibilities for regenerative medicine.

The mouse is the genetic model of choice for those interested in understanding mammalian growth, development, behaviour and physiology. Methods for manipulation of the mouse genome have rapidly advanced and become widespread in their application. They offer a powerful means to investigate gene function; however, in order to understand the mechanisms that underlie the phenotypes of transgenic and knockout mice, the application of a whole range of additional techniques is required. Cell culture describes a set of techniques that have been invaluable in its own right and can be seen as increasingly important when used in conjunction with the resources generated by mouse genetic experiments. This book brings together a number of methods for the culture of specific cells and tissues isolated from the foetal or adult mouse. Methods have been developed for the culture of a wide range of cell types, and this range is still steadily expanding. Indeed, for any tissue or cell type, culture methods continue to evolve. Thus, although we could not hope to be comprehensive in the coverage of different tissues, our main aim in compiling this book was to bring together a selection of the current methods in order to make them available in one convenient source. We have included protocols for the explant of foetal tissues and stem cells that allow developmental processes to be followed ex vivo, as well as protocols for the culture of isolated cell types that allow for the study of relatively homogeneous cell populations. The result is a diverse collection of protocols that cover a number of intensively studied systems using a variety of techniques. These methods should not only be immediately applicable by many researchers but also be useful as a springboard for the development of new tissue culture methods.

Andrew Ward
David Tosh

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Chapter 1

The Culture of Mouse Embryonic Stem Cells and Formation of Embryoid Bodies

Melany Jackson, A. Helen Taylor, Elizabeth A. Jones, and Lesley M. Forrester

Abstract

Embryonic stem (ES) cells are pluripotent cells isolated from the inner cell mass of the pre-implantation blastocyst. They have the capacity to undergo indefinite rounds of self-renewing cell division and differentiate into all the cell lineages of the developing embryo. In suspension culture, ES cells will differentiate into aggregates known as embryoid bodies in a manner similar to the early embryo. This culture system therefore provides a useful model to study the relatively inaccessible stages of mammalian development. We describe methods for the routine maintenance of mouse embryonic stem cells in culture, assays of stem cell self-renewal potential in monolayer culture and the generation of embryoid bodies to study differentiation pathways.

Key words: Embryonic stem cells, embryoid body, in vitro differentiation, leukaemia inhibitory factor, alkaline phosphatase, culture methods.

1. Introduction

Mouse embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of a 3.5 days postcoitum (dpc) blastocyst. Remarkably, when ES cells are placed back into a blastocyst they can contribute to all lineages of the embryo including the germline, even after extensive genetic manipulations and selection procedures (1). It is this property that has resulted in the widespread use of ES cells to make precise alterations to the genome and to study the phenotypic effects of these alterations in vivo (2–4). Originally, explanted blastocysts were

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propagated in vitro by outgrowth on a supporting murine embryonic fibroblast layer (5, 6). However, germline-competent ES cells can be derived de novo and maintained without a feeder layer if leukaemia inhibitory factor (LIF) is added to the media (7–9). De novo derivation and maintenance of ES cells can also be achieved in serum-free conditions with the addition of LIF and bone morphogenic protein (BMP4) to maintain pluripotency (10). Batches of serum are likely to have differences in BMP4 content and/or other factors affecting the growth and differentiation of ES cells, so batch testing of serum for self-renewal and differentiation properties is an essential pre-requisite for ES cell culture. In culture medium containing serum and LIF, mouse ES cells can be propagated as a largely undifferentiated population of stem cells with only minimal spontaneous differentiation (**Fig.1.1a**). ES cell self-renewal can be quantified by plating the cells at low density to generate discrete colonies and the staining of these colonies for alkaline phosphatase activity (11) or by expression analysis of stem cell markers such as OCT4, SSEA1, E-cadherin or Nanog (12–14).

On removal of LIF, mouse ES cells differentiate into a wide range of different cell lineages, a process which has been studied extensively in vitro (15). As this is also a property of human ES cells, they have been proposed as a source of mature cell types that could be used in regenerative medicine as a treatment for many human diseases (16–18). ES cells carrying reporter genes (e.g. *lac*Z or *egfp*) and/or selectable markers (e.g. neomycin^r, hygromycin^r) that are driven from tissue-specific promoters are useful tools to identify and/or enrich specific cell lineages for such therapies (19–22). The differentiation of ES cells that have been mutated by transgenesis or gene targeting also provides a valuable model of the developing embryo for the analysis of gene function (23–29). The differentiation of ES cells into many of the lineages of the embryo requires additional stimuli from exogenous factors such as retinoic acid (30), growth factors (31), from cell–cell contact with stromal cell layers (32) or contact with extracellular matrices (12). When experiments are to be performed to investigate the effects of specific exogenous growth factors on self-renewal or differentiation, a defined serum-free culture medium may be more appropriate. Such serum-free conditions will not be described here and readers are referred to several recent publications on the topic (10, 22, 33–36).

Differentiation into many different cell lineages can be achieved when ES cells are aggregated in suspension culture to form three-dimensional structures, known as embryoid bodies (EBs). Indeed, differentiation of ES cells in the context of EBs mirrors, to some extent, the developing egg cylinder stage of the day 6 postcoitum embryo (37). Similar to the egg cylinder, the outer cells of the EB differentiate into a layer of cells that express

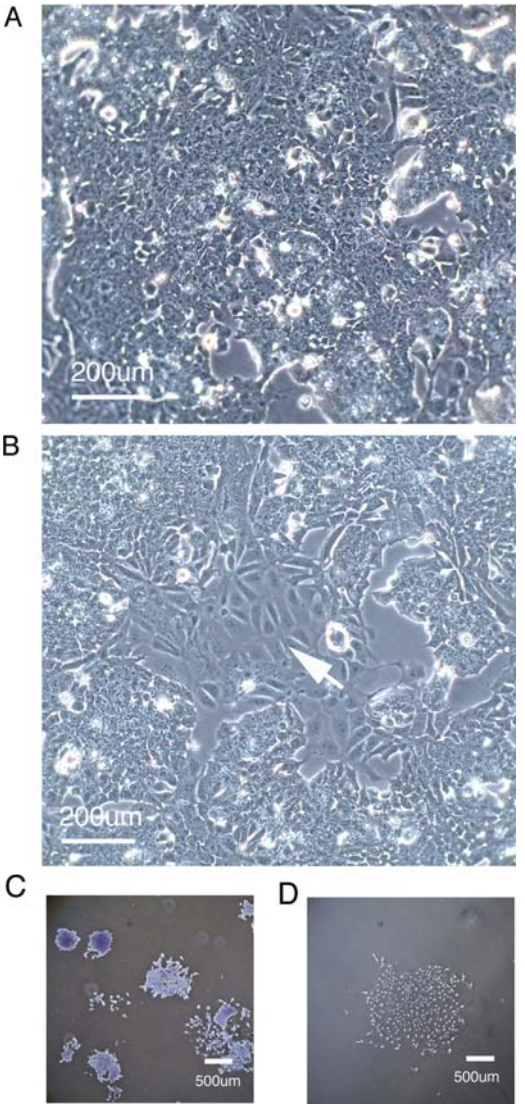

Fig. 1.1. (**a**) Photomicrograph of confluent CGR8 ES cells cultured in ES medium + LIF (100 units/ml) showing predominantly undifferentiated ES cells. (**b**) A confluent culture of CGR8 ES cells cultured in ES medium + LIF (100 units/ml) with a large central area of spontaneously differentiated cells (shown by the *arrow*). (**c**) Leishman's stained undifferentiated ES cell colonies. (**d**) Leishman's stained differentiated colony.

visceral endodermal markers and subsequently a cavity forms within the central core (38). Formation of the primitive streak and cell movements associated with gastrulation do not occur in an EB but nevertheless derivatives of all three germ layers can be generated, including mesoderm (cardiomyocytes) (39, 40), endothelial cells (41), haematopoietic cells (31, 42, 43), adipocytes (44, 45), ectoderm (neurons) (11, 46, 47), endoderm (liver) (20, 34, 48) and pancreas (49). EBs can be generated using various

culture methods including aggregation in hanging drops, aggregation in suspension culture or by culture in semisolid medium such as methylcellulose. The formation of EBs by aggregation in suspension culture is the simplest and easiest method to use. EBs of a wide range of sizes and shapes are produced using this method and it can be used for obtaining large quantities of differentiated cells or for the enrichment of rare cell types. In contrast, more consistent EBs are generated when the hanging drop method is used because each EB is generated from a defined number of cells. This method is therefore more appropriate to use when a quantitative analysis is required such as the assessment of the number of haematopoietic colonies or a defined cell type that is present in each EB. After EB formation, subsequent differentiation can continue in suspension or the EBs can be adhered to gelatin or other extracellular matrices; the method of choice being largely dependent on the lineage of interest. Haematopoietic cells, for example, develop more efficiently in suspension EBs than when adhered to gelatin (50), whereas the efficient differentiation into beating cardiomyocytes requires adhesion to gelatin (39).

Here we describe the methods for culturing and maintaining undifferentiated ES cells and for the generation of EBs. The subsequent differentiation into specific lineages and the identification of defined cell types is not described in detail in this chapter and the reader is referred to more detailed literature (10, 21, 32, 51). Likewise, methods for differentiation of defined lineages in monolayer culture can be found elsewhere (10, 27, 34, 52).

2. Materials

2.1. Cells

Many mouse ES cell lines that are available are mainly derived from the 129/Sv mouse strain (53). Some of these ES cell lines (e.g. D3 (42) and R1 (54)) require routine maintenance on primary mouse embryonic fibroblasts whereas some lines, that were originally derived on a feeder layer, have now been adapted to feeder-free conditions (e.g. E14 (55), CCE (56) and J1 (44)). Cell lines that were derived in feeder-free conditions are also available (e.g. EFC-1 (9), CGR8 (19)). For simplicity, we describe the culture of ES cells using feeder-free conditions but the reader is referred to other publications where feeder cell-dependent culture is described (57).

2.2. Routine ES Cell Maintenance

Chemicals are general purpose reagents unless otherwise stated.

1. Tissue culture plastics: T25 (25 cm^2), T75 (75 cm^2) tissue culture flasks and other tissue culture plastics were obtained from Corning.

2. One percent gelatin stock: Add 5 g gelatin (Sigma G1890) to 500 ml distilled water (Invitrogen 15230089). Autoclave, aliquot into 50 ml and store at 4°C. The working solution of 0.1% gelatin is made by adding 50 ml of 1% gelatin stock to 500 ml sterile PBS. Store at 4°C.
3. 100 mM β-Mercaptoethanol: Add 100 μl β-mercapto ethanol (Sigma M7522) to 14.1 ml distilled water (Invitrogen 15230089). Aliquot into 1 ml, store at 4°C for no longer than 1 month.
4. Foetal calf serum (FCS): Batches of FCS must be screened for suitability in propagating ES cells, i.e. to maintain cells in an undifferentiated state, with minimal spontaneous differentiation (described in **Section 3.6**). FCS batches can also be screened to identify batches that promote the optimal differentiation of ES cells into the desired lineages.
5. ES cell culture medium (ES medium): 500 ml 1 × GMEM with L-glutamine (Invitrogen 21710025), 50 ml FCS, 5 ml sodium pyruvate (Invitrogen 11360039), 5 ml nonessential amino acids (Invitrogen 11140035), 500 μl 100 mM β-mercaptoethanol.
6. Inoculate 5 ml of ES medium into 5 ml tryptose phosphate broth (Gibco 11360-039) and incubate for 2 days at 37°C to check sterility. Infected broths appear cloudy.
7. Complete ES medium: Add 500 μl LIF (1×10^5 units/ml) to 500 ml ES medium (100 units/ml final concentration) for the maintenance of undifferentiated ES cells. LIF-transfected COS 7 cell supernatant (**Section 3.4**) or commercially available recombinant LIF (e.g. ESGRO, Life technologies; Chemicon) can be used.
8. Trypsin solution: Add 0.078 g EDTA to 245 ml PBS and filter through 0.22 μM filter. Add 2.5 ml trypsin (Sigma T4549) and 2.5 ml chick serum (Invitrogen 16110033). Aliquot into 20 ml and store at –20°C.
9. Freezing mix: Add 1 ml DMSO (Sigma D2650) to 9 ml FCS, store at 4°C.

2.3. COS 7 Cell Transfection

1. COS 7 cells (ATCC).
2. Fugene 6 transfection reagent (Roche).

2.4. Testing Efficacy of LIF

1. Leishman's stain (Fisher Scientific).

2.5. X-Gal Staining

1. X-gal fix: 0.2% gluteraldehyde (Sigma G6257), 5 mM EGTA, 2 mM $MgCl_2$ and 100 mM Na_2HPO_4.

2. X-gal wash Buffer: 2 mM $MgCl_2$, 0.02% NP-40 and 100 mM Na_2HPO_4.
3. X-gal staining solution: 5 mM potassium ferricyanide (Sigma P8131), 5 mM potassium ferrocyanide (Sigma P9387), 2 mM $MgCl_2$, 0.02% NP-40 and 1 mg/ml X-gal (Sigma B4252). Stocks of X-gal were made in dimethyl formamide and stored in the dark at –20°C.

2.6. Alkaline Phosphatase (AP) Stain (Sigma Kit 86R)

1. AP fix: Add 25 ml citrate solution from the kit to 65 ml acetone and 8 ml formaldehyde.
2. AP stain: Add 0.2 ml sodium nitrite (Sigma Kit 86R) to 0.2 ml FRV alkaline (Sigma Kit 86R), mix and incubate for 2 min. Add this mixture to 9.4 ml water and add 0.2 ml naphthol (Sigma Kit 86R) (stain should be made immediately before use. It is light sensitive and so should be kept in the dark).

2.7. Generating EBs by Aggregation in Suspension Culture

1. 90 mm round Petri dish (Sterilin 101R20) (*see* **Note 1**).
2. 50 mm deep dish (Sterilin 124).

2.8. Generating EBs by Hanging Drop

1. 12 × 12 cm square Petri dishes (Greiner 688102).
2. 90 mm round Petri dish (Sterilin 101R20).
3. 50 mm deep dish (Sterilin 124).
4. 10,000 units penicillin/10 mg/ml streptomycin (Sigma P0781).

2.9. Disaggregation of EBs

1. Dispase II (Roche 10 295 825 001): 1 ml aliquots stored at –20°C.
2. DNase I (Sigma DN25): 7 mg/ml in water and stored in 1 ml aliquots at –20°C.

3. Methods

All sterile ES cell culture is carried out in a dedicated ES cell tissue culture facility in a class II laminar flow hood. Incubations are at 37°C in a humidified atmosphere of 5% CO_2. All ES cell lines that are introduced into the facility are quarantined and mycoplasma tested before use using a PCR-based method (Cambio. VGM-050).

3.1. Thawing Cells

1. Gelatinise a T25 tissue culture flask by adding 5 ml 0.1% gelatin. Tilt flask to cover the bottom and then aspirate the gelatin after 5 min.

2. Thaw cells quickly by placing the cryovial in a waterbath at 37°C. Immediately, transfer the contents of the cryovial into a final volume of 10 ml warmed complete ES medium using a pastette (it is very important that the cells are removed from the freezing medium as soon as possible).
3. Centrifuge at 1,200 rpm (80*g*) for 3 min. Aspirate the supernatant and resuspend the cell pellet in 10 ml complete ES medium. Transfer the cell suspension into the gelatinised T25 tissue culture flask.
4. Incubate at 37°C and change medium the following day.

3.2. Passaging Cells

It is important to passage the cells when they become confluent (usually every 2 days). If ES cells become over-confluent they will begin to differentiate and their viability will decrease.

1. Warm complete ES medium, trypsin solution, PBS and 0.1% gelatin to 37°C.
2. Prepare a gelatinised T25 flask.
3. Aspirate the media from confluent cell culture and add 5 ml PBS to wash the monolayer of cells.
4. Aspirate the PBS and add 2 ml trypsin solution to the flask closing the lid firmly. Tilt flask to make sure the cell layer is covered.
5. Transfer flask to incubator at 37°C for 2 min to trypsinise. Tap the flask to dissociate the cells (view under microscope to ensure all cells have lifted from flask).
6. Add 8 ml ES medium and pipette up and down to achieve a single cell suspension. Centrifuge the cells at 1,000 rpm (75*g*) for 5 min. Aspirate the media/trypsin from the cells and resuspend in 10 ml of fresh ES medium. Count the cell numbers using a haemacytometer and seed at 1×10^6–1.5×10^6 in a volume of 10 ml complete ES medium into the prepared flask (*see* **Notes 2** and **3**).
7. Loosen the lid of the flask, note the passage number (*see* **Note 4**) and incubate at 37°C. Cells seeded at 1×10^6 are usually ready to be passaged 2 days later when the cells are confluent (**Fig. 1.1a**).

3.3. Freezing Cells

1. Trypsinise the cells (**Section 2.2**) and centrifuge at 1,000 rpm (75*g*) for 5 min.
2. Resuspend in 1 ml of freezing mix cooled to 4°C.
3. Store in cryovials (two cryovials for each T25 flask), noting the passage number on the vial (*see* **Note 4**).
4. Store overnight at –80°C, then transfer to liquid nitrogen or –140°C cell freezer.

3.4. Production of Cell Supernatant Containing Recombinant LIF

1. COS 7 cells (ATCC) are thawed and grown to confluency in a T75 flask in ES medium.
2. Trypsinise and divide the cells from one T75 equally into four 150 mm dishes with 25 ml ES medium and culture until 80% confluent.
3. Aspirate the medium, wash with PBS and add 25 ml ES medium (without the addition of FCS). Transfect 10 μg LIF expression plasmid (pCAGGSLIF a kind gift from Austin Smith) per dish using Fugene 6 transfection reagent (Roche). Add 80 μl Fugene transfection reagent dropwise to 280 μl ES medium (without the addition of FCS) in a 1.5 ml microfuge tube and leave for 5 min at room temperature. To 40 μg plasmid DNA in a fresh tube, add the 360 μl diluted Fugene, gently tap to mix and leave for 15 min at room temperature. Add 100 μl of this transfection mix to each dish and incubate at 37°C.
4. After 24 h replace the medium with ES medium and incubate at 37°C for a further 4 days.
5. Remove medium and filter sterilise. Test for self-renewal activity (**Section 3.5**) and aliquot into 400 μl volumes and store at –20°C.

3.5. Testing Efficacy of Recombinant LIF-Containing Cell Supernatant

1. Make 5 ml dilutions of LIF-containing cell supernatant (**Section 3.4**) from 1/1,000, 1/10,000, 1/50,000, 1/100,000, 1/200,000 to 1/500,000 in ES medium and plate out 1 ml per well in 4 wells of a 24-well plate.
2. Seed duplicate wells of ES cells (two at 1×10^3 per well and two at 1×10^4 per well in 100 μl ES medium – a total of four wells per LIF dilution). Culture cells for 4 days.
3. Aspirate the medium and add 500 μl Leishman's stain per well, leave for 5 min. Aspirate the stain, rinse with 1 ml water and air dry.
4. Undifferentiated ES cells are clearly visible as small tight colonies which are stained more intensely with Leishman's stain (**Fig. 1.1c**). Differentiated colonies are flattened, larger and more dispersed colonies that stain faintly with Leishman's stain (**Fig. 1.1d**).
5. Determine the dilution of LIF at which inhibition of differentiation is still evident. This dilution is defined as 1 unit/ml (9). LIF-containing supernatants with differentiation inhibition activity at a dilution of 1/100,000 can be considered as a suitable preparation to use. Undiluted LIF would then have a concentration of 1×10^5 units/ml.

3.6. Batch Testing FCS for ES Cell Self-Renewal Properties

To screen batches of FCS for their use in ES cell maintenance we have routinely used IOUD2 ES cells which carry an IRES *lac*Z reporter gene targeted to the *Oct*4 locus (19) to mirror OCT4 (pluripotent stem cell) expression upon X-gal staining. Alkaline phosphatase staining of wild-type ES cells could equally be used for this purpose. We routinely test approximately 12 batches of serum in two ways. Part I of the screen is a rapid and simple screen to quickly eliminate batches of FCS that are either toxic to ES cells or that do not support self-renewal of ES cells. Part II of the screen is a more detailed and quantitative assessment of sera that identifies batches that are capable of supporting optimal plating efficiency and that maintain the highest proportion of *Oct4* positive ES cell colonies.

3.6.1. Part I

1. Thaw IOUD2 ES cells and passage once.
2. Gelatinise 12 × 6 well plates and seed 500 IOUD2 cells/well in 5 ml complete ES medium. Incubate for 24 h at 37°C.
3. Prepare 30 ml of ES medium using the different test batches of FCS with either 10 or 30% serum (including known serum as a positive control).
4. Aspirate the medium from ES cells and replace with 5 ml test complete ES medium. Change media 2 days later.
5. On day 4 of culture, aspirate the media and stain with Leishman's stain (**Section 3.5**).

Eliminate any serum batches which are either toxic at 30% or have predominantly differentiated colonies (**Fig. 1.1c**).

3.6.2. Part II

1. IOUD2 ES cells are propagated in the test ES medium in the presence of FCS (selected from phase I) and LIF 100 units/ml. Seed 1×10^6 cells per T25 flask and passage every 2 days.
2. Gelatinise 24-well plates. Seed 1×10^3/well in triplicate with 0, 1, 10, 100 units/ml of LIF. Seed three wells at 5×10^3/well in 0, 10, 100 units/ml of LIF. Change the media on day 2 of culture.
3. On day 4 stain the cultures for *lac*Z expression with X-Gal. Aspirate medium and wash the wells twice with 1 ml PBS. Add 500 μl/well X-gal fix and leave for 5 min at room temperature. Wash wells three times for 5 min each in 1 ml X-gal wash buffer. Add 500 μl/well of X-gal staining solution and leave to develop overnight at 37°C in the dark. Aspirate the stain and replace with X-gal wash buffer. Plates can be stored at 4°C.

4. Count the numbers of blue (*Oct4* positive stem cell colonies), blue/white (semi-differentiated) and unstained (differentiated) colonies per well.
5. Select the serum which propagates ES cells in flasks most efficiently, and which has the most *Oct4* positive stem cell colonies (blue) and least differentiated (white) colonies in the X-gal stained wells.

3.7. Self-Renewal Assay

Alkaline phosphatase activity is a marker of undifferentiated ES cells (11). Using this property in conjunction with the plating of ES cells at clonal density, the proportions of self-renewing stem cells and differentiating cells within a culture can be established. This assay can be used to quantify the changes in self-renewal potential of an ES cell line after, for example, the addition of exogenous factors, or to investigate the role of specific genes in embryonic stem cell biology using genetically engineered ES cells.

1. Trypsinise ES cells to ensure a single cell suspension and count (**Section 3.2**).
2. Plate 1×10^3 ES cells per well in 5 ml ES medium and LIF (100 units/ml) into eight wells of gelatinised six-well dishes. Gently pipette the cell suspension up and down to ensure even plating. Incubate at 37°C for 24 h.
3. Aspirate the medium from the wells and replace with ES medium and LIF at 100, 10, 1 and 0 units/ml (**Section 3.5**). Incubate for 5 days.
4. Alkaline phosphatase activity was carried out at room temperature as follows. Remove medium from wells. Add 1 ml AP Fix (Sigma kit 86R) per well, leave for 30 s and remove.
5. Fill each well with distilled water to rinse, leave for 45 s and remove.
6. Add 1 ml of alkaline phosphatase stain (Sigma kit 86R) per well, and leave in the dark to develop at room temperature for 15 min.
7. Remove stain, rinse briefly in distilled water and air dry at room temperature.
8. View colonies under the inverted light microscopy and score the number of stem cells, mixed and differentiated colonies. Stem cell colonies consist of only pink stained alkaline phosphatase-positive cells, mixed colonies consist of a mix of differentiated, unstained cells and pink stem cells and differentiated colonies consist of little or no alkaline phosphatase stained cells (**Fig. 1.2a**). In experiments using wild-type cells we routinely detect a total of approximately 250 colonies in 100 units/ml of LIF. The majority (70%) of these colonies are undifferentiated colonies, 30% are mixed colonies and only minimal, if any, differenti-

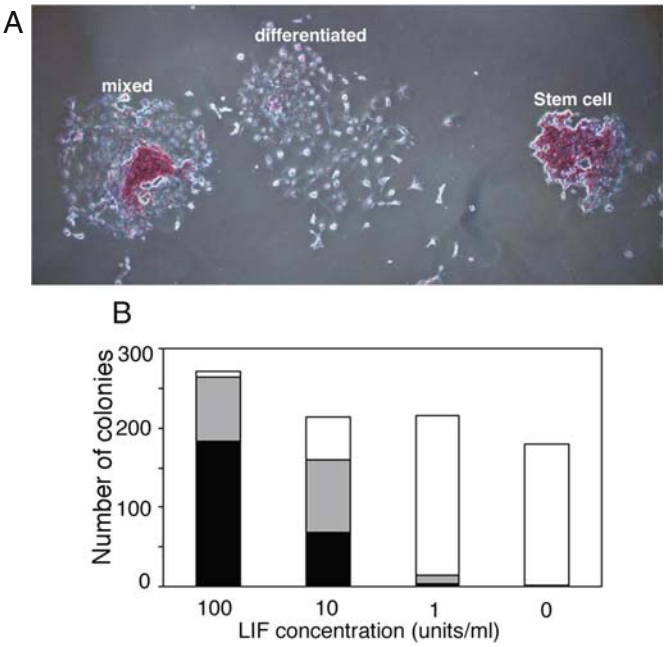

Fig. 1.2. ES cells plated at clonal density for the self-renewal assay and stained with alkaline phosphatase. (**a**) Staining and morphology of stem cell, mixed and differentiated colonies. Stem cell colonies are small, tight, Alkaline phosphatase positive colonies. Differentiated colonies are more translucent dispersed colonies with little or no stain. Mixed colonies often have a central core of undifferentiated ES cells strongly stained for alkaline phosphatase surrounded by differentiated cells. (**b**) Quantification of a self-renewal assay of J1 ES cells when plated in 100, 10, 1 and 0 units/ml of LIF. Numbers of stem cell colonies (*black*), mixed colonies (*grey*) and differentiated colonies (*white*) are shown (mean of triplicate 35 mm wells).

ated colonies are observed (**Fig. 1.2b**). In contrast, in the absence of LIF the total number of colonies is slightly less and the vast majority of these colonies are scored as differentiated (**Fig. 1.2b**).

3.8. Preparation of EBs by Aggregation in Suspension Culture

This is the simplest and easiest way of generating EBs and is appropriate to use in large-scale experiments when the population of EBs will be disaggregated for further differentiation, purification or analysis. However, EBs with irregular sizes and shapes are generated using this method (**Fig. 1.3c**) compared with the hanging drop method (**Fig. 1.3d**). For either method of generating EBs the culture medium should be prepared using FCS that has been pre-screened for its use in the particular differentiation of interest (*see* **Note 5**). Medium using FCS that has been pre-screened in this way is identical to ES medium apart from the serum batch that is used and will be referred to as DIFF medium. (LIF is never added to DIFF medium).

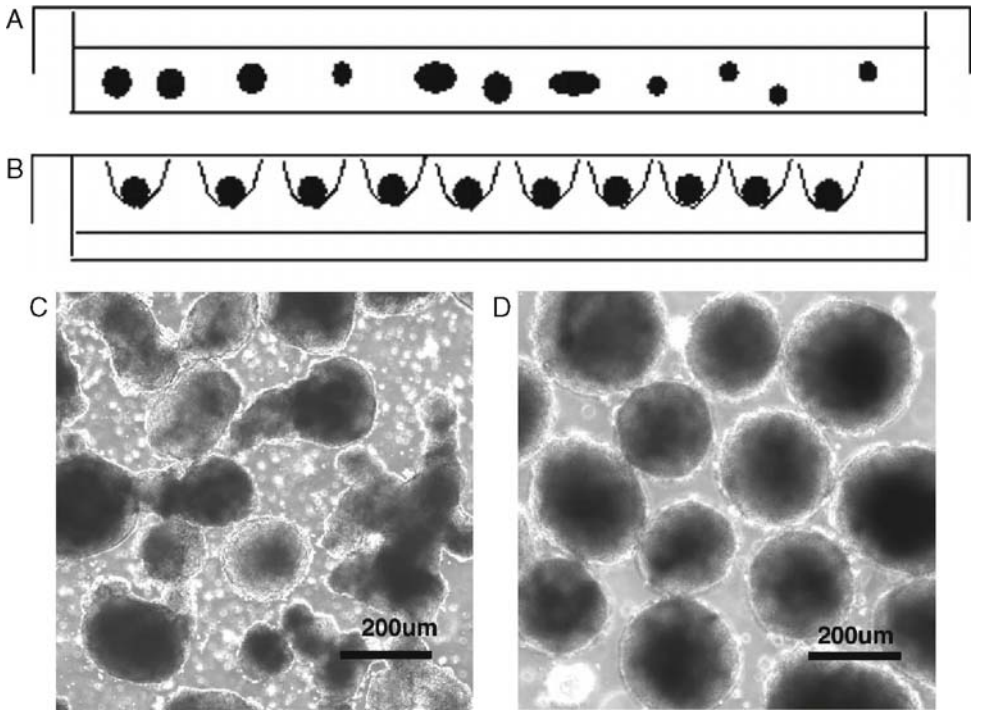

Fig. 1.3. EBs generated by either suspension culture (**a**, **c**) or the hanging drop method (**b**, **d**) after a further 3 days in suspension culture.

1. Trypsinise ES cells (**Section 3.2**) and aliquot 10^3–3×10^4 cells/ml (*see* **Note 6**) in DIFF medium in a sterile bacteriological grade Petri dish.
2. EBs are fed with fresh DIFF medium every 2 days: Using a pipette with a wide bore (ie >5 ml) aspirate some of the medium and, very gently, expel this medium to dislodge any EBs that have become loosely attached to the surface of the dish. Pipette the EBs into a 50 ml centrifuge and allow them to settle by gravity. Aspirate the medium (*see* **Note 7**). Replace with fresh DIFF medium and plate into a new 100 mm bacterial grade Petri dish.

3.9. Preparation of EBs by Hanging Drop Method

This method is more appropriate when a comparison between EBs is made since EBs of more regular sizes are generated (**Fig. 1.3d**).

1. Aliquot 10 ml of sterile water into the bottom of a large (12 × 12 cm) square Petri dishes (Greiner) to maintain a humidified environment.
2. Trypsinise cells and count. Prepare cells at a concentration of 3×10^4 cells/ml (*see* **Notes 6, 8** and **9**) in complete ES medium. Using a multi-channel pipette dispense 10 μl drops onto the up-turned lid of the square Petri dish (**Fig. 1.3b**),

covering the surface with drops. Replace the lid onto the Petri dish so that the droplets are now hanging down. The cells in the suspension will congregate at the bottom of the droplet and will form an EB. Incubate the hanging drops at 37°C for 2 days.

3. To harvest the EBs, tilt the dish and tap gently to collect all of the droplets at the bottom of the dish. Pipette EBs to a centrifuge tube using a wide bore pipette (>5 ml).
4. Centrifuge EBs at 800 rpm (50*g*) for 3 min. Aspirate the media (*see* **Note 7**) and then using DIFF medium transfer the EBs to an appropriately sized Petri dish, e.g. 10 ml of cell suspension requires approximately two to three dishes of hanging drops and is cultured in 10 ml in a 50 mm Petri dish. A volume of 100 ml cell suspension requires approximately 25 dishes of hanging drops and is cultured in 5 × 20 ml volumes in 100 mm Petri dishes.
5. Add penicillin (10,000 units)/streptomycin (10 mg/ml) (Sigma P0781) at a dilution of 1:100. Antibiotics are added only after harvesting the EBs and are not routinely added when replacing the medium.
6. Feed EBs every 2 days as described above.

3.10. Disaggregation of EBs into a Single Cell Suspension

In some experimental situations it will be necessary to disaggregate EBs into a single cell suspension prior to further analysis, e.g. replating in methylcellulose to analyse haematopoietic colony formation, or for the analysis of cell surface marker expression by flow cytometry or the enrichment of specific cell types lineages by cell sorting.

1. Harvest EBs into a 50 ml centrifuge tube and leave to settle by gravity.
2. Add 50 ml PBS, allow EBs to settle by gravity then aspirate PBS almost to the bottom of the tube.
3. Add 1 ml PBS and 1 ml dispase and 20 μl DNase 1 (7 mg/ml stock) and incubate for 1 h at 37°C with gentle (100 rpm) shaking.
4. Aspirate EBs to disaggregate several times with 30 gauge needle.
5. Add 4 ml DIFF medium and plate into 50 mm bacterial grade Petri dishes and leave for 5–10 min at room temperature.
6. Centrifuge at 1,000 rpm (75*g*) for 5 min and aspirate the supernatant.
7. Wash the cell pellet in 10 ml PBS and centrifuge at 1,000 rpm (75*g*) for 5 min.
8. Resuspend in 5 ml PBS and count using a haemacytometer.

3.11. Cardiomyocyte Differentiation

Differentiation of ES cells into beating cardiomyocytes occurs spontaneously in EBs when plated onto gelatinised tissue culture plates. Since no exogenous factors are required and the method is uncomplicated, it is a useful strategy not only for studies investigating cardiomyocyte differentiation pathways, but also for establishing whether an ES cell line can differentiate per se (e.g. selecting clones for further study after genetic manipulation and subcloning of a parental ES cell line).

1. Generate EBs by hanging drop (as per **Section 3.9**) and then transfer EBs to suspension culture for 5 days in DIFF medium.
2. Gelatinise the wells of a 24-well plate. Add 1 ml DIFF medium per well. Using a 1 ml micropipette plate out one EB per well.
3. Incubate at 37°C for a further 10 days, change the medium every 2 days or every day if the medium becomes yellow very quickly.
4. View under inverted microscopy (using both 2.5 and ×10 objectives) and score EBs for beating cardiomyocytes after 24 h and for the next 10 days.

4. Notes

1. EBs are generated and subsequently cultured in sterile Petri dishes which are bacteriological grade and have not been treated for tissue culture use. This reduces the attachment of EBs to the dish.
2. ES cells are sensitive to plating density and appropriate treatment. Passaging every 2 days is usual for most ES cell lines. A 1:5–1:10 split is a good guideline but it is advisable to count cells at all passages to maintain cells in prime condition.
3. If cells have a more than desirable amount of spontaneous differentiation within the cultures (**Fig. 1.1b**) then plating cells at high density may alleviate this, since differentiated cells plate less efficiently than undifferentiated cells.
4. It is important to use ES cells at low passage numbers. Prolonged time in culture may generate chromosomal abnormalities in ES cells of unknown or high passage number, which require karyotyping.
5. It is very important to screen FCS batches for use in routine ES cell culture and for differentiation of ES cells, since there is great variation between batches in their suitability for a

particular use. We screen approximately 12 serum batches for its use in ES cell maintenance. Many of these batches may be unsuitable, in our hands 50% of the batches were eliminated for use in ES medium to maintain ES cells after an initial screen (**Section 2.6** , step 1). The batches of FCS not suitable for ES cell propagation may be screened for use in differentiation assays. Differentiation FCS must be screened for its suitability to use in each individual protocol.

6. EBs can be made in suspension using a range of cell concentrations: 2×10^4/ml for endoderm (20), 1–2×10^3/ml for efficient haemopoiesis (21). Dang et al. (50) observed increased efficiency of EB formation in suspension culture as ES cell concentration decreased (approximately 40% efficiency at 1×10^2 or 1×10^3 ES cells/ml). Interestingly, this study (50) observed that EBs grow to a maximum size (30,000 cells per EB at day 12 of culture) regardless of starting cell number or method of preparation (hanging drops or suspension cultures).
7. When aspirating medium of EBs (particularly small ones) pipette off rather than vacuum aspirate to avoid aspirating all the EBs into the waste.
8. The minimum workable volume to prepare EBs by the hanging drop method is 10 ml, which is approximately two square Petri dishes of hanging drops with approximately 500 hanging drops per dish. A workable large-scale hanging drop preparation would be approximately 400 ml. An electronic repeating multi-channel pipette is useful for large-scale preparations. Alternatively, for large-scale ES preparation of EBs prepared by aggregation in suspension culture a stirred suspension culture system may be considered (13).
9. EBs tend to form efficiently in hanging drops due to cells aggregating due to gravity, in suspension culture smaller aggregates fuse to form larger EBs (50).

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Chapter 2

Derivation of Primary Mouse Embryonic Fibroblast (PMEF) Cultures

Alastair S. Garfield

Abstract

Primary mouse embryonic fibroblasts (PMEFs) have a number of properties that make them an attractive cell culture model. Relative to other primary explant cultures they are easy to establish and maintain, proliferate rapidly and, as a result, large numbers of cells can be produced from a single embryo within several days following explantation. This allows, for instance, for ready comparison of wild-type and knockout cells derived from the same litter of animals. PMEFs can be expanded through several passages before they reach crisis and can be used to establish cell lines following spontaneous transformation or following derivation from strains carrying mutations, such as in the gene encoding the tumour suppressor Trp53. They have been widely used as feeders to support other cultured cell types, notably embryonic stem cells, as well as for the study of a diverse range of cellular phenomena using microscopic, biochemical and molecular biological techniques. Here, we describe a simple and reliable method for the derivation and maintenance of PMEFs.

Key words: Primary culture, mouse, embryonic, fibroblasts, PMEF.

1. Introduction

Cell culture was first demonstrated in 1907 with the in vitro growth of nerve fibres from amphibian neuronal tissue (1). The utilisation of cell culture as a model for more complex in vivo systems has flourished since this groundbreaking event. For all the advantages cell culture affords the modern researcher it has its obvious limitations. At the forefront is the debate as to whether an in vitro system can be accurately representative of the comparative in vivo model. Generating cultures directly from the tissues of interests is held to be the closest approximation of the

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organismal environment. These "primary cultures" can be derived from almost any tissue, including tumours and individual organs, and can represent a myriad of cell lineages. This chapter focuses on the derivation of fibroblast cultures from murine embryos: primary mouse embryonic fibroblasts (PMEFs) (**Fig. 2.1**). Cultures can be readily established and maintained and have proven to be a versatile tool in many branches of cell biology.

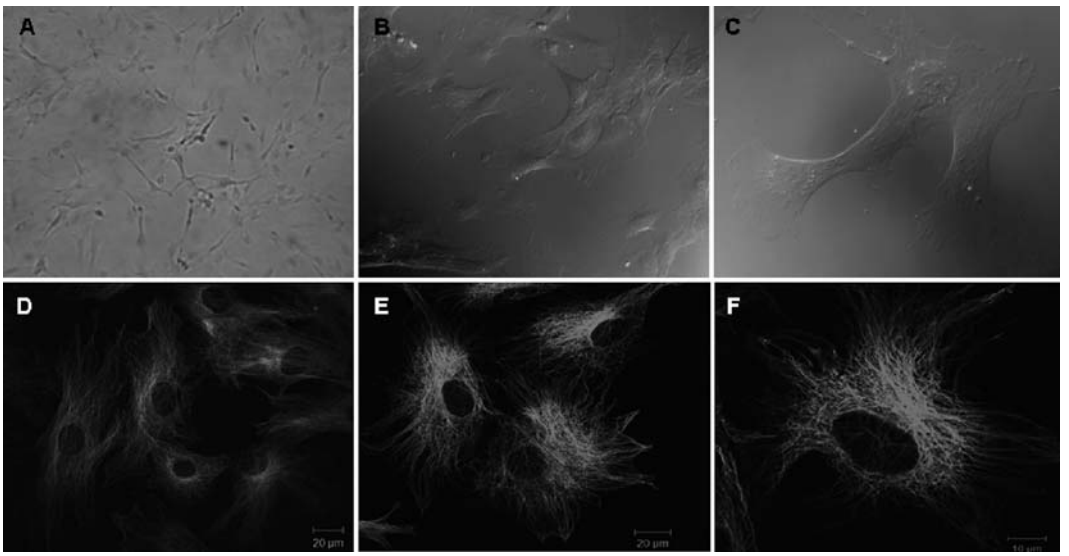

Fig. 2.1. Examples of PMEF cells in culture. (**a**) Cells in a plastic culture vessel viewed with an inverted light microscope (magnification ×100). (**b**) Differential interference contrast (DIC) image (magnification ×200). (**c**) DIC image (magnification ×400). (**d–f**) PMEFs immunofluorescently labelled with an antibody specific to α-tubulin and viewed with a confocal microscope. Magnification ×400 (**d**) and ×630 oil immersion (**e** and **f**).

Fibroblasts are a relatively undifferentiated mesodermally derived cell type found in abundance in connective tissue and responsible for the secretion of a host of extracellular matrix components (2). They represent a relatively low maintenance anchorage-dependent cell system that is easily derived and can be differentiated into a variety of more complex cell types. PMEFs are often used as a tool to aid the analysis of whole organism affects resulting from genetic modification of mice. The growth affects imparted by the dysregulation of a gene's function can be more readily investigated in a primary cell culture system. For instance, in vivo ablation of the murine *Recql4* gene results in 95% mortality within 2 weeks of birth and a reduction in body weight by approximately 60% (3). Given the low survival rate, in situ analysis of growth kinetics is impractical; however, PMEFs generated from embryonic day 14.5 (E14.5) embryos demonstrated retarded proliferation and permitted further analysis of the mechanisms governing the developmental phenotype of the animals (3). Similarly, primary fibroblasts from *Trp53* knockout mice demonstrated enhanced growth, genetic instability and a

transformed phenotype, all of which are consistent with the highly tumourigenic model from which they were generated (4, 5). In vitro analysis in this manner has assisted in successfully delineating the cellular function of Trp53 and provided greater insight into its in vivo role, in particular in tumourigenesis. A second often utilised role for these cells is as a feeder layer. In this capacity PMEFs condition the culture media by secreting the necessary factors required to ensure the healthy development of a second, often low-density cell type present in the same vessel (6). This has been frequently exploited in the culture of embryonic stem (ES) cells. Overall, PMEFs represent a simple yet highly pliable cell type with a variety of in vitro applications.

Despite the discernible advantages primary cultures afford they have certain limitations, principally as a consequence of the crude manner in which they are generated. Foremost, due to the cellular heterogeneity of embryonic tissue, generation of a homogeneous culture is often difficult, although steps can be taken to ensure a greater degree of homogeneity. In addition, primary cultures tend to reach senescence by around passage 12, earlier if cells are cultured under stressful conditions. This must be borne in mind should one require cells with an extended lifespan. In this case, cells can be selected for spontaneous escapes from senescence or, alternatively, transformed cells can be derived directly from strains carrying mutations, such as in the gene encoding the tumour suppressor Trp53 (4). Further to this, such conditions, in particular changes in CO_2 concentrations, can induce unstable expression and in some cases inactivation of certain imprinted genes. These include *Igf2, Cdkn1c (p57), H19* and *Grb10* (7), the dysregulation of which may impart an artefactual phenotype.

2. Materials

2.1. Mice

1. Timed-pregnant mice (*see* **Note 1**) to be killed (*see* **Note 2**) on postcoitum day E14.5, where E0.5 is the day of detection of a copulation plug.

2.2. Media and Solutions

1. Dulbecco's Modified Eagle's Medium (DMEM; Gibco).
2. Foetal bovine serum (FBS; Gibco).
3. Streptomycin (10,000 μg) and penicillin (10,000 units) solution (Gibco).
4. Trypsin–EDTA (0.05% with EDTA.4Na) (Gibco).
5. Dimethyl sulfoxide (DMSO; Sigma Aldrich).
6. Phosphate buffered saline (PBS).

7. Complete medium: DMEM, 10% FBS, 1% streptomycin/penicillin/1 mM L-glutamine.
8. Freezing medium: 90% FBS, 10% DMSO.

2.3. Tissue Culture Plastic and Other Equipment

1. 25 and 75 cm^2 flasks with vented caps (Nunc).
2. Six-well plates (Nunc).
3. Petri dishes (10 cm diameter) (Nunc).
4. Cryopreservation tubes (Nalgene).
5. 1 ml syringes.
6. 15 ml Falcon tubes.
7. 37°C water bath.
8. 37°C incubator (5% CO_2).

3. Methods

The success of culturing any cell type is dependent upon maintaining an aseptic environment at all times during their generation and upkeep. With the exception of the initial embryo harvesting, all steps should be performed in a laminar flow hood. All materials used must be sterilised with 70% ethanol. All solutions that come into direct contact with the cultures should be prewarmed to 37°C to prevent cell lysis and shock. Newly derived primary cultures may have their own particular idiosyncrasies and attention should be paid to these to ensure that the culture technique is tailored to the requirements of the cell type. For example, certain knockout strains of PMEFs demonstrate a density-dependent growth phenotype and are unviable when seeded below this threshold level. If genotypic differences are expected within a litter, each embryo and resulting suspensions must be kept separate from each other to prevent cross-contamination.

3.1. Generation of Primary Cultures

1. Dissect out uterine horns from the pregnant mouse staged to E14.5 of gestation (*see* **Note 3**).
2. Place horns in ice-cold PBS for at least 5 min.
3. Remove each individual embryo from the uterus and separate placentae and yolk sacs (*see* **Note 4**).
4. Place the individual embryos in prewarmed complete medium.
5. Decapitate and eviscerate each embryo and remove as much blood and liver tissue as possible (*see* **Note 5**).

6. Transfer the remainder of the embryos to 1 ml of warm DMEM (*see* **Note 6**).
7. Homogenise the embryos using a 1 ml syringe by drawing them up and down until the disaggregated tissue moves freely in and out of the syringe.
8. Further homogenise using a P1000 pipette, again until the tissue pieces move freely in and out of the disposable pipette tip.
9. Transfer the cell suspension to a 15 ml Falcon tube.
10. The suspension is enzymatically disaggregated by adding 250 μl of trypsin/EDTA.
11. Incubate at 37°C for 30 min and mix by inverting every 5 min (*see* **Note 7**).
12. Allow the remaining tissue to settle and collect the supernatant in a fresh 15 ml Falcon tube (ensure not to disturb the settled tissue).
13. Centrifuge at 180*g* for 4 min to pellet the cells.
14. Resuspend the cell pellet in 1 ml warmed complete media by pipetting up and down using a P1000 (*see* **Note 8**).
15. Count an aliquot of cells using a haemocytometer. It may be necessary to dilute the suspension to facilitate accurate counting.
16. Seed 1×10^6 cells into a 25 cm^2 flask (with vented caps) in a total of 8 ml complete media (*see* **Note 9**).
17. Incubate flasks in a 37°C incubator with 5% CO_2 for approximately 48 h.

3.2. Cell Maintenance and Splitting

Successful propagation of PMEFs requires continued care and maintenance. Cells must be washed and the media replaced every 48–72 h, depending upon cell number and the rate of growth. For continued culture, cells must be split before they reach confluence.

3.2.1. Cell Washing

1. Remove the conditioned medium from each flask using a sterile pipette or aspirator.
2. Wash the cells twice with prewarmed PBS.
3. Add an appropriate volume of prewarmed complete media.

3.2.2. Cell Splitting

1. Remove the conditioned media from each flask.
2. Wash the cells twice with prewarmed PBS.
3. Add 1 ml/25 cm^2 trypsin/EDTA.
4. Incubate at 37°C for 5 min.

5. Check cell disattachment under an inverted microscope (*see* **Note 10**).
6. Replace the flasks in the incubator, if required, for a maximum of 5 min.
7. Remove the flasks from the incubator and add 2 ml complete media for each 1 ml of trypsin.
8. Remove the cell suspension and transfer to a 15 ml Falcon.
9. Centrifuge at 180*g* for 4 min.
10. Resuspend the cell pellet in 1 ml complete media.
11. Divide the cell suspension between an appropriate numbers of flasks (*see* **Note 11**).

3.3. Freezing and Storage of Cells

PMEF cultures tend to be hardier than many cell types and can be readily frozen down for future use. The freezing and thawing process will result in a certain level of cell death, therefore, to ensure the cells are at a sufficient density for recovery at a later date, freeze cells at densities in the order of 5×10^6–1×10^7 cells per ml. Early investigation into the longevity of liquid nitrogen stored cells suggests minimal loss of viability over a 2- to 4-year period (8).

1. Remove the conditioned media from each flask.
2. Wash the cells twice with prewarmed PBS.
3. Add 1 ml/25 cm^2 trypsin/EDTA.
4. Incubate at 37°C for 5 min.
5. Check cell disattachment under an inverted microscope.
6. Replace the flasks in the incubator if required.
7. Remove the flasks from the incubator and add 2 ml complete media for each 1 ml of trypsin.
8. Remove the cell suspension and transfer to a 15 ml Falcon.
9. Centrifuge at 180*g* for 4 min.
10. Resuspend cells in freezing media and transfer to a cryotube (*see* **Note 12**).
11. Place cells in a tissue-padded polystyrene box at room temperature and transfer to –80°C for 24 h (*see* **Note 13**).
12. The next day transfer cells to a liquid nitrogen store.
13. The thawing of cells after storage should be carried out rapidly at 37°C (preferably in a water bath). Once thawed, transfer cells immediately into the appropriate volume of prewarmed complete medium.

4. Notes

1. We have mostly used mice of a mixed inbred strain background (C57BL/6:CBA). While this and similar protocols have been used successfully to derive PMEFs from a number of mouse strains, it is possible that some aspects of the protocol may benefit from further optimisation when used to derive cells from mice of different strain backgrounds.
2. Experiments involving animals must be conducted in accord with the prevailing local and national regulations. For instance, in the UK, this includes the need for local ethical approval and requires that appropriate licences are obtained from the government Home Office.
3. The method works well for E14.5 embryos but can also be adapted for embryos of different stages. With earlier stage embryos the cell yield will be reduced. At later stages, the embryos are notably more fibrous and may need to be chopped using a pair of scalpel or razor blades prior to disaggregation using a syringe.
4. Yolk sacs can be dissected free and used as a source of embryo-derived tissue for genotyping by PCR, if required. Alternatively, any source of embryonic tissue, e.g. tail or limb, can be used.
5. These tissues represent more differentiated cell sources. Their removal will help to generate a more homogeneous cell suspension. However, due to the highly proliferative nature of fibroblasts, under standard culture condition it is likely that other cell types will in any case be diluted out over the course of the cell culture period. Removal of the liver is most easily achieved by using two pairs of Watchmen forceps.
6. This media is *not* supplemented with serum. This is to ensure that serum proteins do not inhibit the trypsin and prevent successful cell disaggregation.
7. Prolonged exposure to trypsin can be detrimental to the survival of the cells. Ensure that homogenates are left no longer than 30 min. Alternative preparations of trypsin or other proteases can be used if a more delicate disaggregation is required, maybe as a result of a genotypic affect on cell viability.
8. Serum is required to inactivate residual trypsin. The cell pellet can be resuspended in less than 1 ml but must be greater than a 1:1 ratio of medium to trypsin in order to ensure total inactivation.

9. Cells derived from genetically modified embryos may require seeding at higher or lower densities, for instance, when the genotype may affect cell survival. It may be necessary to determine the optimum plating density empirically. If in doubt the entire cell suspension can be seeded in one flask.
10. Fibroblasts are strongly adherent cells, banging the flask against the palm of one's hand or bench will help facilitate the disattachment of the cells.
11. The number of divisions and the size of the flasks used will depend upon the nature of the individual cell type. It is advisable to start with fewer divisions into smaller flasks until you have a feel for the proliferative properties of your particular line. For instance density-dependent growth, rate of proliferation and transformed phenotypes will influence the manner in which the line is treated.
12. DMSO acts as a cryoprotectant possibly by permeabilising the cell membrane and allowing the exit of water during the cooling process; this prevents the formation of ice crystals within the cell interior (9, 10).
13. Cells should be frozen at a rate of approximately 1–3°C/min. To prevent damage and subsequent loss of viability cells must not be introduced directly into liquid nitrogen (10, 11). This rate of cooling can be achieved by using a room-temperature polystyrene box padded out with tissue paper (alternatively, there are commercially available insulated vessels designed for this purpose).

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Chapter 3

Embryonic Skeletal Muscle Microexplant Culture and Isolation of Skeletal Muscle Stem Cells

Janet Smith and Deborah Merrick

Abstract

Cultured embryonic and adult skeletal muscle cells have a number of different uses. The microdissected explant technique described in this chapter is a robust and reliable method for isolating relatively large numbers of proliferative skeletal muscle cells from juvenile, adult or embryonic muscles as a source of skeletal muscle stem cells. The authors have used microdissected explant cultures to analyse the growth characteristics of skeletal muscle cells in wild-type and dystrophic muscles. Each of the components of tissue growth, namely cell survival, proliferation, senescence and differentiation can be analysed separately using the methods described here. The net effect of all components of growth can be established by means of measuring explant outgrowth rates. The microexplant method can be used to establish primary cultures from a wide range of different muscle types and ages and, as described here, has been adapted by the authors to enable the isolation of embryonic skeletal muscle precursors. Uniquely, microexplant cultures have been used to derive clonal (single cell origin) skeletal muscle stem cell (SMSc) lines which can be expanded and used for in vivo transplantation. In vivo transplanted SMSc behave as functional, tissue-specific, satellite cells which contribute to skeletal muscle fibre regeneration but which are also retained (in the satellite cell niche) as a small pool of undifferentiated stem cells which can be re-isolated into culture using the microexplant method.

Key words: Skeletal muscle stem cell, embryonic tissue culture, apoptosis, growth factor, proliferation, myoblast, myogenesis, satellite cell, skeletal muscle differentiation, muscular dystrophy.

1. Introduction

Two approaches can be employed to isolate proliferative skeletal muscle cells. In the first muscle tissues are enzymatically digested to isolate single cells prior to plating out (1). The second method is to explant pieces of muscle tissue into culture to allow cells

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to grow out during incubation (2, 3). The second method is described in this chapter. Tissue culture itself has its roots in explant culture. The year 2007 was the 100th anniversary of the classic experiments of Harrison in which he obtained neuron outgrowths by incubating nerve explants in hanging drops of lymph (4). Explant culture techniques have been used and refined in a variety of different contexts in the ensuing 100 years as a means of generating proliferative primary cultures of adult and embryonic cells (4, 5). The principle behind the explant technique, however, remains the same; to minimise the trauma of primary cell isolation by maintaining the three-dimensional structure of the parent tissue during the crucial early stages of cell outgrowth whilst providing the outgrowing cells with a rich nutritive media in which to proliferate. In skeletal muscle there is an additional advantage to using explant culture because the act of cutting up the muscle tissue mimics muscle fibre trauma, the usual trigger for satellite cell activation, migration and proliferation (3, 6). Adult skeletal muscle satellite cells (also called myoblasts) are the proliferative stem cell population responsible for muscle fibre repair and growth (7). Skeletal muscle explants thus mimic the in vivo environment of the regenerating muscle and stimulate stem cell migration and division.

In the embryo, the majority of vertebrate skeletal muscle (trunk and limb muscles) derives from the somites, although somitomeres and branchial arches give rise to the musculature of the head (8, 9). The myotome can be identified as two distinct groups of Myf-5 expressing stem cells located in the dorsal, medial and lateral edges of the differentiating somite, respectively. Respectively, these cells generate the epaxial muscles of the back, which differentiate in situ, and the ventral and lateral hypaxial musculature (limbs, abdomen and respiratory muscles) which require the migration of muscle stem cells from the somite (10). Embryonic muscle stem cell migration is under the control of Pax 3 (11). Myf-5 expression is essential to the establishment of the embryonic musculature and this importance persists to postnatal muscles where over 98% of activated satellite cells express Myf-5 (12). Myf-5 is therefore a reliable and specific marker of the proliferating skeletal muscle stem cell population in both adult and embryonic tissues. Embryonic muscle stem cells (also called muscle cell progenitors, skeletal muscle precursors, myoblasts or even embryonic satellite cells) can be isolated from the somites of early-stage mouse, chick and frog embryos (13). In order to isolate myogenic cultures from the embryonic skeletal muscles of older embryos the authors have adapted the microdissected explant technique for embryonic tissues. A similar approach is used by Cossu et al. (14) to generate clonal cell populations from the embryonic somite (14).

When explants are used in combination with high-density culture to isolate skeletal muscle cells (myoblasts) the soluble factors released by the explant in response to dissection, together with growth factors released by emerging, proliferating myoblasts, serve to "condition" the growth medium and to support the survival and proliferation of skeletal muscle stem cells (SMSc). To inhibit differentiation of these cultures during the later stages of expansion SMSc are proliferated in calcium-reduced medium (3, 15). Conversely, differentiation can be induced in the presence of horse serum and foetal calf serum (FCS)-reduced medium (16). To generate pure populations of proliferative SMSc, the size of the explant is crucial, it must be sufficiently small that media can penetrate to its centre during culture, to prevent necrosis and the release of toxins, but large enough to provide sufficient skeletal muscle stem cells to maintain the culture (3). The microdissection explant method described in this chapter was originally developed to overcome the hypersensitivity of dystrophic myoblasts to enzymatic digestion methods (3). The technique was subsequently used to demonstrate the elevated levels of apoptosis found in dystrophic myoblast populations (17, 18). In 1997 clonal isolates were successfully derived from microdissected skeletal muscle explants and formally demonstrated to be functional SMSc capable of repopulating skeletal muscle in vivo (19). These SMSc cell lines demonstrate all of the key features of an adult tissue stem cell including self-renewal and niche specificity, illustrated by their behaviour when injected in vivo. SMSc can also be isolated from even very aged muscle explants (20). More recently the approach has been modified for embryonic tissue to enable the isolation of embryonic SMSc (eSMSc) as a means of analysing and understanding the origin, behaviour and function of these cells in myogenesis (21) and in dystrophic muscle (22, 23).

2. Materials

2.1. In Vitro Cell Culture of Established SMSc

1. Cryovials containing the appropriate cell line.
2. DF10 culture medium comprising:

2.1. Liquid medium: DMEM/F12 1:1 mix (Dulbecco's Modified Eagles's medium and Ham's F12 medium, 1:1 v/v), DMEM or other media appropriate to cell line.

2.2. 100× Glutamine (200 mM) diluted in medium to a 1× concentration of 2 mM (Glutamine HYBRI-MAX®, Sigma-Aldrich Company).

2.3. Batch-tested FCS, 10% supplement to liquid media.

3. Lab centrifuge capable of reaching 1,000 rpm such as the Heraeus Labofuge 300.
4. 15 ml Falcon centrifuge tubes.
5. Tissue culture plasticware (25, 75 or 175 mm^2 tissue culture vessels; Nunc, Costar or similar).
6. Humidified CO_2 incubator.
7. Sterile hood with laminar air flow.
8. Water bath (37°C).
9. Inverted microscope (e.g. Leica DMIRB, Leica Instruments, UK).
10. 70% Ethanol.

2.1.1. Subculture of SMSc

1. Lab centrifuge capable of reaching 1,000 rpm such as the Heraeus Labofuge 300.
2. 15 ml Falcon centrifuge tubes.
3. Plasticware, as above.
4. Trypsin/EDTA: porcine trypsin (0.2 g/l) in 0.2% ethylenediamine tetraacetic acid (EDTA·4Na), 0.9% sodium chloride (Sigma-Aldrich Company, UK).
5. Calcium- and magnesium-free phosphate-buffered saline (PBS). Cell culture-tested PBS (Dulbecco's formula) is purchased as a ready-mixed powder or in tablet form (Sigma-Aldrich Company, UK) and made up with double-distilled water before sterilization by autoclave. PBS consists of 2.68 mM potassium chloride (KCl); 1.47 mM potassium phosphate monobasic (KH_2PO_4); 0.137 M sodium chloride (NaCl); and 8.1 mM sodium phosphate dibasic (Na_2HPO_4). PBS can be prepared from scratch as follows: 200 mg KCl, 200 mg KH_2PO_4, 8 g NaCl and 1.15 g Na_2HPO_4/l of double-distilled water followed by sterilization by autoclave.

2.1.2. Cryopreservation of Cell Lines and Primary Cultures

1. Lab centrifuge capable of reaching 1,000 rpm such as the Heraeus Labofuge 300.
2. 15 ml Falcon centrifuge tubes.
3. CryoTube™ vials (Nunc).
4. Dimethylsulphoxide (DMSO).
5. Freeze down mix: 10% DMSO in DF10 medium.
6. –80°C Freezer, liquid nitrogen storage tank.

2.1.3. Determining Cell Numbers

1. A Neubauer haematocytometer.
2. Haematocytometer coverslips.
3. Inverted microscope (Leica DMIRB, Leica, UK).

4. Hand counter.
5. Pipettes and hand-held pipetting device.

2.2. Establishing Primary Skeletal Muscle Microexplant Cultures

1. Freshly dissected skeletal muscle from aged (16 months or older), adult (2–15 months) or juvenile (1–8 weeks) mice.
2. Dissection microscope, such as the Stemi 1000 (Ziess, UK).
3. Small sterile hood.
4. Dissection instruments (sterilised by autoclave).
5. 70% Ethanol.
6. Enriched culture medium (DF20): A 1:1 ratio of DMEM: F12 supplemented with 20% heat-inactivated batch-tested FCS, 2 mM glutamine and penicillin/streptomycin.
7. 96-well tissue culture plates.
8. 60 mm Petri dishes.
9. Myoblast-conditioned medium.
10. Calcium-depleted medium.

2.2.1. Clonal Derivation

1. Confluent SMSc or primary explant cultures.
2. 96-well tissue culture plates.
3. Conditioned medium prepared from SMSc or primary explants.
4. 0.2 μm Syringe filter (e.g. Acrodisc® Syringe filter, Gelman Laboratory, VWR International, UK).
5. Myoblast-conditioned medium.
6. Inverted microscope.
7. Marker pen and score sheet.

2.2.2. Karyotyping

1. Colchicine.
2. 0.0075 M Potassium chloride.
3. Prepared glass slides (*see* **Section 3.2.2.1**).
4. Sulphuric acid.
5. Sodium hypochlorite (5%).
6. Leishman's stain (1.5 g Leishman's in 1 l methanol; Sigma-Aldrich, UK)
7. Gurr's buffer pH 6.8 (VWR International, UK).
8. Lab centrifuge (Heraeus Labofuge 300; Kendro Laboratory Products, Germany).
9. Ice bucket and ice.
10. Pasteur pipettes.
11. Cells cultured to exponential phase (~75% confluent).

12. Freshly made fixative (3:1 v/v methanol: glacial acetic acid).
13. Coverslips and DePEX (Merck) mounting fluid.
14. Upright microscope (Nikon Eclipse E600; Nikon, UK).

2.3. Establishing Primary Microexplant Cultures from Embryos

1. Pregnant mice at the appropriate stage of gestation.
2. Sterile dissection instruments.
3. 70% Ethanol.
4. Plastic collecting vessels (7 ml bijou tubes).

2.3.1. Embryo Collection

5. Primary explant culture medium (PECM): DMEM:F-12 supplemented with 20% FCS, 1% glutamine and 1% penicillin/streptomycin solution (Sigma-Aldrich, UK), warmed to 37°C.

2.3.2. Embryo Microdissection

1. Fresh, staged mouse embryos.
2. Warm PECM: DMEM:F-12 supplemented with 20% FCS, 1% glutamine and + 1% penicillin/streptomycin solution (Sigma-Aldrich, UK).
3. Dissecting microscope (Stemi 1000; Ziess, UK).
4. Sterile hood.
5. Jeweler's forceps.
6. 60 mm Petri dishes.

2.3.3. Setting Up Embryo Microexplant Cultures

1. PECM.
2. Dissecting microscope.
3. Sterile hood.
4. 96-well tissue culture plates.
5. Jeweler's forceps.
6. 60 mm Petri dishes.
7. 37°C Humidified incubator.
8. Inverted microscope.

2.3.4. Monitoring Outgrowth

1. Inverted microscope.
2. Embryo explant cultures.
3. Score sheet.
4. For immunostaining reagents, *see* **Section 2.4.4.**

2.3.5. Subculturing Primary Embryonic Explants

1. 0.2 μm Acrodisc® syringe filter.
2. Dispase (50 μg/ml, equivalent to 6 units/mg; available from MP Biomedicals, UK)
3. PECM.

4. 37°C Incubator/5% Co_2.
5. 100 μl Gilson pipette and sterilised yellow pipette tips.
6. 48-well plates or appropriate plastic ware, as above.

2.4. In Vitro Analysis of Skeletal Muscle Stem Cells and Primary Cultures

1. 48-well plates or appropriate plastic ware, as above.
2. Glass coverslips.
3. PBS.

2.4.1. Preparation of Cells

4. 4% Paraformaldehyde prepared in sterile PBS (*see* **Section 3.4.2**).
5. Rocking platform.
6. 4°C Fridge for storage.

2.4.2. Preparation of Paraformaldehyde Fixative

1. Fume hood.
2. Gloves and mask.
3. Weighing scales.
4. Magnetic stirring platform with heated stage.
5. Magnetic stirrers.
6. Glass bottle.
7. PBS.
8. Powdered paraformaldehyde.
9. Thermometer.

2.4.3. Apoptosis and Proliferation Assay

1. Cells fixed onto coverslips with paraformaldehyde (**Section 2.4.1**).
2. 10 μg/ml 4′,6-Diamidino-2-phenylindole, dihydrochloride (DAPI).
3. Vectashield fluorescent mounting fluid (Vector Laboratories).
4. PBS.
5. PBS/glycine (0.05 M glycine in PBS).
6. Silver foil and –200°C freezer for storage.
7. Fluorescent upright microscope with ultraviolet filter (we use a Nikon Eclipse E600; Nikon, UK).
8. Digital camera and imaging software (we use a Coolpix 995 camera; Nikon, UK and OpenLab4.0a software; Improvision, UK).

2.4.4. Immunohistochemistry

1. Cells fixed onto coverslips with paraformaldehyde (**Section 2.4.1**).
2. Primary antibody.

3. Tyramide signal amplification (TSA) kit (PerkinElmer), including proprietary TNB buffer.
4. Sodium citrate buffer: 4.7 g sodium citrate in 1.6 l distilled water, pH 6.
5. Pressure cooker.
6. 30% Hydrogen peroxide (should be less than 3 months old and stored in the fridge).
7. Tween 20.
8. PBS.
9. Appropriate biotinylated second antibody.
10. 3,3′-Diaminobenzidine (DAB) tablets.
11. Foil-covered tray, gloves, bleach and beaker for disposal of waste at the DAB incubation stage.

2.4.5. Differentiation

1. Living cells plated onto coverslips (**Section 2.4.1**).
2. Differentiation medium: DMEM, 0.5% FCS supplemented with 2% horse serum and 1% glutamine.
3. 37°C Humidified incubator.
4. 4% Paraformaldehyde.
5. PBS.

2.4.6. Transfection of SMSc: Expression of Transgenes and shRNAi Constructs

1. 8-well chamber slides (Nunc Plasticware, UK).
2. DF10 medium.
3. Incubator, laminar flow hood, microscope (**Section 2.1**).
4. DNA construct (e.g. shRNAi in pSHAG vector or transgene).
5. Lipofectamine 2000 (Invitrogen).
6. Serum-free DMEM supplemented with 2 mM glutamine.
7. Sterilised Eppendorf tubes.

3. Methods

Microdissected explant cultures can be used to reliably and reproducibly isolate cell populations containing a very high proportion (~85%) of proliferative Myf-5-positive skeletal muscle stem cells (SMSc). Under the rigorously controlled culture conditions described here primary explant cultures can be used to characterise the growth behaviours of genetically mutant mouse SMSc and can be used as a means of generating myotubes for detailed in vitro analysis of differentiation processes. Careful maintenance

and manipulation of these cultures enables long-term culture and expansion. Using the methods described here it is also possible to derive clonal skeletal muscle stem cell lines from explant cultures by means of single cell dilution. To achieve the proliferation of isolated single cells during the cloning procedure, "conditioned medium" is used to mimic the normal requirement of these cells for high-density culture. The method is applicable (with modification) to embryonic, adult and aged-adult tissues and in addition to mouse can be used to isolate cells from the skeletal muscles of other species including human (Rao and Smith, unpublished) and fish (salmon) (24). Clonally derived SMSc can be analysed in vivo by intramuscular transplantation and under these conditions injected SMSc will combine with host myotubes to form hybrid muscle fibres. Intramuscularly injected SMSc do not form tumours and have been found in host muscles in the satellite cell position more than a year after injection, suggesting that they are subject to endogenous control by the satellite stem cell niche. These cells can be re-isolated from injected hosts as proliferative SMSc more than 12 months after host injection (19).

3.1. In Vitro Cell Culture of Skeletal Muscle Stem Cells (SMSc)

SMSc are cell lines of single cell origin which have been clonally derived from primary skeletal muscle explant cultures. They can be cultured using standard tissue culture methodology if sufficient care is taken. Note that, unless indicated otherwise, all manipulations described are carried out under aseptic conditions using a laminar flow hood (Class 1 or Class 2 sterile cabinet) and all culture reagents are warmed to 37°C in a water bath before use.

1. To bring SMSc from liquid nitrogen storage (*see* **Section 3.1.2** for freezing down method) cryovials should be thawed rapidly and the contents transferred into 5 ml pre-warmed (37°C) DF10 culture medium for immediate centrifugation (1,000*g* for 3 min) to remove DMSO. The best method to thaw cells is by means of repeat pipetting of small quantities of pre-warmed culture medium into the vial before transferring to the centrifuge tube. The process of thawing cells should be carried out very quickly since cryopreserved cells contain 10% DMSO which is toxic to cells at room temperature (LD50 approximately 2 min).
2. Following centrifugation the supernatant is removed and the cells are washed by re-suspending the cell pellet in a further 5 ml DF10, then centrifuge as before.
3. The cell pellet is then mixed for the second time with 5 ml of DF10 and the resulting cell suspension is transferred to a small 25 cm^2 plastic culture vessel.
4. Cultures are maintained at 37°C in a humidified incubator containing 5% CO_2 in air. Unless vessels are used with a filtered cap, the cap of the flask must be slightly loosened for

several hours to allow air in the culture vessel to equilibrate with the incubator and acidify the culture medium. pH of the medium is monitored by means of incorporating a phenol red dye pH indicator into the culture medium.

5. Thawed cells must always be monitored 24 h after plating and re-fed with fresh DF10 medium to ensure removal of cell debris and residual toxins (*see* **Notes 1** and **2**).

3.1.1. Subculture

For established SMSc lines, when cells reach approximately 95% confluence, they should be removed from their culture vessel, diluted and placed into a fresh vessel to enable further growth. This subculture procedure can be achieved by means of a number of different enzymatic procedures, trypsin/EDTA being the most frequently used (*see* **Note 3**). It is usual (and good) practice to grow cells at densities which require them to be subcultured on the third day of growth. For most SMS cell lines this can be achieved by splitting cells 1/10 at each subculture. This allows careful monitoring of cells and enables those performing the tissue culture to immediately identify unusual growth behaviour (for example faster growth) which could indicate phenotypic changes to the cell line such as transformation or reduction in apoptosis caused by adaptation to culture conditions. Additionally, a consistent and careful subculturing routine vastly reduces the incidence of such events.

1. For subculture using trypsin (trypsinisation) vessels are removed from the incubator and their medium discarded by aspiration.
2. Cells are then washed twice with sterile calcium- and magnesium-free phosphate-buffered saline (PBS), 10 ml per wash, removed each time by aspiration.
3. To dissociate the cell monolayer (25 mm^2 flask) 1 ml 1× trypsin/EDTA is added and left on the cells at room temperature for 2–3 min until the cells begin to detach (*see* **Note 4**). This can be seen by the experienced user as small holes forming in the slightly opaque monolayer when the flask is held to the light (*See* **Note 5**). Whilst cells should be trypsinised for sufficient time to ensure a single cell suspension, care should be taken not to overexpose SMSc to trypsinisation since this will result in high levels of cell death and poor attachment when cells are re-plated.
4. To stop the trypsin reaction, serum-containing medium (DF10) is added at a minimum of 2× volume (i.e. twice the volume of the trypsin solution). When subculturing a 25 mm^2 flask it is convenient to add 9 ml DF10 at this stage. A 1/10 split of cells can then be easily made by diluting 1 ml of the resulting cell suspension into a new 25 mm^2 culture flask together with a further 9 ml of fresh DF10 medium.

The remaining cells can be used for cell expansion (transfer to a larger vessel), cryopreserved (*see* **Section 3.1.2**) or counted and plated in experimental dishes, wells or plates for proliferation and survival assays, differentiation, growth factor treatment or other purposes (see below).

3.1.2. Cryopreservation of Cell Lines and Primary Cultures

1. For cryopreservation cells are dissociated from their monolayer as described for subculture (**Section 3.1.1**) and pelleted by centrifugation (3 min at 1,000*g*).
2. The supernatant is removed by aspiration and cells are carefully and quickly re-suspended in 10 ml of freeze down mix (10% DMSO in DF10) before they are re-pelleted by centrifugation.
3. This time the pellet is re-suspended in sufficient freeze down mix to allow 0.5 ml of cell suspension per cryovial (*see* **Table 3.1**) and immediately placed into –80°C overnight.
4. Cryovials are transferred to liquid nitrogen the following day for long-term storage (*see* **Note 6**). As with thawing of cells, this process of cryopreservation must be carried out speedily. Whilst DMSO is protective for cell membranes during freezing it is very toxic to cells at non-freezing temperatures.

Table 3.1
Calculation table to estimate the number of cryovials required for maximum cell viability during cell freeze down protocols

Plate/flask/chamber slide	Volume of culture medium	PBS washes	Volume of trypsin	Number of freeze down vials (*see* Section 3.1.2)
96-well	50 µl	2 × 100 µl	25 µl	N/A
48-well	150 µl	2 × 200 µl	50 µl	N/A
24-well	500 µl	2 × 700 µl	200 µl	N/A
6-well	3 ml	2 × 3 ml	500 µl	1
Small (25 cm^2)	10 ml	2 × 10 ml	1 ml	2
Medium (80 cm^2)	10 ml	2 × 10 ml	3 ml	4
Large (175 cm^2)	10 ml	2 × 10 ml	5 ml	8
Chamber well	500 µl	2 × 500 µl	100 µl	N/A

N/A, not applicable; cell numbers were too low to freeze down unless multiple wells were frozen down together.

3.1.3. Determining Cell Numbers

Accurate determination of cell numbers is an essential prerequisite for the setting up of experimental assays using SMSc. It is also a useful way of monitoring the growth of newly established microexplant cultures and cell lines.

1. To determine the cell concentration of a single cell suspension (following subculture) a Neubauer haemocytometer can be used. To ensure accuracy of counting the coverslip must be securely mounted onto the haemocytometer base (*see* **Note 7**).
2. A small drop of cell suspension is then placed close to the edge of the coverslip and will be taken up by capillary action.
3. Cells are then counted using an inverted microscope with phase-contrast illumination. To increase the accuracy of the final cell concentration, the cells in the counting chamber should not overlap, if they do the original cell suspension should be diluted and the cells re-counted. Clumping should be avoided by thorough dissociation of cells during trypsinisation and 100–200 cells over a known surface area should be counted to obtain an accurate estimate of cell number. A convenient method using the Neubauer haemocytometer is to count cells in 2 or more 16 square sets. Cell density per millilitre is then obtained by dividing the total by the number of sets counted (e.g. 2) and multiplying by 10^4. For example, 100 cells counted over 2×16 square sets $= 100/2 = 5 \times 10^5$ cells/ml.

3.2. Establishing Primary Skeletal Muscle Microexplant Cultures

Primary microexplant culture can be used to isolate SMSc from any accessible skeletal muscle including the individual muscles of the fore and hind limbs, diaphragm, back and abdominal muscles. The method for deriving microexplant cultures from juvenile and adult muscles is described in detail by Smith and Schofield (3) and has subsequently been used extensively to derive SMS cells from juvenile, adult and aged mouse muscles. The method can also be used to derive cultured skeletal muscle cells from fish (24) and human skeletal muscle (Rao and Smith, unpublished). Outgrowth of SMSc from a mouse muscle microexplant is illustrated in **Fig. 3.1a, b**. The method has been modified for the isolation of embryonic muscle precursor cells (*see* **Section 3.3**). The basic method is as follows:

1. Aseptic dissection of target muscle(s) from a freshly culled mouse is achieved using sterile instruments, a clean working area and liberal use of 70% ethanol spray.
2. Isolated muscles are washed through two changes of DF20 medium and are placed into fresh DF20 medium in a 60 mm^2 dish. Using a stereo dissection microscope muscles are carefully microdissected under sterile conditions to exclude fat, connective tissue and bone.
3. Cleaned muscle pieces are then cut into 400 μm^3 cubes which, using jeweler's forceps, are placed individually into the centre 60 wells of a 96-well plate containing 50 μl

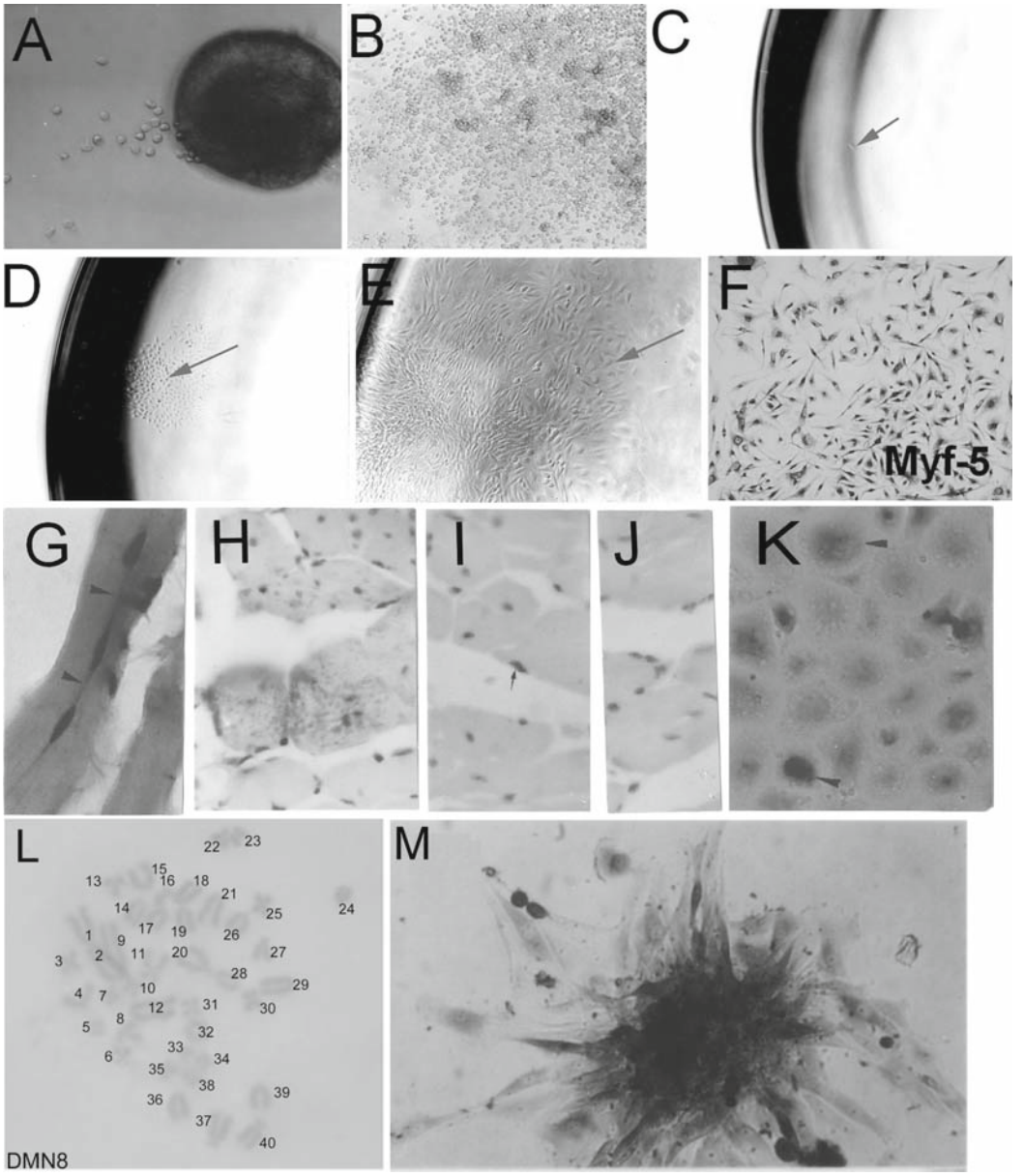

Fig. 3.1. Isolation of skeletal muscle stem cells (SMSc) from microexplants: (**a**) Early outgrowth from explanted adult skeletal muscle (day 2). (**b**) Established explant outgrowth showing aggregated cultures and high cell density. Clonal derivation of SMSc. (**c**) Single cell isolated into a 96 well plate. (**d**) Colony of single cell origin. (**e**) Established clonal population. (**f**) Verification of SMSc identity using Myf-5 immunohistochemistry. Cells derived from SMSc clone PD50A (expressing β-galactosidase) in host mice at 3 months (**g**) and (**h–j**) 14 months post-injection of 2,000 PD50A cells into mouse tibialis anterior muscle. (**g**) Three recently fused (centrally located nuclei) β-galactosidase-positive cells (*blue stain*) in muscle fibre (*longitudinal section*). (**h**) Extensive contribution of β-galactosidase-positive cells (*brown stain*, detected by anti-β-galactosidase antibody) in muscle fibres (*transverse section*). (**i**) β-Galactosidase-positive satellite cell (*brown stain*, detected by anti-β-galactosidase antibody). (**j**) Secondary antibody control (no staining). (**k**) β-Galactosidase-positive cells (*blue stain*) proliferate in culture when isolated from injected host muscles 12 months post-injection. (**l**) Karyotype of a mouse clonal SMSc line (DMN8) showing normal diploid chromosome complement. (**m**) Histochemistry showing β-galactosidase expression in a colony of PD50A cells (**Fig. 3.1 g–k**, reproduced with permission from AACR press, Smith and Schofield, 1997).

DF20 (*see* **Note 8**). Wells are checked under the microscope and placed in the incubator. The outer wells are filled with saline to prevent drying out of wells containing explants (3).

4. Microexplant attachment and outgrowth is scored after 24–48 h incubation and thereafter at 48–72 h intervals (depending on the rate of growth of the muscles being cultured).
5. For expansion and isolation of SMS cells, outgrowth cultures should be individually monitored for cells with a predominantly SMSc morphology, i.e. spherical mononucleate cells with high refractivity which grow in aggregating clusters (*see* **Fig. 3.1b**).
6. Once explant outgrowth is established, individual cells are fed (*see* **Note 9**) by the addition of 50 μl increments of medium when the medium acidifies due to increased cell density. When the well is full and cell culture almost confluent, wells are fed by replacement of 50% of the medium each time to ensure the maintenance of "conditioning" factors secreted by the cells (*see* comments on conditioned medium under cloning; **Section 3.2.1**). To suppress differentiation, at 60–70% confluence primary explant outgrowths are switched into calcium-depleted medium by substituting DF20 medium for calcium-depleted DMEM/F12 (all supplements remain the same) for feeding cells (3).
7. Explant conditioned medium can be prepared from cultures at this stage and stored for use during expansion and cloning of primary SMSc (for cloning method, *see* **Section 3.2.1, Fig. 3.1c–f**). Cultures are subcultured using the dispase method (*see* **Section 3.3.5**).
8. Clonally derived SMSc can be analysed in vivo by intramuscular injection (**Fig. 3.1 g–k**) (19).
9. Karyotyping can be carried out on clonally derived SMSc lines to confirm diploid status (*see* **Section 3.2.2, Fig. 3.1 l**) (25).
10. This method can be adapted for culturing embryonic muscle (**Section 3.3**).

3.2.1. Clonal Derivation

Primary explant myoblast cultures (**Fig. 3.1a, b**) are a useful and accurate tool for establishing a variety of different growth parameters in wild-type and mutant skeletal muscles. Clonal derivation, the isolation of a cell line from a single cell, is an essential step in the isolation of skeletal muscle stem cells and can also be used to subclone SMSc lines transfected with RNAi constructs or transgenes.

Established SMSc and primary explant cultures are highly density dependent and will "crash" (detach from the dish and die) if plated out at too low a cell density. This is because SMSc release soluble factors which are required to maintain growth and cell survival. To simulate a high-density culture and supply these factors during the cloning process, SMSc are cloned in self-conditioned medium. The addition of conditioned medium was found to be essential to allow individual cells to proliferate in an isolated environment.

1. Conditioned medium is prepared from proliferating SMSc cultured for 48 h between 33 and 75% confluence.
2. The media in which these cells are grown is removed after 48 h and filtered using a 0.2 μm syringe filter; this ensures sterility of the conditioned medium and removes all residual cells and debris.
3. This conditioned medium is mixed in a 1:1 ratio with fresh culture medium (cloning medium; *see* **Note 10**) and is used as the culture media for single cell cloning.
4. To achieve single cell dilutions cells are dissociated to a single cell suspension by careful trypsinisation (established SMSc) or dispase treatment (primary explant cultures) and diluted to a concentration of one cell per 100 μl cloning medium.
5. 50 μl of this cell suspension can then be plated into each of the centre 60 wells of a 96-well plate.
6. Cells are allowed to attach by incubation at 37°C in 5% CO_2 for 6 h and each well is then carefully scored for the presence of cells. Wells containing 0 or more than one cell are discounted at this stage.
7. Wells containing one single attached cell are carefully noted and the colony derived from this single cell is carefully monitored (daily during the first few days) to ensure that only one colony, derived from a single cell, is present. Cell lines were only derived from wells containing 1 cell. **Figure 3.1c–e** illustrates the expansion of such a single cell-derived colony.
8. Once the colony reaches confluence in the 96-well plate it can be subcultured into one well of a 48-well plate.
9. The cloned cell lines can then be carefully expanded into 24- and 6-well plates until sufficient cells are available to plate into a 25 cm^2 flask.
10. The skeletal muscle origin of these cultures can be demonstrated by the expression of Myf-5 (**Fig. 3.1f**) or other skeletal muscle-specific markers such as MyoD and Pax 7.
11. At this stage lines are frozen down before further expansion (*see* **Table 3.1**).

3.2.2. Karyotyping

Karyotyping is an important method of monitoring cell phenotype. Cell lines derived by clonal derivation should be karyotyped to ensure that they have retained a diploid chromosome complement without gross chromosomal rearrangements which could affect their phenotype.

1. For karyotyping cells are grown to late exponential phase (80% confluent) in 25 cm^2 culture vessels (2 days after subculturing) to maximise the proportion of mitotic cells in the culture.
2. Twenty-four hours before karyotyping the cells are fed with 10 ml fresh culture medium. 0.2 ml of 10 mg/ml colchicine (*see* **Note 11**) is then added to the cells which are incubated for a further 1 h at 37°C.
3. After 1 h, the cells are subject to the standard trypsinisation subculture procedure except that both the culture medium and PBS washes are retained to maximise the number of mitotic cells harvested.
4. The dissociated cells, retained medium and PBS washes are spun at 1,000*g* for 3 min to pellet the cells and the supernatant removed and discarded into bleach.
5. The cell pellet is then re-suspended in 5 ml of 0.0075 M potassium chloride for exactly 4 min, before cells are again pelleted by centrifugation.
6. Most of the supernatant is aspirated, leaving a small amount (~50–100 μl) in the tube for re-suspension. Resuspend cells by flicking the base of the falcon tube until a cell slurry is achieved. The cells are then placed on ice and fixed in freshly made ice-cold fixative (methanol: glacial acetic acid in a 3:1 ratio) as follows: 10 ml of fixative is slowly added dropwise to the cells using a small glass Pasteur pipette (this prevents cell clumping).
7. Cells are placed on ice for 30 min and then pelleted by centrifugation after which the cell pellet is re-suspended in 0.5 ml fresh fixative.
8. Slides are produced by dropping the fixed cell suspension onto prepared slides (*see* **Section 3.2.2.1**) held at an angle of 45°. To ensure well-spaced metaphase spreads the pipette should be held at least 30 cm above the slide.
9. To visualise chromosomes, slides are stained for 2 min in Leishman's Stain, diluted with three volumes of Gurr buffer pH 6.8 just prior to use.
10. Slides are dried at room temperature and mounted in DePex mounting medium.

3.2.2.1. Preparation of Slides for Karyotyping

1. Glass slides (Premium microscope slides, VWR International, UK) are prepared for use in the karyotyping protocol by placing them overnight in a large (glass) container of sulphuric acid.
2. Slides are then placed under running tap water for 8 h and are then stored in 70% ethanol until required.
3. Before use, slides should be rinsed under running tap water for a further 30 min and air-dried at room temperature for 1–2 h.

3.3. Establishing Primary Microexplant Cultures from Embryos

Three mouse strains were used to validate this method, wild-type (C57BL/10) together with *mdx* and *CAV3KO* (both dystrophic mutants). The dystrophin-deficient *mdx* mouse originated spontaneously in C57BL/10, this line was obtained from the Bullfield laboratory in 1991 and has since been continually maintained in our inbred colony (26). *CAV3KO* dystrophic mice, which contain a mutation in the *caveolin-3* gene, were bred onto the C57BL/10 background for 10 generations before being used in this study (27). Each mouse line generated a robustly reproducible outgrowth, proliferation and survival profile which was embryonic stage specific and different for each strain. The following protocols were adapted for embryos from Smith and Schofield PN (1994) (3), essentially as in Merrick (21).

3.3.1. Embryo Collection

1. To obtain staged embryos, pairs are set up as natural (1:1) matings and females checked every morning for vaginal plugs. On the day of plug detection, embryos are counted as E0.5 days (12 h post-fertilisation).
2. Once vaginal plugs have been detected the males are removed from the cage to ensure accuracy of embryonic staging.
3. When the desired embryonic stage is reached (E11.5–E17.5) the mothers are killed by cervical dislocation, the abdomen is shaved, the skin and surrounding areas are swabbed with 70% alcohol and the uterus is removed via a horizontal abdominal incision made using sterile dissecting instruments.
4. The uterus is then washed once in primary explant culture medium (PECM) before being placed into a small dish containing fresh PECM prior to dissection.
5. E11.5–E17.5 embryos are dissected from the uterus using a dissecting microscope and placed individually into Petri dishes containing PECM in readiness for detailed microdissection.

3.3.2. Embryo Microdissection

1. Individual embryos are further dissected to isolate areas rich in skeletal muscle (*see* **Fig. 3.2a**). Hind and forelimbs (hypaxial skeletal muscles) are dissected out, as well as the upper and lower body wall (predominantly epaxial skeletal muscles). To do this an incision along the length of the thorax, abdomen and pelvis is created to allow the internal organs of the embryo to be removed.
2. To enrich for embryonic skeletal muscle stem cells (eSMSc), the head, spinal cord and all internal organs are then removed.

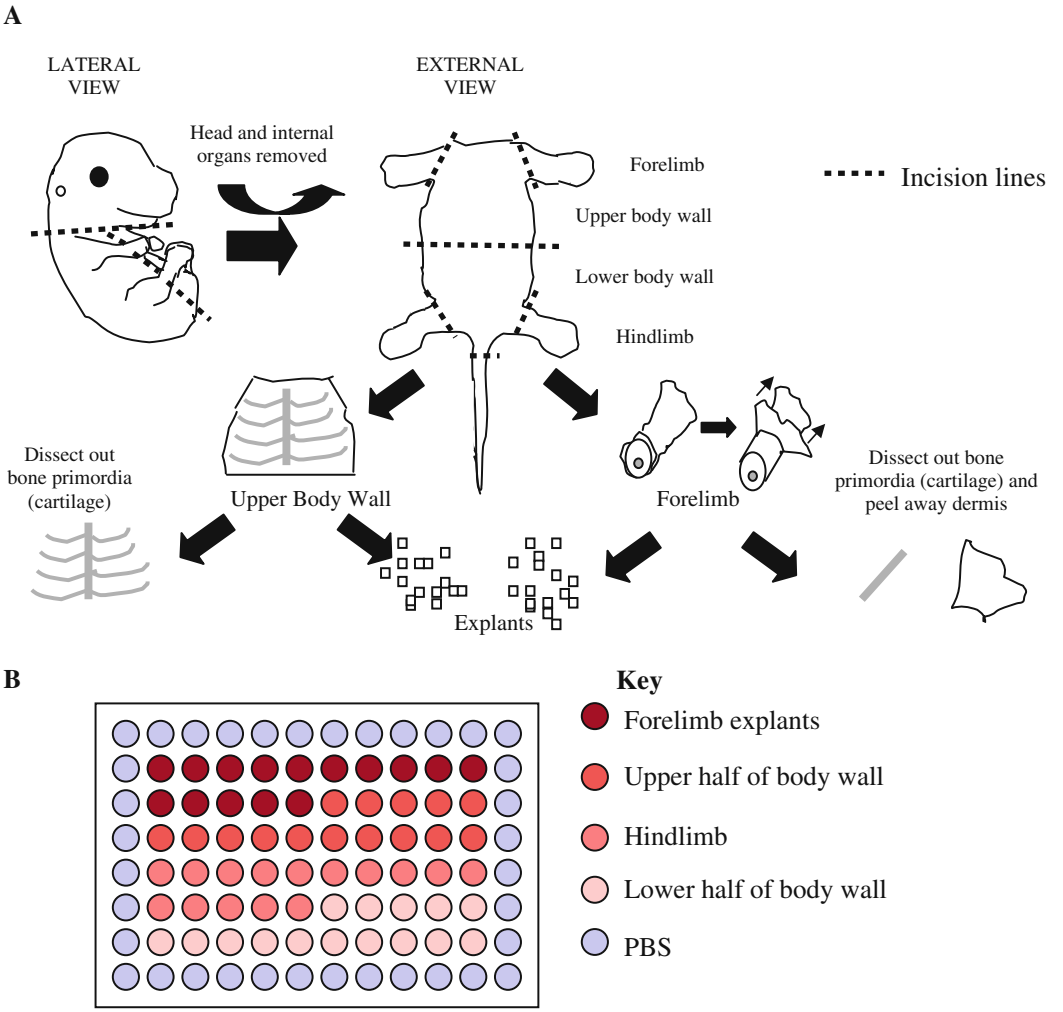

Fig. 3.2. (**a**) Illustration of the embryo dissection process. The figure represents an E15.5 embryo where bone primordia (cartilage) could easily be identified and dissected free of the surrounding skeletal muscle tissue. At this stage, and in later stage embryos (E15.5–E17.5), dermis was also removed to maximise the proportion of skeletal muscle cells obtained. (**b**) Setup of primary explant cultures in a 96-well plate. Each embryo was used to produce one plate as seen above. Usual practice is to plate replicates of three separate embryos (three plates = 180 wells) to establish outgrowth rates.

3. In older embryos (E15.5–E17.5 embryos) it is also possible to remove skin and cartilage/bone again to increase the proportion of muscle cells in the cultures.

3.3.3. Setting Up Embryo Microexplant Cultures

1. Once forelimbs, hindlimbs and upper and lower body walls have been dissected out they are placed into fresh PECM and further microdissected to produce small cubes of tissue of equal size (~0.5 mm^3; **Fig. 3.2a**).
2. These microexplants are then put into the centre 60 wells of a 96-well plate (one explant per well) containing 50 μl PECM per well. A minimum of 60 wells containing 1 explant per well are established, per embryo studied.
3. For culturing embryos the centre 60 wells can be subdivided into regions denoting where the explant was derived from (**Fig. 3.2b**). This design allows 15 wells each containing, respectively, forelimb, upper body wall, hindlimb and lower body wall explants (21).

3.3.4. Monitoring Outgrowth

Outgrowth rate is a reliable measure of the growth rate of embryonic skeletal muscle explants and under the carefully controlled conditions described here is highly reproducible.

1. Explants are incubated at 37°C and 5% CO_2 for 3 weeks and scored on the 3rd, 7th, 14th and 21st day of culturing using an inverted microscope. Explants are scored according to the level of confluence of cells in each individual well (**Fig. 3.3a–e**).
2. Photographic images of cultures can be taken, for example, using an SLR camera attached to the microscope and 100 ASA Fuji (colour) or Kodak TMAX (black and white) professional film (**Fig. 3.3f**).
3. A skeletal muscle-specific antibody specific for Myf-5 can be used to demonstrate the skeletal muscle origin of eSMSc, depending on strain 80–95% of cells isolated using this method are Myf-5 positive. Other markers such as MyoD and Pax 7 can also be used to demonstrate the skeletal muscle origin of these cell populations.

Whilst these cells contain a very high proportion of embryonic skeletal muscle stem cells it cannot be assumed (especially for younger embryos) that they are all of skeletal muscle origin or that they are all stem cells. To isolate pure stem cell populations it is necessary to clonally derive primary explant cultures as described in **Section 3.2.1**.

3.3.5. Subculturing Primary Embryonic Explants

Once confluent, explant cultures displaying the morphological features of SMSc (**Section 3.2, Fig. 3.3f**) can be subcultured as follows (3, 21):

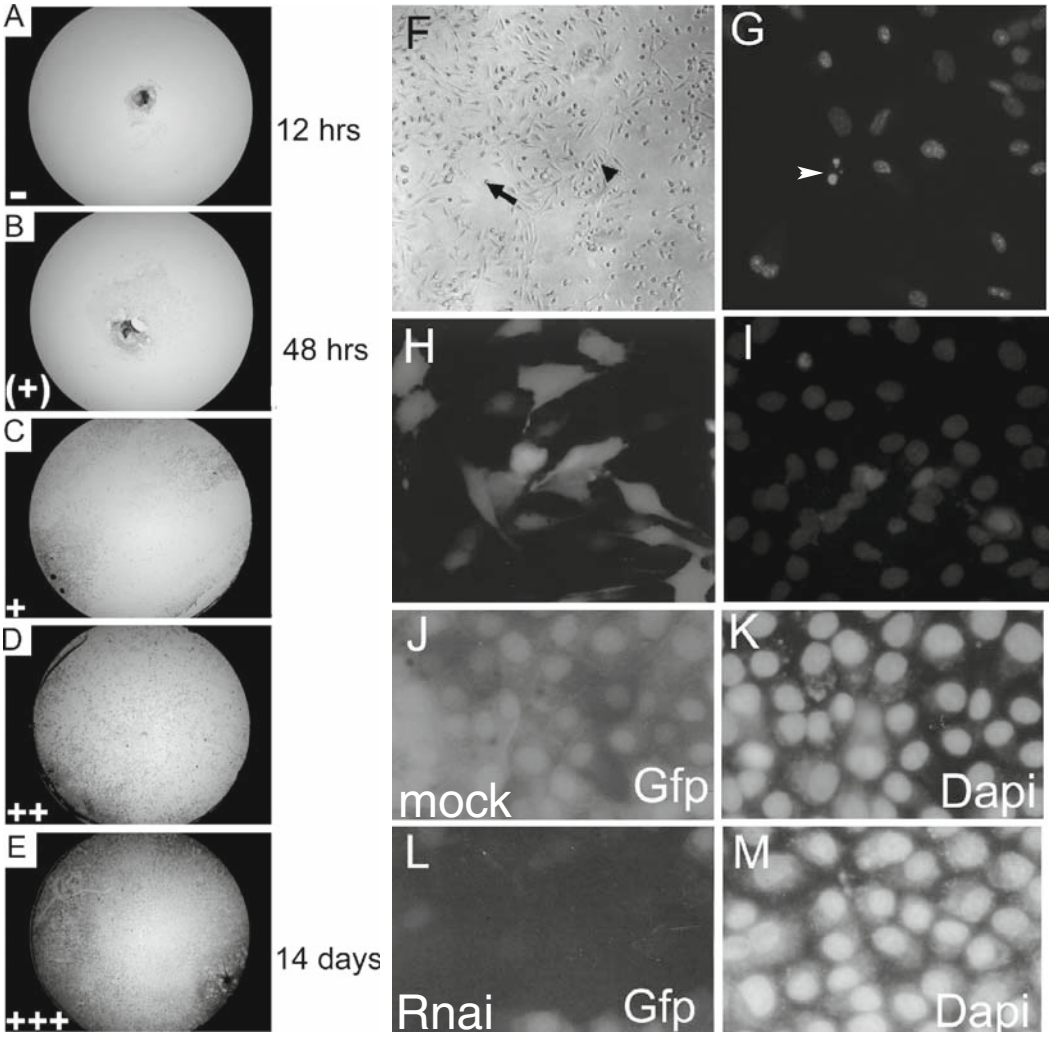

Fig. 3.3. Embryonic primary explant cultures were scored at 3, 7, 14 and 21 days of culture and assigned an outgrowth level representative of confluence level. (**a**–**e**) C57BL10 E15.5 primary embryonic explant cultures stained with Myf-5 to illustrate 0–14% (–); 15–24% (+); 25–49% (+); 50–74% (++); 75–100% (+++) levels of confluence. The proportion of wells showing each level of outgrowth (on each day of scoring) was multiplied by an arbitrary number (– = × 1; (+) = × 2; + = × 3; ++ = × 4 and +++ = × 5) before collating data to give a final outgrowth value. Approximately 85% of wild-type (C57BL/10) primary eSMSc stain for the skeletal muscle cell marker Myf-5. Magnification is ×10. (**f**) Established embryonic cultures have the morphological characteristics of adult SMSc, bipolar cells (*small arrow*) and spherical monomorphic cells (*large arrow*). (**g**) Identification of fragmenting apoptotic nuclei using DAPI staining. (**h**–**i**) High levels (~75%) of transfection of a GFP-expressing construct into SMS cell lines using the optimized Lipofectamine 2000 transfection method. (**i**) Counting of total cell number is aided by DAPI counterstain. RNai using pSHAGshRNAigfp (**j**–**m**) abolishes GFP expression in SMSc (*see* (29) for an example of this construct used as a control). (**j**) Control (mock transfection) showing GFP expression in a GFP SMSc line. (**k**) DAPI control. (**l**) shRNAiGfp 24 h after transfection. (**m**) DAPI control for shRNAiGfp transfected cells in (**l**).

1. Culture medium is removed from the selected wells, filtered using a 0.2 μm Acrodisc® syringe filter and retained for use as conditioned medium. The medium can be stored at 4°C for 1 week.

2. 100 μl of dispase diluted 1:10 in PECM is added to each well and plates then returned to the 37°C incubator for 20 min.
3. A pipette tip is then used to gently scrape the loosened cells from the surface of the well.
4. The cell suspension is then centrifuged at 1,000*g* for 3 min to pellet the cells and the supernatant is removed and discarded.
5. Cells are re-suspended in 200 μl of 1:1 mix of conditioned medium and PECM.
6. The cell mix is transferred to 48-well plates for further expansion.
7. For in vitro analysis cells can be plated at a density of 5×10^3 cells/cm^2 either in 48-well plates (each containing a 9 mm^2 sterile glass coverslip) or in 8-well-glass chamber slides. For differentiation analysis cells are grown overnight to 50–60% confluence before they are transferred to differentiation permissive medium (*see* **Section 3.4** for in vitro method details) for 3 days, before fixation.

3.4. In Vitro Analysis of Skeletal Muscle Stem Cells and Primary Cultures

1. Dispase subcultured (**Section 3.3**) primary embryonic explant cultures are plated in PECM/conditioned medium onto coverslips in 48-well plates at a density of 3×10^3 cells/cm^2 and allowed to attach.

3.4.1. Preparation of Cells

2. For assessment of apoptosis and proliferation coverslips are washed twice in PBS, fixed in 4% paraformaldehyde (*see* **Section 3.4.2**) in PBS for 20 min at room temperature, followed by a further 10 min PBS wash.
3. Coverslips prepared this way can be stored at 4°C for up to 1 week in PBS or PBS/glycine.

3.4.2. Preparation of Paraformaldehyde Fixative

1. In a fume hood, weigh 4 g paraformaldehyde (PFA; Sigma-Aldrich, UK) and add to a glass bottle of 100 ml of sterile PBS with a magnetic stirrer. A face mask and gloves should be worn for protection.
2. In a fume hood, the solution is heated and continually stirred on a magnetic hotplate until the powder dissolves. This takes approximately 5–10 min at 65°C. Care must be taken to prevent the temperature rising above 70°C, as there is a risk of the solution exploding at high temperatures.

3.4.3. Apoptosis and Proliferation Assay

1. Fixed coverslips (prepared as in **Section 3.4.1**) are stained with 10 μg/ml DAPI for 3 min.
2. Coverslips are washed once in PBS (5–10 min) and inverted onto a spot of vectashield mounting medium on a glass slide (17, 18).

3. The edges of the coverslip are sealed with nail varnish (*see* **Note 12**).
4. For storage, slides are wrapped in foil and placed at –20°C.
5. For counting, slides are viewed under fluorescence (UV filter) on an upright microscope and scored for apoptotic and mitotic cells using an eyepiece graticule. Twenty randomly distributed grids are counted (representing ~1,000 cells), and cells are morphologically characterised as non-apoptotic, apoptotic or mitotic (**Fig. 3.3 g**).
6. Mitotic and apoptotic indices are calculated as a proportion of total cells.

3.4.4. Immunohistochemistry

Cells fixed onto coverslips can also be used for immunohistochemistry. For antigen retrieval using a pressure cooker coverslips must be firmly attached to glass slides using standard paper clips. Immunostaining can be used to identify proliferating cells, using an antibody to Ki67 (1/1,000 dilution), to establish identity, using an antibody to Myf-5 (1/1,000 dilution), or to investigate gene expression (*see* **Section 3.4.5**). Immunostaining can be achieved using a number of methods, the following (described in (28, 29)) is used routinely by the authors:

1. Sodium citrate buffer is pre-heated in the pressure cooker. For antigen retrieval, slides containing sectioned tissue are placed into the heated buffer and heated under pressure for 2 min. Pressure is achieved by firmly locking the pressure cooker lid and placing on the weight. Once the 2 min retrieval time has elapsed the pressure cooker is then carefully placed under running cold tap water to reduce pressure. To prevent the buffer boiling up, care should be taken not to remove the lid until pressure has equalized with atmospheric pressure. Pressure is sufficiently reduced when the weight can be removed easily (without force) and the lid removed. Slides are then removed from the buffer and washed in room temperature PBS for 10 min.
2. Slides are pre-blocked by immersing them in 3% hydrogen peroxide/tap water for 5 min and then washed three times in PBS + 0.05% Tween 20 (10 min per wash).
3. Blocking is achieved by a 30 min incubation in TNB blocking buffer (supplied in TSA kit) at room temperature.
4. Primary antibody is diluted in TNB buffer to the appropriate dilution (arrived at by titration, *see* **Note 12**) and incubated overnight at 4°C (or alternatively 1–2 h at room temperature).
5. Following three 10 min washes in PBS + 0.05% Tween 20, slides are incubated for 1 h at room temperature in the

appropriate biotinylated second antibody diluted in TNB buffer.

6. Following a further three 10 min washes in PBS + 0.05% Tween 20, slides are incubated for 30 min in Streptavidin-HRP (provided in the TSA kit) diluted 1:100 in TNB buffer and then washed three times (10 min each) in PBS + 0.05% Tween 20.
7. Biotinyl tyramide (amplification reagent; TSA kit) is then added to each section for between 8 and 15 min (the precise time should be obtained by optimisation experiments).
8. Following amplification, wash slides three times (10 min each) in PBS + 0.05% Tween 20 and then incubate for 30 min in SA-HRP.
9. Following three further washes (10 min each) in PBS + 0.05% Tween 20, visualise using 3,3′-diaminobenzidine tetrahydrochloride chromogen (DAB) for 5–10 min. Then carry out two final washes in water before counterstaining slides in haematoxylin and coverslipping. DAB is a known carcinogen and should be handled with care (as for Colchicine, **Section 3.2.2**).

3.4.5. Differentiation

1. SMSc plated onto coverslips or chamber slides (*see* **Note 13**) can also be differentiated before fixation for myotube analysis.
2. For these experiments cells are plated at a density of $10^4/cm^2$ and allowed to attach for 6–8 h.
3. Cells are then switched in to differentiation permissive conditions for 3 days (*see* **Note 14**).
4. Differentiation medium is composed of DMEM + 0.5% FCS supplemented with 2% horse serum and 1% glutamine. This differentiation permissive culture medium is replaced at 48 h intervals.
5. Coverslips are then fixed in 4% paraformaldehyde as above (**Sections 3.4.1** and **3.4.2**).

3.4.6. Transfection of SMSc: Expression of Transgenes and shRNAi Constructs

Stem cells and primary cultures are refractory to transfection and with a majority of methods the transfection rate in SMSc and primary skeletal muscle cells is very low (<10%), preventing the use of transient transfection methods. To overcome this it has been standard practice in our laboratory to isolate clonal derivates from transgene transfected cultures (*see* **Section 3.2.1**) following transfection with calcium phosphate or lipofectamine. Alternatively cells can be efficiently transfected using infection of virally packaged constructs. **Figure 3.1m** shows stable expression of β-galactosidase in PD50A, a clonal SMSc derivative isolated

under G418 selection following infection with pIRV, a replication defective retrovirus carrying the genes for *neo*/G418 resistance and *β-galactosidase* (19). This cell line was used to formally demonstrate that SMSc behave as functional stem cells in vivo (*see* **Fig. 3.1**). Whilst the generation of a stable clonal cell line expressing a marker gene is desirable for in vivo stem cell transplantation experiments, it is a time-consuming and unsatisfactory method of analysing gene function in vitro. For these reasons the authors have recently developed an optimized modification of the Lipofectamine^{TM} 2000 transfection reagent which is capable of delivering transfection rates of 60–70%. This allows the analysis of gene function using transient transfection of transgenes or RNAi constructs into SMSc or primary explant cultures (**Fig. 3.3h, i**).

The authors use a short hairpin RNAi vector (pSHAG RNAi) (30) to generate shRNAi constructs capable of gene-specific targeting of mRNA expression in SMSc. The success of the shRNAi technique depends on two elements: (a) an efficient transfection method and (b) the design of a short hairpin sequence which specifically recognizes the target gene. A shRNAi construct directed to eGFP can be used to validate the RNAi knockdown method (**Fig. 3.3j–m**).

3.4.6.1. *Optimised Lipofectamine^{TM} 2000 Transfection Protocol for SMSc*

1. Cells are plated at 5×10^4 cells/cm^2 into chamber slides in 250 μl DF10 culture medium and cultured for 18 h to reach 95% confluence (optimal confluence for each cell line was established by assessing transfection rates at different densities).
2. For each well, 0.5 μg of DNA (shRNAi vectors, transgenes) is added to 33 μl of serum-free DMEM supplemented with 2 mM glutamine and mixed gently in a sterile Eppendorf tube.
3. For each well, 1.25 μl of Lipofectamine^{TM} 2000 is separately diluted in a further 33 μl of serum-free medium DMEM + glutamine, gently mixed and retained at room temperature for 5 min
4. The DNA and lipofectamine mixes are then rapidly added together, mixed gently for 60 s by pipetting and then incubated at room temperature for 19 min to allow DNA–Lipofectamine^{TM} 2000 complexes to form.
5. For transfection, 66 μl of complex mix is added to each chamber well and slides are gently rocked for 10 s to ensure equal distribution of complexes.
6. Cells are incubated for 24–72 h at 37°C and 5% CO_2. Depending on the construct, functional gene expression or shRNAi knockdown is first detected between 8 and 24 h posttransfection.

4. Notes

1. When thawed cells have attached at very low cell density it is prudent to replace only half the medium to prevent culture crash.
2. SMSc isolated from dystrophic muscle are susceptible to apoptosis and must be treated with particular care. Dystrophic SMSc (such as the dfd-13 cell line, which was established from skeletal muscle obtained from 5-week-old dystrophic (*mdx*) mice) should be grown at higher cell densities than usual for myoblasts. Such apoptosis-sensitive cell lines are also cryopreserved at higher densities (*see* **Sections 3.1.1 and 3.1.2**) (19).
3. An alternative method of removing cells from a monolayer utilises dispase, which provides a gentler method of cell dissociation, with the advantage that it can be carried out in the presence of FCS and calcium (both present in DF10). Dispase can therefore be used to subculture and expand primary skeletal muscle explant cultures and early-stage SMSc clones (*see* **Sections 3.2 and 3.3**).
4. For larger flasks the quantity of trypsin/EDTA used should be scaled up as follows: 75 mm^2 flask (3 ml trypsin) and 175 mm^2 flask (5 ml trypsin). Similarly for smaller surface areas reduce the amount of trypsin used (*see* **Table 3.1**).
5. Alternatively, dissociation can be monitored using an inverted microscope, this is recommended for beginners.
6. Cryopreservation of cell lines is usually carried out using a confluent large (175 mm^2) plastic vessel. Between 7 and 9 cryovials can be obtained from one such large vessel, depending on the survival profile of the cell line being cryopreserved. Primary cultures and newly established cell lines are often very refractory to freeze down procedures. To improve recovery and success of freezing down such cells two approaches can be used (separately or in combination). (A) The FCS content of the freeze down mixture can be increased from 10% (to a maximum of 50%). (B) The freeze down process can be slowed by placing cryovials in the vapour phase of N_2 for 12–24 h before transferring vials to the liquid phase.
7. A convenient way to check firm attachment of the haemacytometer coverslip is to look for Newton's Rings (rainbow reflections in the glass) on the coverslip or alternatively, to hold the haemacytometer upside down over an open hand.
8. A variation of the explant method can be employed for short-term cultures for use in immunohistochemistry,

proliferation or apoptosis assays. Microdissected explants are placed on glass using 8-well chamber slides. An alternative method is to use 9 mm^2 coverslips placed in 24-well plates. In both cases two explants are transferred to each well in 150 μl DF20 medium. Alternatively, primary cultures can be subcultured by the dispase method and plated out onto coverslips placed in 24-well plates or directly into 8-well chamber slides.

9. For quantitative analysis of outgrowth rates a minimum of 60 wells containing 1 explant per well are established, per muscle/mouse strain. To establish growth parameters for a strain at least three separate animals must be used. Plates and individual wells are not fed whilst outgrowth is being scored.
10. For cloning established SMSc lines it is sufficient to culture single cells in a 1:1 mix of conditioned medium and DF10 medium. For primary explants it is necessary to increase the serum content of the culture medium to 20%.
11. Colchicine is highly toxic and a known carcinogen and should be handled with appropriate care. Double gloving and working in the confines of a designated tray is essential. All disposables (i.e. Gilson tips) are placed in a beaker of bleach (5% sodium hypochlorite) overnight before disposal with copious amounts of water the follo -wing day.
12. We currently use primary antibodies specific to Ki67 and Myf-5 each at 1/1,000 dilution. The optimal dilution for primary antibodies needs to be established empirically for each antibody to be used and ideally also for different batches of the same antibody, even when obtained from the same source.
13. Chamber slides can also be used for this assay. Before fixing cells in 4% PFA, the culture medium is removed and cells washed twice with 37°C sterile PBS. The well chambers, gasket and glue are removed and the glass slides placed in a 50 ml glass Coplin jar containing freshly prepared 4% paraformaldehyde at room temperature, which is then gently rocked on a Gyro-Rocker® shaker for 25 min. Slides are then washed twice in PBS (room temperature) and either used immediately or stored in PBS at 4°C (short term; 1–2 weeks) for immunohistochemical (IHC) analysis.
14. If more extensive myotube formation is required primary skeletal muscle cells can be differentiated for up to 8 days.

Acknowledgments

We thank Patrick Paddison for his gift of the shRNAi shuttle vector. Angela Sloan generated the GFP RNAi image in **Fig. 3.3**. We also thank the following funding bodies for their support: Muscular Dystrophy Campaign grant number RA2/592/2; SPARKS grant number 02BHM04, The Royal Society grant number 574006.G503/1948./JE and BBSRC grant number 6/SAG10077.

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Chapter 4

The Embryonic Kidney: Isolation, Organ Culture, Immunostaining and RNA Interference

Jamie A. Davies

Abstract

Embryonic mouse kidneys develop well in organ culture. This, coupled with the fact that renal organogenesis includes a range of developmental processes, has made cultured kidney rudiments a popular model for the study of organogenesis. Although cultured kidneys do not replicate every event that takes place in vivo, they do allow close observation of events as they happen and they allow easy access for experiments that use drugs, antibodies, exogenous growth factors and interfering RNAs. Renal organ culture therefore offers a much quicker method to address certain problems than would the generation of transgenic mice. Requiring only material from freshly killed healthy animals, it also avoids some ethical problems connected with subjecting living animals to treatments (or the effect of mutations) that are harmful.

Key words: Organogenesis, kidney, renal, organ culture, induction, RNAi, siRNA.

1. Introduction

Organogenesis of the mammalian kidney includes a great range of developmental processes that are important features of animal development in general. These include cell proliferation and death, cell movement, cell differentiation, inductive signalling, cell sorting, boundary formation, branching morphogenesis, epithelial fusion, angiogenesis, vasculogenesis and mesenchyme–epithelial transitions. Furthermore, kidneys will develop very well in organ culture where they are accessible to manipulation by experimentally applied drugs, proteins and nucleic acids. These

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two facts together make the developing kidney an excellent model system in which to study the basic biology of organ formation (1).

Congenital abnormality of the urinary system happens to be one of the most common types of congenital abnormality in humans (2). Study of kidney development therefore attracts strong medical interest. The use of organ culture of mutant kidneys to screen for potentially useful drug targets is a growing area of research and the method is likely to be adapted for high-throughout applications in the near future.

Isolation and culture of embryonic kidneys is a procedure simple enough that we often include it in the research projects of final year undergraduates. It does, however, require practice and demands considerable reserves of patience in the early stages. Different laboratories have developed slightly different techniques. The method I shall describe here has been adapted from that which I learned from that late Lauri Saxén and his assistant Anja Tuomi at the University of Helsinki.

2. Materials

2.1. For Isolation of Kidneys

1. Curved scalpel blades.
2. Fine forceps.
3. Two 1 ml hypodermic disposable syringes (**Note 1**).
4. Supply of disposable orange (fine) hypodermic needles.
5. 150 mm sterile plastic Petri dishes (**Note 2**).
6. 30 mm sterile plastic Petri dishes.
7. Glass Pasteur pipettes.
8. Small glass beaker part-filled with methylated spirits.
9. Spirit or gas burner.
10. Dissecting medium (we use Sigma M5650 Earle's MEM; many labs use sterile PBS).
11. Dissecting microscope (**Note 3**).

2.2. For Culture, in Addition to the Above You Will Need

1. Pieces of fine stainless steel mesh, cut into equilateral triangles with sides approximately 2 cm long, with the corners bent down at 90° to form "legs" approximately 3 mm high (**Fig. 4.1**).
2. Pieces of track-etched polycarbonate filter (Nuclepore or Millipore), of any pore size in the range 0.1–5 μm. Cut these into squares of about 4 mm each side, or into more complex shapes if you wish to use the filter shape to identify a sample.

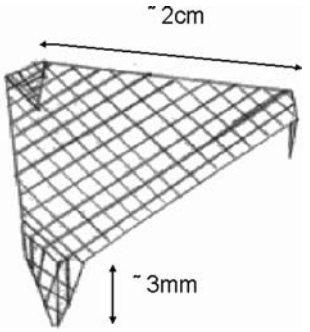

Fig. 4.1. Simple Trowell grids can be produced by cutting stainless steel mesh into triangles and bending down the corners.

3. Culture medium: we use Eagle's MEM with Earle's salts (Sigma M5650) supplemented with 10% heat-inactivated newborn calf serum (NCS/Labtech), penicillin and streptomycin (Sigma) for routine work. Many alternatives exist in the literature.

2.3. For Immunofluorescence

1. 5 ml "Bijou" bottles.
2. Methanol, pre-cooled to –20°C.
3. Phosphate-buffered saline (PBS).
4. A suitable primary antibody (we recommend Sigma polyclonal anti-laminin L9393 for someone wanting to check their culture skills).
5. A suitable secondary antibody (Sigma FITC anti-rabbit is suitable for the above).
6. A mix of 50% PBS:50% glycerol.
7. An epifluorescence or confocal microscope.

3. Methods

3.1. Isolation of Embryonic Kidneys

Most renal organ culture experiments use kidneys isolated from embryos at E10.5 or E11.5 (E0 being the morning of discovery of the vaginal plug in the mother). At E10.5, the ureteric bud has just entered the metanephrogenic mesenchyme but has not branched; choosing this early stage makes separation of bud and mesenchyme relatively easy, but makes finding the kidney rudiment in the embryo quite difficult. By E11.5, the ureteric bud has branched once to make a "T" shape and the kidney rudiment is much easier to find; this stage is therefore very useful for any experiments that do not require separation of bud and

mesenchyme. The technique for dissection is the same. Novices are advised to begin dissecting E12 or even E13 embryos first, where the kidneys are very obvious, and to work backwards to the more difficult stages (**Notes 4** and **5**).

1. Begin by obtaining uteri from mice of an appropriate stage of pregnancy, killing the mice according to the appropriate ethical procedures.
2. Remove the embryos from each horn of the uterus. In this lab, we usually do this in a 150 mm Petri dish in dissecting medium, under the dissecting microscope at low power. At this stage of development, the uterus looks rather like a string of sausages, and one way to remove the embryos is to cut between each "sausage" and gently squeeze the embryo out through the resulting hole by applying pressure externally with forceps (**Fig. 4.2**).
3. Some embryos will emerge without their covering membranes; those that are still covered with their extra-embryonic membranes need to be uncovered manually (fine forceps work well). As soon as they are isolated, the embryos may have to be "killed" by an ethically approved method (**Note 6**).
4. Transfer the embryos to a fresh dish (**Note 7**).

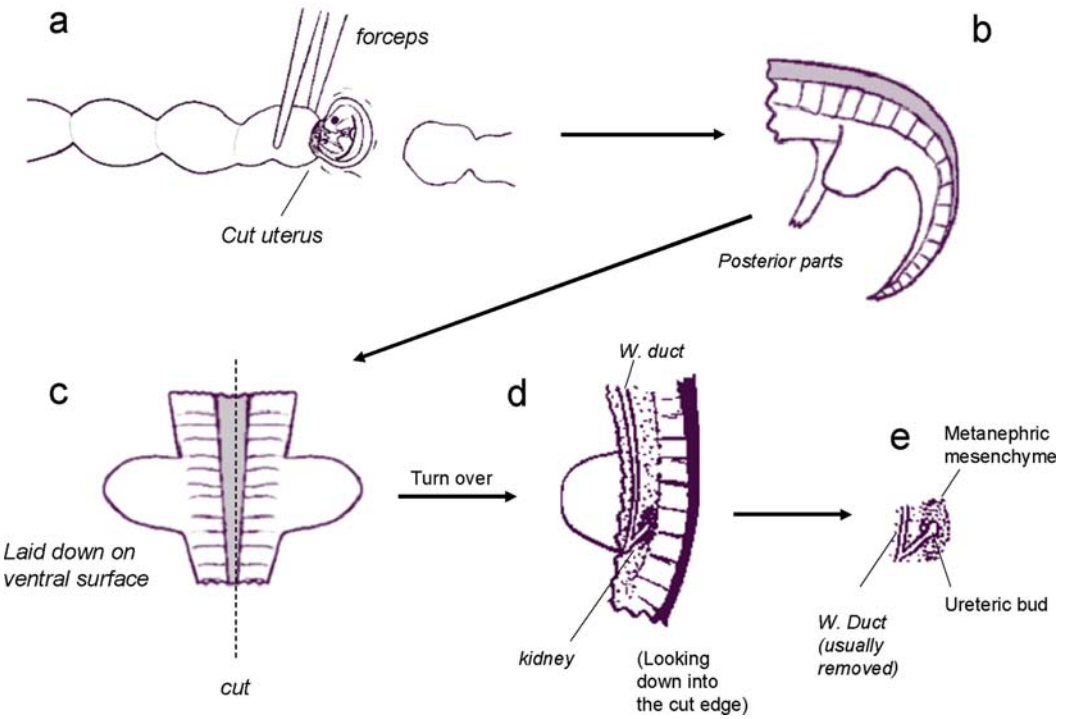

Fig. 4.2. Sequence of operations involved in isolation of kidney rudiments.

5. Cut the embryos transversely through their abdominal regions, about half way between the fore- and hind-limb buds (**Fig. 4.2**).
6. Discard the heads and the trunks. Keep the trunks aside, though, if you need spinal cord for induction experiments (see below).
7. Transfer the posterior parts of the embryos to a 30-mm Petri dish (larger dishes risk too much movement of medium).
8. Cut off the tail a short distance from the posterior limit of the hind-limb buds. The exact place is not important – the cut is performed only to prevent a long tail interfering with the positioning of the embryo fragment for the next stage of the dissection.
9. Place the embryo fragment on its ventral surface and cut it sagitally, through the middle of the spinal cord, so that it is separated into left and right halves (**Fig. 4.2**).
10. Turn each of these over so that it is lying skin-side downwards, and you are looking into the cut side of the embryo (**Fig. 4.2**).
11. Now try to find the kidney. It will be somewhere in the region of the body bounded by the anterior and posterior axial levels of the hind-limb bud; exactly where depends on embryonic age, the position being quite posterior at E10.5 and more anterior thereafter. The Wolffian duct can provide a very useful aid to finding the kidney. The duct will be running at the medio-ventral edge of the intermediate mesoderm; if you can find the duct, you should be able to follow it posteriorly and find the ureteric bud, which runs into the heart of the kidney (and will be branching in it from E11). There are other ducts in that region of the embryo, but only the Wolffian duct carries anything like the ureteric bud. The kidney itself is defined by a slightly whiter and more opaque region of mesenchyme surrounding the bud tip; seeing it is very difficult for novices, but becomes a lot easier with time and practice. If you find it impossible to see the duct and kidney, try removing the hind-limb bud – this will remove a layer of opaque tissue that can make the kidney harder to see, but will also reduce the number of anatomical cues available to you. If all else fails, check the other side of the embryo, because a slight error in the sagittal cut will leave both kidneys on the same half.
12. Isolate the kidney by removing everything that is not the kidney (**Fig. 4.2**). For the final dissections, take care to put

the "holding" needle (**Note 8**) in the waste tissue rather than the kidney, and work gently so that you do not flick the kidney away. Depending on what experiment you are doing, you may want to remove the Wolffian duct or to keep it attached.

13. Transfer the kidneys to another dish (a glass Pasteur pipette is fine for this) so that there is no danger of them getting lost among the debris of dissection.

Throughout the dissection, keep an eye on the colour of your dissecting medium; if it is looking alkaline, replace it with some that has been equilibrated in the CO_2 incubator. Again, the most critical thing is to ensure that all of your kidneys have experienced similar conditions. Finally, check all of the kidneys in your collection to ensure that there are no other pieces of embryo present.

3.2. The Trowell/Grobstein/Saxén Organ Culture System

The Trowell/Grobstein/Saxén culture system depends on kidney rudiments being supported by polycarbonate filters at the gas/medium interface, where surface tension tends to spread them on to the filter (**Fig. 4.3**). The need for this surface tension is not understood, but it does seem to be important and culture systems without it fail.

1. Cut stainless steel mesh into triangles and bend down each corner to form a "leg". Triangles are better than squares because they cannot rock in the way that squares with imperfectly matched legs can. Typical dimensions are shown in **Fig. 4.1**.

2. Using the tip of a pair of scissors, auger out small (2–3 mm) holes in the mesh to make it much easier to view kidneys as they are set up.

3. Sterilise the screens before use either by autoclaving or by flaming. When they become discoloured or rusty they should be discarded, and grids exposed to formaldehyde-containing fixes should be thrown away. In particular, care should be taken that all of the grids used in a particular experiment are in similar condition.

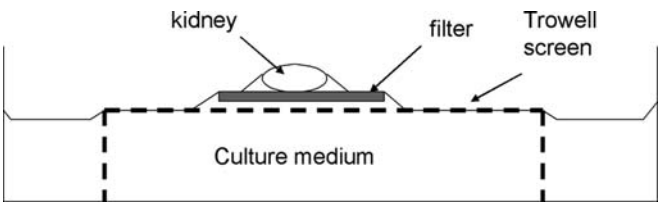

Fig. 4.3. The Trowell/Grobstein/Saxén culture system. The size of the kidney is exaggerated for the purposes of clarity.

4. Place a screen in a 30 mm dish and fill the dish with culture medium until the grid is just wet but the surface of the liquid away from the grid is below the level of that grid. This is important; if the grid is completely submerged, the surface tension effect is lost.
5. Dip small pieces of polycarbonate filter into medium and place them on the grid, across the holes made in step two. Irregular shapes of filter fragment can assist identification of the top and bottom surfaces (which will be important when immunostained kidneys are finally mounted for viewing). Various media are suitable; Sigma Eagle's MEM (M5650) with 10% calf serum, penicillin and streptomycin is a common choice.
6. Transfer the isolated kidneys to the filters and culture them for 1–10 days at 37°C, 5% CO_2. Finely pulled glass pipettes, of a diameter just smaller than the kidneys, are useful for transferring them without also transferring excess medium (they can be pulled manually in a spirit or gas flame).

3.3. Whole-Mount Immunofluorescence of Cultured Kidneys

Immunostaining of cultured kidneys is generally easier if they are fixed on to their filters and remain on them throughout.

1. To fix and permeabilise kidneys in methanol, pipette the culture medium away from underneath the culture grid, tipping the Petri dish gently so that all medium can be removed, then replace it with pre-cooled methanol at –20°C, pouring the methanol in gently so that the filters float up off their grids.
2. Remove the grid with forceps, taking care not to damage the filters floating at the top of the methanol, and tap the dish *gently* so that the filters sink. The kidneys will be visible as opaque, white blobs.
3. Leave the kidneys to fix for at least 15 min (they can be stored indefinitely in methanol at –20°C, for later staining).
4. Wash them in PBS, with blocking agents if they are needed, for at least 1 h.
5. Transfer the filters (using forceps) to PBS in Bijou tubes.
6. Stain with primary antibody solutions at the same concentration you would use for staining cultured cells, but incubate the kidneys in primary antibody for much longer (**Note 9**).
7. Wash in several millilitres of PBS for several hours.
8. Incubate in secondary antibody overnight.
9. Wash in PBS for several hours again.

Cultured kidneys are quite flat but care should be taken, when mounting them, not to crush them with a coverslip. A simple way of doing this is to mount two 22 × 22 mm coverslips at opposite

ends of a microscope slide, mount the specimen between them (kidney side up) and then mount a 22 × 64 mm coverslip over the whole assembly. The small coverslips act as bridge pillars to prevent the larger coverslip crushing the kidney. If you are not certain which side of the filter is "up", build the assembly described above on a 22 × 64 mm coverslip rather than a slide. This whole "cassette" can then be placed on a slide for viewing and, if the filter is upside down, the "cassette" can be inverted easily.

3.4. Spinal Cord-Mediated Induction of Mesenchyme Explants

Some experiments require "uninduced" metanephric mesenchyme to be cultured and induced in isolation from the ureteric bud (typically to check whether a mutation or drug treatment affects the mesenchyme directly or secondarily to an effect on bud development). A common method of inducing its development is contact with spinal cord (the inclusion of 15 mM LiCl in the medium is an alternative (3)). Mesenchyme can be isolated most conveniently from E10.5 kidneys, in which the ureteric bud has not yet branched, although they can also be isolated from E11 kidneys with care. Researchers are divided on the need for enzymatic treatment. If it is not important to remove *all* of the mesenchyme, manual dissection without enzymes is easiest. If all mesenchyme is needed, kidneys should be incubated in trypsin/EDTA, at the same concentration used for routine passage of cultured cells, for 5–15 min. The mesenchyme can then be pulled directly away from the ureteric bud, rather as a ripe raspberry can be pulled away from its core. Trypsinised tissues are, however, adhesive and liable to become stuck to instruments; the addition of DNAse can reduce this tendency.

1. Dorsal spinal cord is most easily isolated from the rostral end of the embryo's trunk, where it is larger.
2. Cut about 10 somites' length of embryo and isolate the spinal cord (by cutting everything else away).
3. Lay the spinal cord down on its floorplate and slip one needle into the spinal canal from the rostral end (**Fig. 4.4**).
4. Stroke the other needle along the first needle to cut through the roof plate of the cord so that it opens like a book, the dorsal parts opening outwards and the floorplate forming the "spine" of the book.
5. Cut along a line parallel to the long axis of the spinal cord, about half way between floorplate and edge and retain the strip of dorsal spinal cord that results from this.
6. Remove any dorsal root ganglia that may be attached to the underside of this strip.
7. Cut the strip into approximately square fragments and transfer them to another Petri dish for storage.
8. For direct, side-by-side induction, transfer a piece of spinal cord to a filter in the Trowell/Grobstein/Saxén culture

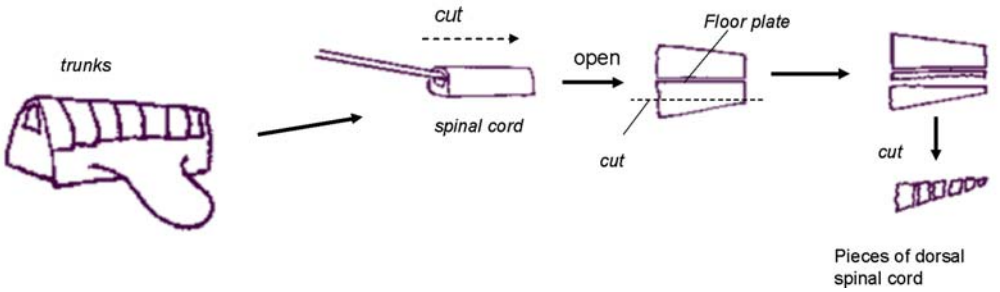

Fig. 4.4. Sequence of operations in isolating pieces of dorsal spinal cord, for use in inducing metanephric mesenchyme.

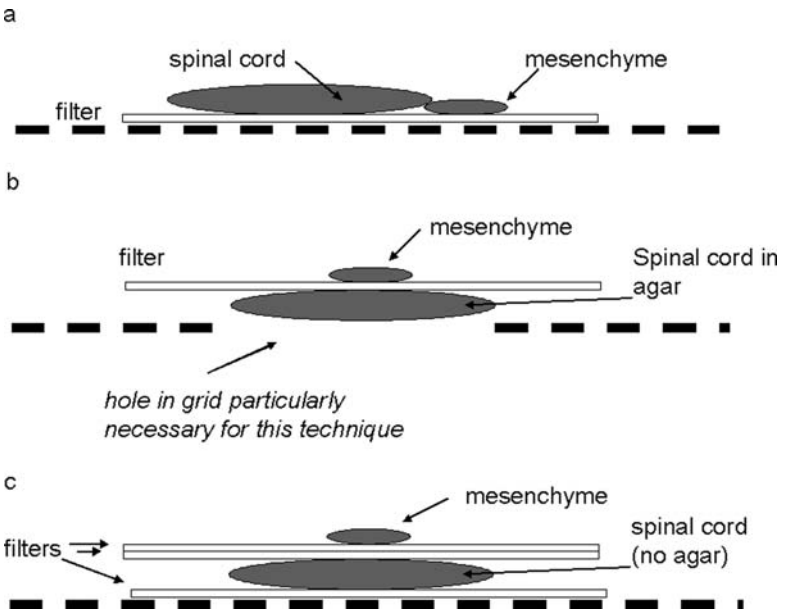

Fig. 4.5. Different methods for inducing mesenchyme with spinal cord. (**a**) Uses simple apposition, which works well as long as the tissues do not have to be separated later. (**b**) Is the traditional Saxén method, which uses agar as an adhesive. (**c**) Uses multiple filters to avoid the need for agar.

system described above and pipette a piece of mesenchyme directly next to it (**Fig. 4.5a**).

There are two ways to perform transfilter induction (in which one or more filters intervene between cord and mesenchyme). In the traditional method, much used in the Saxén lab (**Fig. 4.5b**), spinal cord is placed on the top of a filter and then stuck down with a small drop of low melting point agar, dissolved in warmed medium and cooled so that it is only just still liquid (too high a temperature will kill the spinal cord). When set, the filter is inverted, and a piece of mesenchyme is placed directly over the spinal cord. In this lab, we mount a piece of spinal cord on a filter, place one or two filters on it, then pipette mesenchyme on top

of the last filter, directly above the spinal cord (**Fig. 4.5c**). Both methods work; the first has the disadvantage that the agar can kill the spinal cord and can also sometimes separate it from the filter by a variable distance of solid agar, while the second has the disadvantage that it is difficult to put the second filter on top of the spinal cord without washing the spinal cord away in the process. It has the advantage that, in its two-filter form, it is easy to pull the top filter, carrying the mesenchyme away from the spinal cord at any stage of the experiment.

In all cases, if the interpretation of an experiment depends on the mesenchyme being "uninduced" before culture, it is important to set up some mesenchyme cultures with no spinal cord present to verify that nephrogenesis does not take place.

3.5. Growth Factors, Drugs, Function-Blocking Antibodies, siRNA and Plasmids

One of the great advantages of the organ culture system is that it allows development to be manipulated using drugs, proteins and nucleic acids. In all cases, it is important to verify that the reagent has penetrated to throughout the tissue. This can be aided by rinsing isolated kidneys in the drug (etc.) solution before pipetting it on to the filter, so that the liquid that immediately surrounds it is the same as in the bulk culture medium (and not drug-free dissection medium). For antibodies, it is important to use only heat-inactivated serum in cultures (to avoid complement-mediated cell lysis) and to use an irrelevant antibody that binds the cell surface, in controls. The penetration of the culture by proteins (antibodies, growth factors, etc.) can usually be verified by immunofluorescence for the protein itself or by using labelled versions of the proteins. This approach is more difficult for drugs, but their effects may be possible to measure directly throughout the tissue.

RNA interference can be performed by using exogenous siRNAs complexed with carriers such as oligofectamine. In our experience (4), not all cells take up the siRNA, so the result of performing a knock-down using this technology is a "mosaic" kidney of knocked-down and wild-type cells. For the interpretation of most experiments, it is therefore critical that the effect of siRNA-mediated knock-down is assessed on a cell-by-cell basis (e.g. by immunofluorescence) rather than by assay of a tissue homogenate. The ratio of siRNA to carrier, and the concentration of siRNA required, has to be determined for each siRNA.

Transfection of kidney rudiments with plasmids is also possible using lipophilic carriers such as GeneJuice™ (Merck, Darmstadt, Germany). Again, only a subset of cells is affected, so it is advisable to use plasmids that report their presence in an easily detected manner (for example, by encoding GFP as well as the experimental gene). Uptake is better in cells at the outside of the culture and is also improved after 1 day of culture, by which time the rudiment is already quite flat.

4. Notes

1. Dissecting techniques, and the instruments used for them, vary from person to person and researchers can be surprisingly passionate in their advocacy of a particular method. A frequent point of contention is the decision between using high-precision, delicate instruments, such as platinum needles or iridectomy knives, and using crude and cheap instruments that can be thrown away as soon as they become blunt. Partly for financial reasons and partly because I do not have the patience to do the dissections *and* to look after precision dissection equipment, I do all of my fine dissections with disposable "orange" (25Gx5/8″/0.5 × 16 mm) hypodermic needles held in 1 ml plastic syringes from which the plunger has been removed. The tips of these needles are fine enough and, as soon as they get blunt, they can be replaced at very low cost (for dissection of whole kidneys, one pair of needles will last all day, but for finer dissections of parts of kidneys, I would change to new needles after the whole-kidney stage).
2. Cheap bacteriological dishes are fine throughout; there is no need for tissue culture-grade plasticware.
3. The microscope should be the best that can be procured; any economy made here will be regretted. Different people, even within one lab, prefer different kinds of illumination, so it is sensible to use a microscope that gives a choice (we use the excellent Zeiss Stemi 2000 with a variable illumination stage and a separate light for epi-illumination). I use bright field trans-illumination with no diffusing filters, but have colleagues who prefer the softer lights of diffusing filters, slanted illumination or even epi-illumination.
4. The isolation of embryonic kidneys requires two distinct skills; fine manipulation of dissecting instruments and the ability to spot a whitish, vaguely opaque kidney rudiment amongst the other whitish, vaguely opaque structures in the embryo. To the surprise of most newcomers to the field, who worry about fine manipulations, it is the second of these skills that presents by far the greatest challenge. It comes to all with practice. The Kidney Development Database (http://golgi.ana.ed.ac.uk/kidhome.html) provides extensive resources and includes contact details for workers in the field who are experienced with these techniques.
5. The dissection area should be maintained as sterile as possible, and instruments should be flamed before use. We find

that flow hoods generate too much vibration, so have constructed simple Perspex open-fronted enclosures that at least define a clean area for dissection and keep it separate from the lab. The sanity of the dissector is greatly aided by the presence of a wireless set or CD player. As well as entertaining, it discourages conversation (which, when conducted by the dissector, would spray the tissues with oral bacteria).

6. Workers in the United Kingdom, in particular, should note that the embryos they use for most experiments will generally be at or just beyond half-way through gestation and will be subject to the same legal protections as the adult animal, so need to be subjected to an appropriate "Schedule 1" method of killing.
7. We tend to perform the same stage of the isolation on all embryos together and then move them all on to the next stage, rather than dissect them all the way, one by one. The main reason for this is that it minimises the different conditions experienced by different kidneys; dissecting the earliest embryo quickly all the way to isolated kidneys while the last remains fully intact for, say, an hour before dissection has begun might create unexpected variations in the kidneys' subsequent development.
8. The technique for dissecting with two needles is to "stab" the tissue with one needle (the one in your right hand, if you are right handed) and to angle the needle so that it lies along, and parallel to, the intended line of cut. The other needle can then be drawn through the tissue using the first needle as a guide. With practice, you will find you can make many cuts with no guidance at all. The stabbing, which should obviously be done in the part of the tissue that is to be discarded rather than the kidney itself, works because the needle can just penetrate the plastic of the Petri dish bottom and therefore hold still. This is why plastic dishes are better than glass. Similarly, the cutting needle will cut slightly into the plastic and will therefore go right through the tissue (the scratches left on the dish bear testament to this).
9. We find that for most antibodies, 4°C overnight is best.

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their skills to a rather clumsy newcomer. Work described in this chapter was supported by the BBSRC, AICR and Leverhulme Trust and the EU Framework VI "EuReGene" programme.

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Chapter 5

Explant Culture of Mouse Embryonic Whole Lung, Isolated Epithelium, or Mesenchyme Under Chemically Defined Conditions as a System to Evaluate the Molecular Mechanism of Branching Morphogenesis and Cellular Differentiation

Pierre-Marie del Moral and David Warburton

Abstract

Lung primordial specification as well as branching morphogenesis, and the formation of various pulmonary cell lineages, requires a specific interaction of the lung endoderm with its surrounding mesenchyme and mesothelium. Lung mesenchyme has been shown to be the source of inductive signals for lung branching morphogenesis. Epithelial–mesenchymal–mesothelial interactions are also critical to embryonic lung morphogenesis. Early embryonic lung organ culture is a very useful system to study epithelial–mesenchymal interactions. Both epithelial and mesenchymal morphogenesis proceed under specific conditions that can be readily manipulated in this system (in the absence of maternal influence and blood flow). More importantly this technique can be readily done in a serumless, chemically defined culture media. Gain and loss of function can be achieved using expressed proteins, recombinant viral vectors, and/or analysis of transgenic mouse strains, antisense RNA, as well as RNA interference gene knockdown. Additionally, to further study epithelial–mesenchymal interactions, the relative roles of epithelium versus mesenchyme signaling can also be determined using tissue recombination (e.g., epithelial and mesenchymal separation) and microbead studies.

Key words: Organ culture, lung, morphogenesis, branching, epithelium, mesenchyme, epithelial–mesenchymal separation, gain of function, loss of function.

1. Introduction

Embryonic lung development begins by evagination of the laryngotracheal groove from the foregut endoderm at embryonic

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day 9.5. Bronchial branches arise initially as two distal buds. Each bud consists of three different layers: the epithelium, the surrounding mesenchyme, and the mesothelium. It was appreciated early on that the mesenchyme can induce ectopic branching of the trachea, giving rise to extranumerary lungs (1). Various growth factors, including those from the epidermal growth factor (EGF), fibroblast growth factor (FGF), sonic hedgehog (SHH), bone morphogenetic protein (BMP), and wingless (Wnt) signaling families, are required to mediate the specific mesenchymal–epithelial–mesothelial interactions that are required and regulate respiratory organogenesis. Many of these growth factors have been extensively studied and affect epithelial branching, proliferation, and differentiation (2, 3). In order to determine the key molecules involved in the regulation of these epithelial–mesenchymal interactions, many of these studies have used early embryonic lung organ culture experiments as well as epithelial–mesenchymal separation and recombination.

Briefly, both techniques consist in removing embryos from pregnant females at gestational stages of early branching (e.g., embryonic days E11.5–E13.5) and isolating the lungs from these embryos. Early embryonic lung organ culture experiments then consist in placing the isolated lungs on filters and growing them under serum-free, chemically defined conditions, typically for periods from 12 to 96 h (4, 5). This method allows characterization of the overall effect of a particular growth factor on both epithelial and mesenchymal morphogeneses.

In order to further dissect and characterize the molecular mechanism involved and/or inductive role in branching, in the respective epithelial or mesenchymal response to a specific growth factor, the method of choice would be epithelial–mesenchymal separation and recombination. This technique consists in the enzymatic (e.g., dispase) and mechanical separation of the epithelium from the mesenchyme of E12.5–E13.5 lungs. The isolated explants are then placed in Matrigel^{TM} and grown for 24–48 h (6, 7) or mesenchyme can be replaced in apposition to the epithelium (8).

2. Materials

2.1. Embryonic Whole Lung, Epithelium, and Mesenchyme Isolation

1. Timed-pregnant (C57BL6 wild-type or transgenic) mice to be sacrificed on postcoitum day E11.5–13.5, where E0.5 is the day of detection of a copulation plug.
2. Hanks' balanced salt solution (HBSS) (1X), liquid, without calcium chloride, magnesium chloride, or magnesium sulfate (Invitrogen, Carlsbad, CA) supplemented with 50

units/ml of penicillin–streptomycin (Invitrogen, Carlsbad, CA). Store the HBSS at room temperature and at 4°C after opening. Aliquot and store the penicillin–streptomycin at –20°C.

3. Stereoscopic dissecting microscope (Leica, Wetzlar, Germany).
4. Dissection tools including Dumont® forceps, Fine iris scissors (straight), Noyes spring scissors, and Moria® perforated spoon (Fine Science Tools, Foster City, CA).
5. Insect pin holders and Etched tungsten micro-needles (Fine Science Tools, Foster City, CA).

2.2. Embryonic Whole Lung Culture

1. Dulbecco's Modified Eagle Medium: nutrient mix F-12 (D-MEM/F-12) (1X), liquid, 1:1 (v/v). Contains L-glutamine, but no HEPES buffer (Invitrogen, Carlsbad, CA) supplemented with 50 units/ml of penicillin–streptomycin (Invitrogen, Carlsbad, CA). Keep the DMEM/F-12 bottle away from the light (e.g., covered by aluminum foil) and store at 4°C (*see* **Note 1**). Aliquot and store the penicillin–streptomycin at –20°C.
2. Nuclepore polycarbonate track-etch membrane, 8.0 μm, 13 mm (Whatman, Florham Park, NJ).
3. Nunclon™Δ polystyrene dishes with lids, 4 wells (Rochester, NY).
4. Disposable transfer pipets, sterile (Fisher Scientific, Pittsburgh, PA).

2.3. Isolated Epithelium and Mesenchyme Culture

1. Dulbecco's Modified Eagle Medium: nutrient mix F-12 (D-MEM/F-12) (1X), liquid, 1:1 (v/v). Contains L-glutamine, but no HEPES buffer (Invitrogen, Carlsbad, CA) supplemented with 50 units/ml of penicillin–streptomycin (Invitrogen, Carlsbad, CA). Keep the DMEM/F-12 bottle away from the light (e.g., covered by aluminum foil) and store at 4°C. Aliquot and store the penicillin–streptomycin at –20°C.
2. Dispase (BD biosciences, San Jose, CA). Aliquot the undiluted dispase on ice and store at –20°C.
3. Fetal bovine serum (FBS), certified, heat inactivated (Invitrogen, Carlsbad, CA). Aliquot and store at –20°C.
4. Phosphate-buffered saline (PBS) 7.4 (1X), liquid (Invitrogen, Carlsbad, CA).
5. Nunclon™Δ polystyrene dishes with lids, 4 wells (Rochester, NY).
6. BD Falcon 60 × 15 mm Petri dishes (BD biosciences, San Jose, CA).

7. Matrigel™ (BD biosciences, San Jose, CA). Aliquot on ice and store at –20°C.
8. Glass Pasteur pipets, 5–3/4 in. (Fisher Scientific, Pittsburgh, PA) (*see* **Note 2**).
9. Rubber bulbs for small pipets (Fisher Scientific, Pittsburgh, PA).
10. Diamond scriber (Fisher Scientific, Pittsburgh, PA).
11. Calibrated micropipets, 1–5 μl, with aspirator tube (Drummond, Broomall, PA).

3. Methods

Organ culture and also epithelial or mesenchymal explant cultures are very useful and versatile techniques to study gene and protein expression. These ex vivo culture methods can be used as preliminary or in complement to in vivo studies.

3.1. Embryonic Lung Isolation

1. Timed-pregnant mice are sacrificed on postcoitum day E11.5–12.5 by CO_2 narcosis according to the NIH and OLAW guidelines (*see* **Note 3**). The animal is placed in a chamber and 100% CO_2 is introduced. After the animal is unconscious the CO_2 flow is increased. Clinical death of the animal must be ensured.
2. All the following steps need to be completed under sterile conditions in a laminar flow hood.
3. To remove the uterus, the pregnant females are placed on their back and sprayed with 70% ethanol. An incision is made in the abdomen, and the skin is removed by pulling the skin upward while holding the animal's hind legs. The peritoneal cavity is opened and the uterus is dissected free.
4. The blood is rinsed off by placing the uterus in a 50-ml conical tube filled with cold HBSS, and the tube is gently rocked for 2 min.
5. The uterus is then placed in a Petri dish under the stereoscopic dissecting microscope and the embryos released from the uterus by incising the uterine wall using spring scissors. The embryos are harvested using a perforated spoon and placed on ice in a new Petri dish containing HBSS.
6. Under the stereoscopic dissecting microscope, embryonic lungs are dissected one by one in a Petri dish containing cold HBSS (**Fig. 5.1**). For dissection, each embryo is placed on its left flank. The right forelimb is removed. The forceps

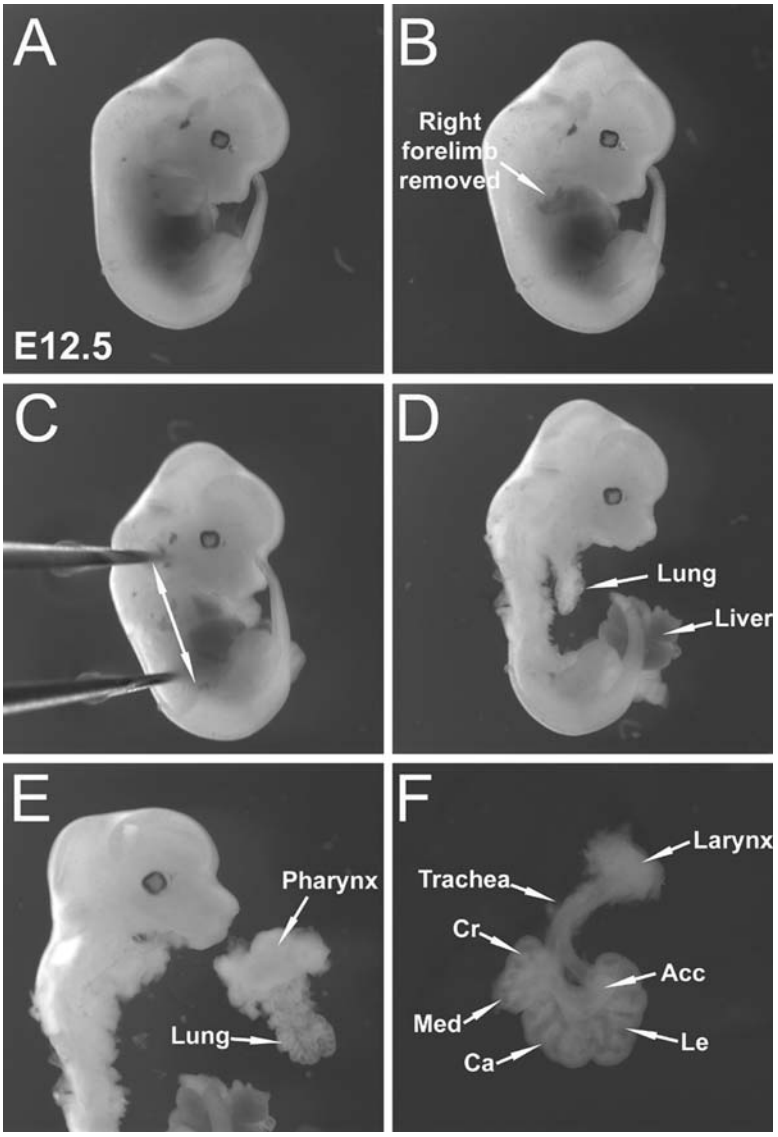

Fig. 5.1. Dissection of E12 embryonic lungs. (**a**) E12.5 whole embryo viewed from the *right side*. (**b**) Right forelimb has been removed from the embryo. (**c**) Forceps held by the left hand holding the embryo steady in the dish. *Arrows* indicate plan of dissection. (**d**) Embryonic lung lies posterior to the heart (removed) and anterior to the spine. This figure shows the lungs after skin and heart removal. (**e**) Pharynx removal allows separation of the intact lung from the embryo. (**f**) Extraneous pharyngeal tissue and esophagus have been trimmed away. Dissected E12.5 embryonic lung with intact trachea and larynx is shown. (Cr) Cranial lobe, (Med) Medial lobe, (Ca) Caudal lobe, (Acc) Accessory lobe, (Le) Left lobe.

held by the left hand is placed with one prong in the head and the other in the hindlimb of the embryo, thus holding it steady in the dish. The forceps held by the right hand is used to open the right flank of the embryo from the abdomen to the neck area. The skin above the heart and the heart

itself are then removed. The lung should be now observable, lying posterior to the heart and anterior to the spine. The neck area should then gently be opened to remove the tissue surrounding the trachea. The pharynx is divided and gently pulled using the forceps. This will allow the removal of the lung with an intact trachea. If any surrounding tissue (e.g., esophagus or other surrounding organs) is present, it should be gently removed from the isolated lung.

7. The isolated embryonic lung is placed on ice in a Petri dish containing HBSS using a sterile transfer pipet.

3.2. Embryonic Lung Culture

1. 500 μl–1 ml per well of D-MEM/F-12 supplemented with 50 units/ml of penicillin–streptomycin is added in a Nunclon^{TM}Δ polystyrene dish.
2. A nuclepore polycarbonate track-etch membrane is placed on top of the media (*see* **Note 4**).
3. A dissected embryonic lung is placed on top of the nuclepore polycarbonate track-etch membrane using a sterile transfer pipet.
4. The embryonic lung position is adjusted using forceps (*see* **Note 5**).
5. The Nunclon^{TM}Δ polystyrene dish is transferred for the desired culture time in a cell incubator. An example of the results produced is shown in **Fig. 5.2**.

3.3. Isolated Epithelium and Mesenchyme Culture

1. 500 μl of undiluted dispase is placed in a well of a Nunclon^{TM}Δ polystyrene dish.
2. E12.5–E13.5 dissected lungs are transferred into the dispase using a transfer pipette and incubated on ice for 25–30 min.
3. Dispase activity is stopped by transferring the samples to pure FBS on ice for 15 min.
4. Samples are transferred into a well containing D-MEM/F-12 media containing 10% FBS, on ice.
5. One embryonic lung is transferred to a 60 mm Petri dish containing DMEM/F12 media supplemented with 10% FBS. Epithelium and mesenchyme are separated under the dissecting microscope using tungsten needles.
6. Isolated epithelium and mesenchyme are transferred using a calibrated micropipet to respective wells (containing DMEM/F12 media supplemented with 20% FBS). The Nunclon^{TM}Δ polystyrene dish is placed on ice.
7. 10–15 μl of Matrigel^{TM} is added to make a dome (*see* **Note 6**).

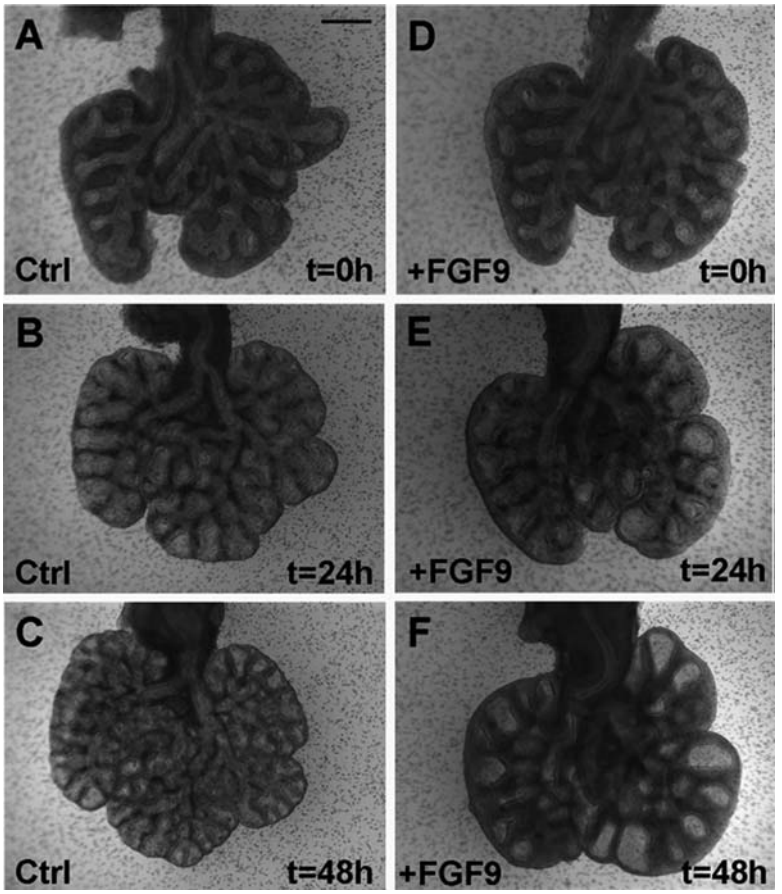

Fig. 5.2. FGF9 induces expansion of the mesenchyme and the dilation of the epithelium in lung grown in vitro (6). (**a–c**) E12.5 lung grown for 48 h in the absence of FGF9. Note the increase in branching over time. (**d–f**) E12.5 lung grown for 48 h in the presence of FGF9. Note the dilation of the epithelium and mesenchyme as early as after 24 h of culture (**e**). After 48 h (**f**), the effect on the epithelium is even more pronounced. Scale bar: (**a–f**) 400 μm.

8. The epithelial or mesenchymal explants are immediately and as quickly as possible added to the Matrigel™ dome using a calibrated micropipet.
9. Once all of the explants are positioned, the Nunclon™ Δ polystyrene dish is placed in the incubator for about 30 min (until it hardens) (*see* **Note 7**).
10. DMEM/F12 media supplemented with 0.5% FBS is added to each well, and the Nunclon™ Δ polystyrene dish is put back in the incubator for 24–48 h.
11. Pictures are taken at time = 0 and every 12 h until the end of the experiment. An example of the results produced is shown in **Fig. 5.3**.

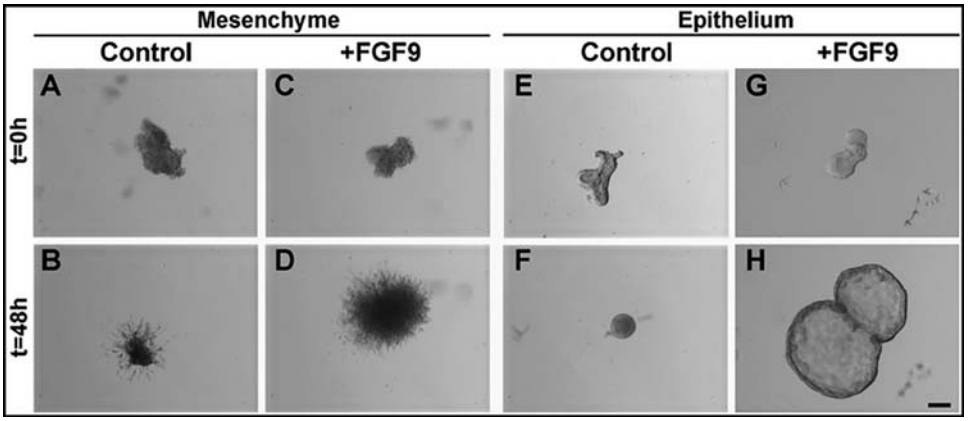

Fig. 5.3. Effect of FGF9 on isolated distal lung epithelium and mesenchyme (6). (**a**, **b**) Isolated distal mesenchyme grown for 48 h in the absence of FGF9 undergoes necrosis (**b**). (**c**, **d**) In the presence of FGF9, the mesenchymal explant grows and many mesenchymal cells invading the Matrigel are observed in the periphery. (**e**, **f**) Isolated distal epithelium grown for 48 h in the absence of FGF9 undergoes necrosis (**f**). (**g**, **h**) In the presence of FGF9, the epithelial explant grows considerably to form a cyst-like structure. Scale bar: (**a**–**h**) 80 μm.

4. Notes

1. BGJb media is no longer used. It has a high osmolarity and salt concentration. Also vitamin C supplementation is required and can interact with calcium signaling pathways.
2. Once the lungs have been digested in dispase, they become very sticky, and it is important to use a glass Pasteur pipet and not a plastic transfer pipet to transfer them to another well or to the Petri dish before epithelial–mesenchymal separation. It is also important to make a larger end to the Pasteur pipet using a diamond scriber to allow a cleaner cut of the glass. Then add at the other end a rubber bulb and use this as transfer tool.
3. Experiments involving animals must be conducted in accord with the prevailing local and national regulations.
4. When the nuclepore polycarbonate track-etch membrane is placed on top of the media, make sure that the shiny side is against the media and rough side is facing upward.
5. Embryonic lung placed on the filter should have an intact trachea and be placed with their trachea having a straight position, thus allowing all lungs to have the same internal pressure.
6. Before making a Matrigel^{TM} dome to culture epithelial or mesenchymal explants, a small flat base of Matrigel^{TM} can be made by adding 2–4 μl of Matrigel^{TM} at the bottom of the

well of the Nunclon™Δ polystyrene dish and by letting it harden for 1 min (no longer). This will prevent the spreading of the epithelial or mesenchymal explant by adhesion to the bottom of the dish.

7. Epithelial or mesenchymal explants grow better when positioned in the middle part of the Matrigel™ dome.

Acknowledgments

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Chapter 6

Isolation, Culture, and Characterisation of Mouse Embryonic Oesophagus and Intestine

Jonathan M. Quinlan, Wei-Yuan Yu, and David Tosh

Abstract

The gastrointestinal tract of vertebrates is lined by epithelium that develops from the endodermal germ layer. The oesophagus and intestine form part of the gastrointestinal tract and studying the normal development of both tissues is difficult due to lack of suitable in vitro model systems. One of the criteria for a reliable culture model includes the ability to carry out real-time observations in vitro. The method we describe here is based on the isolation of embryonic oesophagus and intestine from 11.5-day-old embryos and culture on fibronectin-coated coverslips in basal Eagle's medium and 20% fetal bovine serum. This model permits real-time observations of both tissues and over a few days in culture, markers of differentiation appear. This culture system appears to recapitulate normal oesophagus and intestine development.

Key words: Oesophagus, intestine, culture.

1. Introduction

The gastrointestinal tract of adult vertebrates is lined by different epithelia. For example, the oesophagus is lined by stratified squamous epithelium, whereas the intestine is lined by columnar epithelium. The epithelia are derived from the embryonic endoderm and are patterned by signals from the associated splanchnic mesoderm (1). During development, the epithelial lining of the gastrointestinal tract may be different from that seen in the adult. The embryonic mouse oesophagus is initially lined by a columnar epithelium which gradually becomes replaced by squamous cells from about embryonic day 17 (E17) to form a stratified

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squamous epithelium (2). The stratified squamous cells become keratinised around 1 month postnatally (3). In the developing rodent intestine, the epithelium is initially composed of a pseudostratified layer which converts into a single-layered epithelium overlying nascent villi at around E14.5 (4). Crypt formation occurs during the early postnatal period (5) and maturation of digestive function appears after weaning.

Study of the normal development of both oesophageal and intestinal epithelium has been hampered by lack of suitable model systems. For the intestine, the main problem is related to apoptosis. Once intestinal cells are removed from the basement membrane and underlying stroma, apoptosis is rapidly initiated (within a few hours) (6, 7). Explants of embryonic gut can develop successfully when transplanted under the kidney capsule of syngeneic hosts (8). However, the problem with such in vivo cultures is that the tissues are not easily accessible to study. In vitro alternatives to the in vivo experiments described above include culture of embryonic intestinal explants by attachment to filters (these are called catenary cultures) (9–11).

In this chapter we shall focus on the technique for the isolation and culture of two embryonic gastrointestinal tissues: oesophagus and intestine. These systems can be used for the study of epithelial development and may be applied to other gastrointestinal tissues, e.g. stomach.

1.1. Principles of the Method

The protocols described here for oesophagus and intestine are a modification of previous publications based on culture of CD1 mouse embryonic pancreas (12–14), oesophagus (2), and intestine (15). A reliable culture system must contain all the necessary cell types, allow real-time observations and manipulations in vitro, and permit wholemount immunostaining, thereby providing a three-dimensional visualisation of tissue arrangement. In order to permit wholemount immunostaining, the cultures must not exceed a few cells in total thickness. The key is a suitable substrate (in this case fibronectin) which encourages tissue attachment and epithelial spreading in culture.

1.2. Characterisation of Embryonic Oesophagus

It is possible to use the ex vivo culture system of mouse embryonic oesophagus to study the epithelial changes seen in vivo (2). Cultured oesophageal explants from E11.5 initially express the columnar epithelial markers cytokeratins 8 and 18 (K8,18). K8 and K18 expression decreases in the basal layer of the epithelium around 5 days of culture but persists in the suprabasal layers of the stratified epithelium for several more days. The stratified squamous epithelial marker K14, in contrast, is absent at E11.5 and starts to appear in the basal layer from days 5 to 9 of culture. Co-staining for K8 and K14 demonstrates colocalisation in some cells, suggesting a direct conversion process from columnar to the basal layer of the squamous epithelium.

1.3. Characterisation of Embryonic Intestine

The absorptive surface of the small intestine is increased by projections into the lumen termed villi. Three different cell types populate the intestinal villi. These cell types are known as enterocytes, goblet cells, and enteroendocrine cells. The absorptive enterocytes secrete enzymes such as sucrase–isomaltase (16). Goblet cells secrete protective mucus and enteroendocrine cells secrete hormones (e.g. secretin and glucagon-like peptide-1) (17, 18). At the base of the villi are the crypts of Lieberkuhn – these contain Paneth cells which secrete antimicrobial agents such as lysozyme (19). It is possible to demonstrate the formation of all four differentiated epithelial cell types in the culture system together with other markers including the intestinal transcription factor Cdx2, columnar epithelial marker K8, and mesenchymal marker α-smooth muscle actin (Table **6.1**).

Table 6.1
Markers for characterisation of intestinal cell types in mouse E13.5 model

Marker	Cell type	Detected in culture (day)	References
Cdx2	Intestinal epithelium	1	(20)
Troma-1 (K8)	Columnar epithelium	1	(21)
α-smooth muscle actin	Mesenchyme	1	(22)
Alkaline phosphatase	Enterocyte	7	(16)
Sucrase-isomaltase	Enterocyte	11	(16)
Periodic acid Schiff	Goblet	7	(23)
Chromogranin A	Enteroendocrine	11	(24)
Lysozyme	Paneth	7	(19)

2. Materials

2.1. Embryo Isolation

1. Scissors
2. Forceps
3. Ice-cold sterile phosphate-buffered saline A (PBSA, Oxoid)

2.2. Isolation and Culture of Oesophagus and Intestine

1. Two pairs of fine forceps (size 5)
2. Tungsten needle
3. Sterile PBSA
4. 35-mm tissue culture dishes
5. 22 × 22 mm glass coverslips (Scientific Laboratory Supplies)

6. 2% 3-Aminopropyltriethoxysilane (APTS, Sigma) in acetone
7. Bovine plasma fibronectin (Invitrogen) at 50 μg/ml in sterile water
8. Minimum Essential Medium Eagle (MEM) with Hanks' salts (Sigma) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 2 mM L-glutamine (Sigma), gentamicin 20 μg/ml (Invitrogen), and penicillin (100 units/ml)–streptomycin (100 μg/ml, Sigma)
9. Basal Medium Eagle (BME) with Earle's salts (Sigma) supplemented with 20% FBS, 2 mM L-glutamine, gentamicin 20 μg/ml, and penicillin (100 units/ml)–streptomycin (100 μg/ml)
10. Sterile cloning rings with internal diameter 4 mm (SLS)
11. Dissecting microscope and light source
12. 5% CO_2 incubator at 37°C

2.3. Fixation, Histology, and Immunohistochemistry

1. PBSA
2. MEMFA pH 7.4: 0.15 M MOPS (3-[*N*-morpholino] propane sulfonic acid), 2 mM EGTA, 1 mM $MgSO_4$, 3.8% formaldehyde
3. Acetone/methanol 1:1 at –20°C
4. 1% Triton X-100 (Sigma) in PBSA
5. Citrate buffer pH 6 (Lab Vision Corporation)
6. 2% blocking reagent (Roche) in PBSA
7. Primary and fluorescent secondary antibodies (Table **6.2**)
8. Gel/mount aqueous medium (Biomeda Corp.)
9. Vector blue alkaline phosphatase substrate kit III
10. 100 mM Tris–HCl pH 8.2
11. DePeX (BDH)
12. 1% periodic acid (BDH)
13. Schiff's reagent (Sigma)

3. Methods

3.1. Preparation of Coverslips

1. The glass coverslips are subbed as follows. Rinse the coverslips in 95% alcohol, 0.1% acetic acid, and allow to dry at room temperature. Immerse the coverslips in 2% APTS in acetone for 10 min, rinse twice in acetone and then sterile water, dry at 37°C, and sterilise by baking at 180°C for 3 h.

Table 6.2
Sources of antibodies for immunostaining

Experiment	Primary	Supplier	Secondary	Supplier
Oesophageal characterisation	Rat monoclonal anti-Troma-1 (K8) 1/50	DSHB, University of Iowa	Goat anti-rat Texas Red-conjugated IgG 1/100	Vector
	Mouse monoclonal anti-K14 1/100	NeoMarkers	Horse anti-mouse fluorescein isothiocyanate (FITC)-conjugated IgG 1/100	Vector
	Rabbit polyclonal anti-K14 1/100	Covance	Swine anti-rabbit tetramethylrhodamine isomer R (TRITC)-conjugated IgG 1/100	DAKO
	Mouse monoclonal anti-E-cadherin 1/50	BD transduction laboratories	Horse anti-mouse FITC-conjugated IgG 1/100	Vector
Intestinal characterisation	Rat monoclonal anti-Troma-1 (K8) 1/50	DSHB, University of Iowa	Goat anti-rat Texas Red-conjugated IgG 1/100	Vector
	Mouse monoclonal anti-sucrase-isomaltase 1/200	Gift (Dr Hans-Peter Hauri)	Horse anti-mouse FITC-conjugated IgG 1/100	Vector
	Mouse monoclonal anti-cdx2 1/100	BioGenex	Horse anti-mouse FITC-conjugated IgG 1/100	Vector
	Rabbit polyclonal anti-lysozyme 1/100	DAKO	Goat anti-rabbit FITC-conjugated IgG 1/100	Vector
	Rabbit polyclonal anti-chromogranin A 1/100	DAKO	Goat anti-rabbit FITC-conjugated IgG 1/100	Vector

2. Aliquot 40 µl bovine plasma fibronectin at 50 µg/ml in sterile water onto the centre of each sterile subbed coverslip and allow to dry in a tissue culture hood.
3. Place the coverslips in 35 mm tissue culture dishes, transfer a sterile cloning ring onto the fibronectin-coated area, and add 2.5 ml supplemented BME.

3.2. Embryo Isolation

1. Obtain stage-specific embryos from timed matings based on the observation of a copulatory plug.
2. Sacrifice the pregnant animal by cervical dislocation and open the abdomen to expose the uterus.

3. Dissect the uterus free into ice-cold sterile PBSA.
4. Open the uterus, isolate the embryo, and decapitate it before proceeding to organ isolation.

3.3. Oesophagus and Intestine Isolation and Culture

1. Transfer the decapitated embryo to supplemented MEM (*see* **Note 1**).
2. Open the ventral aspect of the embryo to reveal the heart, lung buds, liver, and gut tube (**Fig. 6.1**).
3. Dissect the liver and heart free using the fine forceps and tungsten needle.
4. Isolate the gut tube from pharynx to intestine and dissect free from the lung buds.
5. Dissect the oesophagus as a single tube from the pharynx and stomach and transfer to the centre of a cloning ring.
6. Isolate the proximal small intestine, remove any associated pancreas which appears as a small bud, divide into two to four segments, and transfer to the centre of a cloning ring.
7. Incubate the cultures in 5% CO_2 at 37°C.
8. After 24 h, carefully remove the cloning ring, decant the BME, and add 2 ml supplemented BME. Change the medium every 2 days thereafter (**Fig. 6.2**).

3.4. Fixation, Histology, and Immunohistochemistry

1. Rinse the coverslips with PBSA three times (*see* **Notes 2** and **3**).
2. Fix for 5 min in acetone/methanol at –20°C for immunostaining of cytoskeleton proteins or 30 min in MEMFA pH 7.4 at room temperature for immunostaining of membrane, cytosolic, and nuclear proteins.
3. Rinse the coverslips with PBSA three times. Samples may be stored in PBSA at 4°C for several days if required.

3.4.1. Immunohistochemistry

1. Permeabilise in 1% Triton X-100 in PBSA for 30 min at room temperature (tissue fixed in acetone/methanol does not need permeabilisation).
2. Perform antigen retrieval using citrate buffer pH 6 for 1 h at 37°C (this is necessary for immunostaining of cytoskeletal proteins).
3. Rinse with PBSA three times.
4. Block non-specific binding sites for at least 1 h in 2% blocking reagent.
5. Apply primary antibodies diluted in 2% blocking reagent overnight at 4°C.
6. Wash with PBSA for 15 min. Repeat twice more.
7. Apply fluorescent antibodies diluted in 2% blocking reagent for 3 h in the dark at room temperature.

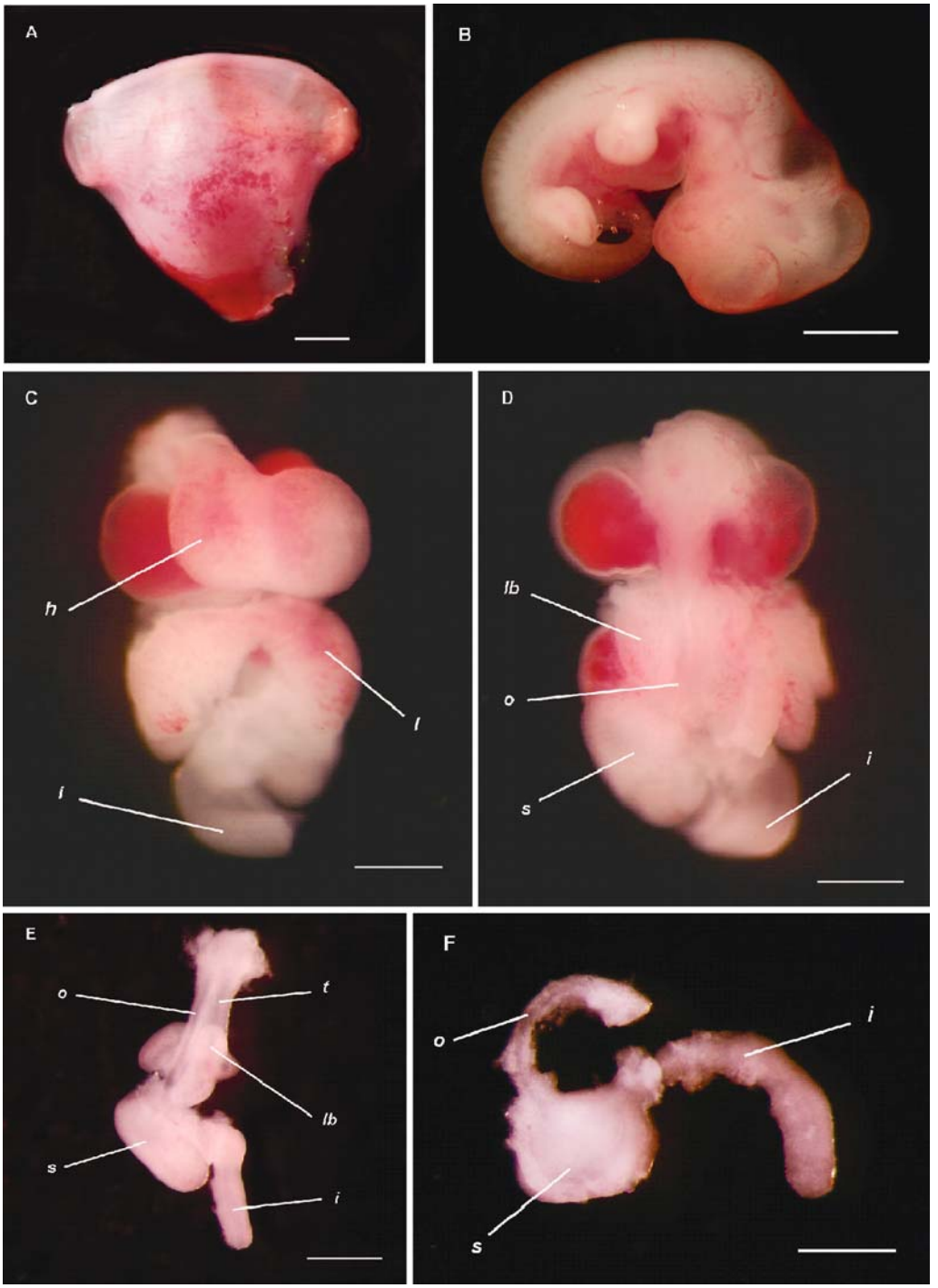

Fig. 6.1. Oesophagus and intestine isolation in CD1 E11.5 mouse (**a**) uterus; (**b**) whole embryo; (**c**) anterior view viscera; (**d**) posterior view viscera; (**e**) gut tube, trachea, and lung buds; (**f**) gut tube; (*h*) heart; (*i*) intestine; (*l*) liver; (*lb*) lung bud; (*o*) oesophagus; (*s*) stomach; (*t*) trachea. Bar = 2 mm (**a**, **b)**; 800 μm (**c**, **d**); 750 μm (**e**, **f**).

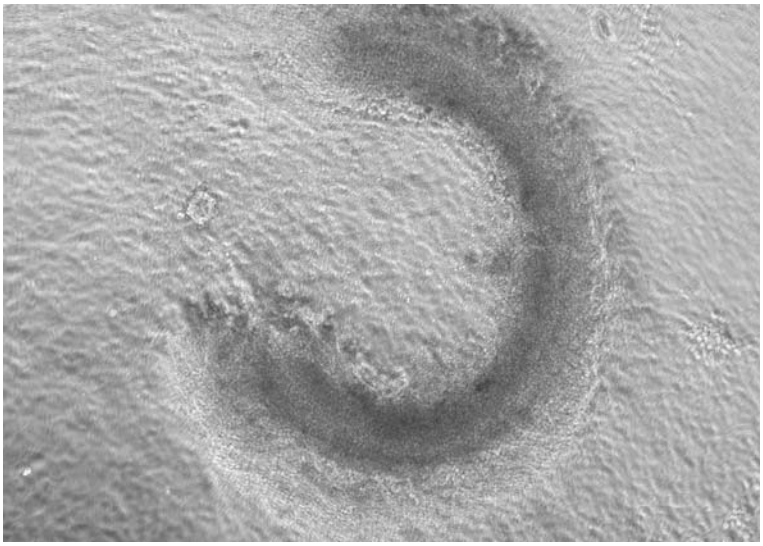

Fig. 6.2. The embryonic intestine attaches to the substratum. The epithelial intestinal tube is surrounded by mesenchyme.

8. Wash with PBSA for 15 min. Repeat a further two times.
9. Mount in Gel/mount and protect from the light.

3.4.2. Histochemistry – Enterocytes

1. This protocol is based on the Vector Blue alkaline phosphatase substrate kit III manufacturer's instructions.
2. Add 2 drops of reagent 1 to 5 ml 100 mM Tris–HCl pH 8.2, followed by 2 drops of reagent 2 and 2 drops of reagent 3.
3. Incubate the coverslips for 30 min at room temperature in the dark.
4. Wash with PBSA for 5 min, rinse in tap water before dehydration and mounting in DePeX.

3.4.3. Histochemistry – Goblet Cells

1. Wash the coverslips in running tap water for 3 min.
2. Add 1% periodic acid for 10 min at room temperature.
3. Wash for 3 min in water.
4. Immerse in Schiff's reagent in the dark for 2 min.
5. Wash in running tap water for 20 min and then 3 min in distilled water.
6. Counterstain with haematoxylin before dehydration and mounting in DePeX.

4. Notes

1. Different tissues may be cultured together to examine epithelial or mesenchymal interactions. To improve contact

between neighbouring tissues, they can be cultured in BD Matrigel Basement Membrane Matrix (BD Biosciences) mixed with an equal volume of supplemented BME. Co-cultures can be maintained in a matrix bilayer overlaid with supplemented BME within 8-well slides (Lab-Tek Chamber Slide System), changing the medium daily.

2. For dual immunostaining, two primary antibodies raised in different species can be applied simultaneously in 2% blocking reagent overnight at 4°C. Avoid species where secondary antibodies may cross-react or use highly cross-adsorbed secondary antibodies.
3. If cultures prove too thick for wholemount immunostaining, fix as described above, cryoprotect in 30% sucrose, embed in OCT compound (Gurr), and section (8 μm) using a cryostat. Mount on APTS-subbed slides and store at –20°C. The slides may be immunostained using the method described above.

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Chapter 7

Isolation and Culture of Embryonic Pancreas and Liver

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Abstract

Culturing embryonic tissue in an in vitro setting offers the unique ability to manipulate the external medium and therefore to investigate the pathways involved in regulating normal organogenesis as well as providing models for developmental disorders. Here we describe a system for the in vitro culture of the dorsal pancreatic buds and liver buds from mouse embryos. The tissues are dissected from day 9.0 or 11.5 mouse embryos. The tissues are placed on fibronectin-coated coverslips in serum-containing medium and allowed to attach. Over the next few days, the buds grow as flattened structures which are thin enough to allow the use of wholemount immunostaining methods.

Key words: Pancreas, liver, embryo, culture.

1. Introduction

In vitro organ culture provides an invaluable tool for studying the mechanisms involved in regulating organogenesis. Embryonic tissues including pancreas, liver, lung, kidney, and salivary glands have been successfully cultured with the developing organs exhibiting many of the morphological features specific to their tissue type (1–5). For example, the lung, pancreas, and salivary gland all undergo extensive branching morphogenesis in vitro, recapitulating the normal in vivo development. Culturing embryonic tissue in this way offers the opportunity for the researcher to manipulate the external medium and therefore to investigate the pathways involved in regulating normal organogenesis as well as providing models for developmental disorders. One advantage provided by adding factors directly to the growth medium is that developmental effects can be monitored directly, thereby

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eliminating lengthy procedures in which animal models have to be generated and maintained. It is also possible to use transgenic or knockout tissue in organ cultures, alone or in combination with wild-type tissues. This adds a further dimension to the ability to understand the effects of the genetic alteration. Here we describe the isolation and culture of two embryonic tissues: liver and pancreas. The isolation procedure serves only as a guide to locating the correct tissues without causing damage; once the researcher is familiar with the location of the embryonic pancreas and liver the procedure can be adapted to individual needs (and applied to other tissues). Many different culture conditions have been used to culture embryonic liver and pancreas; however, the conditions described here support the growth of branched pancreatic tissue.

1.1. Liver Development

The developing liver arises from the anterior foregut endoderm and the mesenchyme of the septum transversum. Specification of the foregut endoderm toward a hepatic fate occurs at around embryonic day 8 (E8) as movement of the head fold brings a region of the endoderm into close proximity to the developing heart. Signals emanating from the adjacent cardiac mesoderm and the mesenchyme of the surrounding septum transversum, namely fibroblast growth factors and bone morphogenetic growth factor 4 (6–8), have been shown to be essential for the correct specification of the liver. Subsequently, the hepatic endoderm undergoes a period of proliferation (~E9.0–9.5) to generate the liver bud followed by outgrowth at E10 as the early hepatoblasts migrate into the septum transversum in a cord-like fashion. At E10.5 the fundamental lobular structure of the liver is apparent and robust expression of liver markers such as albumin and α-fetoprotein is observed. By E11.5, the embryonic liver is already the largest visible organ within the abdomen of the embryo, and hepatic haematopoesis is well established. Several days later at E15.5, the appearance of bile duct precursors around the developing vasculature demarcates the onset of biliary system formation (9). Subsequently, the liver and small intestine become connected through the intrahepatic bile ducts and the liver continues to increase in mass.

1.2. Pancreatic Development

The embryonic pancreas develops initially as two separate buds, the dorsal bud appearing at around E9.0 and the ventral bud around E11. Dorsal and ventral pancreatic buds are exposed to different inductive signals during the early stages of development. It is believed that the ventral pancreatic buds arise from the distal tip of the anterior foregut endoderm that also gives rise to the liver. The ventral buds are initially subject to signals from the lateral plate mesoderm and subsequently with signals derived from the cardiac and septum transversum mesenchyme. Induction of the dorsal pancreatic bud, however, is mediated by notochord

signalling and later through signals from the dorsal aorta (10–13). As development proceeds, the pancreatic buds undergo extensive branching and differentiation and later fuse to give rise to a single organ composed of three functional units: (i) the exocrine cells, responsible for synthesising and secreting the digestive enzymes for breakdown of carbohydrates, fats, and proteins; (ii) the ductal cells, which provide a means of collecting digestive enzymes and transporting them to the intestine; and (iii) the endocrine cells that generate hormones for the regulation of energy homeostasis (e.g., insulin and glucagon).

1.3. Principles of the Method

The protocol is based on the technique described by Percival and Slack (14) and has been applied to studying pancreas development and transdifferentiation in an in vitro setting (15–18). The culture system allows the embryonic pancreatic tissue to grow as a flat branched structure. This approach facilitates analysis of the tissue by enabling efficient wholemount immunostaining without the requirement for sectioning small and delicate samples. Within hours of culture the pancreata adhere to the fibronectin-coated coverslips and then gradually, over the next 1–2 days, flatten as mesenchymal cells spread out to form a monolayer surrounding and covering the epithelium. Differentiation of pancreatic cell types appears to occur normally with the expression of cell-specific markers, such as pancreatic polypeptide (PPY), insulin, and amylase. For example, in vivo, small numbers of both PPY and insulin-producing cells are first detected at E12.5 and increase in numbers thereafter. In vitro, PPY and insulin-positive cells are visible after 1 day of culture, equivalent to an E12.5 embryo. The exocrine marker amylase is expressed at E14.5 in the developing embryo and at the equivalent of E15.5 in cultured pancreata (day 3 in culture). While cell differentiation in the cultures closely resembles that seen in vivo, the overall growth and size of the pancreata is considerably less than that seen in the developing embryo. The procedure described here can be utilised for the culture of many different types of embryonic tissue including kidney, salivary gland, intestine, and oesophageal tissues (*see* **Chapter 6** for culture of embryonic oesophagus and intestine).

2. Materials

2.1. Dissection and Culture of Embryonic Liver and Pancreas

1. Sterile PBS (9.55 g/l in MilliQ dH_2O, autoclaved)
2. Dissection medium: Minimum Essential Medium Eagle (MEME) containing Hank's salts supplemented with 10%

foetal bovine serum (Gibco), 1% L-glutamine (200 mM; Sigma), and 0.2% gentamycin (10 μg/ml; Gibco)

3. Bovine plasma fibronectin (Invitrogen). Dissolve 1 mg fibronectin in 1.0 ml of sterile MilliQ dH_2O and aliquot into 50 μl aliquots and store at –20°C
4. 3-Triethoxysilylpropylamine (APTS; Sigma)-coated coverslips
5. Sterile cylinder cloning rings (Scientific Laboratory Supplies Ltd.)
6. Nalgene Nunc 35 mm culture dishes (Fisher)
7. Culture medium: Basal Medium Eagle (BME) containing Earle's salts supplemented with 20% foetal bovine serum (Gibco), 1% L-glutamine (200 mM; Sigma), and 0.2% gentamycin (10 μg/ml Gibco)
8. Fine dissecting instruments: Jewellers forceps, (Sigma); fine tungsten needle

2.2. Immunostaining of Embryonic Tissue Cultures

1. PBS.
2. MEMFA (10% formaldehyde, 0.1 M Mops, pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$).
3. 0.1% Triton-100 (Sigma) in PBS.
4. 2% Blocking buffer (dissolve 50 g of blocking reagent (Roche) in 100 mM maleic acid, 150 mM NaCl, pH 7.5).
5. Primary (anti-α-fetoprotein 1:100, DAKO; anti-amylase 1:200, Sigma; anti-insulin 1:300, DAKO; anti-glucagon 1:200, Sigma) and secondary antibodies (anti-rabbit fluorescein isothiocyanate (FITC) (Vector Labs), anti-mouse 7-amino-4-methylcoumarin-3-acetic acid (AMCA) (Vector Labs), and anti-guinea pig tetramethylrhodamine isothiocyanate (TRITC) (Sigma), all 1:100).
6. Mounting medium (Biomedia Corp.) and glass slides (Fisher).

3. Methods

3.1. Preparation of Fibronectin-Coated Coverslips

1. Wash the coverslips in hot soapy water, rinse thoroughly in hot water and then in RO (reverse osmosis) water.
2. Rinse in 95% ethanol containing 0.1% acetic acid and allow to dry at room temperature.
3. Place into 2% APTS in acetone for 10 min (*see* **Note 1**).

4. Dip the coverslips twice in acetone for 10 s each followed by RO water for 10 s.
5. Dry at 37°C then wrap in foil and bake for 3 h at 180°C.
6. Allow coverslips to cool before coating with fibronectin.
7. Place sterile-subbed coverslip into 35 mm dish.
8. Dilute bovine fibronectin to 50 μg/ml (50 μl of stock fibronectin) (*see* **Section 2.1**) and 950 μl sterile water, place 40 μl onto the centre of each coverslip.
9. Allow fibronectin to dry at room temperature (preferably in a sterile flow hood), then store coverslips at 4°C.

3.2. Isolation of Embryonic Tissues

Regardless of developmental stage, all embryos are harvested from pregnant female mice in the same way. First, the uterus containing the embryos is located within the body cavity, then using sharp scissors, three cuts are made, one at the base of the uterus and one at each end of the uterus where the uterine horn meets the ovary. The embryo-containing uterus is then placed into sterile PBS, rinsed to remove blood and particulate matter, and then transferred to MEME for dissection.

3.2.1. Isolation of Embryonic Day 9 Liver Buds

1. At this early stage the embryos are quite small; they are, however, clearly visible as bulges within the uterus. The embryos are separated from one another by simply cutting between the bulges with a fine pair of dissecting scissors and gently tearing away the uterine tissue using forceps.
2. Remove the thick protective extra-embryonic tissue (decidua) surrounding the embryo by holding the embryo in place with one pair of forceps and carefully cutting at the surface of the decidua with another (*see* **Note 2**).
3. Gently tease away the amnion and chorion membranes surrounding the embryos and discard.
4. Transfer isolated embryos to a fresh dish of dissecting medium and place on ice while remaining embryos are isolated.
5. Remove the E9.5 liver buds using a tungsten needle. Hold the embryo in position with a pair of forceps and use a tungsten needle to cut around the hepatic region taking care not to remove adjacent cardiac tissue (*see* **Note 3**).
6. Transfer liver bud to a separate dish containing MEME until all liver buds are isolated.

3.2.2. Isolation of Embryonic Day 11 Liver and Pancreas

1. Cut uterus between embryos using a fine dissecting scissors and release embryos from their extra-embryonic tissue and membranes.

2. Decapitate all embryos before proceeding to organ isolation.
3. Use one pair of forceps to hold the embryo in place and another to open up the embryo along the right-hand side of the dermal tissue (*see* **Note 4**).
4. Peel away the dermis to expose the developing internal organs that can be removed by using the heart to pull them away from the rest of the embryo.
5. Remove the liver using forceps, taking care not to damage the remaining organs positioned behind the liver, and dissect into small pieces more suitable for culturing (1–2 mm^2).
6. Rinse liver pieces through several washes of MEME to remove blood and debris before setting up tissue to culture.
7. Using fine forceps and a tungsten needle remove the dorsal pancreatic bud from the base of the stomach and the closely associated duodenum (*see* **Note 5**).

3.2.3. Culture of Embryonic Organs on Fibronectin-Coated Coverslips

1. Place a sterile cloning ring over the fibronectin-coated region of the coverslip and add 2 ml BME culture media (*see* **Note 6**).
2. Using a pipette (Gilson p200) transfer individual tissue pieces to the centre of the cloning ring ensuring the cut edge of the tissue is in contact with the coverslip (*see* **Note 7**).
3. Culture tissue for at least 24 h to allow good attachment before removing cloning ring and changing media.

3.2.4. Immunostaining of Embryonic Cultures on Fibronectin-Coated Coverslips

Fixation of tissues is generally carried out in MEMFA at room temperature for 30 min. This is usually suitable for most hepatic and pancreatic markers. For cytoskeletal markers a 5 min fixation at –20°C in acetone:methanol (1:1) is usually required.

1. Fix specimens with appropriate fixative.
2. Permeabilise tissue in 1% Triton X-100 for 20 min (this step can be omitted if acetone:methanol fixation has been used).
3. Rinse coverslips briefly several times with PBS to remove Triton X-100.
4. Block for 30 min for up to 1 h in blocking buffer. We use a commercially available blocking buffer but species-specific serum can also be used.
5. Add primary antibodies diluted in blocking buffer to the tissue and incubate overnight at 4°C. This helps to reduce non-specific binding.
6. The next day, wash coverslips three times in PBS for 15 min each.
7. Add secondary antibodies, again diluted in blocking buffer, and incubate for 3 h at room temperature.

8. Repeat the washes in step 6 and mount coverslips onto glass slides (*see* **Note 8**). View under fluorescent microscope (*see* **Fig. 7.1**).

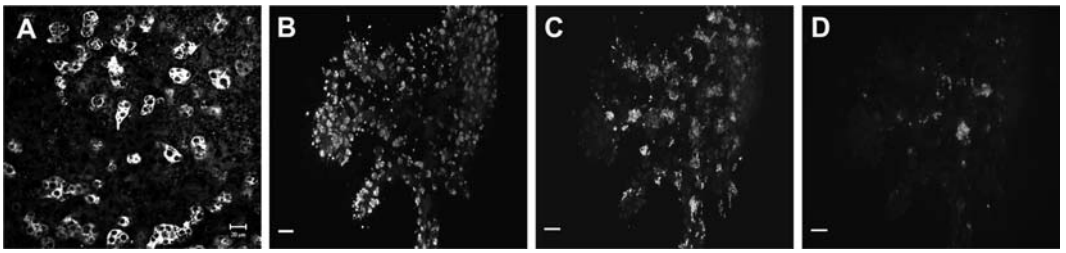

Fig. 7.1. Embryonic liver and pancreas in culture. (**a**) Embryonic liver cultured for 7 days in BME with 20% serum, immunostaining for α-fetoprotein. (**b**–**d**) Embryonic pancreas cultured for 7 days in BME with 20% serum. Immunostaining for amylase (**b**), insulin (**c**), and glucagon (**d**) on the same pancreatic culture.

4. Notes

1. This step should be carried out in a fume hood.
2. At E9.5 the embryo including the extra-embryonic tissue appears slightly triangular in shape with the embryo proper located at the "wider" end. When removing the embryo, hold it in place at the narrow end, which contains the developing placenta and therefore appears quite vascularised, to prevent damage.
3. The most distinct feature in the E9.5 embryo is the beating heart; located below this is the liver bud which appears as a small white thickening surrounded by less opaque tissue.
4. The liver is quite large and red in colour beneath which the pancreas is located on the left side posterior to the stomach. The blood-filled aorta lies dorsal to the body organs; using this as a guide open the embryo along the right-hand side to help prevent damage to the organs of interest.
5. The dorsal pancreatic bud is composed of an epithelial component resembling a bunch of grapes surrounded by the more opaque mesenchyme and is located close to the base of the stomach. Rotate the stomach on to its side, a natural groove between the stomach and dorsal pancreas is visible. Using a tungsten needle, detach the pancreatic tissue from the duodenum first and then follow the line of the natural groove along the base of the stomach to detach the entire dorsal pancreas.

6. Cut the end off the Gilson tip using a razor to prevent damage to tissue during transfer. The cloning ring stops the medium moving around and keeps the explant in the centre of the dish.
7. The cut surface of the tissue adheres well to the fibronectin-coated coverslip, if not orientated properly the tissue is unlikely to attach properly resulting in a free-floating culture.
8. When mounting coverslips take care not to move them or apply too much pressure on them once they are in contact with the slide as this causes the tissue to tear and detach from the coverslip.

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Chapter 8

Isolation and Culture of Mouse Satellite Cells

Antonio Musarò and Laura Barberi

Abstract

Muscle tissue culture provides a system for studying the growth and differentiation of muscle cells in a controlled environment. In mature muscle tissue, terminally differentiated myocytes form multinucleate syncytia in which structural and regulatory genes are expressed and the contractile apparatus is assembled. Adult muscle fibres are characterized by the presence of satellite cells. These are a quiescent population of myogenic cells that reside between the basal lamina and the plasmalemma of terminally differentiated muscle fibres and are rapidly activated in response to appropriate stimuli. This chapter describes protocols used in our laboratory for isolating and culturing satellite cells isolated from mouse skeletal muscles. In particular we discuss the technical aspect of satellite cell isolation, the methods necessary to enrich the satellite cell fraction, and the culture conditions which optimize proliferation and myotube formation of mouse satellite cells.

Key words: Muscle primary culture, isolation satellite cells, cell culture, muscle differentiation, myogenic program.

1. Introduction

The complex program of skeletal muscle development involves an orderly progression of molecular signals that induce primordial muscle precursor cells to be committed in a myogenic fate and subsequently to differentiate into mature muscle.

The maturation of the myogenic phenotype involves an interplay between the intrinsic program of muscle lineage specification and the extrinsic influence, such as innervation and growth factor activity (1). Myogenic cell decisions during embryogenesis require the expression and function of specific transcription factors, which in turn activate genes encoding structural and regulatory muscle proteins, such as myosin, α-actin, troponin I,

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troponin T, β-enolase, muscle creatin kinase (MCK), and the different subunits of acetylcholine receptor (AChR) (2, 3). In particular, skeletal muscle development is mediated by four myogenic determination factors (myf-5, MyoD, myogenin, and MRF4), which are considered master regulatory genes due to their ability to activate the program of muscle gene expression when overexpressed in transfected nonmuscle cell types (2, 4).

The mature skeletal muscle is composed of myofibres, which are multinucleated syncytia arising from mononucleated precursor cells. However, there is a population of mononucleated muscle stem cells, known as satellite cells, that reside at the periphery of muscle fibres between the basal lamina and the sarcolemma. Historically, satellite cells were identified in 1961 using electron microscopy studies by Katz (5) and Mauro (6), who reported that satellite cells might be "dormant myoblasts that failed to fuse with other myoblasts and are ready to recapitulate the embryonic development of the skeletal muscle fibres when the main multinucleate cell is damaged" (6).

The discovery of molecular markers selectively expressed by satellite cells, but not by muscle fibres, has contributed to their characterization. It has been reported that c-Met, M-cadherin, MNF, Pax-7, NCAM, syndecan 3 and 4, CD34, and VCAM-1 are markers expressed by quiescent, activated, and proliferating satellite cells (7). Pax3, the paralog of Pax7, is also expressed in quiescent muscle satellite cells in a subset of muscles (8). In contrast desmin, myf-5, MyoD, and PCNA are expressed in activated and proliferating satellite cells.

The transition from cell proliferation to differentiation involves the activation of other markers such as cardiac and slow-twitch skeletal muscle Ca^{2+}–ATPase (Atp2a2), slow-twitch skeletal muscle troponin T (Tnnt1), Igf2, nicotinic cholinergic receptor alpha polypeptide 1 (Chrna1), fibroblast growth factor receptor 4 (Fgfr4), myogenin, and cardiac/slow-twitch skeletal muscle troponin C (Tncc) (9).

Satellite cells are activated in response to physiological stimuli, such as exercise, and under pathological conditions, such as injury and degenerative diseases, to generate a committed population of myoblasts that are capable of fusion and differentiation (10). Thus satellite cells are able to fuse with existing myofibres, repairing damaged muscle fibres, or alternatively fuse to each other to form new myofibres, participating in muscle regeneration and repair. Satellite cells are therefore the major source of myogenic precursors in mammalian muscle regeneration.

Skeletal muscle is an attractive model to study the regulation of tissue-specific gene expression due to existence of in vitro cell culture systems which spontaneously fuse to form differentiated muscle fibres, activating a battery of muscle-specific genes. Identification of myogenic transcription factors responsible for the

induction of the skeletal muscle phenotype represents a significant breakthrough in our understanding of the molecular pathways underlying skeletal muscle differentiation.

Muscle primary cultures require special conditions for optimal growth and differentiation and in this chapter we will discuss the protocol to isolate satellite cells and the potential experimental limitations.

2. Materials

2.1. Solutions and Media

1. Collagen type I (Sigma C9791): dissolve 10 mg of collagen type I in 10 ml 20% acetic acid. Store at room temperature.
2. Penicillin/streptomycin solution 100x solution 10,000 U/ml penicillin and 10 mg/ml streptomycin (Sigma P0781).
3. L-Glutamine 200 mM, sterile filtered cell culture tested (Sigma G7513).
4. Dulbecco's phosphate-buffered saline (PBS) without calcium chloride and magnesium chloride (Sigma D8537).
5. Collagenase type II (Sigma C6885). Dissolve 1.5 mg of collagenase type II in 15 ml of PBS (0.1 mg/ml).
6. Collagenase/dispase solution (Roche 1097113). Dissolve 15 mg of collagenase/dispase in 15 ml (1 mg/ml) of PBS without calcium and magnesium. Filter and use fresh.
7. Dulbecco's Modified Eagle's Medium (Sigma D6546) supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin.
8. Heat-inactivated horse serum (Sigma H1138).
9. Heat-inactivated foetal bovine serum (Sigma F4135).
10. HEPES 1 M pH 7.0–7.6 cell culture tested (Sigma H0887).
11. Hanks' balanced salt solution (HBSS) (Gibco 041-040209).
12. Chick embryo extract (CEE) (MP-Biomedicals 2850145). Alternatively prepare CEE from chicken eggs as reported below.
13. 10% Goat serum (Sigma G6767) in PBS.
14. Primary antibodies: MyoD (Dako M3512 clone 5.8A; 1:50 diluted in PBS), MyHC (MF-20 Hybridoma Bank, 1:20 diluted in PBS).
15. Secondary antibody anti-mouse fluorescein (Jackson lab, 115-015-100) 1/200 dilution in PBS.

16. Vectashield mounting medium with Hoechst.
17. β-D-arabinofuranoside (Sigma C1768) add 0.3 mg per 100 ml of culture media.

2.2. Preparation of Chick Embryo Extract (CEE)

1. Clean day 9–11 chicken eggs by wiping with ethanol. Harvest the embryo into a large petri dish. Sacrifice the embryo and place in a 10 ml syringe.
2. Pass embryos twice through the 10 ml syringe. Collect the processed embryos in a 50 ml conical centrifuge tube.
3. Add an equal volume of HBSS. Triturate solution with a wide bore 25 ml pipette. Gradually reduce pipette size until solution is able to be drawn into 5 ml pipette.
4. Freeze solution overnight at –20°C.
5. Thaw solution and continue trituration until solution can be passed through a Pasteur pipette.
6. Centrifuge solution in a table top centrifuge at 3,000 rpm for 30 min.
7. Discard pellet, remove supernatant, and freeze this solution in 2 ml aliquots; store at –20°C.

2.3. Culture Media

1. Plating medium consisting of DMEM supplemented with 10% horse serum (Sigma), 0.5% chick extract (MP-Biomedicals), 4 mM L-glutamine (Sigma), and 1% penicillin and streptomycin solution (Sigma).
2. Growth medium (GM): DMEM supplemented with 20% horse serum or 20% foetal bovine serum, penicillin/streptomycin (50 U/ml/50 mg/ml), L-glutamine (4 mM), hepes (10 mM), CEE (3%).
3. Differentiation medium: DMEM supplemented with horse serum (5%), penicillin/streptomycin (50 U/ml and 50 μg/ml), L-glutamine (4 mM), and hepes (10 mM).

3. Methods

Two major methods have been developed to obtain isolated satellite cells. The classical procedure, which will be discussed extensively in this chapter, involves the enzymatic dissociation of skeletal muscles, while the alternative method involves isolation of satellite cells from isolated myofibres. Notably, cells prepared by enzymatic digestion of whole muscle tissue are likely to contain a heterogeneous population of precursor cells, such

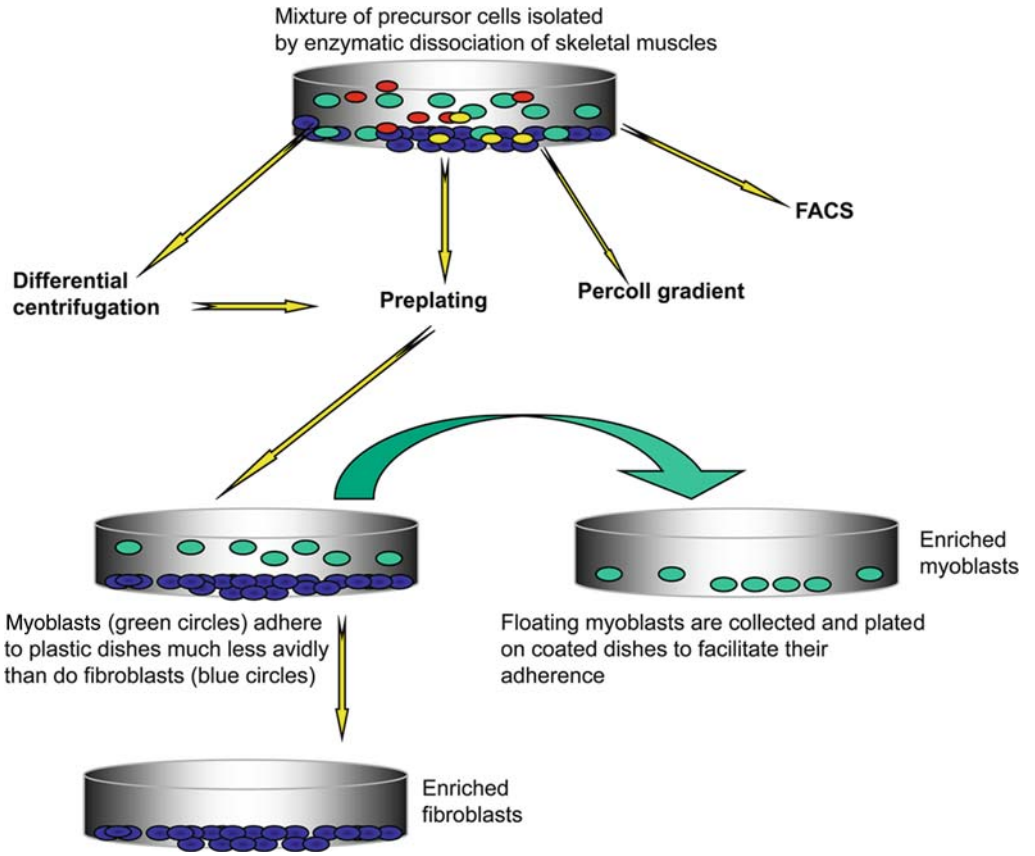

Fig. 8.1. Schematic representation of the isolation of satellite cells by enzymatic dissociation of skeletal muscles. Cells prepared by enzymatic digestion of whole muscle tissue are likely to contain a heterogeneous population of precursor cells. Different methods, such as differential centrifugation, preplating, Percoll gradient, and FACS can be used to enrich satellite cell population (*see* text for details).

as myogenic cells derived both from the satellite cell niche and from other populations in the muscle interstitium and vasculature (**Fig. 8.1**). Nevertheless, different methods, such as fluorescence-activated cell sorting (FACS), differential centrifugation, preplating, and Percoll gradient, can be used to enrich satellite cell population from enzymatic dissociation of skeletal muscles (**Fig. 8.1**). In particular, using relevant molecular markers it is possible to sort and culture the different cell populations by flow cytometry.

In contrast, single muscle fibre preparations, in which satellite cells retain their normal anatomical position beneath the basal lamina, give rise to a more homogeneous population of satellite cells, since they are free of interstitial and vascular tissue and can therefore be used to investigate satellite cell behaviour in the absence of other myogenic cell types.

3.1. Isolation of Satellite Cells from Isolated Myofibres

This method is based on the protocol outlined in Shefer and Yablonka-Reuveni (11) and adapted by Collins et al. (12).

1. Muscles are dissected and digested for 60 min in 0.2% (w/v) collagenase type I in Dulbecco's modified Eagle's medium in a 37°C waterbath.
2. Intact myofibres are suspended in 8 ml of plating medium. Myofibres are triturated for 5 min with a 19G needle mounted on a 1 ml syringe. The suspension is passed through a 40 μm cell sieve to remove the myofibres. The remaining satellite cell suspension is centrifuged for 15 min at 450 RCF, and the resultant pellet is resuspended in growth medium (11). Isolated satellite cells are incubated in growth medium at 37°C, 5% CO_2 in a humidified tissue culture incubator.

In addition, detailed methods for the isolation of viable muscle fibres and for grafting of muscle fibres and their associated satellite cells into mouse muscles to assess the contribution of satellite cells to muscle regeneration have been recently reported by Collins and Zammit (13).

3.2. Isolation of Satellite Cells by Enzymatic Dissociation of Skeletal Muscles

Muscle tissue from hind limbs of one mouse will yield enough cells for ten 35 mm or four 6 cm tissue culture dishes.

This method involves three main steps:

1. Dissection (use of enzymes to liberate satellite cells from cleaned and minced muscle).
2. Enrichment of satellite cell fraction.
3. Plating of satellite cells on selected substratum. Alternatively, satellite cells can be cryopreserved prior to plating.

First, it is important to disaggregate the cells from muscle tissue. The extracellular matrix in animal tissues is a complex mixture of collagens and other extracellular matrix proteins. Therefore a combination of proteolytic enzymes is required for dissociating tissues. The matrix must be effectively broken down to isolate single cells, without alteration of cellular structures. Pronase and trypsin are largely used for this purpose since both easily destroy components of the basal lamina allowing liberation of satellite cells and other intact cells. However, pronase and trypsin are also destructive to satellite cells and this limits the survival of satellite cells (*see* **Note 1**). An alternative protocol is based on the use of collagenase/dispase, which provides a combination of collagenolytic and proteolytic enzymes required for muscle tissue disaggregation, without alteration of cellular structures and without loss of cell viability.

Second, the initial isolated muscle cells contain a mixture of other cell types, mainly fibroblasts, which grow vigorously in culture and predominate if they are not removed (**Fig. 8.1**).

Different methods, such as FACS, differential centrifugation, preplating and Percoll gradient, can be used to enrich satellite cell population (*see* **Note 2**). The myoblast enrichment protocol takes advantage of the fact that myoblasts adhere to plastic much less avidly than do fibroblasts, and therefore the fibroblasts can be removed from the culture by pre-absorption on plastic tissue culture plates. However, this does not guarantee pure myoblast cell cultures. Addition of β-D-arabinofuranoside (AraC) to the differentiation media can improve the culture of myoblasts since it will select against proliferating cell type (i.e. fibroblasts) and generate pure myotube cultures. AraC (0.3 mg/100 ml) should be added after the first day in differentiation media and left on for a 24-h period.

An alternative method to that described above has been proposed by Montarras et al. (14), who reported a direct isolation of satellite cells for skeletal muscle regeneration. The authors used a *Pax3*$^{GFP/+}$ mouse line to directly isolate Pax3-green fluorescent protein – expressing muscle satellite cells – by flow cytometry from adult skeletal muscles, as a homogeneous population of small, nongranular, Pax7+, CD34+, CD45–, Sca1– cells. The flow cytometry parameters thus established enabled the authors to isolate homogeneous satellite cell population from transgenic muscles. This technique discloses also the importance of specific animal models to analyse, for example, the specific effect of homogeneous cell populations on muscle regeneration and repair.

Third, the very characteristics that allow murine myoblast enrichment also require that they ultimately grow on plates coated with specific substrates to facilitate their adherence (**Fig. 8.1**). Different substrates can be used for this purpose (*see* **Note 3**). Matrigel has been used to facilitate myoblast adhesion on plastic dishes. However, cells attached to Matrigel are hard to release. Fibronectin and laminin are more suitable substrates than Matrigel since they are components of the basal lamina. Collagen represents an alternative substratum and it is commonly used in studies on satellite cell proliferation and differentiation. Once these precautions are taken, primary myoblasts are relatively easy to propagate and spontaneously recapitulate the myogenic differentiation process upon reaching confluence or after sufficient time in culture (**Figs 8.2** and **8.3**)

Muscle cells can be cultured in DMEM supplemented with horse or foetal bovine serum and chick embryo extract (CEE). Indeed, mouse primary cultures will grow best with CEE included in the plating media. Mouse myoblasts also experience a long lag period before attaching to the culture plate. For this reason it is important not to change the media for the first 2 days to allow the cells time to adhere to the substrate.

Isolated satellite cells can also be cryopreserved. To this purpose, satellite cells can be resuspended in DMEM supplemented

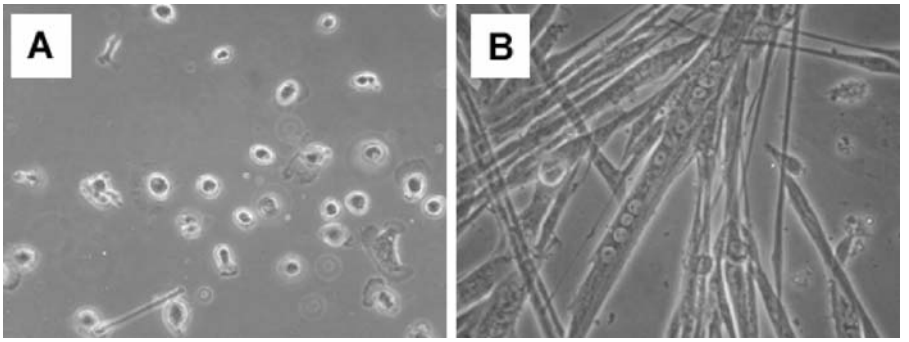

Fig. 8.2. Muscle differentiation of isolated satellite cells. Morphological analysis of (**a**) proliferating satellite cells and (**b**) terminally differentiated myotubes.

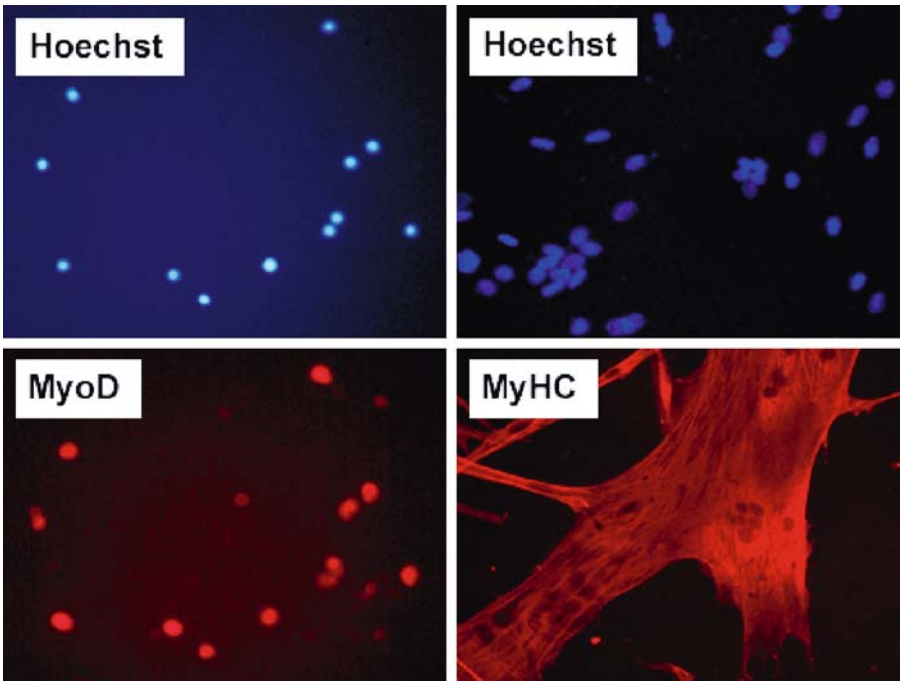

Fig. 8.3. Monitoring myogenic differentiation by MyoD and MyHC expression. Immunofluorescence analysis of MyoD and MyHC expression on proliferating (MyoD panel) and differentiated (MyHC panel) satellite cells. Nuclei were visualized by Hoechst dye (*blue*).

with 20% horse serum and 10% DMSO and aliquoted in cryovials at a density of 1.0×10^6/vial. Aliquoted satellite cells are placed at –80°C overnight and then transferred to liquid nitrogen for long-term storage. When required for experimental use, satellite cells are thawed in a 37°C waterbath, centrifuged at 1,200 RCF for 5 min, and the cell pellet resuspended in growth medium. The differentiation stage can be monitored by analyzing MyoD and myosin expression by immunofluorescence analysis (Fig. 8.3).

3.3. Procedure for Isolation of Satellite Cells by Enzymatic Dissociation of Skeletal Muscles

3.3.1. Dissection of Muscle Hind Limbs from Adult Mice (2- to 3-Month-Old Mice)

1. Prepare plates (6 cm petri dishes) for myoblast culture the day before dissection. Dissolve 10 mg collagen in 10 ml of 20% acetic acid. Cover the surface of each 6 cm dish with 2 ml of collagen solution (1 mg/ml). Aspirate the collagen solution off the plates. Place the plates at 37°C and leave for at least 3 h to dry. Alternatively, take the lid of the plates off and leave the plates under the tissue culture hood to dry.
2. Sacrifice a mouse and place it in a beaker containing 95% ethanol for 5 min. Subsequently, the mouse is placed in a sterilized beaker containing HBSS or PBS for a minimum of 10 min to remove ethanol and wash the tissues. Dissecting tools should be laid on a paper towel and constantly rinsed with 95% ethanol. Allow instruments to air-dry before touching tissue.
3. De-skin the legs and remove hind and fore limbs. Collect the legs in a petri dish containing HBSS or PBS.
4. Remove any visible fat deposits with forceps; remove bones by using tweezers and scissors to pull muscle tissue away from bone.
5. Place the isolated muscle tissue in a new petri dish containing HBSS or PBS. All subsequent manipulations are carried out in a tissue culture hood.
6. Remove HBSS or PBS with the Pasteur pipette. Mince tissue with small surgical scissors.
7. Add 10–15 ml/limb of 0.1 mg/ml collagenase type II and transfer minced tissue to a 50 ml conical tube and incubate at 37°C for 15–20 min on a rocker. This step is useful to dissociate muscle fibres and to dissolve connective tissue. Centrifuge for 3 min at 400–500 rpm; remove supernatant and resuspend the pellet in 10–15 ml of 1 mg/ml collagenase/dispase and incubate in water bath at 37°C for 30 min on rocker (collagenase/dispase, ROCHE, provides a combination of collagenolytic and proteolytic enzymes required for muscle tissue disaggregation).
8. Triturate, by pipetting several times, every 10 min. The tissue should be dispersed with no visible clumps.
9. Add an equal volume of plating media and pipette several time.
10. Filter the homogenate using in sequence 100 μm nylon mesh cell strainer (Falcon 2360), 70 μm nylon mesh cell strainer (Falcon 2340), and 40 μm nylon mesh cell strainer (Falcon 2350) (use fresh strainer as necessary).
11. Collect flow-through in a 50 ml comical tube.

12. Centrifuge in a table top centrifuge at 1,200 rpm for 10 min. Aspirate supernatant and resuspend the pellet gently in 10 ml of plating media (growth medium).
13. Pour the solution into a 100 mm petri dish and preplate in incubator (5% CO_2, 37°C) for 1 h to remove fibroblasts.
14. Carefully remove the solution from the petri dish by tilting it to one side. This solution contains the enriched myoblast population.
15. Plate the enriched myoblast solution in tissue culture dishes coated with collagen.
16. After 48 h change the medium to fresh growth medium. It is important not to change the media for the first 2 days to allow the cells time to adhere to the substrate.
17. At about 3–5 days (the myoblast should present a fusiform phenotype) shift the culture myoblasts from growth medium to differentiation medium and analyse for MyoD and myosin expression (**Fig. 8.3**).

4. Notes

1. The temperature and length of exposure of the minced muscle to the pronase and trypsin enzymes should be monitored during the isolation procedure.
2. Preplating is the most commonly used technique for removing fibroblasts.
3. Different substratum can be used to facilitate the adhesion of satellite cells to the petri dishes after a careful consideration of pros and cons of each of them:

   Matrigel (cells attached to Matrigel are hard to release for counting purpose), fibronectin (it is a component of basal lamina; expensive), laminin (more indicates in study of SC differentiation; very expensive), collagen (commonly used in studies on SC proliferation and differentiation).

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Chapter 9

Analysis of Cardiac Myocyte Biology in Transgenic Mice: A Protocol for Preparation of Neonatal Mouse Cardiac Myocyte Cultures

Nigel J. Brand, Enrique Lara-Pezzi, Nadia Rosenthal, and Paul J.R. Barton

Abstract

We describe a method of isolating and maintaining primary cultures of mouse neonatal cardiac myocytes (NCM). This is derived from the well-established procedure for making NCM cultures from rat neonates by sequential digestion of rat ventricular myocardial pieces using a collagenase/pancreatin mixture. One-day-old mouse neonates are taken and the heart excised. The great vessels, atria, and top section of the ventricular chambers are cut away and the remaining ventricular myocardium is cut into small cubes (about 1–2 mm^3). Heart pieces from at least 30 animals are then subjected to short (15–25 min) digestion in a shaking water bath in the presence of collagenase and pancreatin. Cell supernatants are taken and pooled together for a total of five digestion steps. The cells are then plated on gelatinized culture dishes and allowed to attach overnight. Myocyte cultures were inspected microscopically for up to 4 days, revealing that many myocytes beat throughout this period. This protocol may be of use for making primary cardiac myocyte cultures from transgenic mice and for investigating gene transcription and cell signalling.

Key words: Primary culture, transgenic, neonatal cardiac myocyte, collagenase.

1. Introduction

Transgenic mouse models are proving to be useful for studying many aspects of cardiac biology and pathology. These include models of congenital heart disease (1, 2), cardiac development (3, 4), signalling pathways, and gene transcription (5, 6). The use of surgery or drug treatment to simulate ventricular hypertrophy in a mouse transgenic or knock-out model forms a powerful strategy for elucidating the individual contribution of gene

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function to the disease process, particularly in the study of cardiac regeneration and repair (7). To date, in vitro biochemical and gene transcription data have largely been derived through the use of primary cultures of 1- to 3-day-old rat neonatal cardiac myocytes (NCM) in the absence of a reliable cardiac myocyte-derived cell line. Rat NCM cultures can be maintained in culture for days or weeks and are amenable to transfection with DNA constructs or treatment with pharmacologic agents that induce, or suppress, hypertrophy. Previously we have carried out a detailed examination of the expression of the human cardiac Troponin I gene (TnIc), which is only expressed in cardiac muscle (8) through transfection in rat NCM primary cultures (9–11). We use a protocol adapted from that described by Iwaki and co-authors (12) that employs sequential digestion of rat ventricular myocardial pieces with a mixture of collagenase and pancreatin in order to digest the extracellular matrix and release cells into the supernatant. The cells are collected after a short digestion, the enzymes inactivated by adding serum, then pooled and stored in a CO_2 incubator at 37°C. Following a final wash of the combined cell pellet in culture media, cells are subjected to a pre-plating step to remove as many non-myocytes (principally cardiac fibroblasts) as possible and counted. Finally, cells are plated onto gelatinized culture dishes to allow adherence of cardiac myocytes. We describe here a modified protocol that is suitable for 1-day-old mice.

2. Materials

2.1. Animals

We buy in pregnant females as newborn animals (no older than P1) (*see* **Note 1**). These are essential if myocytes are to adhere in large numbers to plates. Cells from P3 neonates have a high proportion of cells that exhibit an "adult", rod-shaped phenotype: these are contractile but do not attach to gelatinized plastic (unpublished observations).

2.2. Cell Culture and Solutions

1. NCM cultures are maintained in 4:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/l glucose (Sigma D5671) and medium M199 (Sigma M2154). This is supplemented with 10% heat-inactivated horse serum (Sigma H1138); 5% heat-inactivated foetal calf serum (FCS: Sigma F9665); 5 mM HEPES, pH 7.4; 2 mM L-glutamine (Sigma G1146); and 1× penicillin–streptomycin (Sigma P0781). Cardiac fibroblasts, prepared from the pre-plating step, are maintained in the same media or can be grown in media containing 10% FCS.
2. Preparation of 5× Ads solution (500 ml): the following are combined in 450 ml Milli Q grade water (*see* **Note 2**): 17g NaCl, 11.9g HEPES (Sigma H6147), 0.3g NaH_2PO_4, 2.5g glucose, 1g KCl, 0.25g $MgSO_4$. Adjust to pH 7.35 with 1N

NaOH and make the volume up to 500 ml. Filter-sterilize through a 0.2 μm filter and keep at 4°C. All chemicals are AnalaR grade, except where indicated.

3. For the myocyte prep, prepare 250ml 1× Ads from the 5× stock. Adjust pH to 7.35 with HCl or NaOH and filter through a 500 ml filter unit in the sterile environment of a Class II cabinet. Chill on ice.
4. 1% Gelatin solution is made by dissolving 5 g gelatin (Sigma G9391) in 500 ml of Milli Q grade water. Autoclave and store at 4°C. Warm to 37°C prior to use.
5. Enzyme solution: 24 mg of collagenase (Worthington: collagenase Type II, CLS2) and 5ml pancreatin solution (*see* **Note 3**) are dissolved in 50 ml 1× Ads solution prior to prep. Pancreatin solution is made as follows using Porcine Pancreatin (Sigma P3292). Dissolve 2.5 g pancreatin and 0.85 g NaCl in 100 ml Milli Q water. Filter-sterilize and dispense 5 ml aliquots. Freeze these until needed. Filter-sterilize through 0.2 μm filter and chill on ice.
6. 0.4% Trypan Blue solution is made by dissolving 0.4 g of Trypan Blue (Sigma T8154) in 100 ml of Milli Q water.

2.3. Equipment and Facilities Required

1. Tissue culture lab with Class II cabinet, 37°C incubator with 95% air/5% CO_2 (preferably one dedicated for primary culture, to reduce risk of contamination of cell lines by primary cultures), and a standard 37°C water bath for warming media and reagents. Also, a shaking water bath (e.g. Grant SS40-D) that has a shaking platform that moves along the longitudinal axis of the bath (*not* a rotary shaker).
2. Separate lab for dissection. This is not done in the tissue culture lab, in order to minimize infection. We dissect out hearts in an open-fronted bench-top hood that blows HEPA-filtered air across the specimen. Hood and carcasses are disinfected using a 70% IMS (industrial methylated spirits) spray prior to dissection.
3. One or two pairs of small scissors (for stunning/decapitation). Autoclave before use.
4. One pair of small curved dissecting scissors (for cutting through ribcage: VWR233-2123). Autoclave before use.
5. One pair of small curve-tipped forceps (for removing heart: VWR232-0037). Autoclave before use.
6. 4× Scissor-forceps ("spring-bow scissors": VWR233-0019). Autoclave before use.
7. 1× Fine micro-forceps (VWR232-0034). Autoclave before use.
8. 500 ml Filtering unit (0.2 μm).
9. 100 μM Cell strainers (Falcon 352360).

10. 100 mm Petri dishes (e.g. Nunclon/sterilin tissue culture grade) for dissecting heart pieces.
11. 60mm Petri dishes (Falcon "Primaria" 3802) for preplating and myocyte culture.
12. 50 ml Conical bottom Falcon tubes.
13. 25 and 10 ml Graduated disposable plastic pipettes.
14. 6-Well plates (Falcon 3046) for myocyte culture.
15. 75 cm^2 Culture flasks (e.g. Greiner 658175) for dissociating cells from tissue pieces.

3. Methods

3.1. Prior to Dissection

1. Prepare 250 ml of 1× Ads buffer from 5× stock. Dispense 25 ml into a Falcon tube, label it with number of neonates to be dissected, and place it on ice. Heart pieces will be collected into this tube. Take Ads stock and collection tube to dissection area.
2. Prepare the dissection area (as mentioned above, in our lab this is done in an open-fronted Class I hood that blows filtered air towards the operator and across the dissection). Swab work surfaces with 70% IMS. Lay down several stacks of paper towels to soak up blood. Have to hand a beaker of 70% IMS for rinsing dissecting tools and 70% IMS spray wash bottle for rinsing the decapitated bodies.
3. Prepare two 100 mm Petri dishes containing 1× Ads, one for "trimming" hearts of vessels and atria, the other for roughly chopping the remaining ventricular muscle with scissor-forceps to drain the chambers of blood. Place on ice in dissection area.
4. In the tissue culture room, switch on both water baths. Aliquot out 5 × 2 ml FCS into 50 ml Falcon tubes and number these 1–5: these are the *collection* tubes into which the dispersed cells from five successive enzymatic digestions are placed. Place in the static water bath to keep warm. Also, dispense a further 25 ml aliquot of FCS and keep warm. Prepare a 500 ml bottle of culture medium containing serum and additives. Keep two pairs of scissor-forceps aside for chopping up the heart pieces in the Class II hood.
5. Prepare the enzyme solution of pancreatin and collagenase. Filter-sterilize the solution through a 0.2 μm filter unit and place on ice.

3.2. Dissection

1. Collect the mothers and count the neonates before transfer to dissection area room (*see* **Note 4**).

2. Using a pair of scissors or a metal rod, dislocate the neck, then decapitate the animal using a sharp pair of scissors over a stack of paper towels. Rinse the body with the 70% IMS spray and transfer to a paper towel stack in the dissection area.
3. Remove the heart as follows. Grasp carcass between the shoulder blades and pinch the back using thumb and forefinger: this has the effect of throwing the chest outwards. Using a pair of small, curved scissors, cut downwards through the rib cage as far as the umbilicus (*see* **Note 5**). The chest cavity may open up sufficiently now to expose the still-beating heart. If not, press the small of the back with the middle finger and this will open up the chest, causing the heart to protrude through the gap in the chest wall.
4. Using curved forceps, grip the heart under the great vessels and pull away the ventricular mass. The vessels and the atria usually remain attached to the heart and the lungs often come away with the heart at this stage. Place the heart in the "trimming" Petri dish and place the carcass in an autoclave bag or similar for disposal.
5. Hold the heart steady using the fine dissecting forceps. Trim off blood vessels, atria, or lung tissue by cutting through the top of the ventricular myocardium with scissor-forceps.
6. Transfer the ventricular myocardium to the second Petri dish containing 1× Ads buffer and roughly cut the heart with several slices of the forceps-scissors. This is to introduce the Ads buffer to as much of the tissue as possible to allow blood to escape and reduce ischemic damage and clotting. The heart should ideally remain as a single, opened-up piece of tissue. Transfer the heart into the collection Falcon tube containing 1× Ads buffer and keep on ice (*see* **Note 6**). When all the hearts have been collected, proceed to the tissue culture lab for the cell prep.

3.3. The Myocyte Preparation

1. Place a 75 cm^2 culture flask for the enzymatic digestion of the heart pieces in the Class II cabinet hood. Have a few 100 mm Petri dishes to hand and a ready supply of 10 and 25 ml disposable pipettes. In a rack in the hood place a 50 ml Falcon tube containing fresh 1× Ads buffer – this does not have to be chilled and is used for rinsing the pipettes prior to use (*see* Step 2). Also, have a waste collection bottle or several Falcon tubes nearby.
2. Pre-wet a 10 ml pipette with 1× Ads buffer: this is crucial in order to prevent tissue pieces from sticking to dry plastic. Remove 1× Ads buffer from the collection tube containing the heart pieces using this pipette. Rinse the heart pieces with 1× Ads twice more in this manner to remove blood

(*see* **Note 7**). Finally, add 20 ml 1× Ads and tip the heart pieces into a 100 mm Petri dish.

3. Mince the hearts finely into pieces approximately about 1–2 mm^3, using one or two pairs of scissor-forceps. Work quickly. Do not cut the pieces too small and aim to get as many as possible the same size.
4. Pre-wet a 25 ml pipette with 1× Ads buffer and transfer the tissue from the Petri dish to the 75 cm^2 culture flask. If any tissue pieces *are* taken up, stop pipetting: they will quickly sink to the bottom and can then be expelled with a few drops of liquid back into the dish (*see* **Note 8**).
5. Incline the flask on its short edge at an angle to cause the tissue pieces and buffer to congregate at one corner. Take off as much 1× Ads as possible with a pre-wetted 10 ml pipette, but be careful not to remove any tissue: leave some liquid if necessary.
6. Add 10 ml of enzyme solution. Place the flask lengthways in the 37°C shaker water bath and incubate for 5 min at 230 rpm. This step is to remove blood cells that have adhered to the heart pieces and tissue debris (*see* **Note 9**).
7. After 5 min, remove flask from shaker. Using a pre-wetted 25 ml pipette, gently triturate the solution four to five times (i.e. draw the tissue pieces up and down the pipette). This aids release of cells from the tissue pieces. Try not to get bubbles into the pipette when triturating. Incline the flask to let the tissue settle out in one corner. Take off the supernatant, which should be cloudy with blood cells and cell debris, with a 10 ml pipette and discard to waste. Add a fresh 10 ml aliquot of enzyme solution to the flask and return it to the shaking water bath for 20 min. This will form the first lot of cells for collection into FCS tube #1 (*see* **Table 9.1**).
8. After this first digestion, remove flask from shaker and triturate as before with a pre-wetted 25 ml pipette. Incline flask to sediment the tissue lumps and carefully transfer the

Table 9.1
Volumes of enzyme solution used at each collection step

Collection step	1	2	3	4	5
Amount of enzyme solution added (ml)	10	8	8	6	6
Time of shaking (min)	20	25	25	15	20

supernatant with a pre-wetted 10 ml pipette to the first collection tube that contains 2 ml FCS (collection tube #1). Centrifuge the tube for 5 min at 800–1,000 rpm at room temperature (RT). The FCS will adsorb the enzymes, preventing further digestion.

9. Meanwhile, add 8 ml of the enzyme solution to the flask and return flask to the shaker for 25 min for the second digestion.
10. When the cells in #1 collection tube have been pelleted, remove tube to the hood and carefully aspirate off the supernatant. Resuspend the cell pellet, which will be quite loose, in 4 ml pre-warmed FCS, re-label this tube "CELLS", and place in a beaker in a gassed incubator, with the top unscrewed slightly to allow gas exchange. All four subsequent collections of cells will go into this tube (*see* **Note 10**).
11. At the end of 25 min, remove flask from shaking water bath and proceed to collect cells (this is collection #2) following Steps 8–10.
12. Repeat three more times until you have collected the cells from five digestions. You will have 20 ml of pooled cells in FCS at this stage after enzymatic digestions. However, if using a small number of hearts, you may find that only four digestions are necessary (*see* **Note 11**). Discard the flask and move on to the pre-plating stage.

3.4. Pre-plating to Remove Fibroblasts

1. After the final cell collection, spin down the entire 20 ml contents of the "CELLS" tube at 800–1,000 rpm for 5 min at room temperature (*see* **Note 12**). Aspirate the serum carefully and resuspend the cells gently in 25 ml culture medium.
2. Dispense the cells into five Primaria Petri dishes as follows. Pre-wet a 100 μm cell strainer with culture medium. Dispense the cells through this sieve, about 5 ml per dish, to remove any large tissue lumps. If any liquid from the cell suspension is retained on the sieve, then wash through onto the Primaria Petri dishes with some more media. Rock plates back and forth to distribute cells (do not swirl the dishes – you will simply concentrate all the cells in the centre of the dish) and place in incubator for 45 min. During this time, non-myocytes (principally fibroblasts) will adhere: the myocytes will largely remain floating.
3. Remove plates from incubator and gently bang the plate five times to help dislodge any myocytes. Using a 10 ml pipette, take up the solution covering the dishes and expel it back onto the plate. This process is called "sweeping" and helps dislodge weakly attached myocytes. Fibroblasts will remain

firmly attached. Sweep the plate several times and then transfer the supernatant to a clean 50 ml Falcon tube. At the end, wash each plate sequentially with 5–10 ml culture medium and add this to the cells (*see* **Note 13**).

4. Count the myocytes using a haemocytometer chamber. Take 80 μl of the cell suspension and mix with 20 μl 0.4% Trypan Blue solution (*see* **Note 14**). Count viable myocytes: these will be the largest cells visible. Smaller cells (non-myocytes) will also be apparent and are usually ignored during counting. Ignore any clumped cells, but counting pairs, trios, etc., and calculate the average number of cells per quadrant. Multiply this figure by 1.25×10^4 to correct for the dilution factor and to calculate the number of cells per ml of supernatant. The yield may range from 0.3 to 0.8×10^6 cells per heart. Percentage of live cells as judged by Trypan Blue exclusion will be typically in the range 50–70%.

3.5. Plating the Myocytes

The myocytes are plated onto gelatinized culture wells or plates to maximize adherence. Myocytes typically take 16–20 h to attach and spread out on plastic. Gelatinization is conveniently done during the pre-plating stage.

1. Gelatinize plates (60 mm Primaria) or multi-well dishes with 2–3 ml of a 1% solution of gelatin for at least 20 min.
2. Aspirate the gelatin solution and remove all liquid with a sterile glass Pasteur pipette attached to a vacuum line. Let the plates/dishes dry in the Class II hood with lids ajar for 10 min.

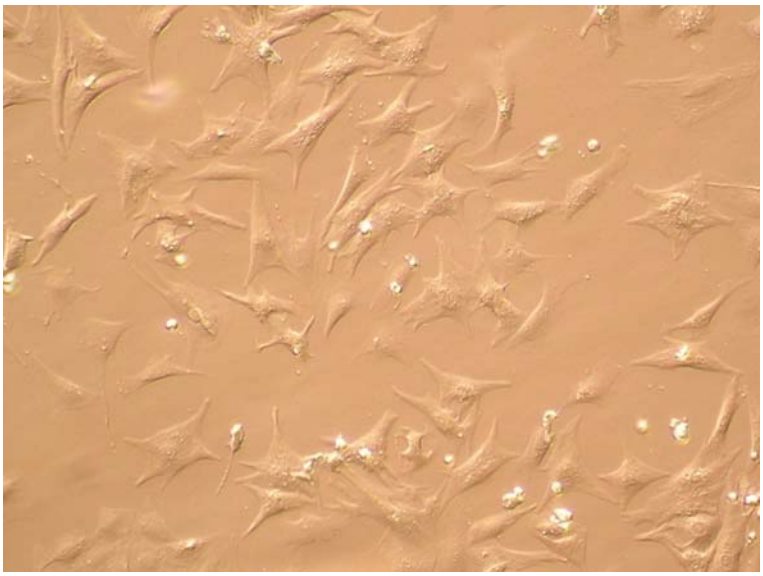

Fig. 9.1. Neonatal mouse cardiac myocyte preparation (P1) 1 day after plating.

3. The cells should be plated fairly quickly at appropriate density in culture medium. Place in the incubator, agitate along longitudinal axis to disperse cells evenly (do not swirl plates), and leave for at least 18 h for the cells to allow the myocytes to settle out and adhere.
4. Next day, check viability by microscopy. The cells can be washed once or twice in a few millilitres of culture medium to remove dead, unattached cells. **Figure 9.1** shows cells that have adhered the day following isolation. Note that many myocytes have a distinct angular appearance at this stage. Myocytes maintained in high serum will hypertrophy within 2–3 days, as shown in **Fig. 9.2.** To prevent this, the percentage of serum may be lowered to 4%.

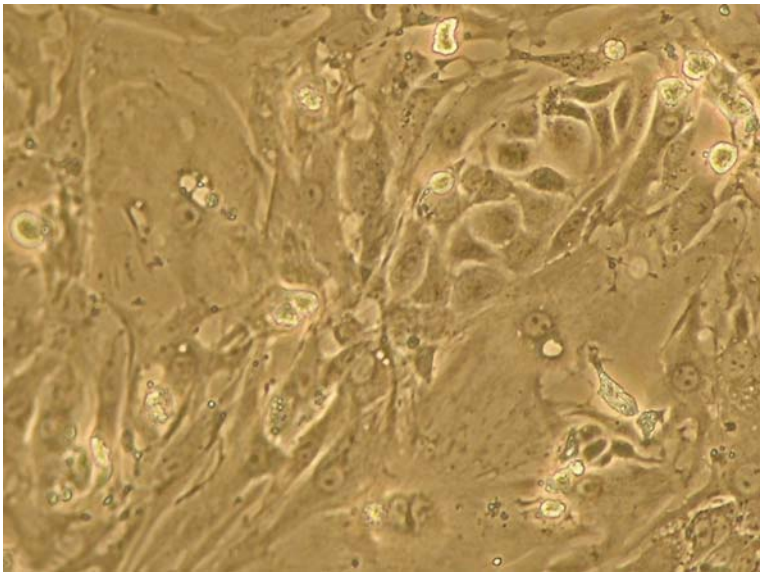

Fig. 9.2. Neonatal mouse cardiac myocyte preparation (P1) 4 days after plating. Myocytes have elongated and aligned themselves along their long axis. Note the cluster of fibroblasts, which display a typical tight "cobblestone" morphology, in the *right* of the view.

4. Notes

1. The age of the animals is absolutely critical. We originally tried using P3 animals and had a poor yield of adherent myocytes. At time of isolation, many of the myocytes appeared under the microscope to resemble adult myocytes, with a rod-shaped, striated morphology. Next day, very few had attached to the culture plates. Fewer rod-shaped cells are apparent in P1 cell preps and most myocytes will attach to gelatinized plastic following an

overnight incubation. Adherence of older cells might possibly be improved by using fibronectin or laminin instead of gelatin. This protocol and the volumes of enzyme quoted (**Table 9.1**) works well with 20–60 hearts. It is inadvisable to use less than 20 hearts with this protocol as the yield will suffer, probably due to the effective concentration of collagenase and pancreatin being too high. We have not tried using lower amounts of enzymes, but this may be feasible for smaller numbers of animals.

2. All solutions for tissue culture are made in Milli Q or equivalent water that has a resistance of 18.2 MΩ-cm or less.
3. Pancreatin helps reduce clumping together of the tissue pieces.
4. It is important to remember that the heart of a 1-day-old mouse is extremely small. Therefore, assuming a yield of no more than half a million cells per heart, large numbers of animals may be needed if planning to do transfection/siRNA studies using luciferase or similar reporter plasmids.
5. We find curved scissors easier to handle: it is essential to keep the tips facing outwards to avoid puncturing the heart. Take care not to cut beyond the umbilicus or you may puncture the intestine or stomach – the latter is full of milk and a potential source of contamination.
6. It is usually helpful and more efficient if one person kills the neonates and removes heart, while a second person trims hearts and chops them in collecting Petri dish.
7. Do not use a pipette on a vacuum line to aspirate solutions during the prep as you may inadvertently lose heart pieces.
8. Do not use a 10 ml pipette as the bore is small enough to allow these small pieces of heart to get stuck in the mouth of the pipette.
9. Place flask so the pieces are shaken along the long axis of the flask.
10. We have used this protocol successfully for over 12 years for making rat NCM cultures and found that most cells are liberated in the second and third digestions, hence the times of digestion described for the various steps reflect this.
11. Four, or even three, digestions may be enough, depending on numbers of animal used. By the later collections, the tissue pieces should be getting very small as the enzymes digest the extracellular matrix and cells are liberated. The tissue also becomes noticeably whiter and more fibrous. Beyond this point, not many cells will be collected. The overall yield might be improved by ceasing trituration for

the final two steps: it is possible that when there are few remaining cells, the stress of triturating combined with the high local concentration of enzymes may affect cell viability.

12. We find that pre-plating significantly reduces the amount of contamination by cardiac fibroblasts or other non-myocytes. To prevent proliferation of surviving fibroblasts 5-bromo-2′-deoxyuridine (BrdU) can be added to the culture media. More fibroblasts can be encouraged to plate down if larger Petri dishes are used for the pre-plating, but this obviously increases the volume of the supernatant.
13. If fibroblasts are required for further experiments, rinse the Petri dishes again with medium, aspirate, and re-feed with 4 ml culture medium. Place in incubator. Fibroblasts can be grown on for several passages and frozen down, if required.
14. Place cells back in incubator whilst counting: it is essential to keep the cells warm in order to maintain maximum viability.

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Chapter 10

Short- and Long-Term Cultivation of Embryonic and Neonatal Murine Keratinocytes

Reto Caldelari and Eliane J. Müller

Abstract

Studies using cultured cells allow one to dissect complex cellular mechanisms in greater detail than when studying living organisms alone. However, before cultured cells can deliver meaningful results they must accurately represent the in vivo situation. Over the last three to four decades considerable effort has been devoted to the development of culture media which improve in vitro growth and modeling accuracy. In contrast to earlier large-scale, non-specific screening of factors, in recent years the development of such media has relied increasingly on a deeper understanding of the cell's biology and the selection of growth factors to specifically activate known biological processes. These new media now enable equal or better cell isolation and growth, using significantly simpler and less labor-intensive methodologies. Here we describe a simple method to isolate and cultivate epidermal keratinocytes from embryonic or neonatal skin on uncoated plastic using a medium specifically designed to retain epidermal keratinocyte progenitors in an undifferentiated state for improved isolation and proliferation and an alternative medium to support terminal differentiation.

Key words: Serum-free mouse keratinocyte cultures, CnT culture conditions, keratinocyte progenitor cells, feeder-free keratinocyte cultures.

1. Introduction

As the dominant cell type of the epidermis, keratinocytes drive the perpetual renewal of the outermost layer of the skin. They arise from mitotic division of epidermal progenitor or stem cells to then go on to build up the stratified epithelium by proliferation

The last author would like to state that she is a co-founder and the president of the Board of CELLnTEC, Advanced Cell Systems AG.

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and terminal differentiation (1–3). When cultured under appropriate conditions, epidermal keratinocytes retain their capacity for proliferation and terminal differentiation in vitro. Early culture protocols for mouse and human keratinocytes from the 1970s and early 1980s required Swiss 3T3 fibroblast feeder layers or fibroblast-conditioned medium in addition to collagen IV coating of the culture dish (4–7). Such early protocols are still in common use today (8, 9). However, the relatively recent development of more complex, growth factor-supplemented, and fully defined media now allows the culture of mouse keratinocytes on plastic without the need for specialized plate coatings or the addition of fibroblasts or fibroblast-conditioned medium (10). The latest generation of media for culturing keratinocytes without the use of serum or fibroblasts was developed in our laboratory in a collaborative project. These media were designed to improve maintenance of progenitor cells in vitro by mimicking the environment of the stem cell niche, thereby activating signaling pathways involved in stem cell retention in vivo.

If handled with care, mouse keratinocytes cultivated on plastic only can be maintained for over 60 passages (approximately 180 population doublings) without losing their ability to terminally differentiate (10). These cultures are therefore suitable for either short- or long-term studies. However, one important aspect to be considered is the tendency of cultured murine keratinocytes toward chromosomal instability (11–13). After about five passages, mouse keratinocyte cultures become increasingly tetra- or polyploid, independent of the culture conditions. However, this does not seem to affect the differentiation capacity of these cells (10, 12, 13) which is in line with the observation that tetraploid mouse embryos develop normally in all organs except the forebrain and associated tissues (14). Nevertheless, outgrowth of single colonies at isolation or during subculture must be avoided, and it is recommended that all experiments for a particular study be carried out on cultures within a defined passage range. By respecting strict culture conditions (passaging cells on a regular basis) in addition to maintaining a heterogeneous cell population by avoiding selection of single clones, we experienced that different passages provide highly reproducible results (10, 13, 15, 16).

Another important aspect of working with keratinocytes and mouse keratinocyte cultures in particular is that terminal differentiation is often initiated when cultures become confluent (13, 17). If this occurs three pitfalls are observed: (1) the subculture of differentiated keratinocytes is hampered, presumably as a result of the failure of the postmitotic cells to re-enter the cell cycle, (2) subculturing postmitotic keratinocytes can provide misleading results due to the remnant expression of terminal differentiation markers after seeding, and (3) the results are not reproducible when repeating experiments. This confirms a gen-

eral rule that experiments with cultured keratinocytes must all be done at an equivalent differentiation stage, which requires a fixed and reproducible culture schedule.

The method to isolate and subculture mouse keratinocytes on plastic outlined below is quite simple, and therefore well suited for investigators working with mouse keratinocytes for the first time, as well as for those wishing to set up numerous parallel keratinocyte cultures, for example, from various genetically modified mouse strains. Using this protocol, wild-type and knockout keratinocyte cultures have been used to gain insight into a wide range of cellular processes, including proliferation, terminal differentiation, and apoptosis, as well as the molecular mechanisms of cancer and other skin diseases (*see*, for example (13, 18–23)).

2. Materials

2.1. Keratinocyte Culture

2.1.1. Mice

1. Source of mouse keratinocytes: skin from C57BL/6 mouse embryos at embryonic days E17–E19 (just before birth) or neonatal C57BL/6 mice (*see* **Note 1**).

2.1.2. Culture Media and Supplements

1. *Keratinocyte medium* (*see* **Note 2**): All media described are from CELLnTEC, Advanced Cell Systems AG (Bern, Switzerland), and are referred to as CnT media in the following protocols. They are serum free and contain low calcium levels (0.07 mM). CnT-07 and CnT-02 are fully defined. According to their needs, investigators can choose the most appropriate medium from the following three distinct formulations:
   a. CnT-57 (cat. no. CnT-57): A progenitor cell targeted medium formulation, specifically designed to retain progenitor cells in an undifferentiated phenotype in vitro. It contains a low amount (6 μg/ml) of bovine pituitary extract (BPE), which is four to eight times lower than in commonly used alternative BPE-containing formulations. It provides maximum isolation efficiency and early passage growth, but may also support growth of melanocytes or fibroblasts in primary culture.
   b. CnT-07 (cat. no. CnT-07): Also a progenitor cell targeted medium, but is fully defined. Provides slightly lower colony forming efficiency at isolation than CnT-57, but is very selective for keratinocyte growth.
   c. CnT-02 (cat. no. CnT-02): Designed to allow unimpeded keratinocyte differentiation. Used instead of CnT-57 or CnT-07 whenever cells are induced to differentiate.

2. Antibiotics/antimycotics are added to all media, except if stated otherwise. The final concentration is 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (cat. no. CnT-ABM, CELLnTEC, Bern, Switzerland).

2.1.3. Solutions and Materials for Culture and Subculture

1. Dissociation solution: (a) Dissolve the appropriate amount of powder at 5 mg/ml dispase (Roche cat. no. 04942078001) in CnT medium and filter sterilize (0.22-μm pore size). (b) Alternatively, CnT-NPD50 can be used.
2. (a) TrypLE Select (Invitrogen cat. no. 12563029) is used straight, no inactivation needed. After detachment of cells it is simply diluted with 2.5 volumes of medium followed by centrifugation. (b) Alternatively, 2.5× trypsin/EDTA (Bio-Concept, Amimed 5-51 KOO–H) can be used. For this purpose, 10× trypsin/EDTA (0.125%/0.05%) is diluted 4× with PBS; inactivation with 10% FCS-containing medium is essential. Soybean trypsin inhibitor may also be used.
3. Cell culture consumables used include various sizes of culture flasks (we prefer Techno Plastic Products – TPP, Trasadingen, Switzerland), glass pipettes, aerosol-resistant filter tips (MBP-ART, Fisher Scientific or Biosphere filter tips, Sarstedt), non-tissue culture-treated Petri dishes (e.g., Falcon 351112).

2.1.4. Solution and Material for Freezing

1. Freezing medium (2×): 40% CnT medium, 40% fetal calf serum, and 20% DMSO. Alternatively, we also recommend the use of a defined freezing medium with better thawing efficiency CnT-CRYO-50 (cat. no. CnT-CRYO-50, CELLnTEC, Bern, Switzerland).
2. NALGENE@ Cryo 1°C freezing container (cat. no. 5100–0001).

2.2. Transfection Reagents

1. Polyethylenimine (PEI, linear, MW-25,000; cat. no.23966 Polysciences, Inc., Warrington, PA 18976, USA).
   a. Dissolve powder at a concentration of 2 mg/ml in water heated to 80°C.
   b. Allow the solution to cool to room temperature.
   c. Adjust pH to 7.0 with 5 M HCl.
   d. Filter sterilize.
   e. Store aliquots at –80°C until further use.

2.3. Reagents and Material for 3D Cultures

1. Millicell inserts (Millipore; 24-well inserts, filter area 0.6 cm^2, cat. no. PIHP0I250; 6-well inserts, filer area 4.2 cm^2, cat. no. PIHP03050) (*see* **Note 3**).

2. Diff-Quik staining solutions I and II (Dade Behring AG, Switzerland).
3. Differentiation medium: we recommend to use CnT-02-3DP (cat. no. CnT-02-3DP, CELLnTEC, Bern, Switzerland).

3. Methods

3.1. Keratinocyte Isolation and Culture

3.1.1. Primary Cultures

a. *Embryos*

1. Open the body of a pregnant mouse (E17–19) with a sterile scalpel.
2. Remove the entire uterus (containing the embryos), (Komma) place in a Petri dish and directly transfer to a laminar flow hood.
3. Open the uterus and dissect free each embryo with a sterile scalpel.
4. Cut the umbilical cord as close as possible to the embryo.

b. *Embryos and neonates*

5. Decapitate using sterile scalpel.
6. Cut off the arms, feet, and tail.
7. Cut skin ventrally from neck to tail using small scissors and peel off the skin in one piece using forceps.
8. Place the skin in a 15-ml centrifuge tube containing 12 ml dissociation solution and 2× antibiotics/antimycotics (for neonatal mice use 5× antibiotics/antimycotics).
9. Continue with the other embryos/mice; one to five skins may be pooled.
10. Incubate the skins in dissociation solution overnight (approximately 15 h) at 4°C. Ensure good contact between skin and dissociation solution by placing the tubes in a horizontal orientation. If multiple skins are pooled, ensure that they are well distributed in the tube.
11. The next morning, pour the skins together with the dissociation solution into a Petri dish.
12. Transfer each skin to a new Petri dish containing CnT medium to wash away excess protease.
13. While holding the skin submerged in medium, gently separate the dermis (pink, opaque, gooey) from the epidermis (whitish, semi-transparent) with two pairs of curved forceps (*see* **Note 4**).

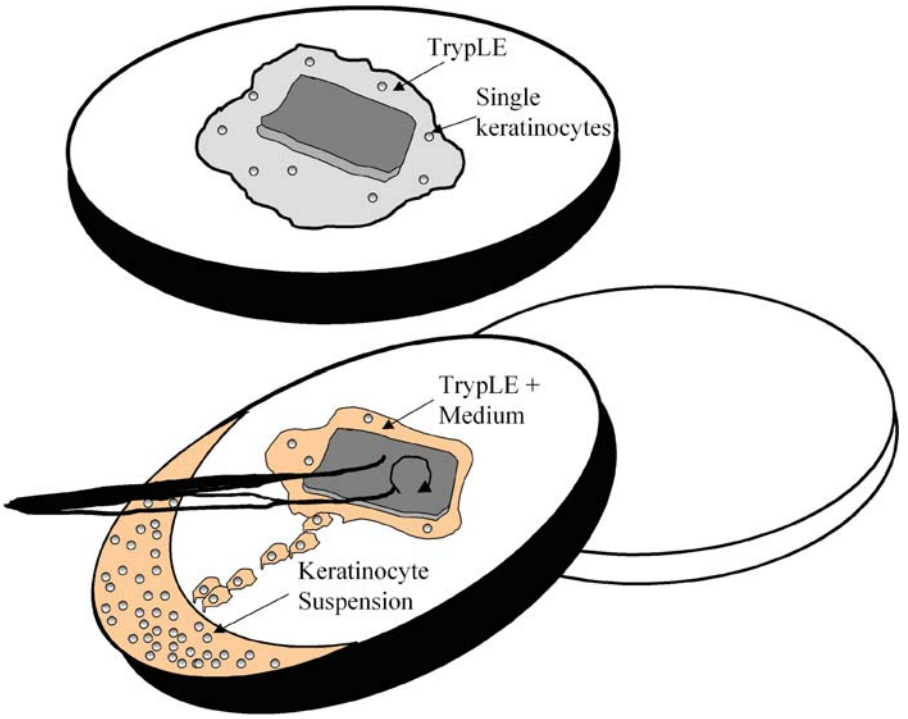

Fig. 10.1. Keratinocyte isolation from epidermis. After incubation on a drop of TrypLE Select, the epidermal sheet is rubbed on the tilted Petri dish in the CnT medium/TrypLE Select mixture.

14. Place a 500-µl drop of TrypLE Select in a new Petri dish. Lift the epidermis out of the CnT medium with one pair of forceps (the tissue will collapse) and slowly transfer it onto the surface of the drop of TrypLE Select with the basal layer downward. The tissue should unfold again and spread out flat on the surface of the drop of liquid, if not, remove any folds by gently agitating underneath the tissue with a second pair of curved forceps (**Fig. 10.1**).
15. Incubate for 20–30 min at room temperature (cover with the lid to prevent evaporation).
16. Tilt the Petri dish at an angle of approximately 30° and add 2 ml of CnT medium to the epidermis (diluting the remaining TrypLE). From this point leave the dish tilted (by lifting one side onto the lid) to minimize expansion of liquid and make collection of the cells more efficient. Gently rub the epidermis on a small area of the base of the Petri dish to gently separate single cells from the cell sheet. The medium will become turbid (**Fig. 10.1**).
17. Use 1–2 ml of fresh medium to wash single cells down to the bottom of the tilted dish.
18. Transfer the single cell solution to a 15-ml centrifugation tube.

19. Repeat rubbing after adding another 2 ml of CnT medium, then collect the cell suspension in the same 15-ml tube.
20. Spin the cells at 160*g* at room temperature for 5 min and discard the supernatant.
21. Re-suspend the cell pellet in 2-ml CnT medium.
22. Count the cells using a hemocytometer.
23. Seed cells at a density of 4–5 $\times$ $10^4/cm^2$ in culture flasks (12.5, 25, or 75 cm^2; to obtain an even distribution square flasks are preferred over Petri dishes).
24. Culture the cells at 35°C and 5% CO_2.
25. Change the medium every 2–3 days.
26. Passage the cells just before they reach confluency which is around 1 week after seeding. If only single colonies grow, discard the flask to avoid clonal selection.

3.1.2. Subculture

1. Aspirate the medium.
2. Wash the cells with PBS (optional).
3. Add 1 ml of TrypLE Select per 12.5 cm^2 flask (for bigger flasks use proportionally more volume) to the cells and incubate for about 10–15 min at 35°C. Check for cell detachment under the microscope.
4. Add 5 ml CnT medium to detach all the cells and pipette 2–3× vigorously up and down to break up clumps (this prevents cells from being trapped in DNA of dead cells).
5. Transfer the cell suspension into a 15-ml centrifuge tube.
6. Spin the cells at 160*g* for 5 min.
7. Aspirate supernatant and re-suspend the pellet in 5-ml CnT medium.
8. Count cells and seed 3 $\times$ $10^4/cm^2$ in culture flasks containing 4-ml CnT medium/25 cm^2.
9. Change medium every 3 days.
10. Trypsinize the cells when they reach confluency (**Fig. 10.2**; *see* **Note 5**).

3.1.3. ***Freeze Cells*** (*not* recommended before passage 3)

1. Subconfluent monolayers are trypsinized as described above.
2. Count the cells and adjust the concentration to 2–3 $\times$ 10^6 cells/ml with *cold* CnT medium (place the cells on ice while counting).
3. Add drop-wise the same amount of *cold* freezing medium, while swirling the tube (final cell number 1–1.5 $\times$ 10^6 cells/ml).

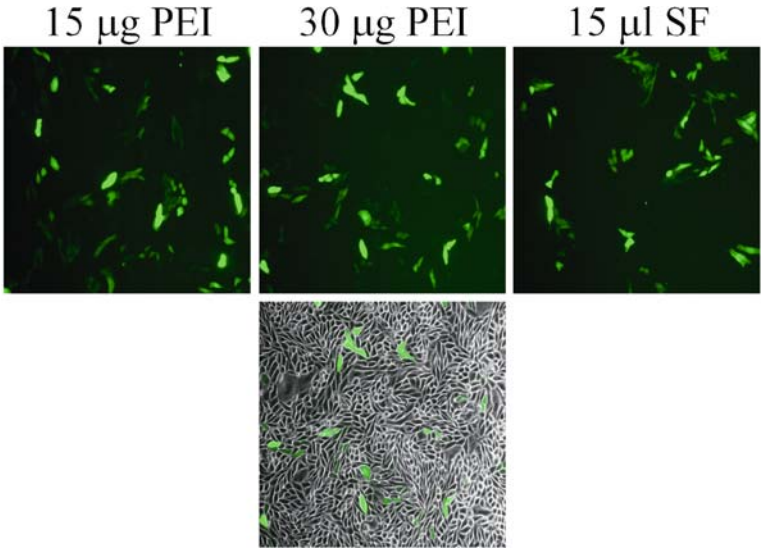

Fig. 10.2. Comparison of transfection efficiency 50 h after transfecting 1.9 μg GFP expression plasmid and 15 μg or 30 μg of PEI or 15 μl of Superfect (SF) per 360,000 β-catenin knockout keratinocytes (*upper panel*). The *lower panel* shows an overlay of fluorescence with a phase-contrast picture. Results were in a similar range for all treatments, with slightly better efficiency using PEI and approximately three times higher efficiency when adding 10% FCS to the overnight culture (data not shown).

4. Add 1 ml cell suspension per labeled cryotube (1–1.5 × 10^6 cells to be seeded in a 25-cm^2 flask).
5. Immediately transfer tubes to a NALGENE® Cryo 1°C freezing container.
6. Place at –80°C overnight.
7. Permanently store tubes in liquid nitrogen.
8. About 1 week after freezing, check viability of cells in one vial by thawing and subculturing.

3.1.4. Thaw Cells

1. Thaw cells in a 37°C water bath until just melted.
2. Immediately transfer the cells into 5-ml CnT medium in a 15-ml centrifuge tube.
3. If cells were frozen in CnT-CRYO-5 freezing medium, seed the cells at 1–3 × 10^4 cells/cm^2 in 25-cm^2 flasks (final volume of 5 ml/25 cm^2 flask). Alternatively, if the freezing medium containing DMSO was used, transfer the cells in 10 ml CnT medium and centrifuge at 160*g* at room temperature. Resuspend pellet in 5 ml CnT medium.
4. Change medium the next day and then subsequently every third day.
5. Passage cells when they are confluent and subculture as outlined above.

3.2. Using Submerged Keratinocyte Cultures for Experimentation

1. Regularly growing mouse keratinocyte cultures from around passage 5 up to passage 40 are routinely used and analyzed in our own experiments. Keratinocytes below passage 5 may also be used, but may proliferate more slowly. Above passage 40 to over passage 100, cells still appear to proliferate normally and terminally differentiate, as judged from the stabilization of adhesion structures and expression of terminal differentiation markers (data not shown). However, extended time in culture increases the chances of selecting aberrant phenotypes, thus it is not recommended to use cells above passage 40.
2. Prior to use in experiments, keratinocytes must be passaged in a strict rhythm, using a consistent seeding density ($3 \times 10^4/cm^2$) and passaging interval. In addition cells should always be seeded at the same density in the experiments themselves (we use $3 \times 10^4/cm^2$ for all experiments except for reporter gene assays where they are seeded at $4 \times 10^4/cm^2$, see below). The latter is of importance as the seeding density defines the interval until cells reach confluency and enter into terminal differentiation.
3. Stimulations must be done at the same stage of confluency in all experiments. As already mentioned, this is of crucial importance as cell density defines the status of differentiation even in the absence of elevated extracellular $CaCl_2$ concentrations (13). To recall the exact density, pictures may be taken (*see* **Note 6**).
4. If intercellular adhesion and terminal differentiation are to be analyzed, differentiation should be triggered by the addition of 1.2 mM $CaCl_2$ to CnT-02, thereby increasing the calcium concentration from 0.07 to 1.27 mM.

3.3. Transfection of Keratinocytes (Well Suited for Reporter Gene Assays)

1. Twenty-four hours prior to transfection, seed keratinocytes at a density of $4 \times 10^4/cm^2$ in either 6-well or 12-well plates (wells smaller than those of 12-well plates are not recommended; *see* **Note 7**).
2. For 360,000 cells (one 6-well plate), mix a maximum of 2 μg DNA with 50 μl of basal medium (CnT medium without supplements, serum, antibiotics, etc.) by pipetting up and down.
3. Add 7.5 μl of a 2 mg/ml PEI stock and pipette up and down.
4. Incubate the mixture for 8 min at room temperature.
5. Add 450 μl CnT medium containing 10% FCS.
6. Apply the DNA-PEI medium mixture to the cells and incubate at 35°C for 2 h while rocking the plate every 10 min.

7. Wash cells 3× with PBS.
8. Add 2 ml of CnT medium containing 10% FCS to enhance transfection efficiency.
9. Incubate overnight.
10. Transfection efficiency will typically be 7–15% (**Fig. 10.2**).

3.4. Other Useful Information for Submerged Cultures

1. Total RNA isolation kits generally yield in the range of 1 μg total RNA from 1.8×10^5 cells (*see* **Note 8**).
2. Approximately 700 μg total protein (depending on confluency) is typically recovered from 10^6 cells (*see* **Note 9**).

3.5. 3D Cultures

1. Place Millicell inserts into a Petri dish. Two sizes are commonly used: 24-well (small) or 6-well (big) *(see* **Notes 3, 10 and 12**). Prepare two spare inserts to monitor confluency later on.
2. Add 9×10^4 cells in 400 μl CnT medium per small insert or 6.3×10^5 cells in 2 ml CnT per big insert (provides a density of 1.5×10^5 cells/cm^2).
3. Add the appropriate amount of CnT outside the inserts, so that the inside and outside are at equal levels, making sure that no air bubbles are trapped underneath the membrane.
4. Place the inserts in a humidified incubator at 35°C and 5% CO_2 (if the incubator is opened frequently, place the dish into a bigger dish containing water).
5. After 3 days examine cell growth/confluency by staining a spare insert with Diff-Quik (*see* **Section 3.5.1**). If the monolayer is confluent, proceed with Step 6, otherwise change CnT in the remaining inserts and cultivate for another day. If confluency is not reached by this time, please discard the inserts and start again (**Fig. 10.3a**).
6. Replace the culture medium with differentiation medium (CnT-02-3DP) in and outside the insert.
7. Place the inserts in the incubator overnight (15–16 h) to allow cells to stabilize intercellular adhesion structures.
8. Initiate 3D cultures by aspirating the entire medium inside the insert and replacing outside medium with fresh differentiation medium up to the level of the membrane (*see* **Note 11**).
9. If a time course study is performed, inserts can be left in the same dish with medium changes every 2–3 days. If not, inserts are placed in 24-well (small inserts) or 6-well (big inserts) plates and the medium is changed daily.
10. After 14–16 days all layers are formed.

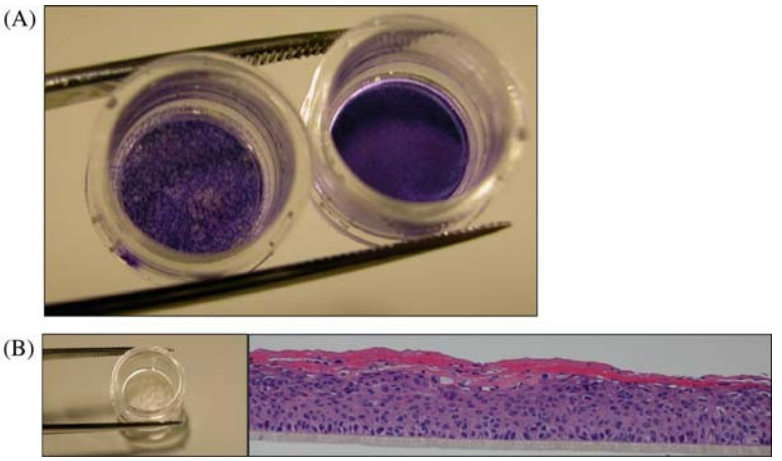

Fig. 10.3. (**a**) Diff-Quik stained inserts. The insert on the *right* shows a continuous staining, indicating a continuous monolayer ready to begin 3D culture. In comparison the one on the *left* is not yet confluent and should be allowed to proliferate further before initiating the 3D culture. (**b**) 16-day-old 3D culture of C57BL/6 mouse keratinocytes. Macroscopic view of an insert before fixation (*left*) and microscope image of the same paraffin-embedded 3D culture, sectioned and stained with hematoxylin and eosin (*right*).

11. Processing:
    a. To proceed to histochemistry, immunohistochemistry, or immunofluorescence analyses, 3D cultures are fixed overnight at 4°C by placing the insert in a separate 24-well plate and filling the well and the inside of the insert with 4% paraformaldehyde/PBS. Cut the skin/membrane out and proceed for paraffin embedding and hematoxylin and eosin staining (**Fig. 10.3d**).
    b. If optimal cutting compound (OCT) embedding is necessary for the preparation of frozen sections, omit fixation. Cutting out the insert needs special care.
    c. If RNA or protein expression studies are to be performed, cells are lysed in the appropriate lysis buffer directly in the insert.

3.5.1. Diff-Quik Staining of Cells

1. Fix cells with 400 µl 100% methanol for 5 min.
2. Pour off methanol.
3. Add 400 µl Diff-Quik I for 5 min.
4. Wash twice with water.
5. Add 400 µl of Diff-Quik II for 5 min.
6. Wash twice with water.
7. Allow to air dry in a cell culture hood.
8. Examine macroscopically and with light microscopy for a completely formed monolayer (**Fig. 10.3a**).

4. Notes

1. Best success in isolating epidermal keratinocytes was achieved from C57BL/6 mice (and different knockout derivatives); however, keratinocytes from mice of 129, FVB, and BalbC genetic backgrounds (and crosses generated from these strains) were also successfully cultured.
2. The method to isolate murine keratinocytes was originally described using defined keratinocyte serum-free medium (KSFM; Invitrogen) (10). To increase isolation efficiency and reproducibility we developed the CnT media in collaboration with CELLnTEC Advanced Cell Systems AG (Bern, Switzerland).
3. We use the Millicell-PCF culture plate inserts (Millipore). However, due to the opaque membrane, the cells cannot be examined under a phase-contrast microscope.
4. If the epidermis does not separate well from the dermis, incubation in dispase solution must be continued for another hour to avoid fibroblast contamination.
5. It is important to subject cells to a strict regime, that is they must be split before reaching confluency and should not, wherever possible, be passaged irregularly. During early passages (1–6) the time needed to reach confluency is longer than after passage 6–8. From this point on a weekly passaging rhythm should be possible.
6. If the keratinocytes are cultured in CnT-07 or CnT-57, the medium must be replaced with CnT-02 for differentiation as CnT-07 and CnT-57 support maintenance of stem cell characteristics at the expense of terminal differentiation.
7. Transfection of keratinocytes with expression plasmids and/or reporter genes is generally dependent on three parameters: (i) the quality of the cells, (ii) the size of the culture device (experimental testing indicates that the size should not be below 3.7 cm^2 or a 12-well plate), and (iii) the transfection agent. We have tested various polyanionic transfection agents such as Fugene (Roche), Lipofectamin 2000 and Superfect (both Invitrogen), and polyethylenimine (PEI). As defined by transfecting a GFP expression plasmid, PEI was found to work best for the keratinocyte cultures described here while Superfect gave slightly lower results (**Fig. 10.2**).
8. We use the Qiagen RNA isolation kit (RNeasy Plant Mini Kit Cat. # 74904 containing QIAshredder spin columns for homogenization).

9. We use 2× Laemmli buffer after the method of Laemmli (24).
10. To prevent daily medium changes, multiple inserts are incubated together in dishes (e.g., eight small inserts/22-cm^2 dish).
11. It is important that the incubator is held at high humidity (above 90%) to prevent evaporation of the medium (dishes can be kept in a tray containing water-soaked tissue paper, gas exchange must be warranted).
12. For an instructional video on the preparation of 3D cultures please consult www.cellntec.com.

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Chapter 11

Isolation, Culture and Analysis of Mouse Mammary Epithelial Cells

Matthew J. Smalley

Abstract

Limited understanding of the cell biology of the breast and breast cancer hampers our ability to develop new therapeutic approaches. Mouse models of mammary gland development and tumourigenesis are key to developing new insights into the biology of both the normal and diseased tissues. Recent advances have enabled the isolation, molecular characterisation and functional analysis of mouse mammary epithelial cell subpopulations from the normal gland, including subpopulations enriched for stem cell behaviour. Application of these techniques both to the normal mammary gland and to tumour models will promote a better understanding of the nature of the different epithelial cell types in the mammary gland, the origins of mammary tumours and the role of tumour stem cells.

Key words: Mammary, breast, isolation, culture, mouse, stem, transplant, CD24, flow cytometry, confocal immunofluorescence, clone, organoid.

1. Introduction

In the period 1982–2002, the age-standardised rate of breast cancer in Europe rose from 80.6 to 116.1 per 100,000 women. In the same period, however, mortality fell steadily from 39.8 to 30.1 per 100,000 women (CRUK Cancer Stats, http://info.cancerresearchuk.org/cancerstats/types/breast/incidence/), thanks largely to hormone-based interventions and improved multidisciplinary patient care. However, the underlying causes of the disease are still poorly understood. One contributory factor to this is that our understanding of the basic cell biology of both the normal breast and the breast cancer is

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limited. It is known that there are two basic epithelial cell layers in the gland: the basal layer, composed mainly of myoepithelial cells which squeeze milk out of the gland during lactation in response to oxytocin, and the luminal layer, which lines the ductal network of the breast and forms the milk-producing cells in the terminal ductal lobuloalveolar units. These cell layers are thought to be maintained by a stem cell compartment (1–6). The presence of stem cells in breast cancer has also been postulated but although direct evidence for such cells has recently emerged (7), the link between breast cancer stem cells and normal breast epithelial stem cells remains unclear.

The mouse mammary gland is the ideal system for improving our understanding of the cellular biology of the breast and clarifying the link between normal and tumour stem cells. Epithelial development in the tissue is characterised by four phases, namely limited pre-natal/pre-pubescent growth, extensive post-pubertal ductal elaboration, extensive pregnancy-dependent alveolar development/differentiation and post-weaning remodelling and apoptosis of the alveolar compartment (involution) (8). As the majority of these stages occur in the adult, they are amenable to study and, at least at the macroscopic level, well defined. Furthermore, in addition to the opportunities that genetic modification presents for tailoring mouse models to answer specific questions of interest, there is also good consensus on how best to report mammary tumour phenotypes in transgenic models (9).

Mechanical and enzymatic isolation of epithelial cells from the mouse mammary gland has been used by a number of groups to establish in vitro models of mammary growth and differentiation (10–18) and, using the powerful cleared fat pad transplantation technique (19) (*see* **Section 3.9**), to study the effects of oncogene over-expression on mammary development (20–22) and to identify and characterise mammary stem cells (1–5, 23–28). The most recent advances in the field have enabled isolation of mouse mammary epithelial cell subpopulations from fresh tissue with no intervening culture period and their analysis by staining for expression of cytoskeletal antigens, molecular characterisation and determination of in vitro and in vivo growth potentials (1–4).

2. Materials

2.1. Media and Buffers

1. L15/10% FCS medium: Leibowitz L15 medium (L15) with L-glutamine (Invitrogen, Paisley, UK) plus 10% v/v heat-inactivated foetal calf serum (FCS) (Invitrogen), penicillin at 100 IU/ml and streptomycin at 100 μg/ml (Invitrogen) (*see* **Note 1**).

2. Serum-free L15 medium with no additives.
3. DMEM/10% FCS medium: Dulbecco's modified Eagle's medium (DMEM) with L-glutamine (Invitrogen) with 10% v/v FCS, penicillin at 100 IU/ml and streptomycin at 100 μg/ml.
4. Joklik's medium: Minimal Essential Medium Eagle (Joklik's modification for suspension cultures) (Sigma, Gillingham, Dorset, UK) with no additives.
5. Versene: 0.8% w/v sodium chloride, 0.02% w/v potassium chloride, 0.115% w/v disodium hydrogen orthophosphate, 0.02% w/v potassium dihydrogen orthophosphate, 0.003% w/v phenol red, and 0.02% w/v ethylenediaminetetraacetic acid (EDTA) (*see* **Note 2**).
6. Trypsin solution: 0.25% trypsin, 0.02% EDTA in Hank's balanced salt solution (Sigma).
7. DNase solution: 5 μg/ml bovine pancreatic DNase I (Type II, ≥ 2,000 U/mg protein; Sigma) in serum-free L15. Store at –20°C in 5-ml aliquots.
8. CT:I:EGF medium: 1:1 v/v DMEM/Ham's F12 mix (Invitrogen) with 10% v/v FCS, penicillin at 100 IU/ml, streptomycin at 100 μg/ml, 4 mM L-glutamine (Invitrogen), 5 μg/ml bovine pancreatic insulin (I) (Sigma, cell culture tested solution), 10 ng/ml cholera toxin (CT) (Sigma) (*see* **Note 3**) and 10 ng/ml epidermal growth factor (EGF) (Sigma, murine submaxillary, cell culture tested). EGF is made up at 10% w/v in PBS then aliquoted in 55-μl aliquots and stored at –20°C. The 50 μl contains 5 μg EGF, which is the correct amount for one 500-ml bottle of medium. The CT, I and EGF are not added directly to the bottle of medium, rather they are added to a 10- to 15-ml aliquot taken out of the bottle, which is then sterile filtered as it is returned to the bottle through a 0.22-μm syringe filter.
9. Dulbecco's phosphate-buffered saline A (PBS): 1.0% w/v sodium chloride, 0.025% w/v potassium chloride, 0.025% w/v disodium hydrogen orthophosphate and 0.1437% w/v potassium dihydrogen orthophosphate.
10. DAPI stain: 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI, Invitrogen) is used at 14 μM (in PBS) for staining methanol:acetone fixed cells on slides for confocal microscopy and at 140 nM (diluted in L15/10% FCS medium and then sterile filtered through a 0.22-μm syringe filter) for flow cytometry.
11. Glutaraldehyde fix for 96-well plates: 0.5% v/v grade I (EM grade) glutaraldehyde (Sigma) in PBS.

12. Crystal violet staining solution: 0.5% w/v crystal violet stain (Sigma) in 25% v/v methanol in water.
13. Mammary wholemount fixative: Methacarn (60% v/v methanol, 30% v/v chloroform and 10% v/v glacial acetic acid). Use glass pipettes and containers to handle the chloroform as it can leech substances out of some plastics.
14. Carmine staining solution: 0.2% w/v carmine red (Sigma) and 0.5% w/v aluminium potassium sulphate in water. Make up 500 ml and boil for 20 min in a fume cabinet. Make the volume back up to 500 ml and filter through 3 M paper. Add 100 mg thymol (Sigma) as a preservative. Store at 4°C.
15. Gentamycin solution (Invitrogen) used at a final concentration of 40 μg/ml.
16. Immunofluorescence wash: PBS with 10% v/v FCS.

2.2. Organoid and Single Cell Preparation

1. For the tissue harvest: Dissection board, 70% ethanol wash bottle to sterilise surface of animals, dissection kit (large needles for pinning out limbs, small needles for holding out skin, round-nosed scissors, two pairs of forceps and #11 disposable scalpels), 50-ml Falcon tube containing 70% ethanol for sterilising instruments and 50-ml Falcon tube containing L15/10% FCS medium for collecting tissue (kept on ice) (*see* **Note 4**).
2. For tissue processing: 50-ml Falcon tube containing 70% ethanol for rinsing fat pads, 50-ml Falcon tube containing L15/10% FCS medium for washing fat pads after ethanol rinse, 35 ml of digestion mix (105 mg collagenase A, 52.5 mg trypsin made up in 35 ml serum-free L15 medium and sterilised by syringe filtering through a 0.45-μm filter) (*see* **Note 5**), red blood cell lysis buffer (Sigma), 40-μM cell strainers (BD Biosciences, Oxford, UK) and a McIlwain tissue chopper (Mickle Laboratory Engineering, Gomshall, Surrey, UK).
3. The McIlwain tissue chopper is sterilised with 70% ethanol and placed in the tissue culture microbiological safety cabinet. Both blade force and speed are set to maximum. Cutting thickness is set to 100 μm. The tools required to set it up (forceps, screwdriver, socket spanner suitable for manipulating the screw, collar and nut which hold the razor blade on the chopping arm; **Fig. 11.1a**) are sterilised in 70% ethanol, as are a pair of cell scrapers used to transfer the tissue after it has been processed. The screw, collar and nut from the arm and two cutting discs are also sterilised in 70% ethanol. A double-sided razor blade with central hole is wiped with acetone to remove lubricants and sterilised

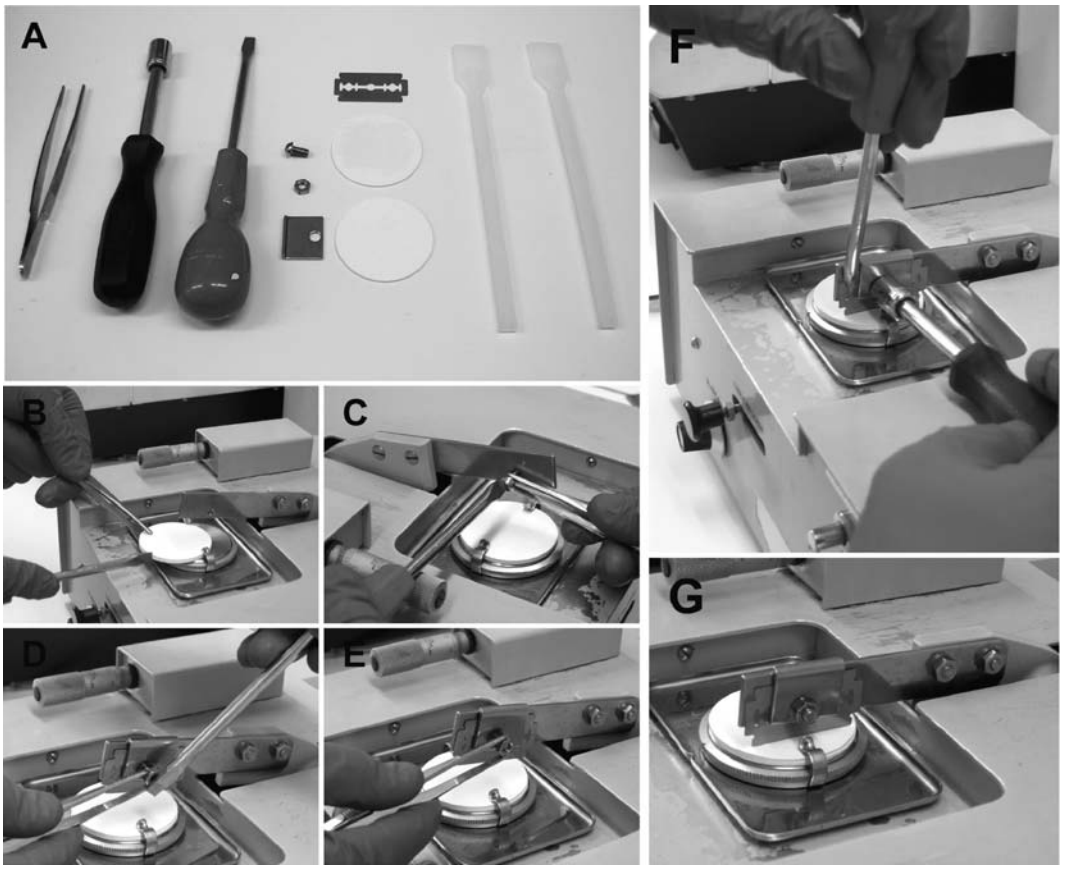

Fig. 11.1. Setting up the McIlwain tissue chopper. (**a**) Required equipment from *left* to *right*: forceps; socket spanner; screwdriver; collar, nut and screw for chopper arm; two cutting discs and razor blade; pair of cell scrapers for transferring chopped tissue from discs to digestion mixture. (**b**) Inserting the discs using forceps and screwdriver. (**c**) Screwing the screw into the arm. (**d**) The initial placement of the nut on the screw thread, having positioned the razor blade and collar. (**e**) Loosely doing up the nut. (**f**) Tightening the nut with the socket spanner whilst holding the blade flat to the discs with the screwdriver. (**g**) The blade in position, absolutely flush against the discs.

with 70% ethanol. Using the sterile forceps and screwdriver, the cutting discs are inserted in the stage clips (**Fig. 11.1b**) (*see* **Note 6**). The screw is then screwed into the chopping arm (**Fig. 11.1c**). The razor blade and then the collar are placed on the screw, and then the nut is put on by holding it in position with the forceps, then using the flat of the screwdriver blade to keep it in place whilst the forceps turn the nut a couple of times to guide it on to the screw (**Fig. 11.1d**). The screw is then loosely done up to the collar using the forceps (**Fig. 11.1e**). The cutting arm is then lowered so that the razor blade lies absolutely flush against the cutting discs. There is a certain amount of front to back movement of the blade as the hole in its centre is larger than the screw. The blade should be moved back so that it is at the limit

of its backward movement but still flush against the cutting disc (*see* **Note 7**). The sterile socket spanner is then used to tighten the nut with the screwdriver holding the blade down flush with the disc at the front (*see* **Note 8**), noting that the blade has a tendency to twist whilst the nut is being tightened (**Fig. 11.1f**). After tightening, check that the blade is still in the correct position (**Fig. 11.1 g**). If it is not, loosen the nut, re-align the blade and re-tighten it.

2.3. Antibodies

1. For flow cytometry using fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated antibodies: Anti-CD24-FITC (clone M1/69, BD Biosciences) and control non-specific IgG2b-FITC (clone MPC-11) are used at 0.5 μg/10^6 cells. Anti-CD45-PE-Cy5 (clone 30-F11, BD Biosciences) and control non-specific IgG2b-PE-Cy5 (clone A95-1) are used at 0.25 μg/10^6 cells.
2. For immunofluorescence and immunocytochemistry: Anti-cytokeratin 14 (CK14) (clone LL002 mouse IgG3, Lab Vision, Suffolk, UK) is used at 2.1 μg/ml. Anti-cytokeratin 8/18 (CK8/18) (clone 5D3 mouse IgG1, Novocastra, Vision Biosystems, Newcastle Upon Tyne, UK) is used at 2 μg/ml. Anti-α-isoform smooth muscle actin (clone 1A4 mouse IgG2a; Sigma) is used at a working dilution of 1:1000 v/v.
3. Secondary antibodies for immunofluorescence and controls: Isotype-specific goat anti-mouse antibodies anti-IgG1-Alexa 633 (A21126; Invitrogen), anti-IgG2a-Alexa 488 (A21131, Invitrogen), anti-IgG2a-Alexa 633 (A21136, Invitrogen) and anti-IgG3-Alexa 555 (A21157, Invitrogen) are used at 10 μg/ml. Lack of non-specific staining by secondary antibodies is confirmed using unlabelled IgG1, IgG2a and IgG3 control primary antibodies (clones 5H6, HOPC-1 and B10, Cambridge Biosciences, Cambridge, UK).
4. Immunofluorescence mounting medium: Vectashield H1000 mounting medium (Vector Laboratories Limited, Orton Southgate, Peterborough, UK).

2.4. Cleared Fat Pad Transplantation

1. Autoclaved dissection instruments: Two pairs of round-nosed forceps, round-nosed scissors, angled spring scissors (Vanna Scissors, 8 cm, 5 mm, angled-on-flat blades, 0.1-mm tip; World Precision Instruments, Stevenage, UK), tools for stitching (depending on personal preference) and cotton buds.
2. Cautery unit: Light duty cautery transformer (set to 5.5 on its power output), cautery handle JA103 and cautery burner tip JA321S (all from RB Medical, Ross-on-Wye, Herefordshire, UK).

3. Sterile PBS, 70% ethanol, suture (4-0 Mersilk; Ethicon, Edinburgh, UK), 25 g 0.5 × 16-mm needles,1-ml syringes, hair clippers, dissection board and 10-μl Hamilton syringes (number 701 N; Sigma).
4. Heat pad, oxygen supply, Transwean (Lilico, Betchworth, Surrey, UK) and analgesic (chosen and administered in accord with local veterinary guidance) for post-operation recovery.

2.5. Miscellaneous Equipment

1. Heraeus BB6060 incubator (Thermo Electron, Basingstoke, UK) equipped with low oxygen electrode.
2. BD FACSVantageSE DiVa flow cytometer (BD Biosciences) equipped with two Coherent Innova 90C-4 argon ion lasers (Coherent, Santa Clara, CA, USA) tuned to 488 and 333.6–333.8 nm. Machine also has a Spectra Physics 127 HeNe laser emitting at 633 nm.
3. Confocal microscope: Leica TCS SP2 with AOBS (acousto-optical beam splitter) and lasers exciting at 405, 488, 543 and 633 nm.
4. Hydrophobic barrier (PAP) pen (ImmEdge; Vector Laboratories Limited); poly-L-lysine-coated slides (Polysine Slide, Fisher Scientific UK, Loughborough, Leicestershire, UK) and 12-mm #1.5 circular glass coverslips (Science Services Ltd, London, UK).
5. RNeasy minispin kit, including proprietary "RLT" lysis buffer, obtained from QIAGen (Crawley, West Sussex, UK).
6. Feeder cells for clonal culture: 3T3 L1 preadipocytes (European Collection of Cell Cultures, Salisbury, Wiltshire, UK). The cells are maintained in DMEM/10% FCS. They are irradiated (25 Gy) whilst still attached as a monolayer and then trypsinised, counted and plated as required for establishing clonal culture conditions.

3. Methods

The protocols below describe how to harvest, isolate and analyse mouse mammary epithelial cells. Methods for bulk cell and clonal cell culture are included. Also described are key immunofluorescence techniques for mammary epithelial cell characterisation. Finally, the method for cleared fat pad transplantation is detailed. This classic assay is applicable both to transplantation of freshly isolated cells to identify populations with stem cell behaviour and to transplantation of short-term primary cultures which have been transduced with a gene of interest to study the effects of the gene

on mammary outgrowth and development. Note that although the transplant protocol has been made as detailed as possible, there is no substitute for being taught the technique in a laboratory in which it is well established.

Mouse mammary epithelial cells show extreme lability of cell type-specific marker expression when placed in monolayer culture. For instance, luminal epithelial cells, which express cytokeratin 18 but not cytokeratin 14 in vivo, express both these markers in monolayer culture after 24–48 h. Gene expression analysis of transgenic mammary epithelium or of mammary tumours should therefore be based around a protocol of flow cytometric isolation of cellular subpopulations and then immediate lysis and isolation of RNA from these subpopulations, as described below. It follows that the option of bulking cells up in culture to obtain more material is not available and the key to this technique is, therefore, the ability to get good cell yields from primary preparations. Experience shows that the key to good cell yields is very fine mincing of the tissue prior to enzymatic digestion. The McIlwain tissue chopper minces far finer than can be achieved by hand, when it is correctly set up, as described.

In vitro culture of primary mouse mammary epithelial cells is well suited to bulking up cells for DNA-based analyses or for experimental cellular manipulation followed by cleared fat pad transplantation. It is also suitable for establishment of models of differentiative potential and for characterising the origins of cellular subpopulations by determining phenotypes of clones they give rise to in culture followed by comparison to previously defined clone types (13).

The flow cytometry staining and analysis protocols describe only the basic procedures for isolating pure basal/myoepithelial and luminal cells on the basis of CD24 expression patterns. For more advanced methodologies for isolating stem/progenitor cells and luminal epithelial subpopulations, researchers should consult recent publications (1, 3, 4, 29, 30).

3.1. Isolation of Mammary Fibroblasts and Epithelial Organoids

1. Fourth mammary fat pads are harvested from 20 female mice (*see* **Notes 9** and **10**). The mice are culled in batches of five and fat pads harvested, working in a microbiological safety cabinet to maintain sterility of the sample. The ventral side of the animal is liberally doused with 70% ethanol, rubbing it against the grain of the fur to ensure that it penetrates into the skin. A ventral midline incision is made using blunt scissors, being careful not to puncture the peritoneum. The incision is extended to the top of the ribcage and down both legs, and the skin is peeled back from the peritoneum using two pairs of forceps. The fourth and fifth fat pads come away from the peritoneum attached to the skin. The skin is stretched out and pinned down taut. Using

a #11 scalpel, an incision is made above the lymph node in the fourth fat pad, and it is popped out of the gland by gently squeezing with forceps (*see* **Note 11**). The connection between the fourth and fifth fat pads is cut and then the fourth fat pad is peeled away from the skin, connective tissue between the fat pad and skin being cut as necessary. The fat pads are placed in L15/10% FCS medium on ice until all are harvested, then transferred to the tissue culture laboratory.

2. To ensure sterility of the sample, the fat pads are briefly rinsed in 70% ethanol by dipping them in and out rapidly three times and then transferring them to fresh L15/10% FCS medium.
3. Fat pads are finely minced (*see* **Note 12**) in batches of 10 (i.e. five animals worth) on the McIlwain tissue chopper. Each batch is chopped three times, with the tissue being stirred and then collected back in the middle of the cutting disc with a sterile Pasteur pipette between each chop. The blade on the McIlwain tissue chopper is examined between each chop to ensure that it is still flush with the disc when in the down position. After two batches (halfway through the tissue sample) the blade is turned so that a fresh cutting edge is used for the remainder of the tissue (*see* **Note 13**). If the chopping has been carried out properly, by the end of the third chop the tissue should form a very fine, semi-liquid slurry with white fat pooling on top of it.
4. The minced tissue is transferred to a 50-ml Falcon tube containing 35-ml collagenase/trypsin digestion mix using the pair of sterile cell scrapers as "shovels". The tube is placed at 4°C until all the tissue has been chopped and added and is then secured horizontally on a shaker platform and incubated for 1 h at 37°C (*see* **Note 14**).
5. After the 1 h incubation, the mixture is centrifuged at 250*g* for 5 min. The fat layer and supernatant are poured off (*see* **Note 15**) into a fresh 50-ml Falcon tube and spun for a second time at 250*g* for 5 min. The fat layer and supernatant are then discarded. The pellets (*see* **Note 16**) from both spins are pooled and resuspended in 10-ml L15/10% FCS medium in a 15-ml Falcon tube and pelleted at 250*g* for 5 min.
6. The pellet is resuspended in 5 ml red blood cell lysis buffer (Sigma), transferred to a fresh 15-ml Falcon tube and incubated for 5 min at room temperature. The sample is then pelleted at 250*g* for 5 min and resuspended in a fresh 5 ml of red blood cell lysis buffer and transferred to a fresh 15-ml Falcon tube. Once again, the sample is incubated for 5 min at room temperature, then pelleted at 250*g* for 5 min.

7. The sample is resuspended in 10-ml L15/10% FCS medium and pelleted at 250*g* for 5 min.
8. To remove most of the mammary fibroblasts from the sample, it is resuspended in 10 ml of DMEM/10% FCS medium and transferred to a T-80 tissue culture flask. The flask is incubated for 1 h at 37°C/5% CO_2/5% O_2. The majority of fibroblasts attach to the tissue culture plastic in this time whilst most of the organoids do not. After the incubation, the flask is shaken in a horizontal plane with moderate vigour to ensure the organoids (which, although they do not attach to the plastic, do settle on it) are in suspension and they are pipetted off. The flask is then rinsed with 10-ml L15/10% FCS medium, shaken as before and then this wash is pipetted off. The organoid suspension and the wash are then spun at 250*g* for 5 min.
9. After this final spin, the supernatants are poured off and the pellets combined by resuspending in 10-ml L15/10% FCS medium to give a sample which is mainly composed of mammary epithelial organoids but also contains tissue lymphocytes and some remaining fibroblasts as well as other cell types.
10. At this stage the epithelial organoids can be plated out to generate bulk cell cultures (see below), they can be immediately processed to give epithelial single cell suspensions (**Section 3.2**) or they can be placed at 4°C overnight and then processed to single cell suspensions the next day (*see* **Note 17**).
11. The mammary fibroblasts remaining behind in the T-80 flask can be kept if required by simply re-feeding the flask with 25-ml DMEM/10% FCS medium and incubating at 37°C/5% CO_2/5% O_2. After 5–8 days, the flask will be confluent and the cells can be passaged to a T-175 flask by differential trypsinisation, to ensure that any epithelial cells from mammary organoids which were not poured off at the end of the differential attachment procedure are not carried over into the new flask. The passage 1 fibroblasts are maintained in 35-ml DMEM/10% FCS medium at 37°C/5% CO_2/5% O_2 as before and again after 5–8 days the flask should be confluent. It may be possible to passage the cells again, but they will usually begin to senesce after three to four passages.

3.2. Preparation of Single Cell Suspensions from Mammary Epithelial Organoids

1. Organoids are washed twice in 5-ml versene by pelleting at 250*g* and resuspension.
2. Organoids are then pelleted at 250*g* and, using a 5-ml plastic pipette, resuspended in 5 ml serum-free Joklik's medium

(*see* **Note 18**). They are incubated for 15 min in a 37°C water bath.

3. They are then pelleted at 250*g* and resuspended in 2 ml of 1 mg/ml trypsin-versene using a 5-ml plastic pipette and incubated for 2 min at 37°C. After this incubation, the tube is gently shaken (*not* by inversion) in the hand causing, if the yield has been good, clumping of DNA released from cells that have been lysed by the trypsinisation. A P1000 pipette is used to shear this DNA clump by vigorously pipetting it up and down three or four times. A total of 5 ml of 5 μg/ml DNase 1 in serum-free L15 (a pre-prepared aliquot thawed to 37°C) is then added and the sample incubated for another 5 min at 37°C.
4. About 7 ml of L15/10% FCS medium is added to stop the trypsin and the sample is then filtered through a 40-μm cell strainer to remove remaining cell clumps and debris.
5. Cells are pelleted at 250*g* at 4°C, resuspended in 5-ml L15/10% FCS medium and counted. If cells have been processed in multiple batches, the samples are now pooled. Single cells are maintained on ice during subsequent procedures.

3.3. In Vitro *Bulk Cell Culture of Mammary Epithelial Cells*

1. If bulk cell cultures of mammary epithelial cells are required (*see* **Note 19**), then staining and flow cytometric separation of cells are not carried out. Instead, either freshly isolated organoids are plated out or single mammary cells are plated.
2. To generate bulk cultures from organoids, after pre-plating to remove fibroblasts, the organoids are resuspended in CT:I:EGF medium and transferred to tissue culture plastic vessels (either flasks or dishes) (*see* **Note 20**). Cultures are maintained at 37°C/5% CO_2/5% O_2 (*see* **Note 21**). Over a period of several days, the epithelial cells mobilise and spread out from the organoids to form a monolayer.
3. To generate bulk cultures from single mammary cells (*see* **Note 22**) without flow sorting, after the single cells have been isolated and counted, they are plated in CT:I:EGF medium in tissue culture plastic vessels (either flasks or dishes) at densities of approximately 4,000/cm^2. Cultures are maintained at 37°C/5% CO_2/5% O_2.

3.4. Staining and Flow Cytometric Separation of Mammary Cells

1. Samples are maintained on ice as much as possible and all washes are performed with cold (4°C) medium (*see* **Note 23**). All centrifugations are carried out at 4°C.
2. Cell density is adjusted to 10^6/ml with L15/10% FCS medium. Samples are aliquoted for control staining procedures. The 5×10^5 cells per control sample are usual but

2.5×10^5 is acceptable if only a small cell yield has been obtained. One single-stained control for each fluorochrome being analysed plus one combined isotype-specific IgG control are required. The minimum number of stains that must be carried out is the following: DAPI to allow for removal of dead cells, CD45 to remove leukocytes and CD24 to define the mammary epithelial cell populations. Thus a minimum of four (three fluorochromes plus one isotype-specific IgG) controls are required. Each additional fluorochrome requires an additional control. The remaining cells, after all the control samples are removed, will form the test sample to which all fluorochromes are added and which is sorted. The staining protocol is summarised in **Table 11.1**.

3. Antibodies are added as detailed in **Table 11.1**. Tubes containing the control and test samples are shaken briefly and gently to mix the antibodies and then placed on ice for 45 min. Every 15 min, the tubes are shaken again and then put back on ice.
4. At the end of the incubation period, samples are pelleted at 250*g* and resuspended in 1 ml cold L15/10% FCS medium with a P1000 pipette. Samples are pelleted at 250*g* again.
5. Test and control samples are resuspended at 10^6/ml in cold L15/10% FCS medium with or without DAPI as described in **Table 11.1**. They are transferred to sterile FACS analysis tubes, put on ice and then taken to the flow cytometry facility.
6. Unstained and single fluorochrome-stained samples are used to set fluorochrome compensation levels. Having established these, the test sample is processed. Cells are first gated on forward and side scatter such that debris and obvious large

Table 11.1
Staining protocol for preparing mouse mammary cells for flow cytometric separation. *n* is the total cell yield for the individual preparation

Sample	Cell number	Antibody/antibodies	DAPI	Purpose
Non-specific staining control	5×10^5	IgG-FITC IgG-PE-Cy5	No	Determine background staining levels
DAPI control	5×10^5	None	Yes	Compensation control
Anti-CD24-FITC control	5×10^5	Anti-CD24-FITC	No	Compensation control
Anti-CD45-PE-Cy5 control	5×10^5	Anti-CD45-PE-Cy5	No	Compensation control
Experimental sample	$n - 2 \times 10^6$	Anti-CD24-FITC Anti-CD45-PE-Cy5	Yes	Sample to be sorted

cell clumps are excluded. Progressive gating excludes dead cells (DAPI bright), leukocytes ($CD45^+$) and non-single epithelial cells. The latter are removed using a time-of-flight approach, where forward scatter height is plotted against forward scatter area. Correct gating on the time-of-flight dot plot, such that only single cells are being included (*see* **Note 24**), is determined for each experiment by sorting 100–200 cells on to a slide and visually examining them. The gate can then be adjusted and checked again until the desired level of purity is achieved. It should be possible to achieve 99% single cellularity.

7. Having excluded all cells except live, single $CD45^-$ cells, the sample is then analysed for CD24-FITC staining. This gives three distinct populations (**Fig. 11.2**): $CD24^{Negative}$, which are non-epithelial (including residual fibroblasts that were not removed by the pre-plating procedure during organoid preparation); $CD24^{Low}$, which are basal cells and which include both the myoepithelial and the mammary stem cell populations; and $CD24^{High}$, which are the luminal cells (*see* **Note 25**).
8. The desired populations are now sorted in one of five different ways, depending on how they will be used, as described below (*see* **Notes 26 and 27**).

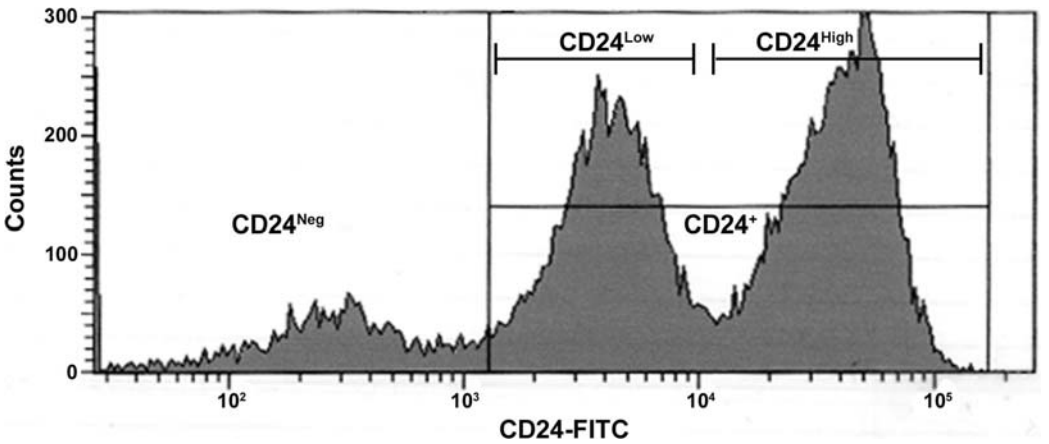

Fig. 11.2. Flow cytometric profile of $CD45^-$ single mammary cells stained with anti-CD24-FITC. The $CD24^{Neg}$ (non-epithelial) and $CD24^+$ (epithelial) regions are indicated, as are the $CD24^{Low}$ (basal/myoepithelial) and $CD24^{High}$ (luminal) compartments within the $CD24^+$ region.

3.5. Characterisation of Antigen Expression Patterns of Mouse Mammary Cell Populations

1. To characterise different mammary cell subpopulations identified by flow cytometric analysis, cells are sorted directly on to poly-L-lysine-coated slides, usually 300 cells per slide.
2. The slides are allowed to air-dry and the droplet of fluid containing the sorted cells is ringed with a wax circle using

a PAP pen (*see* **Note 28**). Once the slides have dried they are stored at –20°C until they are stained.

3. Prior to staining, the slides are transferred to a staining trough containing 1:1 cold (–20°C) methanol:acetone in which they are fixed for 5 min at –20°C (*see* **Note 29**). After fixation, the slides are washed twice in PBS for 5 min per wash.
4. After fixation and washing, the slides are individually and carefully dried with a paper towel. This should completely dry the underside of the slide and the area of the upper side that lies outside the region circled by wax. The wax ring should itself be carefully dried with the edge of the paper towel, but not smudged. Most of the PBS should be removed from the area inside the wax ring using the edge of a piece of paper towel to blot it away, but it should not be allowed to dry completely. The slide is then transferred to a humidified chamber and 100–150 μl L15/10% FCS medium applied to the region inside the wax ring. Once all the slides have been processed, the lid is placed on the chamber and the slides are left at room temperature for 15 min. This incubation is designed to block non-specific protein–protein interactions.
5. After blocking, the slides are placed in a staining trough and washed once briefly in PBS. They are then dried as before, placed in the staining chamber and 100–150 μl of the primary antibody mix at the correct dilution applied to each slide as before. The different primary and secondary antibody combinations used, the antigens they detect and the cell populations that they identify are shown in **Table 11.2**. Slides are incubated with primary antibodies for 40 min at room temperature.
6. After the primary antibody incubation, slides are returned to the staining trough and washed with PBS/10% FCS three times for 5 min per wash.
7. The slides are then dried as before, placed in the staining chamber and 100–150 μl of the secondary antibody mix at the correct dilution applied to each slide as before. The secondary antibodies (goat anti-mouse subclass-specific antibodies) (*see* **Note 30**) are conjugated to fluorochromes (Alexa 555 and Alexa 633) whose excitation and emission spectra do not overlap with those used for flow sorting. The slides are incubated with the secondary antibodies for 40 min at room temperature (*see* **Note 31**).
8. After the secondary antibody incubation, slides are returned to the staining trough and washed once with PBS/10% FCS for 5 min and then twice with PBS only for 5 min per wash.

Table 11.2
Antibody staining combinations for immunophenotyping of cells sorted on to slides. DAPI is used in both these combinations to enable detection of double-negative cells and to distinguish genuine cells from debris which has non-specifically adsorbed the antibodies. K = keratin. SMA = α-isoform smooth muscle actin

Primary antibodies	Secondary antibodies	Antigens	Cell compartments detected
LLOO2 5D3	Anti-IgG3-555 Anti-IgG1-633	K14 K18	Basal epithelial cells ($K14^+/K18^-$) Luminal epithelial cells ($K14^-/K18^+$) Non-epithelial cells ($K14^-/K18^-$)
LLOO2 1A4	Anti-IgG3-555 Anti-IgG2a-633	K14 SMA	Basal epithelial cells ($K14^+/SMA^-$) Myoepithelial cells ($K14^+/SMA^+$) SMA^+ non-epithelial cells ($K14^-/SMA^+$) Other cell types ($K14^-/SMA^-$)

9. The slides are then dried as before, placed in the staining chamber and 100–150 μl of 14 μM DAPI in PBS applied to each slide as before. The slides are incubated for 30 min at room temperature, then returned to the staining trough and washed once with PBS only for 5 min.
10. The slides are then dried for the final time, this time using the edge of a paper towel to remove as much of the PBS from within the wax ring as possible. A total of 5 μl of Vectashield fluorescence mounting medium is then applied to the centre of the area within the wax ring and a 12-mm round glass #1.5 coverslip is carefully placed over the area using a pair of fine forceps. The coverslip should start with one point of its circumference on the wax ring at an angle of approximately 45°. The coverslip is then carefully lowered until it covers the area within the wax ring and should in the process force any bubbles in the mounting medium out from under the sides of the coverslip.
11. The coverslip is pressed down gently until the mounting medium comes completely to its edge all the way around and the edges are then sealed with clear nail varnish.
12. Slides are examined by confocal microscopy within 24 h, although significant fading of the fluorescence will take much longer than that. DAPI staining of the nuclei is used to locate the cells and the antibody staining to determine their phenotype. High-magnification examples of stained cells are photographed but low-magnification non-confocal photographs are also taken for analysis of staining patterns across large cell numbers.

3.6. In Vitro *Culture and Clonal Analysis of Mammary Epithelial Cells in 96-Well Plates*

1. The 96-well plates for clonal analysis are prepared the day before cells are sorted into them. Each well of each plate is seeded with 1,000 irradiated (25 Gy) 3T3 L1 preadipocytes (which will provide a feeder layer) in 200 μl of CT:I:EGF medium. Plates are put at 37°C/5% CO_2/5% O_2 overnight.
2. Cells are sorted directly into 96-well plates at one cell per well using the CloneCyte system. Maximum sterility is maintained at all times. Plates are placed at 37°C/5% CO_2/5% O_2 (*see* **Note 32**).
3. Large clones (3- to 4-mm diameter) are visible at about 8–10 days after plating.
4. Medium is removed from plates by flicking it off into a suitable container and then vigorously blotting the plate on paper towel. The plates are then fixed in glutaraldehyde fix (*see* **Note 33**) by immersion in a bath of the fix, allowing the wells to fill and then removing the plates and draining off the excess back into the bath.
5. After 1 h, the fix is removed in the same way as the medium and the plates are then washed in PBS by the same immersion method.
6. Once all the plates are fixed and washed, one at a time, the PBS is flicked off each plate, it is blotted and then 100 μl crystal violet stain is added to each well using a multichannel pipette. Plates are left to stain overnight.
7. After staining, excess crystal violet is removed by multichannel pipette and the plates are rinsed under running water until the stained cell colonies are clearly visible and the water coming off the plate runs clear.
8. The plates are blotted and allowed to dry. The plates are then analysed by eye for the number of clones present to determine the cloning efficiency of the population sorted into that plate and microscopically to look for differences in clonal morphology (or changing frequencies of clones with different morphologies) between the different populations examined.

3.7. In Vitro *Culture and Analysis of Mammary Epithelial Cells on Coverslips*

1. The 24-well plates with coverslips for analysis of cytoskeletal expression patterns of mammary epithelial cell clones are prepared the day before cells are sorted. A sterile 12-mm round glass #1.5 coverslip is placed in each well of the plate (*see* **Note 34**). Each well is then seeded with 5,000 irradiated (25 Gy) 3T3 L1 preadipocytes in 2 ml of CT:I:EGF medium

with added gentamycin. Plates are put at 37°C/5% CO_2/5% O_2 overnight.

2. Cells are sorted into 2 ml L15/10% FCS medium in standard collection tubes (*see* **Note 35**).
3. Collected samples are pelleted at 4°C and counted and then cells are plated at 500 cells per well of the 24-well plates. Plates are placed at 37°C/5% CO_2/5% O_2.
4. About 8–10 days after plating, coverslips are fixed in situ in their wells. Medium is removed from the wells of one plate and 1 ml PBS carefully added. The PBS is then removed from six wells only and replaced with 1 ml 1:1 cold (–20°C) methanol:acetone. The plate is put at 4°C for 3 min. After this time, the fix is removed and quickly, but carefully, replaced with 2 ml PBS in each well (*see* **Note 36**). The remaining wells on the plate are then fixed in an identical manner, six at a time, until all have been fixed and contain 2 ml PBS.
5. The wells are then washed a second time by removing the PBS and replacing it with a fresh 2 ml aliquot in each well. The plate is then left for 20 min, after which the PBS is changed in each well for a third and final time.
6. After the final change of PBS is added, coverslips can be stained immediately or the plate can be sealed with Parafilm and kept at 4°C, enabling the coverslips to be stained at a later date (*see* **Note 37**).
7. Coverslips are stained by routine methods using anti-cytokeratin antibodies, mainly by double staining with anti-cytokeratin 14 (clone LLOO2, mouse IgG3) and anti-cytokeratin 18 (clone 5D3, mouse IgG1) or by triple staining with LLOO2, 5D3 and anti-α-isoform smooth muscle actin 1A4, together with goat anti-mouse subclass-specific second antibodies as described in **Section 2.3** (*see* **Table 11.3** for a summary of the relevance of the different clonal cytoskeletal staining patterns).

3.8. Extraction of RNA from Freshly Isolated Mammary Cells

1. For RNA isolation from sorted mammary epithelial cell subpopulations, 1.5 ml screw-cap Eppendorf collection tubes are prepared by rinsing with L15/10% FCS medium. Each tube has 1 ml of medium pipetted into it and the cap is screwed on tight. The tube is inverted and then the medium removed and discarded. The tube is then spun briefly in an Eppendorf centrifuge to collect the remaining medium in the bottom of the tube (*see* **Note 38**).

Table 11.3
Summary of typical staining patterns of mouse mammary-derived in vitro clones 1 week after plating. α-SMA = α-isoform smooth muscle actin. – = no staining. + = strong staining in >90% of cells in the clone. –/+ = variable staining, both in terms of strength (ranging from weak to strong) and percentage of cells stained (ranging from 0 to >75%)

Keratin 14 (LL002 IgG3)	Keratin 18 (5D3 IgG1)	α-SMA (1A4 IgG2a)	Typical clonal morphology	Clonal origin
–	–	+	Irregular clone composed of elongated, spindly cells with poor cell–cell contact	Non-epithelial/fibroblast
+	–/+	–/+	Irregular clone composed of few, large spread-out cells with good cell–cell contact, often with cell processes overlapping	Basal/myoepithelial
+[a]	+	–/+	Regular clone composed of many small cells of ovoid, cuboidal or polygonal shape[b], usually with good cell–cell contact	Luminal

[a]In most luminal-derived clones, all cells strongly double stain for both keratin 14 and keratin 18. However, a number of such clones have, in addition, one or more large, spread-out keratin 14-only cells which lie underneath the double-stained bulk of the clone.
[b]Luminal-derived clones may be composed solely of cuboidal or polygonal cells or may be of mixed composition (13). Note that the majority of cells which grow out in in vitro mouse mammary epithelial culture are of luminal origin. The basal/myoepithelial cells have very poor in vitro growth ability.

2. If the tube will be used to collect a cell population which is expected to give a large yield (approximately >50,000 cells) then the remaining medium is left in the collection tube, which is placed on ice.
3. If the tube will be used to collect a cell population which is expected to give a small yield (approximately <20,000 cells) then the remaining medium is removed and 350 μl of QIAgen RLT buffer containing β-mercaptoethanol (as recommended by the manufacturer) is placed in the tube, which is placed on ice.
4. Cells are sorted directly into the collection tubes. After sorting, the low-yield population samples, sorted directly into RLT buffer, are placed at –80°C until RNA isolation.

5. Collection tubes containing high-yield population samples (often more than one tube for each population; *see* **Note 39**) are marked with a vertical line close to the base of the tube. The tubes are loaded into a fixed angle rotor bench top Eppendorf centrifuge with the vertical line on the outside.
6. Tubes are spun at 700*g* for 5 min. At the end of the spin, the vertical line marked on the tube points at the cell pellet, facilitating removal and discarding of the supernatant without disturbing the pellet (*see* **Note 40**).
7. Pellets are resuspended in 350 μl of QIAgen RLT buffer containing β-mercaptoethanol, pooling samples as necessary so that there is only one sample per population, and then the samples are placed at –80°C until RNA isolation.
8. RNA is isolated using QIAgen RNeasy mini-columns (according to the manufacturer's instructions) and then gene expression analysed by standard quantitative real-time rtPCR and/or cDNA microarray techniques (*see* **Note 41**).

3.9. Analysis of Mammary Epithelial Stem Cell Activity by Cleared Fat Pad Transplantation

1. For collection of mammary epithelial cell subpopulations for cleared fat pad transplantation analysis, 1.5 ml screw-cap Eppendorf collection tubes are prepared by rinsing with L15/10% FCS medium. Each tube has 1 ml of medium pipetted into it and the cap is screwed on tight. The tube is inverted and then the medium removed and discarded. The tube is then spun briefly in an Eppendorf centrifuge to collect the remaining medium in the bottom of the tube.
2. Cells are sorted directly into the collection tubes. After sorting, collection tubes are marked with a vertical line close to the base of the tube. The tubes are loaded into a fixed angle rotor bench top Eppendorf centrifuge with the vertical line on the outside.
3. Tubes are spun at 700*g* for 5 min. At the end of the spin, the vertical line marked on the tube points at the cell pellet, facilitating removal and discarding of the supernatant without disturbing the pellet.
4. Pellets are resuspended in 1 ml PBS, pooling samples as necessary so that there is only one sample per population.
5. Samples are placed on ice whilst the number of cells in each sample is determined by visual counting on a haemocytometer slide. At least 100 and preferably 200 cells are counted per sample – if necessary, more than one aliquot is counted to achieve this. Estimates are also made of the numbers of doublets and higher order clumps in the sample after sorting (*see* **Note 42**).

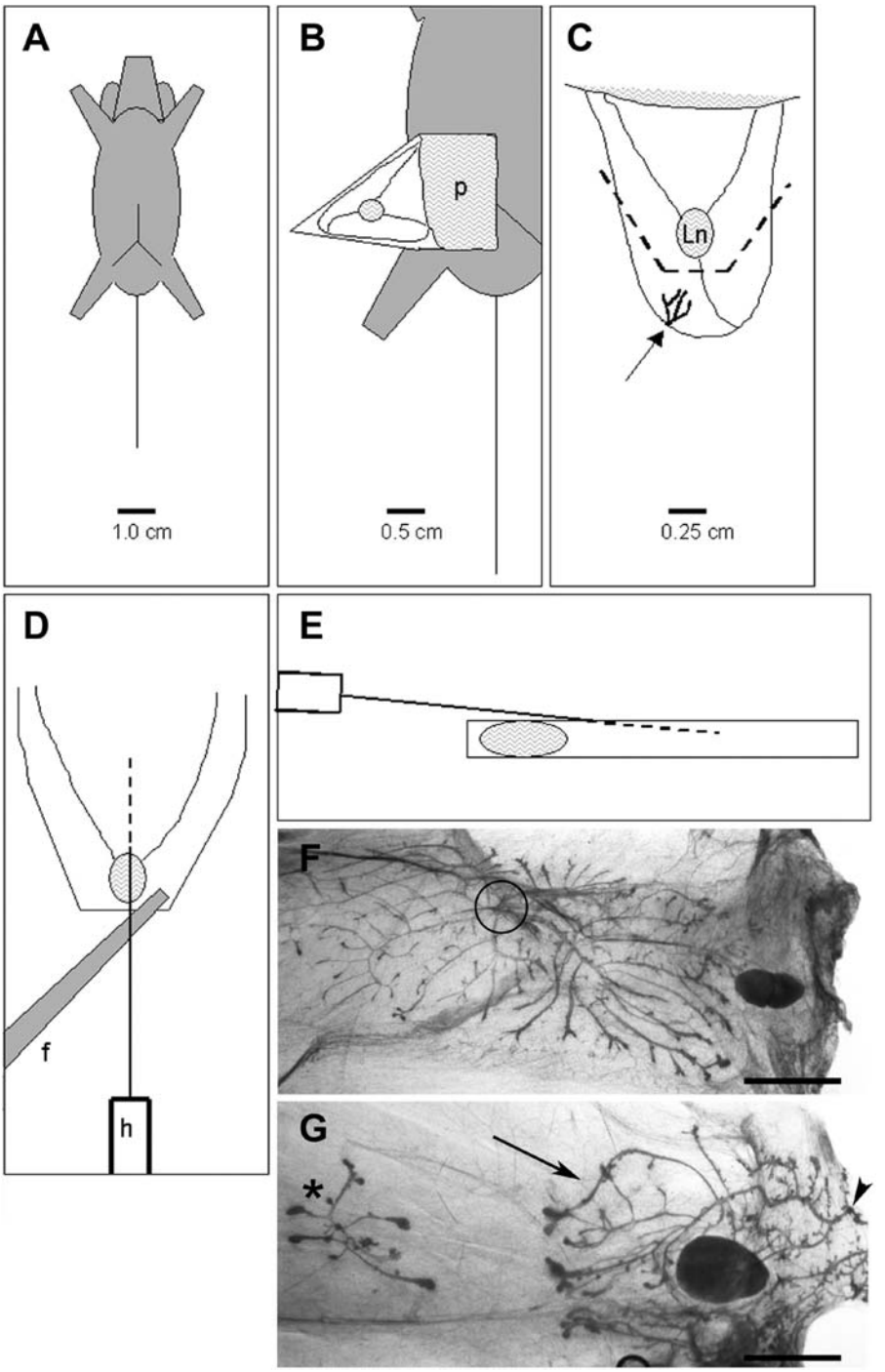

Fig. 11.3. Cleared fat pad transplantation. (**a**) Position of the ventral incisions. (**b**) Deflection of the skin flap reveals the peritoneum (p) and fourth fat pad with central lymph node. In reality, the skin flap cannot, and should not, be pulled out so far. (**c**) Clearing the fat pad. The *dotted line* indicates the line to cut along below the lymph node (Ln) to remove the portion of the fat pad containing the endogenous epithelium (*arrow*). (**d**) and (**e**) *Top* and *side views* of positioning the needle of the Hamilton syringe (*h*) for injection. The forceps (*f*) hold the fat pad out straight and also provide a rest to help support the needle. The *dotted portion* of the needle indicates the amount actually in the fat pad. (**f**) Carmine-stained

6. After counting, cells are pelleted a second time. Again, the tubes are loaded in the rotor such that at the end of the spin, the vertical line points at the cell pellet.
7. The PBS is carefully removed and the pellets resuspended in cold (4°C) serum-free L15 medium, at a density such that 10 μl of medium contains the number of cells it is intended to transplant in one fat pad (*see* **Note 43**). The samples are put on ice and taken to be transplanted.
8. Fat pad clearing (*see* **Note 44**) is carried out under general anaesthesia (*see* **Note 45**) using aseptic techniques. Transplant host animals are 21-day-old (or 9–11 g) FVB mice (*see* **Note 46**). The animals should be weaned from their mothers when 20 days old to allow 1 day to recover before surgery.
9. Once anaesthesia is induced, the animals are placed ventral side up on the dissection board and the limbs are secured using tape, being careful not to trap the whiskers when the forelimbs are secured. The ventral fur is shaved and the ventral surface washed with 70% ethanol, using a cotton bud, to sterilise and then with sterile PBS to prevent drying out of the skin.
10. Three ventral incisions are made using blunt-nosed scissors, being very careful not to pierce the peritoneum. Each incision radiates from a point approximately 2 cm posterior to the rib cage and is approximately 1 cm long (**Fig. 11.3a**).
11. The skin bisected by the incisions is blunt dissected back using forceps to hold the skin and a cotton bud moistened with sterile PBS to get between the skin and peritoneum to reveal the fourth mammary fat pad (**Fig. 11.3b**). The skin flap is pinned to the dissection board using a 25 gauge needle (*see* **Note 47**).
12. The fourth and fifth mammary fat pads are separated by cautery. Any bleeding is controlled by cautery and the cut end of the fifth fat pad is cauterised to prevent endogenous epithelium from the fifth fat pad growing into the cleared fourth fat pad, should they come into contact again after surgery.

◄

Fig. 11.3 (continued) wholemount preparation of a successful cleared fat pad transplant with transplant-derived ducts radiating outwards from a central point (*circled*). Bar = 0.5 cm. (**g**) Carmine-stained wholemount preparation of a failed clear. The transplanted cells have begun to grow (*asterisk*) but endogenous epithelium (*arrow*) has either been left behind by the clearing operation or been grown in due to contact with the fifth fat pad. The origin of the endogenous outgrowth is clearly seen at the cut edge of the fat pad (*arrowhead*). In 1–2 weeks, the endogenous outgrowth would have completely obscured the transplanted structure. Such failed clear cases must be excluded from the analysis. Bar = 0.5 cm.

13. The fourth mammary fat pad is freed from its connection with the skin by lifting it and then carefully dissecting through the strands of connective tissue joining the inside of the skin with the fat pad. The inside of the skin surface is very carefully and lightly cauterised, especially around the nipple area (*see* **Note 48**).
14. Using Vanna scissors, the portion of the fat pad distal to the lymph node is cut away (**Fig. 11.3c**). Cautery is used to stop bleeding where necessary.
15. Test cells in a 10 μl volume are loaded into the Hamilton syringe in preparation for injection.
16. The remaining portion of the fat pad is pulled taut, gently, in a horizontal plane using forceps in one hand. The other hand is then used to insert the syringe needle, with the bevel facing up, into the fat pad in the horizontal plane such that the tip is placed centrally within the remaining portion of the fat pad (**Fig. 11.3d, e**). The cells are injected and the needle withdrawn (*see* **Note 49**).
17. The procedure is repeated on the contralateral fat pad and then the skin flaps are repositioned and sutured together (*see* **Note 50**). Tissue glue can be used to close small gaps between sutures.
18. After the procedure the animals are placed upon a heated pad and given oxygen until they fully recover conciousness and then returned to their cage where they are given Transwean and/or wet diet to aid recovery (*see* **Note 51**).

3.10. Wholemount Analysis of Transplanted Fat Pads

1. Transplant hosts are culled 8 weeks after transplantation for analysis of ductal outgrowth (*see* **Note 52**).
2. Fourth mammary fat pads are dissected out as in **Section 3.1** but leaving the lymph node intact (*see* **Note 53**). They are stretched out gently but firmly, "skin side" down, on to a glass slide with a pair of forceps, ensuring that they are stretched as thinly as possible (*see* **Note 54**). The slides are then gently placed in methacarn fix in a staining trough and fixed overnight at room temperature (*see* **Note 55**).
3. The slides are washed once in 70% ethanol for 30 min, rinsed in water and then stained in carmine aluminium overnight.
4. The carmine solution is poured off (*see* **Note 56**), the fat pads rinsed twice with water and then they are dehydrated through 70% ethanol for 4 h, 90% ethanol for 4 h and finally placed in 100% ethanol overnight.
5. Fat pads are cleared in methyl salicylate for 2–4 h, examined and photographed (**Fig. 11.3f, g**). They are scored as negative for outgrowths if no epithelial structures can be observed. They are scored as "failed clears" if they contain an

epithelial ductal network which can be seen to have grown in from one edge of the fat pad and in which the majority of ductal branching have the same directionality (**Fig. 11.3 g**). However, if outgrowths can be seen to have originated from a central region of the cleared fat pad and the directionality of the ductal branching is different in different parts of the fat pad, they are scored as successful transplants. The morphology of the outgrowth is noted (i.e. ductal or alveolar, the presence or absence of terminal end buds). An estimate is also made of the amount of the fat pad filled by the outgrowth.

6. The fat pads are returned to 100% ethanol. Once it is confirmed that good photographic records have been made of the wholemounts, they are put through two more changes of 100% ethanol for 24 h each to remove all traces of salicylate (*see* **Note 57**).
7. Successful transplants have a region of epithelial outgrowth dissected out under a binocular microscope for paraffin embedding by routine methods and routine immunocytochemistry to detect α-isoform smooth muscle actin (using the 1A4 clone antibody). This stains the myoepithelial layer, allowing confirmation that epithelial structures have both myoepithelial and luminal cells.
8. The remaining, unembedded portion of the gland and negative/failed clear fat pads are eased from the slides using a #11 scalpel and transferred to a 5-ml tube containing 100% ethanol for storage.

4. Notes

1. L15 medium is an air-buffered medium and as such is extremely useful as a general purpose washing or "holding" medium.
2. All solutions are made up with water that has a resistivity of 16–18 MΩ and total organic content of less than 5 parts per billion.
3. Cholera toxin inhibits residual fibroblast overgrowth and promotes epithelial growth.
4. All the equipment and reagents required are prepared the day before tissue harvest for convenience. Media aliquots are kept at 4°C overnight.
5. Enzyme aliquots are weighed out but not dissolved and filtered until after the tissue has been harvested.

6. Two cutting discs are needed to ensure the stage clips grip tightly (when the equipment is new, one may be sufficient). Even when apparently gripped tightly, the discs do still have a tendency to vibrate free during chopping, especially when new and still shiny. Use the forceps and screwdriver to reposition them during the chop as necessary.
7. This seems to keep the blade in position better during chopping.
8. This procedure is quite tricky and should be practiced several times.
9. Experiments involving animals must be conducted in accord with the prevailing local and national regulations. For instance, in the United Kingdom, this includes the need for local ethical approval and requires that appropriate licences are obtained from the government Home Office. Waste from the protocols described below should be disposed of according to approved laboratory practices.
10. We routinely use 10- to12-week-old virgin female FVB mice. The protocol is applicable to any age or strain of mouse and to any developmental stage of the adult gland, except lactating animals, in which case the fatty content of the epithelial cells prevents efficient organoid centrifugation. To isolate larger cell numbers, more than 20 animals can be used, in which case they should be processed as separate batches and the cells pooled only after the single cell suspensions have been generated, prior to flow cytometric staining.
11. Efficient removal of the lymph nodes takes practice but should be persevered at as the more lymph node tissue is removed, the better the results of the flow cytometric analysis.
12. The fineness of the chop is the absolute determinant of the cell yield obtained at the end of the procedure, and correct setting of the blade, as described in Section 2, is the prime determinant of a fine chop. It is also important, however, to stop the tissue spreading out during the chop so that the blade does not miss tissue, which can especially happen towards the front edge of the disc. Use sterile disposable plasticware, such as loops for microbiological work, to bring any escaping tissue back into the path of the blade.
13. Turning the blade is accomplished using the sterile forceps, screwdriver and socket spanner, much as described for setting up the tissue chopper (**Section 2.2**). Loosen the screw with the socket spanner, using the forceps and screwdriver to free the collar and then turn the blade through 180° and finally replace the collar and screw. Once again,

ensure good contact between the blade and cutting disc is achieved both before and after the screw is tightened.

14. The mix should be shaken at such a speed that you get a good "wave" travelling up and down the tube, but should not be too fast. Note that some users of this method have reported to the author that use of trypsin in the mix results in loss of some cell surface antigenicity and reduced cell viability at the end of the procedure. Whilst we have not observed such a reduced cell viability, if this occurs it may be necessary to substitute the trypsin for an alternative or use collagenase only.
15. After pouring off the fat and supernatant layer, the 50-ml Falcon tube containing the pellet should be laid on its side so that fat stuck to the side of the tube does not run back down into the pellet. The pellet should be immediately transferred to the fresh 15-ml Falcon tube in 5-ml L15/10% FCS medium whilst the supernatant digestion mix is being spun a second time. Again, once the supernatant has been poured off after the second spin, the 50-ml Falcon tube should be kept lying on its side until the pellet can be taken up in 5-ml L15/10% FCS medium and added to the material from the first spin. This prevents fat which has stuck to the side of the tube, and not been poured off as waste, from sliding back down into the organoid pellet.
16. The pellet consists of epithelial organoids, red blood cell fragments of blood vessels as well as contaminating fibroblasts and other cell types.
17. We have found no deleterious effects of storing organoids overnight at 4°C followed by processing to single cells compared with immediate processing. Overnight storage has a big advantage in terms of logistics of experiments, as it enables single cell processing to be started early the next day. This allows single cell processing, staining, flow cytometry and, if required, cell transplantation to be all completed in one working day. It is very demanding to carry out all these processes as a continuous procedure if one intends to go straight from organoid harvest to single cell preparation.
18. Incubation in Joklik's medium for suspension cultures helps disrupt Ca^{2+}-dependent cell–cell attachments and begins the process of dissociating the epithelial cells from each other.
19. Organoid-based bulk cultures are suitable when large numbers of epithelial cells are required for, for instance, analysis of mammary-specific Cre-*LoxP*-based gene knockout or for transplantation of epithelial cells from one mouse strain into the cleared fat pads of a second strain.

20. Exact plating density depends upon organoid yields and suitable densities should be determined empirically by the user. Ideally, one does not want to passage mouse mammary epithelial cells as many do not survive the process. Passaging also increases the risk of overgrowth by contaminating fibroblasts and may, also, promote fibroepithelioid dedifferentiation by the epithelial cells. Ideally, therefore, organoids or cells for bulk culture should be plated such that the vessel they are in is about 90% confluent at the time they are to be harvested, for the use they are required for, without the need for intervening passage.
21. Low-oxygen conditions are not strictly required for bulk mammary cell culture. However, we feel that even bulk primary mouse mammary cultures maintained at low oxygen look healthier than those maintained at ambient oxygen.
22. Single cell-based cultures are suitable when primary cultures will be manipulated by retroviral infection and the maximum number of proliferating cells is desired in the culture for retroviral integration. However, retroviral infection of primary mammary epithelial cells is notoriously difficult and single cell bulk cultures are more prone to fibroepithelioid dedifferentiation. We would currently recommend a lenti- or adenovirus-based approach using organoid cultures for in vitro manipulation of primary mouse mammary epithelial cells.
23. CD24 staining of mouse mammary epithelial cells appears to be extremely temperature sensitive, and allowing single cells to sit at room temperature for long periods of time results in significant reduction in intensity of CD24 staining. A chiller unit must be used on the sample whilst it is being sorted on the flow cytometer and, preferably, on the separated subpopulations as they are being collected.
24. The gating for this approach was determined by us empirically, in other words, by gating an area, sorting it and then determining by eye whether single cells, doublets, triplets or larger clumps were isolated. It is recommended that a user setting this system up for the first time should set the gates in a similar manner.
25. Splitting of $CD24^{+}$ cells into distinct $CD24^{Low}$ basal cells and $CD24^{High}$ luminal cells occurs efficiently and reliably with FITC conjugates, but not with PE. The reason for this is not clear. Other fluorochromes have not yet been extensively tested. It should also be mentioned that this separation method has so far only been used extensively with virgin animals and has not yet been optimised for other developmental stages.

26. Note that bulk cultures can also be established from cell populations as described in **Section 3.3**.
27. After sorting it is recommended, if enough cells are available, to re-analyse the sorted samples to ensure that the separated samples are of high purity and not contaminated by one of the other populations. Note that if the yield is big enough then the sort can be used for several assays, for instance, 96-well plate clonal analysis, sorting on to slides and collection for harvesting RNA.
28. This enables the cells to be located after drying, freezing, fixing and staining.
29. Methanol:acetone fixation must be used as keratin 18 is sensitive to other fixation methods.
30. Goat anti-mouse subclass-specific second antibodies do not cross-react with primary antibodies of different subclasses, allowing the use of multiple indirect immunofluorescence analysis with mouse antibodies on mouse cells. Nevertheless, when setting the system up for the first time or when introducing a new primary antibody combination, the appropriate controls must be carried out.
31. There is no need to carry this out in the dark or at low light levels.
32. The low-oxygen environment is key for successful mammary epithelial single cell cloning.
33. Glutaraldehyde is a respiratory irritant. Work involving glutaraldehyde should be carried out in a fume cabinet wherever possible using appropriate personal protective equipment.
34. Epithelial cells attach less well to glass than to tissue culture plastic. If required, therefore, cloning efficiencies can be improved by giving the coverslips a coating which promotes attachment but is inactive biologically, such as poly-L-lysine.
35. The sorting method used with these cultures is the one most likely to produce contamination and consequently gentamycin can be added to the medium. If fungal contamination becomes a problem, fungizone can also be added.
36. Fixing only six wells at a time prevents drying out (which can result in poor staining) when the methanol:acetone is being removed.
37. If the coverslips are to be stored for later staining, it is best to carry out the fixation in a tissue culture hood and keep the procedure sterile, in order to prevent contamination during long-term storage.

38. Washing in this way neutralises the charge on the plastic which would otherwise cause cells, as they are being sorted in charged water droplets, to be in many cases deflected and bounced out of the tubes as they are sorted. Washing therefore increases yield and reduces contamination.
39. One 1.5-ml screw-cap Eppendorf tube will hold up to 4×10^5 sorted cells.
40. Use a P1000 and, if necessary, a P200 pipette. However, it is better to leave a little bit of liquid in the bottom of the tube rather than risk losing the cell pellet.
41. Note that depending on how good a yield of cells one gets, it is possible to sort on to slides, to set up 96-well and 24-well plates and to sort for RNA all in one preparation.
42. Epithelial cells are "sticky" and will tend to stick together after flow sorting, even if the flow sorting procedure itself was isolating single cells at very high purity. However, in most cases post-sort samples are at least 90% single cells. This data is important as cells transplanted as clumps may have a better chance of forming outgrowths than pure single cells and may thus bias estimates of the number of stem cells in a population.
43. For instance, if one intends to transplant 1,000 cells per fat pad and one has 46,000 cells of a particular population, the cells are resuspended in 460 μl serum-free L15. This gives enough material to transplant 46 fat pads. This is impossible to do in one transplant session, so the most practical option is to take, for instance, one-third of the sample to be transplanted, depending on how many transplants one intends on doing, and to pellet the remaining sample, resuspend it in RLT buffer as in **Section 3.8** and use it for RNA isolation.
44. All animal work must have appropriate local ethical approval and be in accordance with relevant national legislation.
45. Consult your veterinary surgeon for recommendations on anaesthesia and analgesia for the procedure. IP injection is preferable. Inhalation anaesthesia is not appropriate due to the need to constantly turn the dissection board to get the best possible angle at which to work. Approximately 45 min of anaesthesia at a sufficient depth to operate is required.
46. FVB host mice are used when FVB cells are being transplanted (syngeneic transplant). Obviously, if using a different inbred strain it is best to use the same strain as a host. However, when transplanting cells of a mixed back-

ground there are two options open. If the background of the mice providing the tissue is relatively simple (e.g. C57 and Balb/c) you can transplant into F1 C57×Balb/c hybrids. If, however, it is more complex, you need to transplant into immune-suppressed animals. Typically *Rag*$^{-/-}$ or *Rag*$^{-/-}$; $\gamma^{-/-}$ knockout mice are used. If transplanting into immune-suppressed animals, extra care must be taken with sterile technique and all procedures must be carried out in a microbiological safety cabinet.

47. Do not stretch the skin too taut, in mice this young you can easily tear it, creating extra difficulty when the time comes to close the wound.
48. As skin in these young animals is very thin, you must be extremely careful when carrying this out. However, it is important as endogenous epithelium contained in tissue left behind on the skin can grow into the surgically altered fat pad and cause a failed clear.
49. With luck, you will see a bubble form in the fat pad as you inject, showing that the cells have gone into the correct place. However, if you do not see a bubble, it does not necessarily mean that the cells have not gone into the fat pad. Some workers co-inject sterile dextran blue beads with the cells to confirm that injection has gone into the fat pad.
50. Stitching is preferable to using clips in such young animals, and furthermore, once the wounds are healed, the mice will remove the stitches themselves.
51. Animals should be monitored carefully by animal care workers on a daily basis for the week following surgery to ensure integrity of stitches and that post-operative infection has not occurred. Stitches occasionally (<1% of the time) come loose following transplantation before wounds have had time to heal. Provided the wound is only partially open, and if prior ethical approval has been obtained, the animal can be re-anaesthetised and re-stitched. If a wound has completely re-opened then the animals should be culled. If stitches fail a second time the animals should be culled.
52. For analysis of pregnancy-dependent alveolar outgrowth, transplant hosts are mated 6 weeks after transplant and then culled and wholemount preparations made 2 weeks after plugging.
53. This dissection is much more tricky and should be done with more care than when the fat pads are being harvested to prepare primary cells. This is because of the reduced size of the cleared fat pads and the amount of fibrosis that develops following healing of the transplant incision.

Particular care should be taken not to get muscle from the peritoneum stuck on to the fat pad as, once carmine stained, this will obscure underlying structures.

54. Provided the fat pads are quite dry they will stick easily to the slide. If they get wet during the dissection (e.g. if the femoral artery is accidentally cut) they will need to be blotted on tissue before stretching on the slide.
55. As an alternative, fat pads can be fixed for 4 h in 4% paraformaldehyde in PBS, washed three times in PBS for 30 min per wash and then carmine stained.
56. Carmine aluminium can be reused several times.
57. If required, wholemount preparations can be cleared again in salicylate and re-photographed.

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Chapter 12

Isolation and Culture of Mouse Pancreatic Islets for Ex Vivo Imaging Studies with Trappable or Recombinant Fluorescent Probes

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Abstract

The endocrine pancreas contains small clusters of 1,000–2,000 neuroendocrine cells termed islets of Langerhans. By secreting insulin, glucagon, or other hormones as circumstances dictate, islets play a central role in the control of glucose homeostasis in mammals. Islets are dispersed throughout the exocrine tissue and comprise only 1–2% of the volume of the whole organ; human pancreas contains about 10^6 islets whereas rodents have approximately 2×10^3 islets. The isolation of islets from the exocrine tissue usually begins with digestion of the pancreas with collagenase. Collagenase-containing medium is either injected into the pancreatic duct, and the organ left to digest in situ, or added after isolation of the pancreas and its dissection into small pieces ex vivo. Islets can then be separated from the exocrine tissue by gradient density or by handpicking. The islets obtained can either be used intact, for example, to measure insulin or glucagon secretion or be dispersed into single cells with a Ca^{2+}-free medium or with trypsin/dispase. The latter facilitates the introduction of recombinant or trappable probes and microimaging studies of, for example, changes in cytosolic-free Ca^{2+} concentration or the dynamics of individual organelles or proteins.

Key words: Pancreas, islets, β-cells, α-cells, calcium, total internal reflection fluorescence microscopy.

1. Introduction

Each islet of Langerhans is composed from a few hundred to several thousand endocrine cells of at least four different types: α, β, δ, and PP cells which secrete glucagon, insulin, somatostatin, and pancreatic polypeptide, respectively. In rodents, β-cells

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(65–80% of islet cells) are mainly localized in the core of the islets and are surrounded by a mantle of non-β-cells. The endocrine pancreas is richly innervated by cholinergic, adrenergic, and peptidergic fibers (1, 2) and receives blood via a capillary network, derived from the celiac and mesenteric arteries, and which provides "inside–out" perfusion of the β-cell core followed by α- and other non-β-cells (2).

The isolation of rodent islets of Langerhans is most conveniently achieved by collagenase digestion of the whole pancreas or pancreatic fragments. This chapter will detail step-by-step the methods that we use in the laboratory to separate mouse islets from the exocrine tissue and to dissociate them into clusters or single cells (3–5), but detailed methods can also be found elsewhere (6–8). We will also briefly detail how isolated islet cells can be infected with adenoviruses as a means to image changes in the concentration of signaling molecules such as free ATP (9, 10) or cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) (5, 11) or to track the localization of individual proteins (12) or organelles (13–15) at the single cell level.

2. Materials

2.1. Reagents

1. Collagenase type V (~1 U/mg) (SERVA, Heidelberg, DE) (*see* **Note 1**).
2. DNase I (Roche, Mannheim, DE).

2.2. Media and Solutions

1. Prepare 600 ml of Krebs–Ringer bicarbonate buffer (KRB) for islets isolation (make fresh), containing 120 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 24 mM $NaHCO_3$:

   Gas with O_2/CO_2 for 10–15 min.

   Check the pH, it should be 7.2.

   Add to the following concentrations: 1 mg/ml BSA, 5 mM HEPES, and 10 mM glucose.

   Adjust pH to 7.30–7.35 with 1 N NaOH.

   Supplement with penicillin at 100 IU/ml and streptomycin 100 μg/ml and filter sterilize 400 ml of the KRB into a sterile glass bottle with a 0.2-μM filter.

   Add a few micrograms (does not need to be accurate) of DNase I to the remaining KRB (~200 ml) and filter into another sterile glass bottle (KRB + DNase I).
2. RPMI 1640 medium containing 11 mM glucose (BioWhittaker™/Cambrex, Verviers, Belgium) supplemented with 2 mM L-glutamine, 10% heat-

inactivated fetal calf serum (FCS), 100 IU/ml penicillin, and 100 μg/ml streptomycin.

3. Histopaque-1119, -1083, and -1077 for separation based on density (Sigma-Aldrich, St Louis, MO).
4. Cell dissociation buffer enzyme-free Hanks'-based to disperse islets into clusters and single cells (Invitrogen, Paisley, UK).
5. 0.25% Trypsin-EDTA (Invitrogen, Paisley, UK).
6. Fura-2 acetoxymethylester (Fura-2 AM). Prepare a 2 mM stock solution in DMSO. Dilute to 2 μM in 2 ml of KRB.

3. Methods

The digestion of the pancreas with collagenase can be achieved with two methods. The first method is based on cutting the pancreas into small pieces before the digestion with collagenase (4, 5) whereas the second method depends upon distension of the pancreas with the collagenase injected through the pancreatic duct (3, 16). Both techniques are used in this laboratory, and we recommend the latter method if one needs to isolate more than 150 islets per mouse. None of the steps listed below need to be done in a flow hood but sterile materials are used. Depending on the method used to digest the pancreas (i.e., with or without injection of collagenase through the pancreatic duct) the maximum yield is 100–250 islets per mouse, which is approximately 10% of the total available islets. Ideally, in this laboratory we use 3- to 5-month-old mice (e.g., CDI, C57/BL6, ad libitum fed adult animals).

3.1. Surgery and Pancreas Digestion

3.1.1. Collagenase Digestion of the Pancreas Injected with KRB

This method is derived from the procedure developed in the laboratory of Henquin (4, 17).

1. Kill the animal by cervical dislocation according to locally approved procedures. Soak the fur with ethanol and using coarse surgical scissors and large forceps open the abdomen along the middle of the sternum.
2. Locate the pancreas, which is diffuse when compared to human (**Fig. 12.1a**), and inflate it by injecting approximately 10 ml of KRB with 25-gauge needle. Inject KRB into several regions of the pancreas. The more the one injects the greater the inflation of the pancreas the more efficient will be the isolation of the islets from connective tissues. The inflated pancreas should look like a filled transparent bag displaying opaque islands (**Fig. 12.1b**).

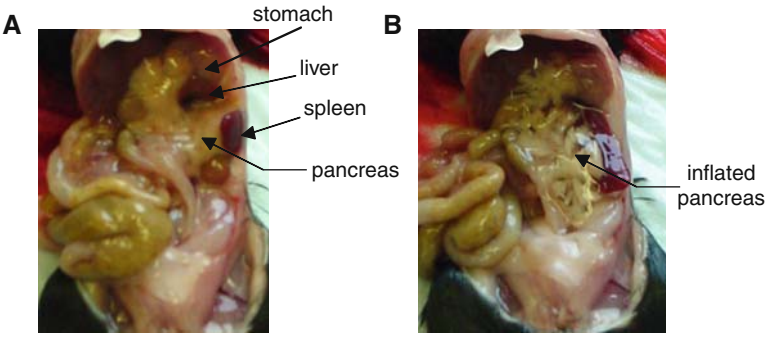

Fig. 12.1. Abdominal cavity of mice before (**a**) or after (**b**) injection of pancreas with KRB.

3. Once inflation is complete, excise the pancreas. Start by removing the spleen, and then cut out the pancreas following the intestine moving anti-clockwise, pull up the intestine. Cut out the pancreatic tissue moving along the line of the intestine then cut out deeper attachments.
4. Cut three to four times the inflated pancreas in a 20-ml plastic/glass vial containing KRB. If you have more than one pancreas to dissect, start from step 1 and then pool all pancreata in the vial (assuming that mice are from the same strain).
5. Cut all the pancreata into small pieces with scissors. Cut about 100 times to yield pieces of approximately 1–2 mm in size and transfer into a 10-ml plastic/glass tube.
6. Prepare 2 mg/mouse of collagenase (type V; ~1 U/mg; SERVA, Heidelberg, DE) (*see* **Note 1**). Add 1–2 ml of KRB and vortex.
7. Remove the supernatant in your 10-ml plastic/glass tube and wash twice with KRB to remove floating fat and blood. Remove supernatant and add the 1–2 ml of collagenase you have prepared in step 6 (filter the collagenase with a 0.2-μm filter). Finally, adjust the volume of collagenase in KRB to a similar volume occupied by the pellet (i.e., if four pancreases give 4 ml of material in the tube, add 8 mg of collagenase in 1–2 ml of KRB and top up with KRB to 8 ml).
8. To digest the pancreas, shake the preparation by hand at 37°C in a water bath (recommended) for 5–10 min or alternatively use a shaking water bath (200 spm) for 10–20 min (*see* **Note 2**).
9. Once digestion is complete (the pellet should now form a milky solution with few rare clumps), top up the tube with KRB + DNase I to wash out the collagenase.

10. Centrifuge the digested pancreas (~300*g* for 10 s to spin down tissue).
11. Pour off the supernatant, top up the tube with KRB + DNase I.
12. Resuspend the pellet.
13. Repeat steps 10–12.
14. Distribute the solution evenly between 6 and 8 plastic/glass tubes of 10 ml.
15. Top up each tube with KRB + DNase I and store in the fridge until islets isolation.

3.1.2. Digestion of the Pancreas After Injection of Collagenase into the Duct

Alternatively, if more than 150 islets are required per pancreas, this can be achieved by perfusion of the pancreatic duct and in situ collagenase digestion instead of inflating the pancreas by injecting KRB into several regions (*see* **Note 3**).

1. Begin as in step 1 of **Section 3.1.1**. Then, using small forceps, grab the uppermost loop of the small intestine (closest to the liver).
2. Spread the web of the pancreas within the loop of the intestine and locate the pancreatic duct running from the intestine loop to the liver.
3. Grip the intestine at the point of duct attachment and clamp the duct at the end nearest to the intestinal tract. We use serrefine clamps (Hammacher HSE 003-35 or HSE 001-28).
4. Holding the clamp in one hand to keep the duct taut, slowly inject 3 ml of filtered collagenase in KRB (1 mg/ml) into the pancreatic duct with a 30-gauge needle. Inflation of the duct should be clearly visible at the injection of the first milliliter. Care must be taken at this point not to move the needle to avoid damaging the duct (*see* **Note 4**).
5. Once inflation is complete, excise the pancreas. Start by removing the spleen, and then cut out the pancreas following the intestine moving anti-clockwise, pull up the intestine. Cut out the pancreatic tissue moving along the line of the intestine then cut out deeper attachments.
6. Cut three to four times the inflated pancreas in an empty 7–10 ml glass vial. If you have more than one pancreas to dissect start from step 1 and you can pool all pancreases in the vial (if mice are from the same strain).
7. Cut pancreata into small pieces with scissors. Cut about 100 times to yield pieces of approximately 1–2 mm in size and transfer into a 20- to 50-ml plastic/glass tube.

8. Rinse the empty glass vial with KRB to collect remaining pieces of pancreas stuck inside and transfer into the 20- to 50-ml plastic/glass tube. Finally, top up with KRB to a similar volume formed by the tissue pellet (i.e., if four pancreases give 4 ml of material in the tube, top up with KRB to 8 ml).
9. Incubate for 11 min at 37°C in water bath without shaking (*see* **Note 2**). Once digestion is complete, add 10 ml of ice cold KRB and disrupt the pancreas by vigorous hand shaking for 1 min.
10. Top up the tube with KRB + DNase I to washout the collagenase.
11. Centrifuge the digested pancreas (~300*g* for 10 s to spin down tissue).
12. Pour off the supernatant, top up the tube with KRB + DNase I.
13. Resuspend the pellet.
14. Repeat steps 11–13.
15. Distribute the solution evenly between 6 and 8 plastic/glass tubes of 10 ml.
16. Top up each tube with KRB + DNase I and store in the fridge until islets isolation.

3.2. Isolation of Islets from Exocrine Tissue

3.2.1. Selection by Size

The islets can be selected by size after filtering the cell suspension through a 100-μm mesh (18). This method will select only the smallest islets. This is useful if you need to infect intact islets with an adenovirus, and you want to infect most of the cells within the islets (i.e., not just those cells at the periphery) (19). However, we have found this method is not completely satisfying because a lot of islets can be lost, especially if the pancreas is under-digested or if clumps of exocrine tissue surround the islets; in this case the islets become clogged in the mesh. Instead of using this method immediately after digestion, it can still be used after the method detailed in **Section 3.2.2**.

3.2.2. Separation Based on Density

Islets separation can be achieved based on density using Ficoll or Histopaque gradients (3). This method will not be suitable if only one pancreas is available or if the islets are to be used immediately after isolation. If either of these criteria applies, the method detailed in **Section 3.2.3** should be deployed.

1. After following **Section 3.1.1** or **Section 3.1.2**, filter the digested pancreas through a sterile 500-μm mesh cone into a 50-ml polypropylene tube (the equivalent of two to three pancreases per tube). Wash the tube to collect any remaining islets by adding a further 10 ml of KRB to the tube and pool.

2. Centrifuge at 300*g* for 2 min to spin down the islets.
3. Remove all the supernatant leaving only the solid pellet.
4. Resuspend the pellet in 6 ml of histopaque solution 1.119 g/l.
5. Gently layer the other gradient solutions over this. First, 6 ml of histopaque 1.083 g/l followed by 6 ml of histopaque 1.077 g/l and then 6 ml of KRB.
6. Centrifuge at 800*g* for 20 min with no break.
7. At the end of the centrifugation step, the islets should be observed at the upper most surface between the KRB and 1.077 g/l layer. Ideally these should appear clean with little or no exocrine tissue (*see* **Note 5**).
8. Harvest the islets from this upper phase by drawing them up into a disposable sterile pipette. Place the islets into a 10-ml polypropylene tube.
9. Wash the islets with KRB. Centrifuge at 300*g* for 5 min. Remove supernatant and resuspend the islets in RPMI 1640 containing 11 mM glucose and supplemented with 2 mM L-glutamine, 10% (v/v) FCS, penicillin 100 IU/ml, and streptomycin 100 μg/ml. Culture the islets in the medium above for at least 3 days at 37°C with 95% air and 5% CO_2 or disperse them into clusters of cells or single cells (*see* **Section 3.3**).

3.2.3. Handpicking the Islets

This method is usually the method of choice especially where islet quality rather than yield is the uppermost priority (4, 5, 17). The handpicking of islets under a stereo dissecting microscope (e.g., Nikon SMZ645, Olympus SZ61, Zeiss Stemi 2000) is the least traumatic method for the islets (*see* **Note 6**), it has the advantage in comparison to the density-based gradients, of not damaging them, so they can be used freshly isolated. However, the disadvantage of this method is that it is time consuming for the investigator, and needs some practice in order to distinguish confidently the islets from exocrine tissue and to avoid damaging them during the isolation process (*see* step 3 below).

1. Remove the supernatant containing DNase I from one tube and resuspend the pellet into KRB (without DNase I).
2. Pour the entire suspension into a sterile glass Petri dish (6 cm diameter).
3. Under a stereo dissecting microscope islets appear as opaque spheres (from 50 to 500 μm) against a grayish background (containing exocrine acini and cell debris) (**Fig. 12.2a**). You should distinguish islets from contaminating lymph nodes which are yellowish, more compact, and bigger (>600 μm) than islets. Handpick the islets with a sterile fire polished

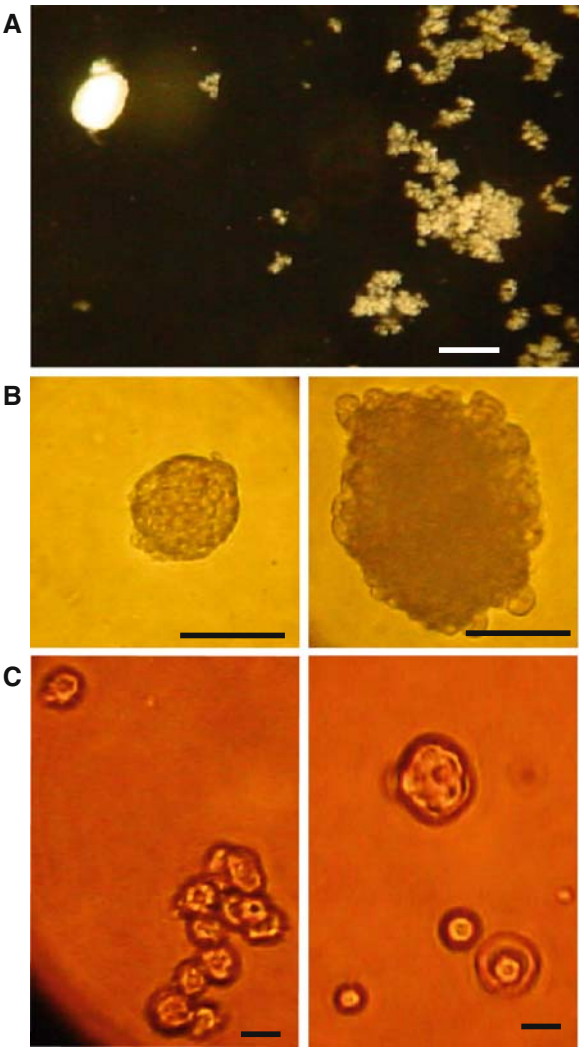

Fig. 12.2. (**a**) Visualization of an islet (*top left corner*) and exocrine tissue (*right*) from a collagenase-digested pancreas under a dissecting microscope. Scale bar = 200 μm. Note that some exocrine tissue is still attached on *top left* of the islets. Exocrine tissue can be distinguished from islets by their somewhat greater translucence and their slightly paler appearance (**b**) Healthy small (*left*) and larger necrotic (*right*) islets after 1 day in culture. Scale bar = 100 μm. (**c**) One single cell and a group of cells with a "bunch of grapes" configuration immediately after dissociation of islets (*left*) and three single cells with a 10-cell cluster one day after culture (*right*). Scale bar = 10 μm.

siliconized glass Pasteur pipette or with a Gilson/Eppendorf pipette and yellow tip (*see* **Note 7**). Transfer islets into another sterile Petri dish placed on ice and containing KRB. If some islets are still attached to vessels/exocrine tissue gently pull the islets away by using two 25-gauge needles attached to 5-ml syringes (use them as a tool to pull the

islets/exocrine tissue and not to suck off the islets!) (*see* **Note 8**).

4. At the end of the isolation, the islets can be used either fresh or cultured overnight at 37°C with 95% air and 5% CO_2 in RPMI 1640 containing 11 mM glucose and supplemented with 2 mM L-glutamine, 10% (v/v) FCS, penicillin 100 IU/ml, and streptomycin 100 μg/ml or dispersed into clusters of cells or single cells.

After overnight culture, take special care when selecting islets for experiments because some may display a necrotic core (*see* **Note 7**) (**Fig. 12.2b**).

3.3. Dispersion of Islet Cells into Clusters and Single Cells

This method will give a mixed population of cells containing β, α, δ, ductal, and vascular cells such as fibroblasts.

3.3.1. Dispersion of Cells with Ca^{2+}-Free Medium or Trypsin

Dispersion of cells with Ca^{2+}-free medium has to be done immediately after the isolation of islets. If you want to dissociate the islets after an overnight culture use either trypsin or dispase.

1. To disperse the islets into clusters of cells or single cells, reassemble all of the isolated islets in the middle of the Petri dish (try to keep the islets free from any exocrine/cell debris).
2. Transfer all of the islets with a 20-μl pipette into a 10-ml polypropylene tube containing 5 ml of either dissociation buffer enzyme-free Hanks'-based (Invitrogen, Paisley, UK) or 0.25% trypsin-EDTA (Invitrogen, Paisley, UK).
3. Wait for 5 min.
4. Centrifuge at 300*g* for 5 min.
5. Remove 4 ml of supernatant and dissociate the islets by resuspending the pellet very aggressively with a 1-ml Gilson pipette and long tips, from 10 (to obtain mainly clusters) to 15–20 (to obtain mainly single cells) times. You should see by eye that the islets are becoming smaller during the process.
6. Top up to 10 ml with RPMI 1640 to wash the solution and centrifuge at 300*g* for 5 min to spin down the cells.
7. Remove all of the supernatant (because Ca^{2+}-free medium and trypsin are both toxic after a while for cells).
8. Resuspend cells in RPMI. If you need to have cells attached to a coverslip, add 100 μl of RPMI with the equivalent of 15 islets per coverslip (these proportions are for 24-mm coverslip) (*see* **Note 9**). So from 150 isolated islets, you can prepare 10 coverslips and you need to add 1 ml of RPMI to resuspend your cells.

9. Put 100 µl of cell suspension in the middle of a sterile coverslip (no need to coat coverslips when you use a Ca^{2+}-free medium or 0.25% trypsin-EDTA to disperse cells) (*see* **Note 10**).
10. Leave cells in the incubator for 15 min to allow cells to decant and be in contact with the coverslip, and then gently (because cells are not attached to coverslips yet) add 2 ml of medium in each well with a 1-ml Gilson/Eppendorf pipette. After dissociation, the cells will be either single or form groups that look like a bunch of grapes (**Fig. 12.2c**). Leave cells overnight in the incubator as they will adhere to the coverslip. They will either stay isolated or form clusters of cells if you have observed a lot of bunches of grapes. Note that bunches of grapes will change shape to give round/ovoid clusters the day after as the cells will re-aggregate (**Fig. 12.2c**).

3.3.2. Dispersion of Cells with Dispase

A detailed protocol has been published by Josefsen et al. (20) and yields a preparation consisting principally of fully dissociated single cells (20).

The released cells can be either used immediately or plated on glass coverslips coated with fibronectin (50 ng/ml; Sigma, St Louis, MO) and cultured at 37°C in RPMI 1640 medium.

3.4. Islets Infection with Recombinant Adenoviruses and Loading with Ca^{2+} Probes

One day after culture, cells can be infected with an adenovirus (*see* **Note 11**) to express a protein of interest at a multiplicity of infection of 30–100 infectious particles per cell for 4 h (3, 5, 19, 21). This level of infection has previously been shown to exert no deleterious effects on β-cell function or viability (19).

3.4.1. Recombinant Adenoviruses

For example, to identify primary mouse α-cells within dispersed islet cells (<20% of islet cells), an adenovirus was generated to express the red fluorescent protein (mRFP) under the control of the preproglucagon gene promoter. It should be stressed that it is very difficult to discriminate an α-cell from a β-cell, even though the latter is slightly larger and displays different responses to stimuli including glucose and adrenaline (22).

The co-infection of cells with a second adenovirus to express the enhanced green fluorescent protein (EGFP)-tagged PKCβII (EGFP-PKCβII) then allowed the measurement of the intracellular localization of PKCβII in identified α-cells dynamically and in real time using total internal reflection fluorescence (TIRF) microscopy (**Fig. 12.3b**).

3.4.2. Loading of Cells with Ca^{2+} Probes and Measurement of Cytosolic Ca^{2+} Concentrations

Ca^{2+} signals are most often measured using Ca^{2+}-sensitive fluorescent dyes such as Fura-2, Fluo-3, or Indo-1 (23). In the following steps we will take the example of the use of Fura-2.

1. Prepare KRB and Fura-2 AM solutions (*see* **Section 2.2**).

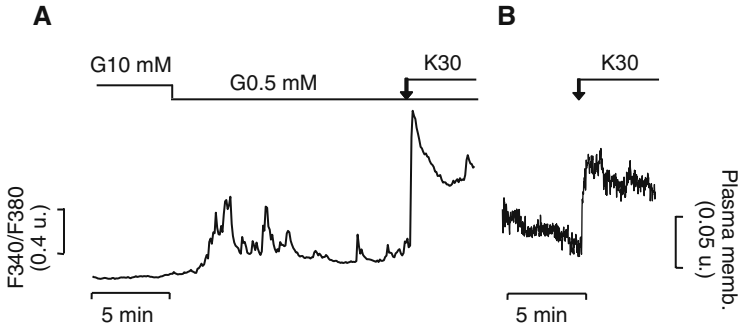

Fig. 12.3. Measurements of $[Ca^{2+}]_c$ (**a**) and PKCβII translocation to the plasma membrane (**b**) in identified α-cells expressing mRFP under the control of the preproglucagon promoter. (**a**) Cells were perifused in the presence of 10 mM glucose (G10 mM). As expected a decrease in glucose concentration to 0.5 mM (G0.5 mM) induced spontaneous $[Ca^{2+}]_c$ oscillations in the α-cell, whereas 30 mM KCl (K30 mM) induced a sustained increase. (**b**) Cells were infected with an adenovirus to express EGFP-PKCβII. An increase of $[Ca^{2+}]_c$ with 30 mM KCl (K30 mM) induced a translocation of PKCβII to the plasma membrane.

2. Remove RPMI 1640 from cells, add Fura-2 AM in KRB, and incubate them in the cell culture incubator at 37°C for 30–40 min.

   In our case, the infection of dispersed islet cells with the adenovirus to express mRFP under the control of the preproglucagon promoter allowed us to measure changes in cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_c$) in identified α-cells using Fura-2 (**Fig. 12.3a**).

4. Transfer cells plated on coverslip into a thermostatted chamber (37°C). In this laboratory we use a thermostatted chamber mounted on the stage of an inverted microscope used in the epifluorescence mode with a 40× objective (UAPO/340 40×/1.35; Olympus). Cells are constantly perifused at a flow rate of 1.5 ml/min with KRB maintained at 37°C in a water bath.

5. Excite the cells alternately with UV light at 340 and 380 nm. In this laboratory we use a monochromator (Polychrome IV; TILL Photonics), and emission signals are detected at 515 nm with a cooled charge-coupled device camera (TILL Photonics, Grafelfing, Germany) controlled by TILLvisION software (TILL Photonics) (**Fig. 12.3a**) (15).

3.4.3. Measurement of PKCβII Translocation to the Plasma Membrane by Total Internal Reflection Fluorescence Microscopy

Total internal reflection fluorescence (TIRF) microscopy is a powerful optical technique that allows extremely thin optical sectioning with excellent signal-to-noise ratios. It is often employed to study cellular membrane activities or the dynamics of actin filaments, along with such varied phenomena as cellular adhesion, movement, single molecule events, or vesicle and protein tracking (14, 15, 24, 25).

1. Prepare KRB for your experiments (*see* **Section 2.2**).
2. One to two days after infection, transfer the adenovirally infected cells expressing EGFP-PKCβII and mRFP under the control of the preproglucagon promoter into a thermostatted chamber (37°C) (*see* step 4 in **Section 3.4.2**).
3. In this laboratory we use a TIRF microscope in conjunction with an objective lens having a numerical aperture of 1.45 (PlanAPO 100×/1.45 TIRFM; Olympus). To observe EGFP or mRFP fluorescence images, a 488 laser (argon ion laser, 30 mW, Spectra-Physics) is used for total internal reflection illumination, with a long pass filter (515 nm for EGFP, 600 nm for mRFP). Images are collected with a cooled charge-coupled device (CDD) camera (640 × 480 pixels, IMAGO, TILL Photonics; operated with TILLvisION software, TILL Photonics) (**Fig. 12.3b**) (15).

4. Notes

1. In this laboratory we use the collagenase from Serva, but alternatively one can use the collagenase type V from Sigma-Aldrich (St. Louis, MO).
2. The length of this incubation period may vary depending on the individual performing the procedure, activity/batch of collagenase. It is therefore advisable to carry out a range of incubation time points to establish optimum incubation times.
3. This method which gives more islets is more laborious and therefore needs a lot of practice to settle.
4. You will find it easier to do the injection into the pancreatic duct under a stereo dissecting microscope.
5. If the pancreas is under-digested you will not have any islets at the interface. They will be either lost (clogged into the mesh) or in the pellet at the bottom of the tube. If they are in the pellet you can still handpick them and separate them from the exocrine tissue as described in **Section 3.2.3**.
6. Epi-illumination is required using a fiber-optic light source which is bright and non-heating. Islets should be observed against a black background (either a glass Petri dish painted black or in a clear glass Petri dish placed on the black plate supplied with some stereo dissecting microscopes).
7. If islets are to be cultured after isolation, it is best to avoid taking the biggest islets (>300 μm) because they will develop a necrotic center overnight; these islets are not well

oxygenated in the center because of inefficient irrigation of the islet core ex vivo.

8. If the pancreas is under-digested, most of the islets will be left trapped in exocrine and connective tissue. In contrast if the pancreas is overdigested the islets will be damaged and disrupted. Instead of appearing opaque they will appear grayish.
9. Use 15 islets per 24-mm coverslip to have a lot of cells in the same field. If you need sparse cells you can reduce to 10 islets per 24-mm coverslip. Note that not all cells will attach on the coverslip, some will be damaged during the islet dispersion, only healthy cells will attach to the coverslip.
10. At that point you should check the confluency of the cells on the coverslip. If cells are too confluent (i.e., all cells are in contact together) they will not attach to the coverslip, so you will need to dilute them before plating the others coverslips.
11. You can find a method to generate adenoviruses at the following URL: http://www.coloncancer.org/adeasy.htm.

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Chapter 13

Isolation and Culture of Adult Mouse Hepatocytes

Wan-Chun Li, Kate L. Ralphs, and David Tosh

Abstract

The liver performs a multitude of functions including the regulation of carbohydrate, fat, and protein metabolism, the detoxification of endo- and xenobiotics, and the synthesis and secretion of plasma proteins and bile. Isolated hepatocytes constitute a useful technique for studying liver function in an in vitro setting. Here we describe a method for the isolation of hepatocytes from adult mouse liver. The principle of the method is the two-step collagenase perfusion technique which involves sequential perfusion of the liver with ethylenediaminetetraacetic acid and collagenase. Following isolation, the cells can either be used for short-term studies or, alternatively, maintained in culture for prolonged periods to study long-term changes in gene expression. The protocol for mouse hepatocyte isolation may be applied to both normal and transgenic mice.

Key words: Hepatocytes, primary culture, transgenic, collagenase.

1. Introduction

Techniques for studying liver functions include isolated perfused liver (1), liver slices (2), or freshly isolated and cultured hepatocytes (3) (*see* **Table 13.1**). Each of these techniques offers a number of advantages and disadvantages (*see* **Table 13.1**). In this chapter we describe the procedure for isolation and culture of adult mouse hepatocytes.

The technique for hepatocyte isolation is based on the two-step collagenase perfusion technique first developed by Berry and Friend for the isolation of rat hepatocytes (4). Prior to the introduction of enzymatic digestion with collagenase, different non-enzymatic mechanical separation methods were used to obtain liver cells (5). These included liver homogenization, trituration

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Table 13.1
Comparison of in vitro systems for studying liver function (*see* (1, 14, 21, 22))

	Hepatocyte suspension	Liver slices	Hepatocyte cultures
Pros	1. Standard preparation protocol	1. Relatively quick and easy to prepare	1. Extended survival periods
	2. Acquirement of large numbers of hepatocytes	2. Preservation of liver architecture and includes all liver cell types	2. Standard preparation protocol
	3. Similar response to in vivo pharmacological and toxicological effects	3. Possibility for cryopreservation	3. Possibility for lengthy and complex experiments
Cons	1. Disruption of intercellular contacts and cell communications	1. Short survival period (~ few days)	1. Contamination by non-hepatic cells
	2. Loss of cell polarity and shape	2. Impaired preservation of metabolizing enzymes (e.g., cytochrome P450)	2. Loss of hepatic phenotype (e.g., metabolizing enzymes, transportation systems)
	3. Damage to receptors	3. Slower metabolizing rate for certain metabolites	3. Time consuming (e.g., concentration of additives in the medium should be tested prior to experiment)
	4. Short survival periods (~few hours)		

of liver samples, and filtration of the tissues through fine sieves. However, under these conditions, marked cellular damage was observed. Besides collagenase, other digestive enzymes used to acquire a single cell suspension include trypsin, pronase, and lysozyme (6, 7), but these did not produce large numbers of viable hepatocytes. By the late 1980s, collagenase was widely used for dissociating hepatocytes from the liver (4, 8).

1.1. Principles of the Two-Step Collagenase Perfusion Technique

To date, the two-step collagenase perfusion technique is the method of choice for the isolation of large numbers of viable adult hepatocytes. In the first step, a calcium-free medium containing a calcium chelator is perfused through the liver. Removal of calcium ions (by EDTA, EGTA, or citrate) from epithelial cells results in the rapid destruction of intercellular junctions with the consequence that the cell–cell contacts are lost (9). The second step is the introduction of the enzyme collagenase into the liver lobes causing disruption of the supporting extracellular matrix. The procedure is carried out in situ using the animals' own circulatory system to perfuse the liver via the portal vein or vena cava (retrograde perfusion).

1.2. Factors Affecting Efficiency of Hepatocyte Isolation

The following factors are regarded as crucial during the two-step collagenase isolation procedure (7).

(A) *Role of Ca^{2+}*: Ca^{2+} plays a dual role in the hepatocyte isolation protocol. Removal of Ca^{2+} is essential to disrupt the Ca^{2+}-dependent E-cadherin molecules between adjacent hepatocytes and hence improve both the quantity and the quality of dispersed hepatocytes. The presence of Ca^{2+} is also required for the enzymatic activity of collagenase during perfusion (1.0–5.0 mM final concentration of Ca^{2+} is sufficient to sustain collagenase activity for liberation of hepatocytes (10, 11)). Regarding these properties of Ca^{2+}, both the concentration and the timing of reintroduction of Ca^{2+} back into liver during the hepatocyte preparation process are critical for acquiring intact cells. Seglen demonstrated the two phases of calcium requirement: Ca^{2+} is first removed from the liver by pre-perfusion with EGTA or EDTA medium and is then added into the perfusion medium with collagenase to obtain a large quantity of intact, viable cells (7).

(B) *Concentration and quality of collagenase*: Collagenase is a key element in hepatocyte isolation. It is therefore necessary that the user tests the activity of each collagenase batch. Different batches of collagenase exhibit differing efficiencies during preparation of hepatocytes (12). Seglen showed that crude collagenase and purified collagenase displayed similar activity on liver cell dispersion in the presence of same concentration of calcium ions (7).

(C) *pH and buffering:* The pH value of the perfusion medium should be kept around 7.4. The detection of a rapid drop in pH during perfusion is due to either the breakdown of glucose in the medium or the generation of bicarbonate from the dissolution of carbon dioxide in water. Therefore a strong buffering system is essential to ensure healthy cells. Several buffering agents have been suggested to maintain pH including sodium hydroxide (NaOH), bicarbonate, and the organic buffer 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES). Among the buffers, the addition of HEPES into perfusion medium has proved to be the simplest and most practical (7).

(D) *Other factors:* In addition to the points raised above, the production of isolated hepatocytes can also be affected by other factors, such as the perfusion flow rate (optimum of 5 ml/min for mouse) or the force of centrifugation following isolation (optimum of 50–100*g* for mouse liver cell isolation).

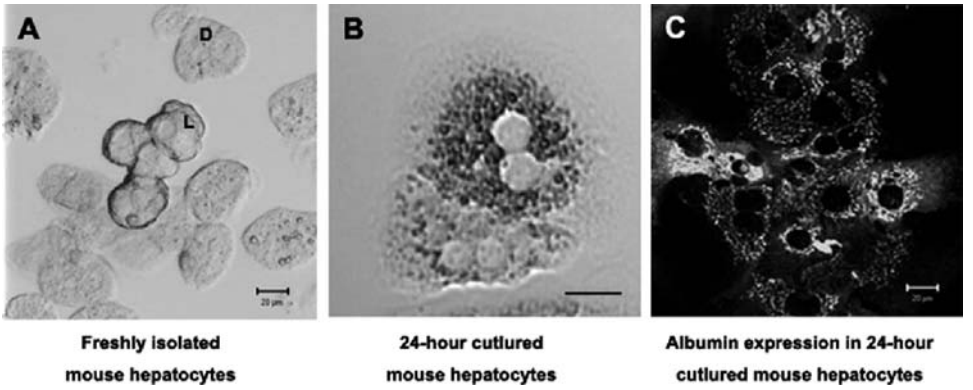

Fig. 13.1. (**a**) Freshly isolated hepatocytes from adult CD1 mouse showing both live (L) and dead (D) cells. Note the refractile property of membrane of the live cells. (**b**) Hepatocytes isolated and cultured for 24 h. Some of the hepatocytes are binucleate. (**c**) Hepatocytes cultured for 24 h and immunostained for albumin. Scale bar is 20 μm.

1.3. Cell Viability Tests

After the liberation of hepatocytes from the intact liver, monitoring of cell viability should be performed. Several techniques for viability testing have been suggested and mostly depend on an intact cell membrane (**Fig. 13.1**). Examples include trypan blue exclusion, measurement of ATP content (13), detection of the reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (the so-called MTT test) (14), monitoring of leaking lactate dehydrogenase, or detection of cell-specific biomarkers such as one of the aminotransferase enzymes (aspartate aminotransferase or alanine aminotransferase) or total bilirubin (15).

1.4. Primary Hepatocyte Culture

Hepatocytes in suspension demonstrate short-term viability and show rapid loss of their differentiation status after isolation (16). This is presumably due to the loss of expression of liver-enriched transcription factors. Therefore, after a standard hepatocyte isolation protocol was established, attempts were made to produce suitable culture conditions for hepatocytes. Recently, Mitaka has compiled an extensive review on the development of conditions for maintenance of hepatocytes in culture (3). At present, there is still no consensus on the best conditions for culturing isolated hepatocytes and this will ultimately depend on the experiments to be performed. **Table 13.2** lists commercially available media used for hepatocyte culture. Although most groups declared that they had successfully found a suitable medium for human or rodent hepatocyte culture, only a limited number of liver-specific functions were demonstrated in individual studies.

It is common to add supplements to hepatocyte culture media in order to help maintain the hepatic phenotype. The supplements are divided into two categories: complete hepatocyte mitogens and co-mitogenic growth factors (17). The complete

Table 13.2
Summary of media used for hepatocyte culture

Year	Authors	Medium	Supplements
1995	Kojima et al. (23)	L-15	0.2% BSA, 20 mM HEPES, 0.5 mg/l insulin, 10^{-7} M dexamethasone, 5.5 mM galactose, 30 mg/l proline, 20 mM $NaHCO_3$, 5 mg/l transferrin, 0.2 mg/l $CuSO_4 \cdot 5H_2O$, 0.5 mg/l $FeSO_4 \cdot 4H_2O$, 0.75 mg/l $ZnSO_4 \cdot 7H_2O$, 0.05 mg/l $MnSO_4$, 5 μg/l Na_2SeO_3, 10 ng/ml EGF
1998	Miyazaki et al. (24)	DMEM	2 g/l BSA, 2.25 g/l glucose, 2 g/l galactose, 0.1 g/l ornithine, 0.03 g/l proline, 0.305 g/l nicotinamide, 0.544 mg/l $ZnCl_2$, 0.75 mg/l $ZnSO_4 \cdot 7H_2O$, 0.2 mg/l $CuSO_4 \cdot 5H_2O$, 0.025 mg/l $MnSO_4$, 146.1 mg/l glutamine, 1 g/l ITS, 10^{-7} M Dex, 40 ng/ml HGF, 20 ng/ml EGF
1999	Lilja et al. (25)	Ham's F12 medium: WE (1:1)	0.25% bicarbonate, 15 mM HEPES, 65.5 μM ethanolamine, 100 μg/ml transferrin, 0.6 g/l insulin, 10^{-7} M dexamethasone, 10 nM glucagon, 32.25 mg/l proline, 7.18 μM linoleic acid, 7 mM glucose, 0.4 mM sodium pyruvate,0.1 mM ascorbic acid
2000	Runge et al. (26)	DMEM (no glucose): DMEM (with glucose): MEM = 1:1:2	1 g/l albumin, 5.5 mM galactose, 0.5 mM glutamine, 0.3 mM ornithine, 0.13 mM proline, 5 mM HEPES, 2.5 ng/ml Na-selenite, 2.5 μg/ml transferrin, 0.2 mM $ZnCl_2$, 0.1 mM $MnSO_4$, 2.75 μg/ml insulin, 50 nM dexamethasone, 40 ng/ml HGF, 20 ng/ml EGF
2001	Mizuguchi et al. (27)	DMEM	10% FBS, 20 mM HEPES, 10 mM nicotinamide, 1 mM ascorbic acid-2-phosphate, 10^{-7} M dexamethasone, 1 mg/ml galactose, 30 μg/ml proline, ITS, 10 ng/ml EGF
2002	Katsura et al. (28)	WEM/KSFM (contain FBS or human serum)	10 mM nicotinamide, 30 mg/l proline, 1 mM ascorbic acid 2-phosphate, 0.5 μg/ml insulin, 10^{-7} M dexamethasone, 10 ng/ml EGF
2003	Iocca et al. (29)	DMEM-F12 based	2% DMSO, 1 μM [+]-alpha-tocopherol, 25 ng/ml EGF
2004	Vanchaecke et al. (30)	DMEM	0.5 U/ml insulin, 7 ng/ml glucagon, 1% antibiotics, 10% FBS
2005	Gardmo et al. (31)	WE+glutamax or Weymouth 752 medium	10 nM insulin

Table 13.2 (continued)

Year	Authors	Medium	Supplements
2005	Siendones et al. (32)	WE	1 μM insulin, 0.6 μM hydrocortisone, 15 mM HEPES, 2 mM glutamine, 26 mM $NaCO_3$
2005	Noreault et al. (33)	WE	10^{-7} M dexamethasone, 10^{-8} insulin, 10^{-7} sodium selenite, 0.3 mM ascorbate
2005	Kimura et al. (34)	Ham's nutrient mixture F-10	1 nM dexamethasone
2005	Abdelmegeed et al. (35)	Modified Chee's medium	2 mM L-glutamine, 6.25 μg/ml transferrin, 0.1 μM dexamethasone, 1 μM insulin
2005	Schmidt et al. (36)	DMEM	20%FBS, 0.1 μM dexamethasone
2007	Li et al. (37)	KSFM	Pituitary gland extract, EGF

DMEM: Dulbecco's modified Eagle's medium; L-15: Leibovitz L-15 medium; MEM: minimum essential medium; WE: William's medium E; KSFM: keratinocyte-stimulating factor medium; ITS: insulin–transferrin–selenium mixture; EGF: epidermal growth factor; HGF: hepatocyte growth factor; BSA: bovine serum albumin; FBS: fetal bovine serum.

hepatocyte mitogens include epidermal growth factor (EGF), hepatocyte growth factor (HGF), and nicotinamide and are capable of preserving the proliferative capacity of in vitro cultured hepatocytes in various chemically defined media (**Table 13.2**). As well as using mitogenic factors to drive hepatocytes to replicate, it is also essential to retain hepatic differentiation properties during the culture period. To achieve this, some laboratories have added trace metal ions (such as copper, iron, zinc, and manganese), the synthetic glucocorticoid dexamethasone (usually in micromolar concentrations), hormones (e.g., insulin), amino acids (e.g., proline), differentiation factors such as dimethyl sulfoxide (DMSO) as well as fetal bovine serum (FBS) into hepatocyte culture systems. In addition, the presence of an extracellular matrix (ECM) may support the survival rate of cultured hepatocytes and improve cell growth. Culture of hepatocytes on supporting materials such as collagen (18), fibroblasts (19), or matrigel/collagen combination (20) showed that proliferative and hepatic functions could be maintained for several days. Intact liver parenchymal cells from adult animals can be obtained and cultured for short periods of time. This technique presents benefits for therapeutic potential in drug discovery and regenerative medicine fields. The protocol for mouse hepatocyte isolation is based on the method developed by Seglen (7) for rat hepatocyte isolation with slight modifications.

2. Materials

2.1. Isolation Equipment

1. Water bath set to 39°C.
2. Peristaltic pump.
3. Tubing containing a bubble trap (a sterile administration "giving" set can be used: non-air vented, filter 200 μm, rotary luer lock; refer Baxter ref: RMC2071B).
4. Cannula (e.g., 18 GA venflon, Becton and Dickinson).
5. Matchsticks.
6. Dissection instruments (one sharp pair of scissors, two pairs of forceps one sharp one blunt, and a clamp).
7. Sutures.
8. Dissection tray.
9. Tape.
10. Syringes.
11. Measuring cylinder.
12. Petri dishes.
13. 50-ml centrifuge tubes.
14. Gauze mesh filter (pore size of 40 μm; Gore-Tex).
15. Hemocytometer and trypan blue.

2.2. Isolation Solutions

1. Buffer A: 1 M HEPES (pH 7.4) and 5% (w/v) KCl, sterile filtered.
2. Perfusion medium I: 500 ml sterile phosphate-buffered saline (PBS), 5 ml buffer A, 2.5 ml 1 M glucose (sterile filtered), 0.5 ml 200 mM EDTA, 1 ml phenol red solution (Sigma). pH adjusted to 7.4 with 2 M NaOH.
3. Perfusion medium II: 500 ml sterile PBS, 5 ml buffer A, 10 ml 1 M HEPES, 2.5 ml 1 M glucose (sterile filtered), 1 ml 500 mM $CaCl_2$, 1 ml phenol red solution (Sigma). pH adjusted to 7.4 with 2 M NaOH.
4. Collagenase solution: 150 ml perfusion medium II collagenase (the source, type, and amount have to be determined by the end user) (*see* **Note 1**).
5. PBS.
6. Ethanol 70% (v/v).
7. Anesthetic as appropriate.

2.3. Cell Culture

1. Culture dishes.
2. Attachment medium: Williams E medium (Sigma), 1% (v/v) penicillin/streptomycin (10,000 U/μg/ml; Sigma),

1% (v/v) 200 mM L-glutamine (Sigma), 1% (v/v) nonessential amino acids (Gibco), and 10% (v/v) heat-inactivated fetal bovine serum (Gibco).

3. Culture media: Williams E medium (Sigma), 1% (v/v) penicillin/streptomycin (10,000 U/μg/ml; Sigma), 0.5% (v/v) gentamycin (10 mg/ml; Gibco), 0.04% (v/v) fungizone (amphotericin B, 250 μg/ml; Gibco), 1% (v/v) 200 mM L-glutamine (Sigma), 1% (v/v) nonessential amino acids (Gibco).

3. Methods

3.1. Preparation of Equipment

1. Pre-warm 500 ml of perfusion media I and II in the water bath.
2. Meanwhile, attach the tubing to the pump and connect to a bottle of PBS. Flush through with 100–200 ml PBS and set the pump to approximately 5–10 ml/min.
3. Ensure the cannula is clear by attaching it to the tubing and pumping a small amount of PBS through it.
4. Change the PBS to the 500 ml bottle of perfusion medium I. Run the medium through the tubing and remove any air bubbles.
5. Add the collagenase to perfusion medium II.

3.2. Hepatocyte Isolation

Anesthetize the mouse. When the mouse is no longer responsive, place the mouse dorsal side down onto a tray and proceed as follows

1. Secure the animal to the tray by taping down the limbs.
2. Check the mouse is fully anesthetized by using the pedal reflex. Pinch the tip of the hind legs with your fingers, there should not be any reflexes. If there are reflexes the mouse needs further exposure to the anesthetic.
3. When the mouse is fully anesthetized spray a small amount of 70% ethanol to wet the fur on the abdomen. This should reduce the possibility of infection. Make a "U"-shaped incision through the skin of the lower abdomen to the lateral aspect of the rib cage and fold back the skin over the chest. Move the intestines to the right to reveal the portal vein and the vena cava.
4. Using hooked forceps, gently push under the vena cava and use this as a guide to place the matchstick under the vena cava. Remove any adipose tissue with a pair of blunt forceps (*see* **Note 2**).

5. Loosely tie a suture around the vena cava prior to inserting the cannula.
6. Insert the cannula into the vena cava. Secure the suture around the cannula. Remove the inner needle of the cannula and allow perfusion medium I to perfuse through the liver. Then, cut the portal vein immediately. The liver should clear of blood within a few seconds.
7. Perfuse with perfusion medium I for approximately 10–15 min.
8. Change perfusion medium I for the collagenase solution and perfuse for approximately 10 min, the solution can be recycled. The liver will swell slightly as the collagenase digests the connective tissue in the liver. When the liver is adequately digested it should leave a small indentation when touched with a cotton bud.
9. Remove the entire liver to a petri dish containing perfusion medium II at room temperature. Cut out the gallbladder and in a tissue culture hood dissociate the liver lobes using two pairs of forceps.
10. Filter the crude hepatocyte preparation through a gauze mesh filter (100 μm in diameter) and transfer the resulting cell suspension into two 50 ml sterile tubes and centrifuge at 50*g* for 2 min. The parenchymal hepatocytes sediment while the non-parenchymal cells and dead parenchymal hepatocytes remain in the supernatant.
11. Discard the supernatant and repeat the wash procedure three to four times by resuspending the pellet each time in fresh perfusion medium II and centrifuging at 50*g* for 2 min.
12. Following the wash procedure the hepatocyte yield should be between 100 and 400 million cells. Resuspend the hepatocytes in 30 ml of attachment medium and check cell numbers and viability using trypan blue and a hemocytometer (*see* **Note 3**).

3.3. Hepatocyte Culture

1. We commonly use a final cell density of 0.4×10^6 cells per ml. The cells are inoculated into 35 mm tissue culture dishes by adding 1.5 ml of hepatocyte suspension per dish.
2. Following inoculation, gently rock the dish to ensure even distribution of hepatocytes and check them under the microscope. Place them in an incubator with 5% CO_2 at 37°C for approximately 6–8 h.
3. After 6–8 h the cells should have attached and formed a monolayer. Remove the attachment medium and replace with 1.5 ml culture medium with any additions depending

on the experiment. Return the cells to the incubator to continue culturing until required.

4. Notes

1. Although some companies sell collagenase specifically for hepatocyte isolation it is worth batch testing and buying in bulk.
2. Although not essential, the matchstick is useful during the initial cannulation of the vena cava. It serves to elevate and hold the vena cava and allows better control during the cannulation.
3. The cells should be inoculated as quickly as possible to prevent hypoxia.

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Chapter 14

Isolation and Culture of Mouse Intestinal Cells

Charles Frederick Campbell

Abstract

Complex cell signal transduction mechanisms regulate intestinal epithelial shape, polarity, motility, organelles, cell membrane components as well as physical and mechanical properties to influence alimentary digestion, absorption, secretion, detoxification and fluid balance. Interactions between the epithelial cells and adjacent mesenchyme are central to intestinal homeostasis although the key regulatory molecules of specific differentiation steps remain unclear. Isolation and primary culture of heterotypic murine intestinal cells provides a model system for elucidation of essential molecular cross-talk between epithelium and mesenchyme that may provide several biological and practical advantages over transformed cell lines. An in vitro primary culture system for neonatal rat or mouse intestinal cells has been established that forms monolayers, expresses intestine-specific epithelial features including intestinal brush borders and appropriate hydrolase enzymes. Our studies confirm the promise of this method which may advance our understanding of heterotypic cellular interactions implicated in intestinal function and may provide important insights into the pathobiology of disease.

Key words: Epithelium, mesenchyme primary culture, enterocyte, feeder layers.

1. Introduction

The polarized epithelium of the human intestine accommodates various differentiated phenotypes, including enterocytes whose apical brush border provides an expanded membrane surface for transport, hydrolysis and secretion, as well as goblet cells, paneth cells and the enteroendocrine lineages. This differentiated state is regulated by heterotypic cell–cell communications, local tissue microenvironment and secreted proteins. Regulatory cues of epithelial function are also provided by the mesenchymal cell compartment, with bidirectional signalling across the basement membrane, whose formation requires close cell–cell

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contacts (1, 2). Indeed, numerous recombination experiments have shown that intestinal cytodifferentiation is dependent upon reciprocal interactions between epithelium and mesenchyme (3–6). For example, in co-culture experiments of endodermal micro-explants seeded on top of confluent monolayers of mesenchymal cells enabled attachment, migration and polarization (7). Key basement membrane proteins including laminins are developmentally regulated and modulate the behaviour of intestinal epithelia (8, 9).

The function and signalling pathways mediated through specific basement membrane molecules at the epithelial–mesenchymal interface are still poorly understood. Recent technological advances may shed light on this area. The use of primary cultures of intestinal cells (10) from wild-type, transgenic or knockout mice and chimeric models, assays of quantitative or qualitative changes in the gene transcription profile by array-based differential gene expression analyses, siRNA interventions and other methods may help elucidate the fundamental mechanisms of gut homeostasis that may be deregulated in intestinal disease. This chapter provides a methodological description for isolation and primary culture of murine intestinal epithelium, culture conditions for selective promotion of epithelial or mesenchymal growth and characterization studies that may be of value in intestinal biology research.

2. Materials

2.1. Animals

1. C57Bl/6 J or Balb/CJ mice (Charles River Laboratories Kent, UK).
2. Wistar rats or AO rats (Charles River Laboratories Kent, UK).

2.2. Materials for Tissue Retrieval and Cell Isolation

1. Hank's buffered salt solution (Northumbria Biologicals, Ltd) with 2% glucose (Sigma) plus penicillin 60 μg/ml (Glaxo, Greenford, England, UK) and streptomycin 60 μg/ml (Sigma) (HBSS–glucose).
2. Dissecting scissors and sharp scalpel blades.
3. Digestive enzymes: dispase type 1, 0.1 mg/ml (Boehringer Mannheim, Lewes, England, UK) and crude collagenase, 300 U/ml (type XI; Sigma, Poole, England, UK).
4. DMEM plus 5% fetal calf serum and 2% sorbitol (DMEM–sorbitol).
5. Plasticware including pipettes, rubber pipette bulb, T25 ml flasks, 25 ml universal containers, cell scrapers.

6. Inverted light microscope (Carl Zeiss).
7. Sorvall low-speed refrigerated centrifuge.
8. Halothane (BDH, Poole, England, UK).

2.3. Materials for Primary Culture

1. Multiwell plates (24 well; Costar, High Wycombe, England, UK).
2. Dulbecco's modified Eagle's medium (DMEM; single strength stock, Gibco, Uxbridge, England, UK) with 1–10% fetal calf serum (FCS; Gibco, Paisley, Scotland, UK), sodium pyruvate 0.11 g/l (Sigma, Poole, England, UK), glutamine 0.58 g/l (Gibco), insulin 0.25 IU/ml (CP Pharmaceuticals, Wrexham, England, UK), penicillin 100 IU/ml (Crystapen; Glaxo, Greenford, England, UK), streptomycin 60 μg/ml (Sigma), insulin 0.25 IU (Sigma) and epidermal growth factor 20 ng/ml (EGF, Sigma).
3. Porcine mucosal heparin 50 μg/ml (Sigma H8514).
4. Types I and III bovine dermal collagens (Vitrogen Imperial Laboratories, Andover, England, UK).
5. Multiwell (24-well) plates (Costar, High Wycombe, England, UK) coated with 300 μg/well of bovine dermal collagens 200 μg/ml (Vitrogen Imperial Laboratories, Andover, England, UK) and air-dried for 24 h prior to commencing primary culture experiments (10).

2.4. Assessment of Cell Growth and Differentiation

1. Crystal violet 0.1% (Sigma) pH 2.5.
2. 5% glutaraldehyde in HBSS (Sigma).
3. 75% ethanol (Sigma).
4. 0.1% 5 N HCl solution (Sigma) pH 2.5.
5. 10% acetic acid (Sigma).
6. 96-well microtitre plate (Costar, High Wycombe, England, UK).
7. Automated TitreTek Multiscan (MCC/340) plate reader.
8. Phase contrast microscope (Carl Zeiss).
9. Vector Red substrate kit (Vector laboratories, Peterborough, England, UK) in Tris–HCl buffer at pH 8.2.
10. Glass coverslips, 1 cm diameter (BDH, Poole, England, UK).
11. Methanol:acetate solution (50:50 v/v) (Sigma).
12. Phosphate-buffered saline (Sigma).
13. 10% goat non-immune serum (Sigma).
14. Monoclonal antibodies against specific epithelial and non-epithelial markers, e.g. anticytokeratins 8, 18 and 19 (gifts from Professor Birgit Lane, Department of Anatomy,

University of Dundee); smooth muscle α-actin (clone 1A4; Sigma Poole).

15. 0.5% bovine serum albumin (BSA, Sigma).
16. 0.2 mg/ml diaminobenzidine HCl (DAB) (Sigma).
17. 0.8 mg/ml nickel chloride (Sigma).
18. 0.003% hydrogen peroxide (BDH, Poole, England, UK).
19. Xylene (Sigma).
20. Xylene mountant XAM (BDH).
21. 0.2 M cacodylate-buffered 2% glutaraldehyde (pH 7.4).
22. Osmium tetroxide (Electron Microscopy Sciences, Ltd, Hatfield, PA 19440).
23. Epoxy resin (Electron Microscopy Sciences, Ltd, Hatfield, PA 19440).
24. Uranyl acetate and lead citrate (Electron Microscopy Sciences, Ltd, Hatfield, PA 19440) (11).

3. Methods

3.1. Tissue Harvest and Cell Isolation

1. For adult animals, kill animals by cervical dislocation (*see* **Note 1**).
2. Make a midline incision and remove intestines from the duodenum to the ileum.
3. Clean the intestines free of mesentery and suspend in ice-cold HBSS–glucose (4°C) (12).
4. Fetal small intestine is typically harvested at day 14 from pregnant dams, under terminal Halothane anaesthesia. After cervical dislocation, fetal small intestines are retrieved through a midline laparotomy, using an operating microscope (*see* **Note 2**).
5. Entire small intestines are retrieved from each fetal donor, from the duodenum to the ileum.
6. Briefly wash intestines in HBSS–glucose.

3.2. Intestinal Cell Isolation Procedures

3.2.1. Postnatal Intestine

Kinetic models of cell renewal in the small intestine identify stem, transit amplifying and terminally differentiated cell populations that differ in their cycle time and pluripotency (13) (*see* **Note 3**). In the current application, the aim is to isolate the proliferative progenitor epithelial population from the depths of intestinal crypts. To achieve this objective, an enzymatic isolation procedure

is used with a combination of collagenase and dispase digestive enzymes for both fetal and neonatal intestines (10, 12, 14).

1. Small intestines are opened along the length of the antimesenteric borders, cut into 3–5 mm lengths and washed in HBSS–Glucose. This step is repeated five times with fresh changes of HBSS–Glucose for each wash.
2. The small intestinal fragments are then diced using a sharp scalpel blade and resuspended in 20 ml HBSS–glucose–dispase–collagenase solution.
3. The diced intestines are placed into T25 flasks with the HBSS–glucose–dispase–collagenase solution and agitated on a shaking platform for 25 min at 25°C.4. The digested tissue is further disaggregated by hand pipetting for 3 min.
5. Samples are inspected under an inverted microscope to ensure separation of the various tissue components. At this stage the tissue digest consists of muscle fragments, multicellular epithelial aggregates, single cells and debris. When >70% of epithelial crypt/villus units are separated from the intestinal fragments, the enzymes may be neutralized by addition of 50 ml DMEM–sorbitol.

3.2.2. Purification of Epithelial Aggregates

On completion of the enzymatic digestion, a purification step is carried out to enrich the concentration of multicellular epithelial aggregates from crypt bases, which contain the intestinal epithelial stem cell fraction.

1. Separate unwanted muscle fragments under gravity, by sedimentation for 1 min in the T25 flasks.
2. Remove four 10 ml aliquots of the supernatant and place in four universal containers (25 ml) (*see* **Note 4**).
3. Single cells and contaminants are removed from the supernatant by a series of slow speed centrifugation steps at 300 rpm, for 3 min.
4. After each centrifugation, the supernatant containing single cells, debris and contaminants is removed by aspiration, leaving a "pellet" of cells at the bases of centrifugation flasks.
5. Resuspend the cell pellets in 20 ml DMEM–sorbitol and repeat the slow speed centrifugation step five times. These steps enrich for epithelial cell aggregates (**Fig. 14.1**) (*see* **Note 5**).

3.3. Isolation of Fetal Intestine

Procedures for isolation of intestinal epithelium from 14- to 20-day fetal mouse or rat intestine is similar in principle to that outlined for postnatal intestine.

1. Fetal small intestines are harvested after hysterectomy, opened along their length, cut into 3 mm segments and washed three times for 2 min in fresh HBSS–glucose solution.

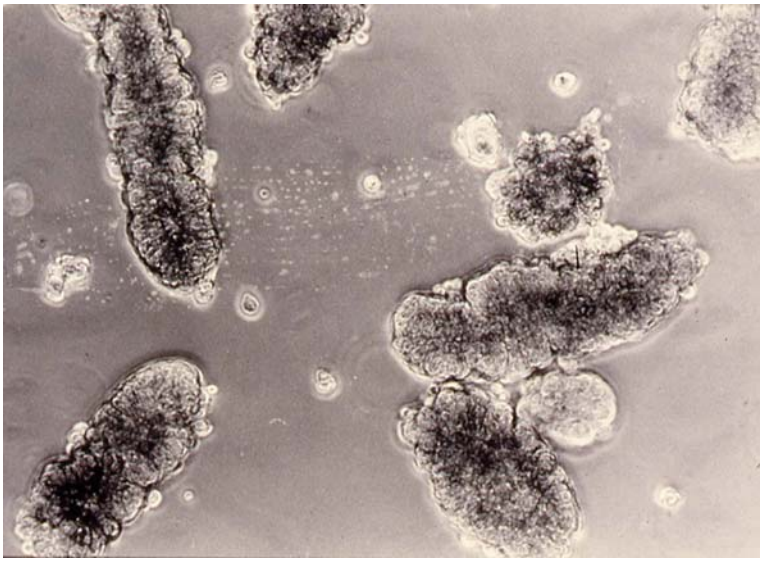

Fig. 14.1. Intestinal crypt cell aggregates isolated from neonatal rat intestine.

2. The diced fragments are incubated in 20 ml HBSS–glucose–dispase–collagenase solution, for 15 min at 25°C.
3. Hand-pipette the suspension for 2 min.
4. Neutralize the enzymes by addition of 50 ml DMEM–sorbitol.
5. Five sequential slow speed (300 rpm for 3 min each) centrifugation steps are carried out in fresh DMEM–sorbitol.
6. Resuspend the final cell pellets in growth medium ready for plating.

3.4. Primary Culture of Intestinal Cells

All small intestinal primary cultures are maintained at 37°C in a humidified atmosphere with 7.5% CO_2, in a mammalian cell incubator (*see* **Note 6**).

1. Small intestinal epithelial cell aggregates are seeded into 24-well plates. Epithelial cells at the periphery of cell aggregates attach and spread over the surface (**Fig. 14.2**). After 2–3 days, small cobblestone colonies appear which enlarge and ultimately coalesce. Attachment and growth may be enhanced by pre-coating plates with types I and III bovine dermal collagens (10) (*see* **Note 7**).

3.5. Assessment of Cell Growth in Intestinal Cell Cultures

Cell growth may be assessed by a number of methods. We routinely use a low pH crystal violet dye-binding assay which provides a linear relationship between epithelial cell number and dye concentration (15).

1. Remove the growth media and wash the cells in HBSS, then fix in 5% glutaraldehyde in HBSS for 30 min.

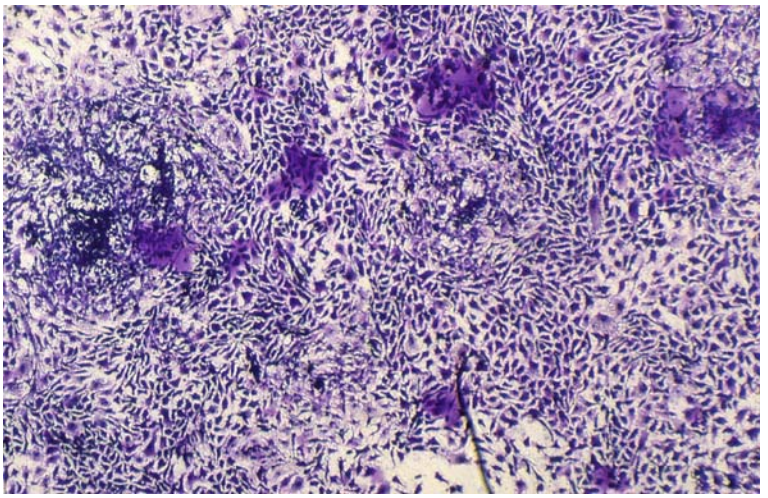

Fig. 14.2. Cell aggregates attach in culture and grow to form monolayers.

2. Add 75% ethanol (4°C) for 15 min and then allow to air-dry.
3. To stain cells with crystal violet solution add 300 µl/well for 20 min at room temperature.
4. Remove excess dye by washing in 0.1% 5 N HCl solution and then air-dry.
5. Solubilize the dye by adding 400 µl per well of 10% acetic acid in distilled water for 30 min at room temperature.
6. Transfer the contents of each well to a 96-well microtitre plate.
7. Measure the optical density at 595 nm using an automated plate reader (TitreTek Multiscan MCC/340 or similar).

3.6. Cell Characterization Studies

3.6.1. Morphological Characterization

1. Monolayer cultures may be fixed and counterstained with crystal violet and viewed by phase contrast microscopy, for rapid assessment of morphology.
2. For histochemical detection of the brush border enzyme alkaline phosphatase, the Vector Red substrate kit may be used. Primary cultures are fixed in 70% ethanol, the substrate applied according to manufacturer's instructions and the product visualized by normal and fluorescent microscopy.

3.6.2. Immunohistochemistry

Cultures are best prepared for immunohistochemistry by growing on sterile collagen-coated glass coverslips.

1. Fix cultures in 50:50 methanol:acetate for 10 min at 4°C.
2. Wash twice in PBS.

3. Incubate the cultures in 10% goat non-immune serum in PBS (*see* **Note 8**).
4. Apply primary antibodies, e.g. against cytokeratins, at appropriate dilutions of 0.5% bovine serum albumin in PBS for 2 h at 25°C.
5. Wash the excess antibody from the cultures using PBS.
6. Incubate cultures in secondary antibody, e.g. goat anti-peroxidase, at 1/75 dilution in PBS and 0.5% BSA.
7. Wash cultures in PBS and incubate with the chromogenic substrate until the staining develops.
8. Wash the cultures with PBS and then dehydrate in graded alcohols, clear in xylene and then mount with the xylene mountant, XAM.
9. Non-epithelial cells may be identified by immunohistochemistry using a monoclonal antibody to smooth muscle α-actin at 1/250 dilution.

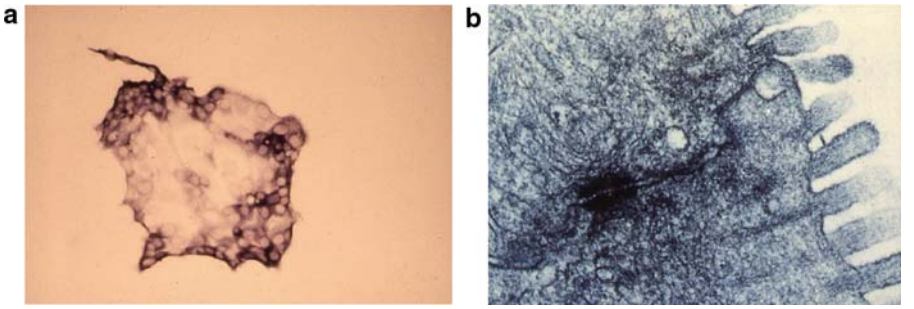

Fig. 14.3. Intestinal cells in culture show electron microscopy features consistent with an epithelial origin including cytokeratin expression (**a**) and intestinal brush borders (**b**).

3.6.3. Electron Microscopy

1. Fix specimens for electron microscopy in 0.2 M cacodylate-buffered 2% glutaraldehyde (pH 7.4) at 4°C.
2. Postfix samples in osmium tetroxide, dehydrate and embedd in Epoxy resin.
3. Prepare ultrathin sections and stain with uranyl acetate and lead citrate for ultrastructure studies (**Fig. 14.3b**).

4. Notes

1. Experimental studies may be carried out using common strains of rats or mice. Animals should be maintained as breeding colonies for production of neonates and fetuses, housed under a 12 h light/dark circle and given food and

water ad libitum. Fetal (14–20 days) or suckling (6 day postnatal) rat or mouse intestine may be used. Experiments with animals should be conducted in accordance with local ethical guidelines and laws.

2. Primary cultures from fetal intestine are rather more robust and easier to establish. Fetal gestational age is calculated according to observation of a vaginal mucus plug, designated day zero and recognizing a mean gestation period of 21–22 days.
3. On average, four neonatal or fetal intestines provided sufficient donor material for each in vitro culture experiment.
4. At this stage, the supernatant should contain multicellular epithelial aggregates, as well as single cells and some debris and contaminants. It is important to remove the debris and contaminants.
5. Pellets may be pooled from different animal strains for chimeric experiments, if required (16).
6. Porcine mucosal heparin [50 μg/ml; Sigma (H8514)] can be added to all cultures to stimulate epithelial proliferation and to inhibit mesenchymal growth (10, 17).
7. Inclusion of EGF and insulin in growth media helps promote proliferation of intestinal cells in primary culture. Addition of sulphated polysaccharides such as heparin (in doses up to 50 μg/ml) to growth medium containing 2.5% serum, with insulin and EGF, may preferentially enhance epithelial proliferation while inhibiting proliferation of smooth muscle-like cells (17).
8. Immunostaining may use a series of monoclonal antibodies against specific epithelial and non-epithelial markers to identify specific epithelial cell populations in vitro.

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Chapter 15

Derivation of Primary Choroid Plexus Epithelial Cells from the Mouse

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Abstract

Choroid plexus epithelial cells form an integral and important part of the barrier between blood and cerebrospinal fluid. Culture of choroid plexus epithelium in vitro has been achieved from several mammalian species and this provides opportunities for the study of choroid plexus development and function, including the capacity of the epithelial cells to control the movement of bioactive molecules, such as novel drug candidates, from the bloodstream to the brain. Here we describe a method for the derivation of primary cell cultures from mouse choroid plexus epithelium, together with characterisation by immunofluorescence using antibodies specific to markers of mature choroid plexus epithelial cells. With this method, relatively pure choroid plexus epithelial cell monolayers are established using the DNA synthesis inhibitor cytosine arabinoside (Ara-C), which is cytotoxic to contaminating cell types such as fibroblasts, but not the epithelial cells. These cells are shown to express the diagnostic choroidal marker, transthyretin (TTR), as well as markers of epithelial cell differentiation and are thus suitable for studies that address the transport and barrier functions of the choroid plexus.

Key words: Choroid plexus, mouse, primary culture, epithelium, transthyretin, barrier.

1. Introduction

The choroid plexus is a highly folded vascular structure that projects into each of the ventricles of the brain and forms an interface that connects the two major physiological circulatory systems: the peripheral blood circulation and the cerebrospinal fluid (CSF) bulk flow of the central nervous system (CNS). There are four choroid plexuses in the brain, one in each of the two lateral

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ventricles, another in the third ventricle and a morphologically distinct structure in the fourth ventricle (1). The functions of the choroid plexus can be broadly subdivided as follows: (i) it is the major site of synthesis and secretion of CSF although a small percentage (10–20%) is secreted by the capillary endothelium of the brain; (ii) the regulation of blood–CSF exchange and homeostasis, the blood–CSF barrier (2).

The choroid plexus is formed by the apposition of two tissue layers, a convoluted outer secretory epithelium and an inner stromal core containing an extensive network of fenestrated capillaries which are intimately associated with the basal (blood-facing) surface of the epithelium (1, 2). The choroid plexus epithelium is continuous with the ependyma, a cuboidal epithelium that lines the lumen of the cerebral ventricles; however, despite a common embryological origin (3), these two cell types are quite different. Choroid plexus epithelial cells possess unique phenotypic properties, setting them apart from ependymal cells. Some of these distinguishing characteristics, together with their functional significance, will be briefly described here. First, choroid plexus epithelial cells are drawn tightly together by a dense band of impermeable tight junctions near their apical (CSF-facing) surface. These tight junctions restrict the passive diffusion of molecules between the cells (4) and, together with the underlying fenestrated capillary endothelium, form the structural basis of the blood–CSF barrier. Second, the differentiation of choroid plexus epithelial cells from their neuroepithelial precursors during embryogenesis is accompanied by the expression of several "liver-type" enzymes and secreted proteins, of which the most significant is the thyroid hormone transport protein, transthyretin (TTR) (5, 6). Expression studies indicate that TTR mRNA accumulates to extremely high levels in the mature choroid plexus epithelium (~20- to 50-fold more abundant than in liver), yet it is not at all expressed in related ependymal cells or elsewhere in the CNS and has proven a highly sensitive diagnostic marker for this tissue (6). Thirdly, choroid plexus epithelial cells express specific receptors which act to transduce key humoral signals between the blood and the CNS, including growth hormone, insulin, insulin-like growth factors 1 and 2, prolactin, vasopressin and leptin (7). As such, the choroid plexus indirectly influences the neuroendocrine regulation of diverse physiological functions such as appetite, metabolism and fluid homeostasis. Finally, choroid plexus epithelial cells also express a vast array of specific transporters and ion channels upon their apical surface. The polarised distribution of these transport systems is critical for the directional movement of complex bioactive molecules such as peptides, organic anions and solutes across the blood–CSF barrier and for clearance of waste substances and toxins out of the CNS (2).

The transport and barrier properties of the choroid plexus epithelium, besides participating in the control of brain homeostasis and endocrine–humoral signalling, have received increasing recognition as the main factors establishing the cerebral availability of drugs. Large numbers of novel drug compounds with the desired attributes of high specificity and bioactivity/unit mass are continuously generated by the pharmaceutical industry, yet to achieve therapeutic benefit in the CNS these molecules must also be able to cross the blood–CSF barrier. Drugs that are effectively transported by the choroid plexus are rapidly distributed throughout the CNS by the bulk flow of ventricular CSF. On the other hand, many of these compounds are either not taken up by the choroid plexus or they are actively removed from CSF by "liver-like" drug metabolising enzymes or transporters of the multidrug resistance (MDR) class, both of which occur at high levels in choroid plexus epithelial cells (8, 9). For these reasons, targeting meningeal tissues with antibiotics or antiretroviral drugs that enter the CNS via the choroid plexus epithelium remains a challenge in the treatment of infectious diseases like meningitis or HIV/AIDS, respectively (8, 10). In summary, while it is clear that the choroid plexus epithelium plays a pivotal role in the regulation of brain development and homeostasis, further study will also lead to a better understanding of the role played by blood–CSF transport mechanisms in drug uptake, dispersal and metabolism.

A range of invasive techniques has been employed to explore the transport functions of the choroid plexus; however, the fragility of choroid plexus tissue, its small mass and its inaccessible location renders it one of the most difficult to isolate in vivo. The utility of other approaches including the use of ex vivo incubated whole organ systems (11) or in situ vascular perfusion (11, 12) has been tested with some success. Nevertheless, the widespread uptake of these techniques has been limited by their inherent transiency (once isolated only the choroid plexus remains viable for a few hours), technical complexity and ethical constraints prohibiting the use of large numbers of experimental animals.

In vitro models overcome many of the limitations discussed above and provide a simple and cost-effective alternative to the use of live animals. Primary choroid plexus epithelial cell cultures have been successfully derived from a range of mammalian species including the cow, pig, sheep, rat mouse and rabbit (13–18). These systems retain the differentiated properties of the choroid plexus epithelium, including transport and barrier functions, and have proved invaluable to investigators seeking to model the uptake and clearance of organic substrates, peptides and novel drugs across the blood–CSF barrier (8, 17, 18). Although significant progress has been made, the development of these systems has not been without its challenges. Inexperienced users find dissection of the choroid plexus rather demanding since it presents

as a small membranous tissue and, without prior knowledge of brain anatomy, is quite difficult to locate. Like other primary cell types, acutely isolated choroid plexus epithelial cells exhibit limited proliferative potential and their viability decreases rapidly after dissection. Prompt transfer to the in vitro environment is therefore critical for the establishment of viable cultures. Finally, co-isolation of contaminating cells (e.g. fibroblasts), released from the stromal mesenchyme during enzymatic digestion, cannot be avoided and is problematic particularly since fibroblasts proliferate rapidly and may even overrun the slower growing epithelial cells in some cultures.

Here we describe a protocol for the isolation and culture of primary choroid plexus epithelial cells from the mouse. The protocol contains detailed instructions and diagrams enabling the user to correctly locate and dissect the choroid plexus, to recover the epithelial cells and to derive primary cultures without any prior knowledge. Cells prepared with this method are plated directly onto tissue culture plastic and are viable for several weeks with correct maintenance. To inhibit fibroblast proliferation we have used the nucleoside-arabinose analogue, cytosine arabinofuranoside (Ara-C), a potent DNA synthesis inhibitor. Nucleoside transport systems on choroid plexus epithelial cells do not recognise arabinose residues as substrates whereas fibroblasts (and many other cell types) cannot distinguish between nucleosides with ribose or arabinose residues and thus incorporate Ara-C into DNA (18). Since Ara-C does not affect the growth or differentiation of choroid plexus epithelial cells, these cultures form confluent monolayers that are largely free of fibroblast contamination and are thus suitable for studies that address the barrier function of this tissue. We also describe a confocal immunofluorescence protocol to examine the expression of choroid plexus differentiation markers in situ. Using this protocol we demonstrate synthesis of the diagnostic choroidal marker TTR (5, 6) and also confirm the expression of the classical epithelial cell markers E-cadherin and cytokeratins in the cultured epithelial cells.

2. Materials

2.1. Isolation and Primary Culture of Choroid Plexus Epithelial Cells

1. Dulbecco's modified Eagle's medium (DMEM) nutrient mix F-12 supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, 50 μg/ml streptomycin, 2.5 μg/ml amphotericin (Gibco/BRL) stored at 4°C.
2. Hank's buffered salt solution (HBSS) (Sigma).

3. Phosphate buffered saline (PBS) made by dissolving PBS tablets (Sigma) in water (*see* **Note 1**) then sterilised by autoclaving and stored at room temperature.
4. Pronase enzyme (nuclease-free, isolated from *Streptomyces griseus*; Calbiochem) is dissolved at 2 mg/ml in HBSS and sterilised by passage through a 0.22 μm filter unit (Millipore). Prepare fresh immediately prior to the dissection (*see* **Note 2**).
5. Cytosine β-D-arabinofuranoside (Ara-C) (Sigma) is dissolved in water at 20 mM (1000× stock), sterilised by passage through a 0.22 μm filter unit, stored in aliquots at –20°C. Ara-C is added to complete growth medium at a final concentration of 20 μM.
6. Trypan blue stain is prepared by dissolving trypan blue (Sigma) at 0.4% (w/v) in PBS. Store at 4°C.
7. Dissection instruments: four pairs of fine straight-point watchmaker forceps (#5) (*see* **Note 3**), standard dissection scissors, fine bowspring scissors and a small curved-head spatula (*see* **Note 4**) to be used as a "brain scoop".
8. Slide haemocytometer (BDH) and microscope coverslips (22 × 22 × 0.15) (Fisher).
9. Mice: 15–20 neonatal (1–3 days post-partum) pups (*see* **Note 5**).

2.2. Confocal Immunofluorescence for Differentiation Markers

1. 3-Aminopropyltriethoxysilane (APTES)-treated glass microscope coverslips (22 × 22 × 0.15 mm): prepare by washing coverslips in hot soapy water (*see* **Note 6**), rinse thoroughly in distilled water, rinse once in acetone for 5 min, coat by immersing in 2% APTES (Sigma) in acetone for 5 min, rinse twice in acetone for 5 min each and rinse thoroughly in distilled water. The coverslips are dried at 37°C then foil wrapped and sterilised by baking at 180°C.
2. PBS: prepared by dissolving PBS tablets (Sigma) in water, sterilised by autoclaving and stored at room temperature.
3. Paraformaldehyde fixative (Sigma): prepare a 4% (w/v) solution in PBS fresh for each experiment. The solution will need to be carefully heated in a fume hood to fully dissolve (*see* **Note 7**).
4. Acetone/methanol (1:1 v/v) fixative is prepared by mixing equal volumes of acetone and methanol (Fisher) fresh for each experiment and chilled to –20°C before use.
5. Citric acid buffer is prepared fresh for each experiment by making a 10 mM citric acid solution in water.
6. Permeabilisation buffer: 0.1% (w/v) Triton X-100 (Sigma) in PBS.

7. Maleic acid buffer: 100 mM maleic acid, 150 mM NaCl in water and adjusted to pH 7.5 with NaOH.
8. Blocking reagent (Roche): prepare a 10% (w/v) stock solution in maleic acid buffer. The solution will need to be heated (microwave oven or heated block) to dissolve. Sterilise by autoclaving and store at –20°C.
9. Primary antibodies: rabbit polyclonal anti-human transthyretin (DAKO); mouse monoclonal anti-mouse E-cadherin (Signal Transduction Laboratories); mouse polyclonal anti-mouse pan-cytokeratin (Santa Cruz Biotechnologies).
10. Secondary antibodies: goat polyclonal anti-rabbit IgG-fluorescein-isothiocyanate (FITC) conjugate; goat polyclonal anti-mouse IgG-FITC conjugate (Vector Laboratories).
11. Gelvatol mounting medium: prepared by dissolving 20 g of polyvinyl alcohol in 80 ml of 10 mM Tris (pH 8.6) and 3 g of *n*-propylgallate in 50 ml of glycerol (all Sigma) followed by mixing and centrifugation at 7,000*g* to remove any undissolved particles. Store at 4°C (*see* **Note 8**).

3. Methods

3.1. Isolation and Culture of Choroid Plexus Epithelial Cells

1. Prior to commencing the dissection, in **Section 3.1** of step 5, a heated block should be set at 37°C (*see* **Note 9**) and an illuminated dissecting microscope are set up in a sterile workspace, e.g. a laminar flow hood to exclude airborne contaminants. Ensure that all surfaces have been thoroughly swabbed with disinfectant and all dissection instruments washed, sterilised and kept under ethanol until required.
2. Approximately 15–20 neonatal mice aged 1–3 days postpartum (*see* **Note 10**) are killed by decapitation with scissors (*see* **Note 11**), the heads placed in a sterile Petri dish and transferred to the laminar flow hood (*see* **Note 12**).
3. Dissection of the brain and the removal of the constituent choroid plexus as described in **Section 3.1** of step 10 are summarised in **Fig. 15.1**. To remove the brain intact (*see* **Note 13**) from each animal, cut longitudinally through the skin covering the skull from the neck to the snout using fine dissection scissors. Using forceps the skin folds are then peeled aside to expose the dorsal surface of the skull. Using forceps to steady the head, insert the tips of the bowspring

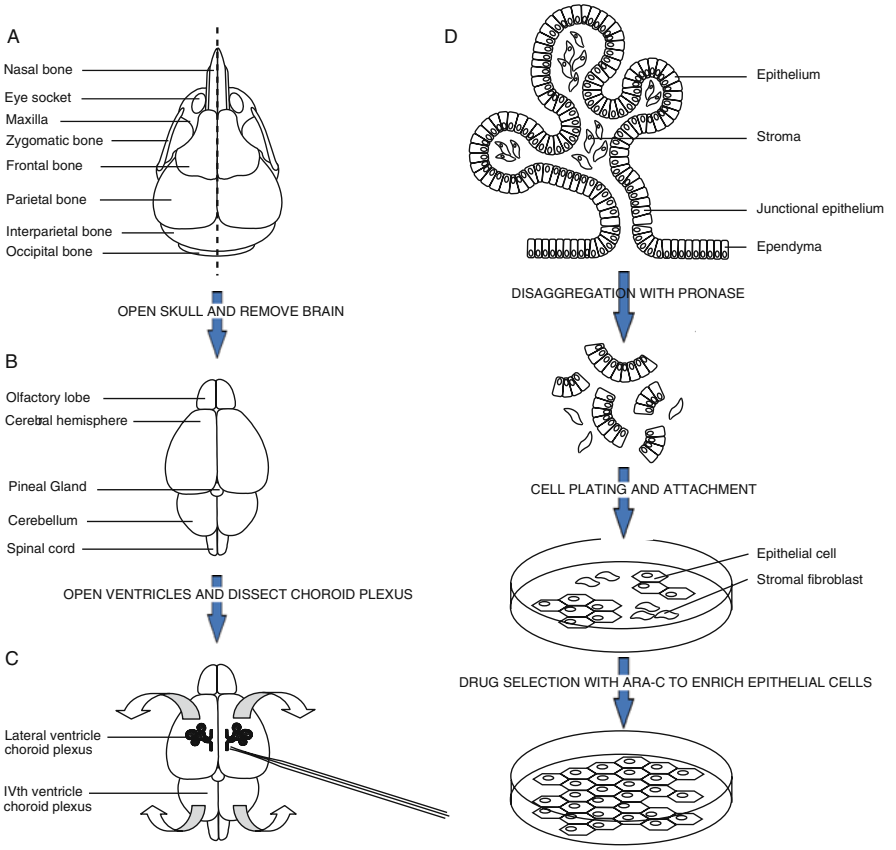

Fig. 15.1. Mouse choroid plexus dissection method. (**a**) Dorsal view of the mouse skull with the relevant bone plates annotated to the *left*. The skull is opened by cutting from the occipital bone plate at the posterior aspect of the skull along the natural sagittal sutures (indicated by a *broken line*) to the nasal bone, the resulting skull halves then peeled back to expose the brain. (**b**) Dorsal view of the exposed mouse brain with relevant subregions annotated to the *left*. (**c**) Each of the two cerebral hemispheres is peeled outwards from its medial aspect (*upper pair* of *curved arrows*) to expose the interior of the lateral ventricle and its choroid plexus. The fourth ventricle and its choroid plexus are located underneath the cerebellum and are exposed by peeling back the cerebellar hemispheres (*lower pair* of *curved arrows*). (**a** and **b**) The skull and brain are orientated with anterior to the *top*. (**d**) Isolated choroid plexus tissue is disaggregated and cultured as shown.

scissors into the opening of the foramen magnum located in the occipital bone and moving in a posterior to anterior direction, cut through the parietal and frontal bones to the nasal bone (*see* **Note 14**) across their dorsal aspect using the natural sagittal sutures (**Fig. 15.1a**). With a pair of forceps in each hand gently ease the cut halves of the skull apart to expose the brain (**Fig. 15.1b**).

4. Insert a small curved-head spatula underneath the brain and gently lift from the skull (*see* **Note 15**) and transfer immediately to a covered Petri dish containing pre-warmed complete growth medium and hold fully immersed therein until required.

5. For the choroid plexus dissection transfer one brain at a time to a fresh reservoir of pre-warmed complete growth medium on a dissecting microscope stage. A choroid plexus is located within the lateral ventricle of each of the two cerebral hemispheres. Using the watchmaker forceps open up each of the lateral ventricles in turn by peeling back the parenchymal tissue in a medial to lateral direction to expose the choroid plexus. The fourth ventricle choroid plexus is located almost directly underneath the cerebellum and immediately posterior to the pineal gland (**Fig. 15.1c**). Systematically dissect out the choroid plexus from both lateral ventricles and the fourth ventricle (*see* **Note 16**) and transfer to pre-warmed HBSS.
6. Collect the dissected choroid plexus tissue by centrifugation at 1,000*g* for 2 min. Aspirate the HBSS, taking care to not disturb the pelleted material, add 2 ml pre-warmed pronase/HBSS solution and incubate at 37°C for 5 min (*see* **Note 17**).
7. Add 10 ml of complete growth medium to stop the digestion (*see* **Note 18**) and collect the digested tissue by centrifuging at 1,000*g* for 2 min. Aspirate the supernatant taking extreme care to not disturb the pelleted tissue and then using a 1 ml pipette, resuspend in 1 ml of complete growth medium by gentle repeated pipetting up and down (15–20 times) to generate a suspension of single cells and cell aggregates. The tissue should visibly dissociate to form a cell suspension almost immediately (*see* **Note 19**).
8. Next perform a viable cell count. Transfer a 20 μl aliquot of the cells to a sterile microtube (*see* **Note 20**), add 20 μl of 0.4% trypan blue stain, mix by gentle flicking and incubate for 5 min at room temperature. Fill both chambers of a haemocytometer with the stained cell preparation, count the viable cell number in each chamber and take the arithmetic mean of both values. Typically 5×10^6–1×10^7 cells are recovered from 15–20 pups.
9. Add 11 ml complete growth medium to the cell suspension, mix by pipetting up and down twice with a 10 ml serological pipette and seed into individual 35 mm plates at 1×10^6 cells per plate (5×10^6 cells are generally sufficient for a single 75 cm^2 flask), then incubate overnight at 37°C, 5% CO_2/air to allow for cell attachment.
10. The medium containing unattached cells and debris is aspirated and the cells gently washed twice with pre-warmed PBS. Fresh complete growth medium supplemented with 20 μM Ara-C (*see* **Note 21**) is added to the cultures to suppress fibroblast proliferation. The medium is changed

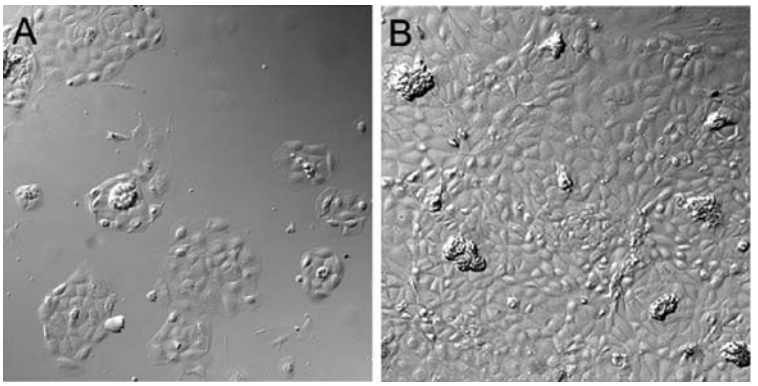

Fig. 15.2. Morphology of choroid plexus epithelial cells in culture. Primary choroid plexus cultures were examined by light microscopy. Epithelial-like colonies with characteristic closely opposed cobblestone morphology are typically observed at 48 h after plating (**a**). These colonies expand to cover large areas after 1 week in culture (**b**). Magnification ×200.

every 48 h thereafter until the cells become fully confluent (7–10 days). Examples of the epithelial cells after 24 h and 7 days in culture, respectively, are shown in **Fig. 15.2**.

3.2. Indirect Immunofluorescence for Choroid Plexus-Specific Markers

1. Choroid plexus epithelial cells are prepared essentially as described in **Section 3.1**, except that the cultures are grown (in 25 mm plates) on APTES-treated glass coverslips.
2. The cells are washed briefly in PBS (*see* **Note 22**) and then fixed either in 4% paraformaldehyde solution for 15 min at 4°C (transthyretin staining) or in acetone/methanol (1:1 v/v) at –20°C (E-cadherin and pan-cytokeratin staining).
3. The fixatives are safely discarded (into appropriate hazardous waste containers) and the cells washed three times for 5 min each with PBS.
4. The cells are permeabilised by incubating in 0.1% Triton X-100 in PBS for 30 min at room temperature and then washed three times more for 5 min each with PBS.
5. When staining for E-cadherin or pan-cytokeratin, an antigen retrieval step is included at this stage of the procedure by incubating the cells in 10 mM citric acid for 1 h at 60°C and then washed three times in PBS for 5 min at room temperature.
6. The cells are pre-blocked by incubation in 2% blocking buffer (prepared by dilution of the 10% (w/v) blocking reagent stock in PBS) for 1 h at room temperature.

7. The coverslip is removed from the blocking buffer using forceps (any excess buffer is drained away *see* **Note 23**) by blotting the edge of the coverslip against paper towel then placed with the cells facing up on a solid support (we use upturned flat plastic lids).
8. Antibodies are applied to the coverslips: anti-transthyretin (1:100), anti-E-cadherin (1:100) or anti-pan-cytokeratin (1:200) in 200 μl of 2% blocking buffer (*see* **Note 24**) and incubated overnight at 4°C (*see* **Note 25**).
9. The primary antibodies are decanted and the coverslips washed three times for 15 min each in large volumes of PBS with a magnetic stir bar activated.
10. Secondary antibodies are applied to the cells: goat polyclonal anti-rabbit IgG-FITC or goat polyclonal anti-mouse IgG-FITC (1:200) in 200 μl 2% blocking buffer and incubated in the dark (*see* **Note 26**) for 3 h at room temperature.
11. The secondary antibodies are decanted and the coverslips washed essentially as described in **Section 3.2** of step 9 but kept under aluminium foil with the lights dimmed.
12. Using forceps the stained cells are mounted by inverting and carefully lowering the coverslips into a drop of gelvatol on a glass microscope slide (*see* **Note 27**) then left on a flat surface to dry (and kept in the dark until required).
13. Cells are initially viewed by phase-contrast microscopy (to identify the correct focal plane) and then by confocal microscopy under green (FITC) fluorescence to visualise cellular staining distributions. Computational support is used to merge the phase-contrast and fluorescence images. Typical subcellular distributions for transthyretin, E-cadherin and pan-cytokeratin staining in primary choroid plexus epithelial cells are shown in **Fig. 15.3**.

4. Notes

1. Unless stated otherwise, all reagents and solutions are prepared in Millipore grade water with an electrical resistance of 18.2 MΩ/cm. This is referred to as "water" in this text.
2. Pronase may "self-digest" once in solution thus compromising its activity. The pronase/HBSS solution should therefore be prepared not more than an hour in advance of the dissection and only pre-warmed to 37°C a few minutes before use.

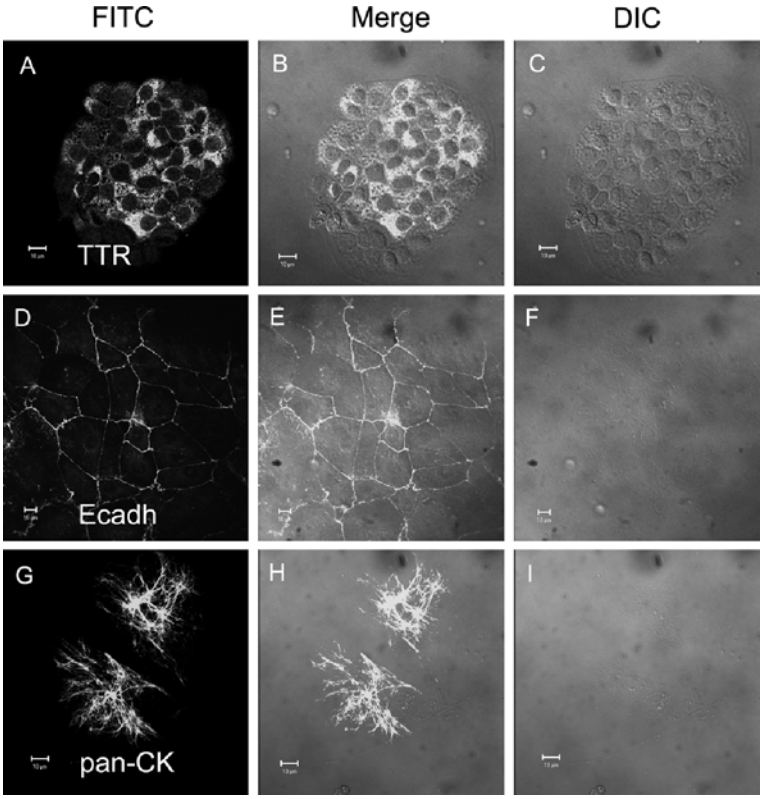

Fig. 15.3. Expression of choroid plexus-specific proteins by confocal immunofluorescence. Shown are examples of choroid plexus epithelial cell cultures following fixation and staining for TTR (**a**–**c**), E-cadherin (**d**–**f**) and pan-cytokeratin (**g**–**i**). Images under green fluorescence (FITC) and brightfield (DIC) conditions were collected by laser scanning confocal microscopy and merged (Merge). These proteins were not detected within the fibroblastic component of the cultures (not shown). Magnifications ×740. Abbreviations: TTR, transthyretin; Ecadh, E-cadherin; Pan-CK, pan-cytokeratin.

3. Ensure that the forceps points are flush at the tips when closed together (inspect under a dissecting scope if necessary). This is essential for the effective dissection of choroid plexus tissue, which is highly membranous and may tear if not properly grasped.
4. An optician's lens spoon is ideal for this purpose.
5. We have mostly used mice of a mixed inbred strain background (C57BL/6:CBA) and some aspects of the protocol may therefore need to be optimised when used to derive cells from mice of different strain backgrounds.
6. The use of a metal coverslip rack allows a number of coverslips to be processed simultaneously and simplifies transfer between the reagents and washes.
7. In an alternative method, heat a PBS solution to 60°C (check with a thermometer) in a microwave oven, transfer

to a fume hood, add the PFA and then place on a stir plate. The subsequent addition of a single NaOH pellet will promote dissolution of the PFA almost immediately. Cool and adjust to pH 7.4 with concentrated HCl.

8. Gelvatol partially solidifies upon refrigeration and must be warmed to room temperature well in advance of use.
9. This is required to pre-warm and maintain at temperature a Petri dish filled with complete growth medium in which the brains dissected in **Section 3.1** of step 4 are held prior to the removal of their respective choroid plexuses.
10. In early neonates, the choroid plexus appears compact and highly vascularised with a noticeable reddish colouration and is readily identified amongst brain parenchymal tissue, whereas in slightly older animals it is more diffuse in appearance, is less well vascularised and may take much longer to locate.
11. Experiments involving animals must be conducted in accord with the prevailing local and national regulations. For instance, in the UK, this includes the need for local ethical approval and requires that appropriate licences are obtained from the government Home Office.
12. Tissue viability decreases rapidly from the moment the donor animal has been killed. When dealing with large litters, kill and dissect brains from not more than 10 animals at once to minimize the time elapsed between donor animal killing and subsequent tissue collection.
13. Damage sustained to the internal physiology of the brain during its removal may hinder the subsequent location and dissection of the choroid plexus in **Section 3.1** of step 5.
14. Failure to cut completely through the nasal bone to its tip will hamper subsequent removal of the skull. Only the very tips of the scissors should be used for this step to avoid cutting into the underlying brain tissue.
15. The brain will be attached to the spinal cord via the brain stem. If care is not taken during its removal from the skull the hindbrain (containing the fourth ventricle choroid plexus) may tear apart.
16. The fourth ventricle choroid plexus is particularly fragile and should be detached from the ependyma at its respective left and right lateral extremities before its dissection in one whole piece.
17. Cell aggregate size affects plating efficiency and must be carefully optimised. We have found this incubation period to be optimal for dissociation of the epithelial sheet into a mixture of small cell aggregates (typically less than 100

cells per aggregate) without significantly compromising cell viability.

18. Serum in the growth medium inhibits pronase activity and its addition provides a means to precisely control the timing of the tissue digestion.
19. We have determined this number of pipetting actions required to produce optimal cell dissociation generating small cell aggregates (that attach efficiently to the culture vessel) at high frequency, with relatively few isolated cells (that attach inefficiently).
20. Generate a single cell suspension by further pipetting of the cell aliquot before performing the haemocytometer count.
21. Cytosine β-D-arabinofuranoside (Ara-C), a nucleoside analogue with an arabinose residue substituting the ribose sugar, is a potent DNA synthesis inhibitor and is used to suppress the proliferation of contaminating stromal fibroblasts. Nucleoside transport systems on choroid plexus epithelial cells do not recognise arabinose-based nucleosides whereas many other cell types including fibroblasts cannot distinguish ribose and arabinose residues. Uptake and incorporation of Ara-C into genomic DNA blocks the proliferation of stromal fibroblasts and substantially enriches the cultures for epithelial cells (10).
22. In **Section 3.2** of step 9 leave each coverslip in its respective culture plate; the latter will act as a reservoir for the addition of the various reagents and washes (typically 2 ml volumes).
23. Drain as much of the blocking buffer as possible to avoid dilution of the primary antibody solution that is subsequently applied to the coverslip. Importantly do not let the cells dry out at any point during the immunostaining process as this may lead to an increased background.
24. For economy only 200 μl diluted antibody is required at this step. Surface tension will retain this volume as a meniscus on the coverslip.
25. Ensure that the specimens are set down on a level surface to prevent the cells staining unevenly or drying out.
26. Excessive exposure of the samples to light from this step onwards may cause photo bleaching of the fluorophore conjugate resulting in decreased fluorescence signals.
27. Air bubbles in the gelvatol mounting medium are undesirable and should be avoided. If bubbles are present following the application of gelvatol to the slides they should be drawn off with a pipette tip before mounting the coverslips.

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Chapter 16

The Preparation of Primary Cortical Neuron Cultures and a Practical Application Using Immunofluorescent Cytochemistry

Carla Sciarretta and Liliana Minichiello

Abstract

Traditionally, cultures of primary cortical neurons are prepared from embryonic animals because at prenatal stages neurons have not yet developed extensive axonal and dendritic arbors and are not highly innervated, thus rendering the cells less susceptible to damage during dissociation of the neuronal tissue. The appropriate developmental age for preparing primary cultures of any cell type is determined by the time at which the cells of interest are generated and abundant. Most cerebral cortical neurons are generated between embryonic days (E) 11 and 17 in the mouse (embryos being considered 0.5 days old when a vaginal plug is detected in the morning). Here we describe a method to obtain short-term cultures of mouse primary cortical neurons at E15.5 and a practical application using fluorescent immunocytochemistry.

Key words: Mouse primary cortical neurons, embryonic stages, signal transduction, receptor activation, phospho-MAP kinases, fluorescent immunocytochemistry, confocal microscopy.

1. Introduction

A primary focus of our laboratory is the study of signalling mechanisms and gene regulation in the nervous system. We are focused on signals elicited by neurotrophins, such as brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), and neurotrophin 4 (NT4), which are a family of polypeptide growth factors that use specific receptor tyrosine kinases (the Trk family) to exert their diverse functions in the developing and mature nervous system (1, 2). We have begun to dissect the signalling

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pathways downstream of the TrkB receptor that mediate the diverse biological functions of its ligands, by an approach that combines sophisticated genetic tools, which allow for the interference of single phosphorylation sites on a large receptor protein, with biochemical and immunocytochemical approaches. This is a relatively clean way to tease out the specific roles of the various signalling players involved in TrkB-dependent functions (3–5). Our in vitro model to examine these processes is based on primary mouse cortical neuron cultures. We routinely prepare cultures using cortices isolated from E15.5 mice for a number of reasons. Our gene of interest is abundantly expressed in the cortex at these ages (6). Moreover, the composition of the culture obtained is almost purely neuronal, since astrocytes and oligodendrocytes are generated after E13 and E14, respectively; thus their presence is negligible (7). In addition, the number of cells obtained from one embryo of this age is sufficient for performing histological and/or biochemical assays. Finally, the meninges and connective tissue sheaths are easier to remove cleanly from cortices at these stages of development. A caveat worth noting, however, is that although the cell preparation obtained is almost exclusively comprised of neurons, a number of different subtypes are present (7). Also, with this method the neurons do not divide in culture, but do extend processes that form contacts.

This chapter describes the dissection of cerebral cortices from E15.5 mouse embryos and their processing to obtain a single cell suspension of neurons. In addition, a practical application is provided that involves the culturing of the cortical neurons on chamber slides, their stimulation with brain-derived neurotrophic factor (BDNF), and a subsequent examination of MAP kinase phosphorylation and cellular translocation using immunofluorescence and confocal microscopy.

2. Materials

2.1. Tissue Dissection and Cell Culture

1. Medium-sized scissors and standard forceps for opening abdomen of female and extracting uterine horns.
2. Small scissors for cutting open uterine horns and decapitating embryos.
3. Dumont #7 curved forceps.
4. Dumont #5 standard tip forceps.
5. Vannas spring scissors.
6. Chamber slides (Lab-Tek 2-well chamber slide with cover, Nunc 177429).

7. Flame source.
8. Borate buffer (pH 8.5): 50 mM boric acid (Sigma), 12.5 mM borax (Sigma). Store at 4°C.
9. Poly-L-lysine (Sigma, poly-L-lysine hydrobromide P2636). Store at –20°C.
10. 1 M HEPES–NaOH (pH 7.25) solution (Gibco 15630-056).
11. Solution of penicillin/streptomycin (100×) (Gibco 15140-114).
12. Hank's balanced saline solution (HBSS) (Invitrogen 24020-091) supplemented with 1× penicillin/ streptomycin, 7 mM HEPES–NaOH (pH 7.25).
13. Solution of trypsin (0.25%) and ethylenediaminetetraacetic acid (EDTA) (1 mM) without Ca^{2+} or Mg^{2+} (Gibco, 25300-054). The contents of the bottle are thawed at 4°C overnight, then supplemented with 10 mM HEPES–NaOH (pH 7.25), and 1× penicillin/streptomycin, mixed well, and stored in 5 ml aliquots at –20°C.
14. Insulin (Sigma I5500) (*see* **Note 1**) is dissolved at 5 mg/ml in 0.01 N HCl to make a 1000× stock. Store at –20°C in 1 ml aliquots. Use within 12 months.
15. Progesterone (Sigma P01301) is first prepared as a 2 mM solution in absolute ethanol, and then diluted 1:100 in H_2O to obtain a 20 μM (1000×) stock solution. Store in 1 ml aliquots at –20°C. Use within 12 months.
16. Putrescine (Sigma P7505) is dissolved at 100 mM in water. Store this 1000× stock solution in 1 ml aliquots at –20°C. Use within 12 months.
17. Selenium dioxide (Sigma S9379) is first dissolved at 3 mM in H_2O, and then diluted 1:100 in H_2O to obtain a 30 μM (1000×) stock solution. Store in 1 ml aliquots at –20°C. Use within 12 months.
18. Transferrin, APO-, Human Plasma (Calbiochem, 178481; 100 mg).
19. Chicken egg albumin (Sigma A5503) is dissolved at 1% (w/v) in N-MEM (see below) and filter sterilized. Store at 4°C if used within 2 weeks, otherwise store in 5 ml aliquots at –20°C and use within 6 months.
20. Glucose (Merck, D(+) Glucose, 1.040.74) is prepared at 20% (w/v) in H_2O, autoclaved and stored at room temperature.
21. Pyruvate (Sigma P2256) is dissolved at 100 mM in H_2O. This 100× stock is filter sterilized and stored at 4°C. Use within 3 months.

22. Glutamine solution (200 mM) (Gibco 25030-024). The contents of the bottle are thawed at 4°C, and then divided into 5 ml aliquots and stored at –20°C. Use within 6 months.
23. Minimal essential media (MEM) Earl's salts with Glutamax (Invitrogen 41090-028).
24. Horse serum (HS) (Gibco 16050-098) is heat inactivated at 56°C for 30 min and stored in 50 ml aliquots at –20°C.
25. MEM–HS: MEM is supplemented with 0.6% glucose, 2 mM glutamine, and 10% horse serum. Store at 4°C
26. N-MEM: MEM is supplemented with 0.6% glucose, 2 mM glutamine, and 1 mM pyruvate. Store at 4°C.
27. N2 supplement: 100 ml is prepared with 1× insulin, 1× progesterone, 1× putrescine, 1× selenium dioxide, and 100 mg transferrin in N-MEM (*see* **Note 1**). Filter sterilize and store in 5 ml aliquots at –20°C. Use within 6 months.
28. N2-MEM: 50 ml is prepared by combining 40 ml of N-MEM with 5 ml of N2 supplement and 5 ml of 1% chicken egg albumin. Store at 4°C and use within 10 days.

2.2. Detection of MAP Kinase Phosphorylation by Immunofluorescence Following BDNF Stimulation

1. Phosphate buffered saline (PBS).
2. Paraformaldehyde (PFA) is prepared at 4% (w/v) in 0.1 M phosphate buffer (pH 7.4). Working in a fume hood, warm the phosphate buffer to 70°C, and add the PFA. The solution is stirred with warming until the PFA is dissolved. Allow the solution to cool to room temperature and adjust the pH if necessary. Filter the solution using a Whatman 3 mm chromatography paper filter, aliquot, and store at –20°C.
3. Glycine is prepared fresh for each experiment at 10 mM in PBS.
4. Nonident P-40.
5. Normal horse serum (NHS) (Vector S-1200).
6. Carrageenan lambda (Sigma C3889).
7. Triton X-100.
8. Tris-buffered saline (TBS): 50 mM Tris (pH 7.4), 150 mM NaCl. Store at room temperature.
9. Blocking solution: 10% NHS, 0.3% carrageenan lambda, 0.5% Triton X-100 in TBS (*see* **Note 2**). Store for a limited time at 4°C.
10. Primary antibody dilution buffer: 1% NHS, 0.3% carrageenan lambda, 0.5% Triton X-100 in TBS. Store for a limited time at 4°C.

11. Primary antibody: phospho-p44/42 (Thr202/Tyr 204) E10 monoclonal (Cell Signalling Technology 9106).
12. Secondary antibody: fluorescein-conjugated goat anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories 115-015-100).
13. Vectashield mounting medium with DAPI (Vector Laboratories H-2000).
14. Confocal microscope.

3. Methods

Dissected cortices are incubated with trypsin/EDTA, washed, and triturated to give a single cell suspension. Cells are cultured for 18–24 h in serum-free medium, then stimulated with BDNF at two time points and examined for MAP kinase phosphorylation and cellular translocation using immunofluorescence. In order to perform immunofluorescent staining of cells (*see* **Note 3**), it is necessary to culture them on chamber slides (*see* **Note 4**). Sufficient cortical neurons are obtained from one E15.5 embryo to be plated on four chamber slides (i.e. eight wells). This produces cultures that are less dense, allowing for easier examination of cell bodies and processes, but not so sparse as to hinder cell survival.

3.1. Tissue Dissection and Cell Culturing

1. Coat chamber slides with poly-L-lysine the day before they are required. Prepare 1 mg/ml poly-L-lysine in borate buffer and sterilize the solution using a 0.22 μm filter. Cover the chamber slides with the poly-L-lysine solution (1 ml per well) and leave at room temperature (UV-light protected) or in a 37°C incubator for at least an overnight or up to 2–3 days, ensuring that the surfaces remain covered with solution.
2. On the day of cell culture preparation, remove the poly-L-lysine by aspiration and wash the chambers twice with sterile water (do not let the surfaces dry between washes). Add 1 ml of MEM–HS to each chamber well and place them in an incubator (5% CO_2, 37°C) for at least 3 h to equilibrate.
3. Kill the pregnant female by cervical dislocation. First clean (with ethanol, for example) and then open the abdomen and carefully remove the uterine horns containing the embryos. Place them in a 50 ml Falcon tube containing HBSS. From this point on, work in a laminar flow hood and use sterile technique. In addition, unless stated

otherwise, the HBSS and all media are used cold; do not pre-warm. Remove the HBSS and transfer the uterine horns to a dish containing fresh HBSS. Cut along the length of the uteri to expose the embryos enclosed within their membranes. Snip each embryo away from the uterine horn and then remove the surrounding membrane using Dumont #5 forceps (*see* **Note 5**).

4. Processing embryos one at a time remove the head and transfer it to a dish containing fresh HBSS (the lid of a tissue culture dish works best). Using a dissecting microscope, remove the brains from the head. This is most easily done by bracing the head through the eyes with curved Dumont #7 forceps, and using Vannas scissors to cut open the skull, down the mid-line from the base of the head towards the front, just past the olfactory bulbs (**Fig. 16.1a**). Create two lateral incisions towards each ear and peel away the skull exposing the brain (**Fig. 16.1b**). Lift the brain out of the head from underneath the olfactory bulbs and cut it away at the spinal cord (**Fig. 16.1c**).
5. Using curved forceps, hold the brain down onto the dish behind the cerebral cortices (**Fig. 16.1d**). Using the Vannas scissors remove the olfactory bulbs and the two cerebral cortices (**Fig. 16.1e**). Strip away the thin meninges from the cerebral cortices using Dumont #5 and #7 forceps. This is most easily done by positioning the cortex such that the inner side of the cortex is facing upwards and

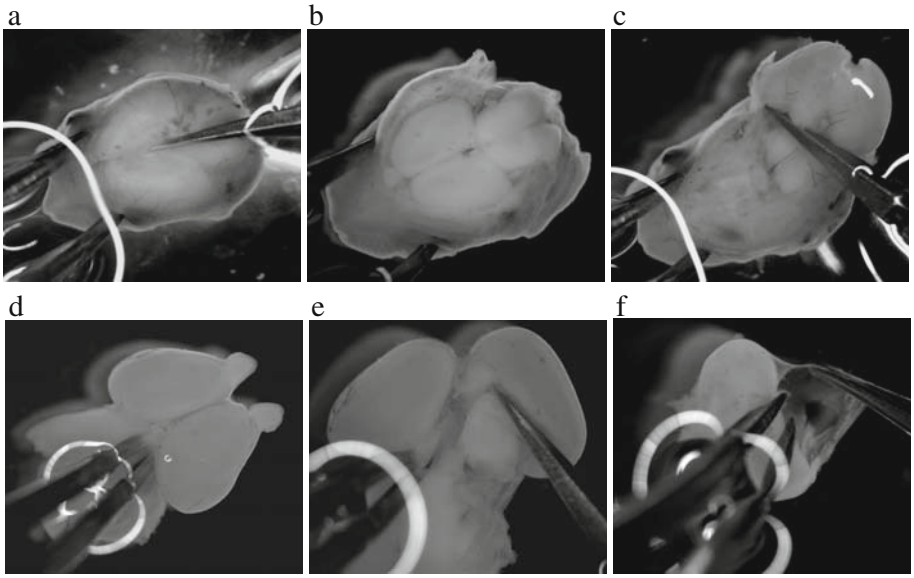

Fig. 16.1. Dissection of cerebral cortices from an E15.5 mouse embryo. See **Section 3** for details.

grasping the meninges with the #5 forceps. Then, using the #7 forceps gently "roll" the cortex out of the membrane covering (**Fig. 16.1f**) (*see* **Note 6**). With curved forceps, gently lift the two cortices and transfer them into a 15 ml tube containing HBSS. When the genotypes of the embryos are not known, collect the cortices of each embryo in separate tubes, each numbered according to the number of the embryo, otherwise pool the cortices in one tube.

6. Once all the cortices have been collected, carefully aspirate away the HBSS and add 5 ml of trypsin/EDTA that was thawed and pre-warmed in a 37°C waterbath. Incubate the cortices in the trypsin/EDTA in a 37°C waterbath for 15 min.
7. Stop the trypsinization process by adding 5 ml MEM–HS to the tube. Carefully remove the solution by aspiration and repeat the wash step with MEM–HS once more. Resuspend the cortices in approximately 3 ml of MEM–HS.
8. Dissociate the tissue by gentle titration through two Pasteur pipettes whose tips have been fire polished (*see* **Note 7**). Pass the cortices through the first pipette approximately four to five times to dissociate the tissue and then use the second pipette to obtain a single cell suspension (*see* **Note 8**).
9. Add sufficient MEM–HS to the cell suspension such that the cells of one embryo can be plated in eight wells (i.e. four chamber slides) at 1 ml per well, which already contain 1 ml of medium. Incubate the cells (5% CO_2, 37°C) for 2–3 h (the time required for them to attach to the chamber surface).
10. After 2–3 h, remove the MEM–HS and replace it with 2 ml per well of N2-MEM, which was pre-warmed to 37°C, and return the cells to the incubator (5% CO_2, 37°C) for 18–24 h (*see* **Fig. 16.2** for an example of cortical neurons in culture) (*see* **Note 9**).

3.2. Cell Stimulation and Immunofluorescent Staining

1. To perform cellular stimulations, remove 1 ml of the conditioned medium from each well and add BDNF to it at a final concentration of 50 ng/ml. Aspirate away the remaining medium from each well and replace it with the BDNF-containing, conditioned medium (*see* **Note 10**). Return the cells to incubator during the period of exposure to BDNF. For our purposes, the phosphorylation of MAP kinase and its translocation to the nucleus are best visualized after 5 and 30 min, respectively, of BDNF stimulation. The unstimulated control cells receive conditioned medium containing only vehicle.

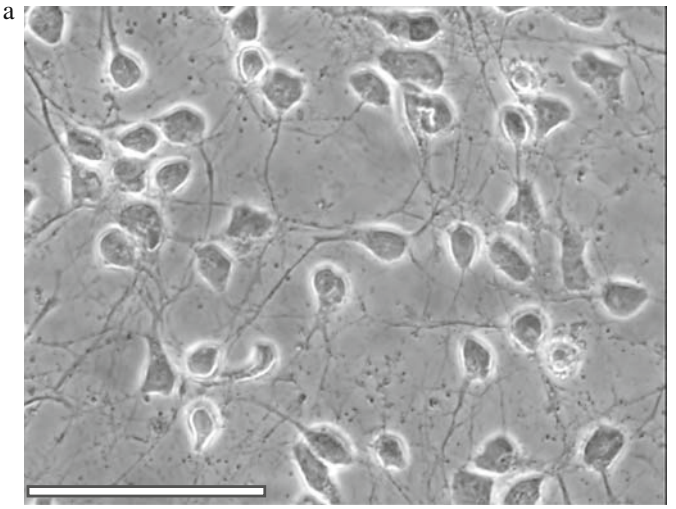

Fig. 16.2. Brightfield image of E15.5 cortical neurons after 18 h in culture. Scale bar: 30 μm.

2. Following the period of incubation with BDNF, remove the stimulation medium from the wells by aspiration and wash the cells at least twice with PBS (*see* **Note 11**).
3. Fix the cells for 20–25 min at room temperature with 1 ml/well 4% PFA that was pre-warmed at 37°C.
4. Remove the fixative (discard into a hazardous waste container) and wash the cells with 1–2 ml of 10 mM glycine/PBS to neutralize the PFA.
5. Wash the cells 2 × 10 min with 10 mM glycine/PBS.
6. To permeabilize the cells add 1 ml/well of 0.5% NP-40 in PBS and incubate for 5–10 min.
7. Wash the cells once with PBS.
8. To prevent non-specific binding of the antibodies, add 1 ml of blocking solution per well and incubate for 1 h.
9. Remove the blocking solution and add 500 μl per well of the primary antibody, which for our purposes is diluted 1:200 in the primary antibody dilution buffer.
10. Incubate the chamber slides for 24–72 h in a humidified chamber (*see* **Note 12**) at 4°C.
11. Wash the cells three times for 10 min each in TBS.
12. Add 1 ml of secondary antibody diluted 1:200 in TBS and incubate for 2 h at room temperature in the dark.
13. Wash away the unbound secondary antibody with TBS as follows: 1 × 10 min, 1 × 15 min, and 1 × 30 min.

14. After removing the final wash, peel away the chamber from the slide, add two drops of Vectashield mounting medium with DAPI per slide, and carefully overlay with a coverslip (*see* **Note 13**).
15. Analyse the cells using a confocal microscope (*see* **Fig. 16.3** for examples of immunofluorescent staining of phosphorylated MAP kinase in cortical neurons following 5 and 30 min of BDNF stimulation).

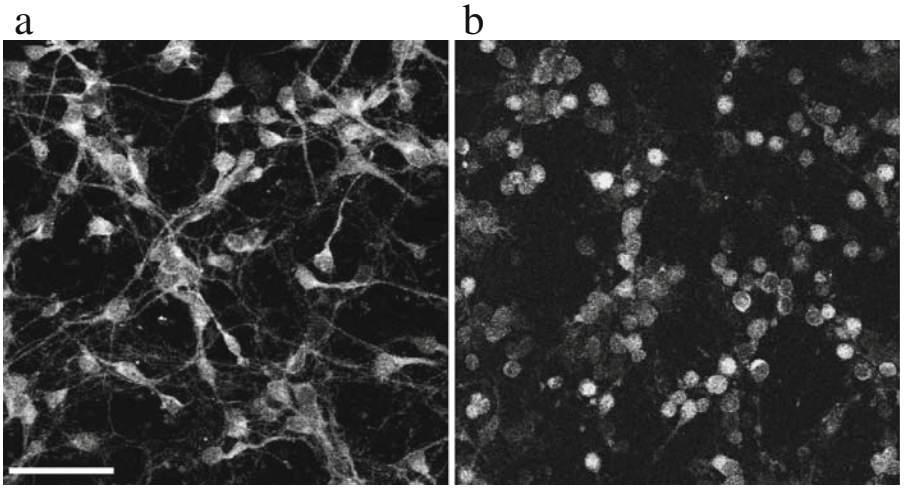

Fig. 16.3. Confocal images of phosphorylated MAP kinase (p42/44) in E15.5 cortical neurons following 5 min (**a**) or 30 min (**b**) of stimulation with 50 ng/ml BDNF. Note that BDNF stimulation after 5 min induced a pMAPKs signal homogenously throughout the dendrites and cell bodies of the cortical neurons, whereas after 30 min there was a clear redistribution of the pMAPK signal to the cell bodies. Scale bar: 50 μm.

4. Notes

1. Insulin is omitted in our N2 supplement because it activates signalling molecules whose regulation is of interest to us.
2. We usually prepare 25 ml of both the blocking solution and primary antibody dilution buffer, adding the carrageenan lambda to the liquid to make its dissolution easier.
3. We have chosen to use immunofluorescence to examine activated MAP kinase; however, you may also perform immunostaining using a horseradish peroxidase-conjugated secondary antibody and diaminobenzidine (DAB) as substrate.
4. Chamber slides, though more expensive than coverslips, are very convenient, since all the washing and incubation steps can be performed in the wells. Moreover, at the end of

the procedure the chamber is easily pulled off exposing a microscope slide, which can be directly mounted.

5. When the genotypes of the embryos are not known, it is best to transfer them into individually numbered 3 cm dishes containing HBSS. The tails or a limb can be used for preparing genomic DNA for genotyping.
6. The removal of the meninges from the cortices is necessary for the complete dissociation of the tissue, and is arguably the most difficult part of the dissection. You will know that they have been successfully removed when the two cortices are uniformly white, with no visible blood vessels.
7. The diameters of the tips of the Pasteur pipettes are reduced by passing them in and out of a flame while simultaneously twirling the pipette. The tip of the first Pasteur pipette should be roughly half its normal diameter and the tip of the second pipette half that of the first.
8. Assess the progress of tissue disassociation by examining a drop of the cell suspension on a Petri dish using an inverted phase contrast microscope. A single cell suspension has been obtained when clumps are no longer visible. If this is not achieved by the sixth passage of cells through the second pipette, it is best to prepare a new pipette with a smaller bore size.
9. In order to obtain RNA, protein etc., cells can be cultured in tissue culture dishes whose surfaces have been treated with poly-L-lysine as described for the chamber slides. Sufficient cells are obtained from one E15.5 embryo to be plated on one 6 cm dish. To increase the number of experimental conditions, one embryo per two 3 cm dishes can be used. When using 6 cm dishes, culture the cells in a total of 3–4 ml of medium. When using 3 cm dishes, 2 ml of medium is sufficient.
10. Multiple wells or dishes of cells of the same genotype may be stimulated simultaneously. Pool the conditioned medium that is removed from each well or dish, add the stimulus to this medium, aspirate away the medium that remains in the well or on the dish, and then aliquot the conditioned medium containing your stimulus onto the cells. For 6 cm dishes, it is best to perform stimulations in 1.5 ml of medium to ensure that the surface of the dish remains covered for the duration of the stimulation. For a 3 cm dish, 500 μl to 1 ml of medium should be used. Unless the time points of stimulation are very short, return the cells to an incubator.
11. Unless stated otherwise, all incubations and washes are performed at room temperature.

12. A humidified chamber can be created using a box with a tight closing lid. Line the bottom of the box with a few layers of Whatman 3 mm chromatography paper. Create a platform on top of which the chamber slides are placed (pieces of 2 ml pipettes narrowly spaced and taped to the Whatman paper work well). Saturate the paper with water, allowing an excess to accumulate in the box.
13. Holding the coverslip at one edge with forceps, place the opposite edge on the slide at a 45° angle. Gently lower the end held by the forceps, spreading the mounting medium slowly over the cells, without creating bubbles, until the coverslip is completely on the slide.

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Chapter 17

Cell Culture of Primary Cerebellar Granule Cells

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Abstract

Cerebellar granule cells are often used as a model system for the study of neuronal development, function and pathology, including the analysis of activity-dependent survival/apoptosis of neurons and the mechanisms of neuroprotection. Cerebellar granule cells are generated postnatally and constitute the largest homogeneous neuronal population of the mammalian brain. In addition, cerebellar granule cells cultured in vitro develop characteristics of mature cerebellar granule cells seen in vivo, such as an extensive neuritic network, expression of excitatory amino acid receptors and production and release of -Lglutamate. Taken together, these features make cerebellar granule cells a unique model system that has been extensively characterised and used for in vitro studies.

Key words: Mouse cerebellar granule cells, homogeneous neuronal cell culture, activity-dependent survival, central nervous system, postnatal development.

1. Introduction

For many experimental questions, an in vitro cell-culture system is required that possesses particular characteristics necessary for it to serve as a useful model: (i) it should closely resemble the nature and behaviour of neurons in vivo; (ii) it should be comprised of an almost homogenous cellular population to allow for whole-culture biochemical and molecular biology studies and (iii) the cells should be easy to manipulate. Many of the presently available neuronal cell lines are not suitable due to changes in their phenotype when compared to the cells from which they originate. An alternative is found in primary cultures, which can serve as an adequate model of highly differentiated neurons. A drawback of using primary cells, however, is that most of these cultures

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consist of mixed neuronal populations. Also, in particular circumstances, the presence of non-neuronal components (mainly astrocytes) may be problematic, as they possess different functional and metabolic characteristics compared to neurons. As a compromise, cerebellar granule cells are often used. Cerebellar granule cells are of a high purity; over 95% of the cells in culture are cerebellar granule cells (1) and mature very late (postnatal) in the development of the central nervous system. These features allow cerebellar granule cells to be isolated from newly born animals and to be cultured in vitro. As with all neuronal cultures, the circuitry of the brain and the connections between cells are lost in this model. The input cerebellar granule cells received from mossy fibres and the connections they form with Purkinje neurons in vivo are disrupted in an in vitro system. To overcome this problem, synaptic activity is mimicked by an exogenously applied depolarising agent such as KCl (2, 3). The resulting depolarisation of cell membranes is thought to activate voltage-gated calcium channels, and thereby increases cytoplasmic calcium levels, which leads to an activation of gene transcription that prevents cell death (4).

2. Materials

Hank's balanced saline solution (HBSS) (Gibco) supplemented with 3 mg/ml bovine serum albumin (BSA; Sigma) (*see* **Note 1**).

1. Complete DMEM: Dulbecco's modified Eagle's medium HEPES modification (Gibco; 42430-025) supplemented with 100 μg/ml sodium pyruvate (Sigma), 100 μg/ml gentamycin (Sigma) and 10% fetal calf serum (FCS; Gibco or Sigma).
2. DMEM without FCS: Dulbecco's modified Eagle's medium HEPES modification (Gibco; 42430-025) supplemented with 100 μg/ml sodium pyruvate (Sigma) and 100 μg/ml gentamycin (Sigma).
3. Solution of 50 mg/ml poly-L-lysine (Sigma) dissolved in sterile ddH_2O and kept in aliquots at –20°C. Working solutions are prepared by adequately diluting the stock solution in sterile ddH_2O and filter sterilisation.
4. Solution of trypsin (2.5%; Sigma): single use aliquots of 100 μl are prepared and kept at –20°C.
5. Potassium chloride is dissolved at 2 M in sterile H_2O and filter sterilised.
6. Solution of cytosine arabinofuranoside (Sigma) is prepared at 10 mM in sterile H_2O and filter sterilised. Aliquots are kept at –20°C.

3. Methods

Dissected cerebella from early postnatal mice are digested with trypsin and triturated to obtain a single cell suspension. Cells are plated at an average density of either 1.5×10^6 cells/ml for biochemical purposes, such as protein or RNA extraction, or at 600,000 cells/ml for immunocytochemistry. Fresh medium containing cytosine arabinofuranoside is added the next day, together with any treatment compounds (e.g. inhibitors, KCl).

1. Prior to starting the cell extraction, six-well plates are coated with 50–200 μg/ml poly-L-lysine (*see* **Note 2**) for 10–15 min at 37°C. The poly-L-lysine solution is washed off using DMEM without FCS (important as addition of FCS will prevent cells from adhering) and plates are incubated at 37°C for at least 2 h in DMEM without FCS (*see* **Note 3**).
2. Six-day-old mice (*see* **Note 4**) are killed by decapitation using a pair of scissors large enough to ensure the head is removed in one clean cut. The heads are transferred to a clean Petri dish where the skin on top of the head is cut down the midline (*see* **Fig. 17.1**), folded down on both sides and used to hold the head (*see* **Fig. 17.1**).

Fig. 17.1. Steps to follow prior to the dissection of the cerebellum from a P6 mouse head. *See* **Section 3** for details.

3. Starting from the back of the head, the skull is cut along one side. Then the top of the skull is lifted with a sharp pair of forceps (*see* **Fig. 17.1**). The cerebellum is now visible on top of the spinal cord. Using a pair of curved forceps (*see* **Note 5**), lift out the cerebellum and place it in HBSS/BSA (*see* **Fig. 17.2a, b** for comparison of the brain before and after removal of the cerebellum).
4. The cerebella are kept in HBSS/BSA while the meninges are carefully removed. To do so, the blood vessels that are visible on the surface of the cerebellum are pulled

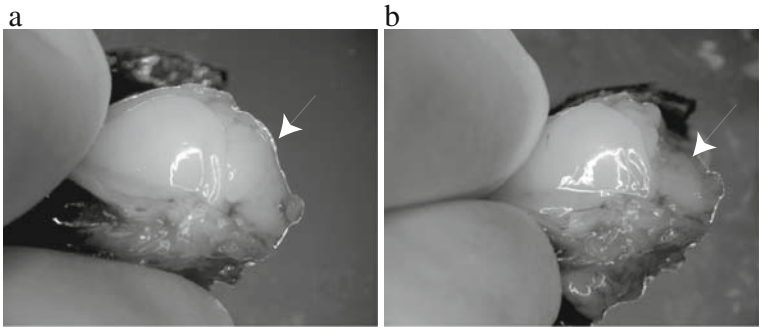

Fig. 17.2. Position of the cerebellum in a P6 mouse head (**a**, *arrow*). Same position is shown in **b** (*arrow*) after dissection.

with very thin forceps until no blood vessels visibly remain (*see* **Note 6**).

5. The cerebella are then transferred into a sterile hood and washed at least three times with fresh HBSS/BSA (*see* **Note 7**).
6. The tissue is transferred into a small droplet of HBSS/BSA in a 10 cm Petri dish and minced with a razor blade into small cubes (ca. 0.5 mm^3). The minced tissue is transferred into a Falcon tube containing 10 ml of fresh HBSS/BSA (max. 4–6 cerebella per tube). The tissue is allowed to settle down to the bottom of the tube and the supernatant is carefully removed and replaced with a fresh 10 ml of HBSS/BSA; this step is repeated at least three times.
7. The tissue is digested by incubation with 0.025% trypsin in HBSS/BSA (100 μl aliquot of 2.5% sterile trypsin in 10 ml HBSS/BSA) in a 37°C water bath for 15 min (exactly) with constant mixing of the tissue (*see* **Note 8**). The digestion is stopped by addition of 20 ml of complete DMEM.
8. A single cell suspension is obtained by triturating the tissue with cotton plugged, sterile Pasteur pipettes. All the tissue (with very little medium) is pipetted into a new Falcon tube and repeatedly pipetted up and down; the triturated tissue is then topped up to approximately 10 ml with medium from the original Falcon tube (*see* **Note 9**), and the tissue is left to settle to the bottom of the tube (approximately 2 min). The majority of the supernatant, containing the single cell suspension, is then transferred back to the original tube. The trituration is repeated until no tissue remains visible and all the cells have gone into suspension.
9. The cells are centrifuged at 650*g* for 5 min and resuspended in complete DMEM. Cells are counted and 2 ml per 3 cm dish (one well of a six-well plate) are plated at

approximately 1.5×10^6 cells/ml (for immunocytochemistry plate at 600,000 cells/ml) (*see* **Note 10**). Cells are incubated overnight at 37°C and 5% CO_2.

10. The next day the medium is replaced with fresh DMEM containing 10 μM cytosine arabinofuranoside to kill dividing cells, e.g. any endothelial cell contamination as well as microglial cells. Granule cells will not be affected by this treatment as they are no longer dividing. In addition, 20 mM KCl is added to the culture medium as cerebellar granule cells require mild depolarisation for survival (complete DMEM already contains 5.3 mM KCl so the final concentration is approximately 25 mM) (*see* also **Notes 11–14**).

4. Notes

1. Since filter sterilising BSA by hand is not an easy task we usually dissolve 1.5 g BSA in approximately 40 ml of HBSS, then filter sterilise and add it to the remaining 460 ml HBSS.
2. Alternatively plates can be incubated for longer periods (e.g. overnight) at room temperature. A volume of 50 μg/ml of poly-L-lysine is used routinely for cell culture for biochemical purposes (protein or RNA extraction), while 200 μg/ml is used for immunocytochemistry. Incubate six-well plates with 1 ml per well; if using other cell culture plates adjust the volume accordingly (e.g. 500 μl per well for 12-well plates).
3. Instead of using DMEM without FCS, plates may be washed twice with sterile water and allowed to dry; in both cases care should be taken to remove excess poly-L-lysine completely to increase the yield of the cell culture.
4. To culture cerebellar granule cells from rats, 7- to 8-day-old pups are used. The use of younger pups is also possible but significantly reduces the number of cells obtained. Using older animals (older than P7 for mice or P8 for rats) is not recommended as cells will start to extend axons and form dendrites and synaptic connections, rendering the cells more susceptible to damage during the purification. Generally, the development of fur is a good indicator of age. Avoid using animals that have developed a proper fur as they are too old to obtain a successful primary culture.

5. Using serrated forceps helps to lift out the cerebellum without accidentally cutting into or through the tissue as might happen when using sharp forceps.
6. Pulling the blood vessels will usually lead to removal of the entire meninges as well. We usually do not use a dissecting microscope to do this, but when preparing these cells for the first time it might be helpful to check under a microscope that the meninges have been removed successfully.
7. A good way of doing this is to pipette small droplets of HBSS/BSA into a 10 cm Petri dish and to transfer the cerebella from one droplet to the other.
8. We normally do not use a rocking water bath but instead shake the tissue by hand for the entire 15 min; if the use of a rocking water bath is preferred it is still advisable to stir the tissue by hand occasionally.
9. Try to avoid generating too many bubbles during this procedure as this will kill cells and decrease the yield. However, being too gentle will make it difficult to achieve a proper single cell suspension. It all comes down to the right balance between being forceful and fast enough without killing too many cells (and you might have to try a few times to find this balance).
10. The cerebellum of one mouse will yield about half a six-well plate (three wells) while that of a rat yields approximately one six-well plate. For immunocytochemistry, we find that plating less than 600,000 cells/ml decreases survival of the cell culture drastically.
11. Do not give up on your cell culture just because it looks quite bad the first day after plating. Although the FCS in the medium prevents cell debris from sticking to the plate it is still swimming around. Give the cells time to recover; about 48 h after the medium change (addition of cytosine arabinofuranoside plus high potassium levels) cells will look much healthier. Granule cells will have formed dendrites and will form a network with each other.
12. Medium is changed only one time (i.e. approximately 24 h after plating), after which the cells remain in their medium. Any treatments (e.g. stimulation with growth factors or incubation with specific inhibitors) should be done when the medium is changed.
13. Purkinje cells, another major cell type of the cerebellum, do not adhere to tissue culture plates when cerebella are harvested at this "late" stage of development. Therefore, any Purkinje cells present in the cell suspension will be washed away when the medium is changed.

14. We have cultured cells for up to 9 days in vitro without any problems or onset of cell death. Longer periods have not been tested by us, but we have been told that cells can be cultured successfully for 2 weeks or longer (personal communication).

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Chapter 18

Isolation and Generation of Neurosphere Cultures from Embryonic and Adult Mouse Brain

Henrik Ahlenius and Zaal Kokaia

Abstract

Neural stem cells are defined as cells that either gives rise to or derives from the cells of the central nervous system and have the unique properties of stem cells, i.e. self-renewal and multipotentiality. One of the widely used methods of expanding neural stem cells under culture conditions is based on the capacity of these cells to divide continuously when cultured in serum-free medium supplemented with various growth factors. One common method used is to grow neural stem cells as free-floating aggregates of cells called neurospheres. Neurospheres can be generated from several structures of the embryonic and adult mammalian brain. Although viable lines can be generated from crude extracts of brain, a precise dissection is crucial to get a pure population of cells. Here we describe methods for dissection, isolation and generation of neurospheres from embryonic ganglionic eminences and adult subventricular zone of mice and rats.

Key words: Neurospheres, dissection, LGE, MGE, SVZ.

1. Introduction

Almost all formation of neuronal tissue is completed during embryogenesis. However, neurogenesis continues throughout adult life in discrete locations of the adult mammalian brain namely the hippocampus and the subventricular zone (SVZ). In 1992, Reynolds and Weiss (1) dissociated enzymatically the striatum of adult mice and cultured as a cell monolayer in defined serum-free medium. In cultures with epidermal growth factor (EGF), a small percentage of cells detached, divided and formed

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multicellular spheres, which they called neurospheres. These heterogeneous spheroid structures contain neural stem cells (NSC), progenitor and differentiated cells embedded in a complex extracellular matrix organized three dimensionally. In the core of neurospheres there are differentiating GFAP- and β-tubulin III-positive cells surrounded by nestin, epidermal growth factor receptor (EGFR) and α-1-integrin-positive undifferentiated cells (2). Neurospheres are useful to evaluate NSC multipotentiality (through the characterization of cell phenotypes that arise from a differentiating sphere) and to analyze NSC self-renewal using repetitive neurosphere formation under clonal conditions assuming that only isolated true stem cells can generate new spheres.

When neurospheres are dissociated into single cells, some of them divide to form new spheres. Serial propagation by repetition of this cycle is possible and allows expansion of neurosphere culture. Upon plating on an adhesive substrate, neurospheres differentiate into mixed cultures containing cells with the morphology and antigen-expression profile of neurons and glial cells. However, many cells remain in an undifferentiated state.

Neurospheres can be generated from several different structures of the embryonic and adult brain. Several ways of isolating and culturing of neurospheres have been developed and the method described here is based on several previously published studies (3–5). Viable cell lines can be generated from very imprecise dissections or without any dissection at all. However, a precise dissection is crucial for obtaining a homogenous, reproducible population of cells. Here we describe the dissection, isolation and culture method of neurospheres from the adult subventricular zone, embryonic lateral and medial ganglionic eminences of rat and mouse, which is routinely used in our and many other laboratories.

2. Materials

2.1. Dissection

1. Dissection instruments; #5 and #55 forceps and extra fine spring scissors (Fine Science Tools).
2. Rat and mouse brain matrices and microthome blades (World Precision Instruments).
3. Leibowits L-15 dissection media (Invitrogen cat. no. 11415-09).
4. Sterile Petri dishes.
5. Dissection microscope.

2.2. Isolation, Generation and Expansion of Neurospheres

2.2.1. Embryonic Neurospheres

1. DMEM/F12 basic medium: DMEM/F12 medium (Invitrogen 21331-020) supplemented with N2 (Invitrogen 17502-048), 0.6% glucose (Sigma G8769), 2 mM glutamax (Invitrogen 35050-038), 1.125% sodium bicarbonate (Invitrogen 25080-060), 15 mM HEPES (Invitrogen 15630-056), gentamicin (0.05 mg/ml) (Invitrogen 15710-080). Mix, filter sterilize and store at 4°C for 1–2 weeks.
2. Recombinant human EGF (R&D systems 236EG): prepare 100 μg/ml stock solution in sterile 10 mM acetic acid with 0.1% BSA. Keep at –20°C and avoid repeated freeze thawing.
3. Recombinant human FGF basic (R&D systems 232FB): prepare 20 μg/ml stock solution in sterile PBS with 0.1% BSA, keep at –20°C, avoid repeated freeze thawing.

2.2.2. Adult Neurospheres

1. Neurobasal basic medium: neurobasal medium (Invitrogen 21103-049) supplemented with B27 (Invitrogen 17504-044), 2 mM glutamax (Invitrogen 35050-038), gentamicin (0.05 mg/ml) (Invitrogen 15710-080). Mix, filter sterilize and store at 4°C for 1–2 weeks.
2. Heparin (Sigma H3149): prepare 0.1 mg/ml stock in basic medium, filter sterilize and keep at 4°C for up to 1 month.
3. Recombinant human EGF (R&D systems 236EG).
4. Recombinant human FGF basic (R&D systems 232FB).
5. Dissociation buffer: HBSS (Invitrogen 14170-070) with 0.015 M HEPES, 5.4 mg/ml D-glucose, 1.33 mg/ml trypsin (Invitrogen 25300-054), 80 U/ml DNAse (Sigma D4527), 0.7 mg/ml hyaluronidase (Sigma H3884) and 0.2 mg/ml kynerenic acid (Sigma K3375), pH 7.5. Prepare fresh.
6. HBSS–sucrose: dissolve 30 g of sucrose in 50 ml of HBSS, bring volume to 100 ml with ddH_2O and pH to 7.5. Filter sterilize and store at 4°C for up to 1 month.
7. BSA–EBSS–HEPES: dissolve 4 g of BSA (Sigma A4503) in EBSS (Invitrogen 14155-048), add 2 ml of 1 M HEPES, bring volume to 100 ml and pH to 7.5. Filter sterilize and store at 4°C for up to 1 month.
8. Cell strainer, 40 μm (BD Falcon 352340).
9. Accutase (PAA Laboratories L11-007).

2.2.3. Differentiation of Neurospheres

1. Poly-L-lysine (Sigma P6282): prepare 1 mg/ml stock in ddH_2O and store at –20°C
2. DMEM/F12 basic medium.

3. Fetal bovine serum, FBS (Invitrogen 10108-165).
4. N2 or B27.

2.2.4. Cryopreservation of Neurospheres

1. DMEM/F12 basic or neurobasal basic medium.
2. FBS.
3. DMSO (Sigma D5879).
4. Cryovials (Nunc).
5. Cryobox with isopropanol.
6. –80°C and nitrogen freezer.

3. Methods

3.1. Isolation, Generation and Expansion of Neurospheres from Embryonic Tissue

1. Anesthetize and kill (by cervical dislocation) a pregnant female mouse or rat at embryonic age 13.5 or 15.5 days postcoitus, respectively (*see* **Note 1**).
2. Spray animal with 70% ethanol.
3. Make a transverse incision on the lower part of the belly and continue up along the body sides to expose the peritoneal cavity. Grasp the end of one of the uterine horns. Cut out and transfer the entire uterus with intact embryos into a 50 ml tube with L-15 medium. Keep on ice for the rest of the dissection procedure (*see* **Note 2**).
4. Remove all embryos from uterus and transfer to Petri dishes with L-15 medium.
5. Under a dissection microscope decapitate embryo by cutting at the plane between eyes and nose as indicated in **Fig. 18.1a**, and place upright on dish (**Fig. 18.1b**). Remove scull and meninges by gently (be careful not to damage brain) tearing the tissue with forceps.
6. When the brain is cleared of scull and meninges (**Fig. 18.1c**), make a longitudinal incision with microscissors close to the fissure on one of the hemispheres as outlined in **Fig. 18.1d**. Fold the tissue to the side to expose the ganglionic eminences (**Fig. 18.1e**).
7. Start by cutting between LGE and MGE to separate the two structures. Remove the LGE by cutting along the bottom of the structure (**Fig. 18.1f**). Take care not to cut too deep so the LGE will be free of underlying tissue. Transfer the LGE to an Eppendorf tube with fresh L-15 medium.
8. When the LGE is removed the MGE can be dissected by simply cutting below the structure and picking it straight

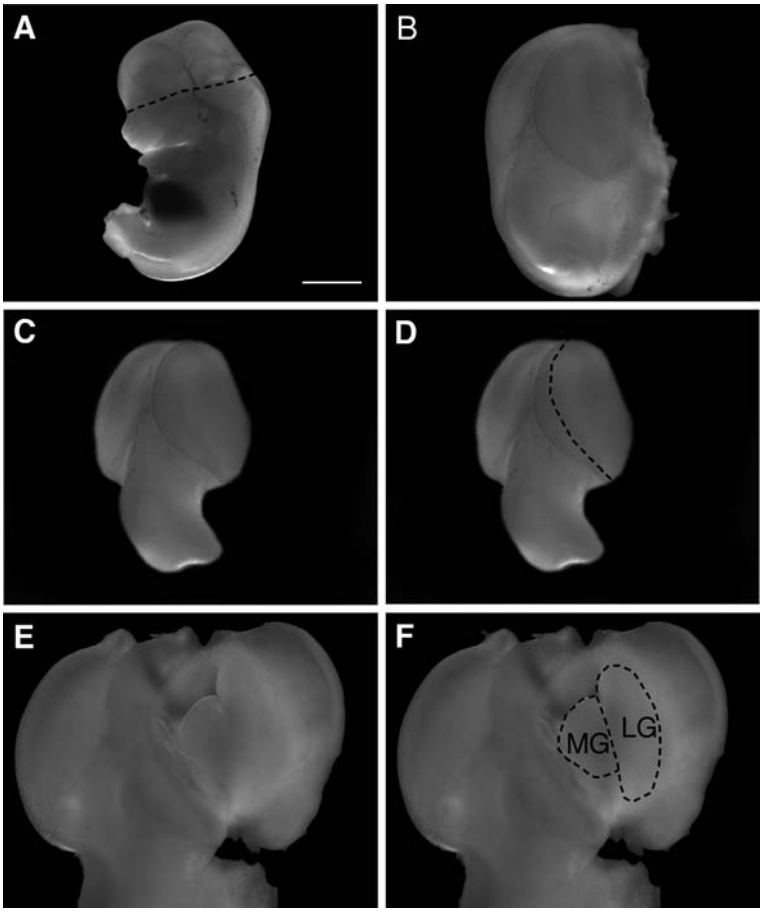

Fig. 18.1. Schematic illustration of the dissection procedure for embryonic LGE and MGE. The brain is removed from the body by making a cut at the level of between eyes and nose as depicted by a *dotted line* in (**a**). Then the brain is placed *upright* on the dish (**b**) and the meninges carefully removed by peeling them off using forceps. When the brain is cleared of the meninges and debris it should look as depicted in (**c**). Start the dissection by making an incision along the hemisphere close to the fissure as indicated by the *dotted line* in (**d**). Fold the hemisphere to the side to reveal the eminences (**e**). Remove the eminences by cutting along the *dotted lines* as shown in (**f**). Start by cutting between the eminences to separate them. Then cut under the LGE to remove it from surrounding tissue. The MGE is removed by cutting under the structure and simply lifting it straight up. Scale bar = 2 mm (**a**), 1 mm (**b**), 0.8 mm (**c**, **d**), 0.5 mm (**e**, **f**).

up. Transfer to Eppendorf tube with fresh L-15 medium. Repeat steps 6–8 on the other hemisphere. Each individual structure can be cultured separately (e.g. when genotyping of transgenic fetuses need to be performed) or LGEs and MGEs, from several animals, respectively, can be pooled together to get more cells.

9. Gently remove the L-15 from the Eppendorf with the tissue and replace with 200 μl of DMEM/F12 basic medium.

Incubate in humidified incubator at 5% CO_2 and 37°C for 15 min.

10. Gently triturate suspension with 200 μl pipette tip 10–30 times or until a single cell suspension is made. Count cells and plate in uncoated culture flasks at density 10–50 cells/μl in DMEM/F12 basic medium supplemented with 20 ng/ml EGF and 10 ng/ml FGF.
11. Culture in humidified incubator at 37°C with 5% CO_2. Feed cells by adding 0.5–2 ml fresh DMEM/F12 basic medium supplemented with 20 ng/ml EGF and 10 ng/ml FGF (depending on flask size) twice a week. Passage when spheres are around 200–300 μm in size but before the inner core of spheres becomes non-transparent when observed under phase-contrast microscope (**Fig. 18.3c**).

3.2. Isolation, Generation and Expansion of Neurospheres from Adult Tissue

1. Anesthetize adult mouse or rat and kill by decapitation. Remove brain and transfer to a beaker with ice-cold L-15 medium. Let brain cool down 3–4 min.
2. Transfer the cold brain to appropriate coronal brain matrix (which should be precooled and kept on ice).
3. Fix the brain in the matrix by cutting and leaving in place a blade at the border between the cerebellum and cerebrum.
4. With two other blades work from rostral to caudal direction by cutting 1 mm thick slices of the brain, always leaving the blade closest to the brain in the matrix. The slices normally stick to the blades and can gently be slided into ice-cold L-15 medium using a forceps. Discard the most rostral and caudal slices and keep the ones containing the SVZ (mostly the striatum, **Fig. 18.2a–d**) (*see* **Note 3**).
5. Under a dissecting microscope using forceps (to support slices) and microscissors (to cut tissue) remove as closely as possible the lateral aspects of the ependymal zone and SVZ, around 1–2 mm from lateral wall of lateral ventricle as indicated on slices in **Fig. 18.2a–d**. Collect tissue pieces into a Petri dish with L-15 medium. Usually it is possible to get tissue from three to four slices per animal (*see* **Note 4**).
6. Cut the tissue into smaller pieces (1–3 mm^3) and transfer to 15 ml tube.
7. Let tissue pieces settle and gently remove the L-15 medium. Add 10 ml of dissociation buffer to the tissue pieces.
8. Incubate at 37°C for 15 min.
9. Gently triturate 10 times with a 5 ml pipette.

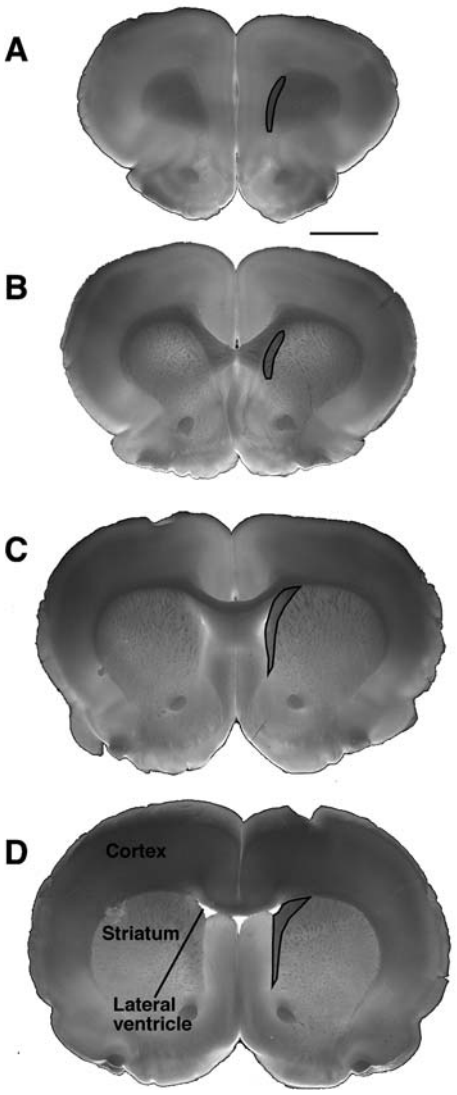

Fig. 18.2. Schematic illustration of the dissection procedure for rat SVZ. Usually it is possible to isolate the SVZ from three to four 1 mm thick slices (**a**–**d**). The SVZ is visible as a brighter area, without any striations, lining the striatum and the lateral ventricle. Illustrated here on the *right side* as a *shaded area* (**a**–**d**). It is removed by cutting it from surrounding tissue. Scale bar = 4 mm.

10. Incubate for an additional 10 min. Total incubation should not be longer than 30 min and times vary from each preparation of dissociation buffer (*see* **Note 5**).
11. Gently triturate solution until most of the tissue pieces are fully dissociated, immediately pass the solution through a 40 μm cell strainer into a 15 ml tube.

12. Centrifuge at 1,500 rpm for 5 min and remove the supernatant. If low number of animals is used or if there is no need for purification continue with step 15 (*see* **Note 3**).
13. Add 1–4 ml (depending of the amount of tissue) of HBSS–sucrose to the pellet and mix by pipetting. Divide solution into one to four of 1.5 ml Eppendorf tubes and centrifuge at 2,000 rpm for 10 min (*see* **Note 6**).
14. Remove supernatant and dissolve pellets in 200 μl EBSS each. Carefully add the 200 μl on top of 1.2 ml of BSA–EBSS–HEPES in new Eppendorf tubes. Centrifuge at 1,500 rpm for 7 min.
15. Remove supernatant, pool all tubes, count the number of viable cells using the trypan blue exclusion method and plate in neurobasal basic medium supplemented with 2 μg/ml heparin, 20 ng/ml EGF and 10 ng/ml FGF at a density of 10–100 cells/μl in uncoated flasks.
16. Culture in humidified incubator at 37°C with 5% CO_2. Feed cells by adding, 0.5–2 ml depending on flask size, fresh medium twice a week. Passage when spheres are around 200–300 μm in size but before the inner core of spheres becomes non-transparent when observed under phase-contrast microscope (**Fig. 18.3c**) (*see* **Note 7**).

3.3. Passage of Embryonic Neurospheres

1. Collect spheres and centrifuge at 1,000 rpm for 5 min. Remove supernatant, filter sterilize and save if conditioned media is needed.
2. Add 1 ml of basic medium to the pelleted spheres and triturate 20–30 times with a 1 ml pipette, then attach a 200 μl pipette tip to the 1 ml tip and triturate another 20–30 times. Pass the suspension through a 40 μm cell strainer (*see* **Note 8**).
3. Count cells and replate in basic medium with growth factors (use 1:3 conditioned media if needed for clonal assays).

3.4. Passage of Adult Neurospheres

1. Collect spheres and centrifuge at 1,000 rpm for 5 min. Remove supernatant, filter sterilize and save if conditioned media is needed.
2. Add 200 μl of prewarmed accutase to the pelleted spheres. Triturate 10 times with a 200 μl pipette and incubate at 37°C for 10–15 min, mix after half of the incubation. Triturate again 20 times, add neurobasal basic medium to 5 ml and centrifuge at 1,000 rpm for 5 min.
3. Remove supernatant, resuspend in neurobasal basic medium, count and replate cells in medium with growth factors (use 1:3 conditioned medium if needed).

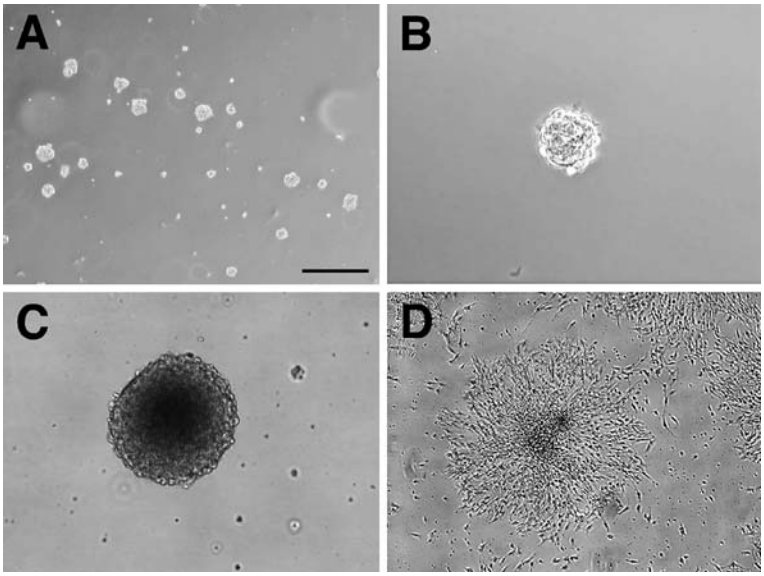

Fig. 18.3. Rodent neurospheres at different time points. (**a**) Representative picture of an SVZ neurosphere culture after 3 days in vitro (DIV). Small spheres of different sizes have formed. (**b**) Magnified picture of a healthy proliferating sphere at three DIV. If allowed to grow for extended periods of time spheres will become so big that nutrients will not reach the core of the spheres and the cells within the sphere will start to die. (**c**) Picture of a sphere where cells in the core have started to die; note the nontransparent appearance of the core. If spheres are transferred to an adhesive surface they will attach and start spreading and if growth factors are withdrawn the cells will start to differentiate. (**d**) Picture of a neurosphere that have been let to attach, for differentiation, to a PLL-coated chamberslide and started to spread. Scale bar = 50 μm (**a**, **b**), 150 μm (**c**, **d**).

3.5. Differentiation of Neurospheres

1. Coat appropriate flasks, wells or chamber slides with poly-L-lysine. Dilute PLL in ddH_2O to 10 μg/ml working concentration and add to culture vessel until bottom is covered. Incubate at room temperature for 1–2 h, remove PLL solution, rinse with ddH_2O three times and let dry completely under sterile conditions (*see* **Note 9**).
2. Collect neurospheres with diameter approximately 100–200 μm and plate in PLL-coated vessels in expansion medium without heparin. When spheres have attached and started to flatten (*see* **Fig. 18.3d**), usually after overnight incubation, the medium is changed to DMEM/F12 or neurobasal basic medium supplemented with 1% FBS and 1% N2 or B27, respectively.
3. Incubate at 37°C in humidified incubator for 3–10 days, change half of the medium one to three times during the differentiation.
4. Cells are now ready to be processed for immunohistochemistry, RNA isolation or other applications.

3.6. Cryopreservation of Neurospheres

3.6.1. Freezing of Cells

1. Freeze cells after one to three DIV after passage, when cells have formed small spheres (**Fig. 18.3a**). The survival of frozen single cells or big neurospheres after thawing is very poor. Collect spheres by centrifugation at 1,000 rpm for 5 min.
2. Discard supernatant and gently dissolve pellet, without triturating spheres, in basic medium containing 10% FBS and 7.5% DMSO. Transfer 1 ml aliquotes containing at least the equivalent of 1–2 million cells, into 1.5 ml cryovials.
3. Place cryovials in a cryobox, filled with isopropanol, which was kept at room temperature. Within 5–10 min, transfer the cryobox to –80°C freezer. For long-term storage after 24 h transfer vials to a nitrogen freezer.

3.6.2. Thawing of Cells

4. Transfer cryovial directly to 37°C water bath, swirl vial to thaw as quickly as possible.
5. Spray vial with ethanol. Transfer the cells to 9 ml of prewarmed DMEM/F12 basic (for embryonic spheres) or neurobasal basic (for adult spheres) medium and centrifuge for 5 min at 1,000 rpm.
6. Discard supernatant and resuspend cells in DMEM/F12 basic medium with growth factors (embryonic spheres) or neurobasal basic medium with growth factors and heparin (adult spheres) and plate in appropriate flask (*see* **Note 10**).

4. Notes

1. Usually to set up breeding for embryo dissections a male mouse or rat is placed in the cage with two females in the evening. The morning after, males and females are separated and females are checked for vaginal plugs. If the plug is detected, females are considered as E0.5. For LGE and MGE dissections mice at E13.5 and rats at E15.5 are most suitable.
2. The entire uterus with intact embryos or the brains of adult animals are removed in the animal facility, then transferred to the lab where the rest of the dissection is performed in open lab before the cells are transferred to sterile environment in the cell lab. This procedure does not cause any problems with contamination but if problems will arise antibiotics can be added to the dissection medium.
3. Normally SVZ cells are not isolated from less than four animals because of limits in the number of cells. It is possible

to isolate cells and generate neurospheres from four or less animals, but in this case the purification step should be omitted.

4. Sometimes it is needed to flip the sections of the brain in order to see the SVZ (depending on the frontal–caudal location). It is also good to gently pull the tissue (laterally–medially) on sections were the SVZ is not visible. This procedure will reveal the best way of starting the dissection.
5. To obtain as many viable cells as possible, tissue should be a little underdigested. Solution should become turbid but small pieces of tissue should still be visible.
6. If preferred the cell suspension can be purified on a 22% percoll (Amersham Bioscience) gradient. If so desired add 3.9 ml of PBS and 1.1 ml of 100% percoll (1 ml of 10× PBS is added to 9 ml percoll to obtain a 100% percoll solution) to the pellet. Centrifuge at 1,500 rpm for 15 min. Remove myelin, debris and the rest of the percoll. Wash once with PBS, 1,000 rpm, and proceed as above.
7. Especially neurospheres generated from adult tissue have a tendency to adhere to tissue culture plastics. Usually it is enough to knock on the flasks a couple of times every week to keep the spheres from attaching. However sometimes, especially when grown in small wells this is not enough to keep spheres free floating and use of ultra-low attachment plates (Corning) or coating the surfaces with an anti-adhesive (poly(2-hydroxyethylmethacrylate), Sigma) is recommended.
8. Sometimes it is necessary to count cells before filtering the cell suspension because if spheres are hard to dissociate one might lose a lot of cells as intact spheres when filtering.
9. Usually PLL coating is sufficient for differentiation assays. However, if needed polyornithine or a combination of polyornithine or poly-D-lysine and laminin can be used.
10. To ensure survival of recultured cells we normally plate spheres at a higher than normal concentration when thawing. When cells have recovered after thawing they can be plated at more appropriate concentrations.

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