Ethics of Science and Technology Assessment | Volume 34

Genetic Engineering in Livestock

New Applications and Interdisciplinary Perspectives

M. Engelhard
K. Hagen
M. Boysen
Editors

Springer

Ethics of Science and Technology Assessment
Volume 34

Book Series of the Europäische Akademie zur Erforschung
von Folgen wissenschaftlich-technischer Entwicklungen
Bad Neuenahr-Ahrweiler GmbH
edited by Carl Friedrich Gethmann

M. Engelhard • K. Hagen • M. Boysen (Eds.)

Genetic Engineering in Livestock

New Applications and Interdisciplinary Perspectives

Springer

Series Editor
Professor Dr. Dr. h.c. Carl Friedrich Gethmann
Europäische Akademie GmbH
Wilhelmstraße 56, 53474 Bad Neuenahr-Ahrweiler
Germany

Editors
Dr. Margret Engelhard
Kristin Hagen, Ph.D.
Europäische Akademie GmbH
Wilhelmstraße 56, 53474 Bad Neuenahr-Ahrweiler
Germany

Dr. Mathias Boysen
Berlin-Brandenburgische Akademie der Wissenschaften
Jägerstraße 22/23, 10117 Berlin
Germany

Desk Editors
Katharina Mader, M.A.
Friederike Wütscher
Europäische Akademie GmbH
Wilhelmstraße 56, 53474 Bad Neuenahr-Ahrweiler
Germany

ISBN: 978-3-540-85842-3 e-ISBN: 978-3-540-85843-0

Ethics of Science and Technology Assessment ISSN: 1860-4803
e-ISSN: 1860-4811

Library of Congress Control Number: 2008936031

© Springer-Verlag Berlin Heidelberg 2009

This work is subject to copyright. All rights are reserved, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilm or in any other way, and storage in data banks. Duplication of this publication or parts thereof is permitted only under the provisions of the German Copyright Law of September 9, 1965, in its current version, and permission for use must always be obtained from Springer. Violations are liable to prosecution under the German Copyright Law.

The use of general descriptive names, registered names, trademarks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.

Cover design: eStudio Calamar S.L.

Typesetting: Lambertz Druck, Köln, Germany

Printed on acid-free paper

9 8 7 6 5 4 3 2 1

springer.com

EUROPÄISCHE AKADEMIE
zur Erforschung von Folgen wissenschaftlich-technischer Entwicklungen
Bad Neuenahr-Ahrweiler GmbH
Direktor: Professor Dr. Dr. h. c. Carl Friedrich Gethmann

The Europäische Akademie

The *Europäische Akademie zur Erforschung von Folgen wissenschaftlich-technischer Entwicklungen GmbH* is concerned with the scientific study of consequences of scientific and technological advance for the individual and social life and for the natural environment. The Europäische Akademie intends to contribute to a rational way of society of dealing with the consequences of scientific and technological developments. This aim is mainly realised in the development of recommendations for options to act, from the point of view of long-term societal acceptance. The work of the Europäische Akademie mostly takes place in temporary interdisciplinary project groups, whose members are recognised scientists from European universities. Overarching issues, e.g. from the fields of Technology Assessment or Ethic of Science, are dealt with by the staff of the Europäische Akademie.

The Series

The series Ethics of Science and Technology Assessment *(Wissenschaftsethik und Technikfolgenbeurteilung)* serves to publish the results of the work of the Europäische Akademie. It is published by the academy's director. Besides the final results of the project groups the series includes volumes on general questions of ethics of science and technology assessment as well as other monographic studies.

Acknowledgement

The symposium "New Applications of Genetic Engineering in Livestock" ("Neue Anwendungen der Gentechnologie bei Nutztieren") was jointly realised by the Europäische Akademie together with the Berlin-Brandenburgische Akademie der Wissenschaften, Berlin. It was conducted in the course of the project "Pharming. Gentechnisch veränderte Pflanzen und Tiere als Arzneimittel-Produktionsstätten der Zukunft? Vergleich von Innovationshemmnissen und Durchsetzungschancen" of the Europäische Akademie and was supported by the Federal Ministry of Education and Research (Bundesministerium für Bildung und Forschung, Förderungskennzeichen 16|1547). In addition, it was supported by the Hermann und Elise geborene Heckmann Wentzel-Stiftung at the Berlin-Brandenburgische Akademie der Wissenschaften, Berlin. The authors of this study are responsible for the content.

Preface

The Europäische Akademie deals with the scientific study of the consequences of scientific and technological advances for individuals and society, as well as for the natural environment with the lifesciences being an important focus of its work.

The genetic engineering in animal species traditionally used as livestock has experienced a renaissance in recent time caused by the huge advances in the methods of animal biotechnology and by an increasing amount of sequencing information available. Applications reach from the use of transgenic animals for the production of biopharmaceuticals, to the alteration of the meat or milk quality or to the improvement of disease resistance of the animals. Besides these expected beneficial traits, livestock genetic engineering also raises a number of complex moral, legal and social questions as well as questions on the animal welfare of transgenic livestock that yet have not been thoroughly discussed. To initiate discussion the Europäische Akademie and the Berlin-Brandenburgische Akademie der Wissenschaften jointly organized a symposium on "New applications of livestock genetic engineering" that took place from 21st to 22nd of September 2007 in Berlin. The results of the symposium are published in these proceedings. The symposium was carried out in connection with an interdisciplinary project group of the Europäische Akademie on 'Pharming'. The results of this project are published in parallel to these proceedings in the same book series (volume 35): "Pharming. Promises and risks of biopharmaceuticals derived from genetically modified plants and animals".

I would like to thank the authors Joseph Carnwath, Ph.D. (Neustadt, Germany), Professor Dr. Louis-Marie Houdebine (Jouy en Josas, France), Professor Dr. Matthias Kaiser (Oslo, Norway), Dr. Wilfried Kues (Neustadt, Germany), Professor Dr. Heiner Niemann (Neustadt, Germany), Professor Angelika Schnieke, Ph.D. (Munich, Germany), Dr. Cornelius Van Reenen (Lelystad, The Netherlands), Professor Gary Walsh, Ph.D. (Limerick, Republic of Ireland) and the scientific coordinators of the symposium Dr. Margret Engelhard (Bad Neuenahr-Ahrweiler, Germany), Kristin Hagen, Ph.D. (Bad Neuenahr-Ahrweiler, Germany) and Dr. Mathias Boysen (Berlin, Germany) for their commitment to this project. Especially I would like to thank one of the authors, Professor Schnieke, for her great support during the planning phase of the symposium. In addition, I thank Katharina Mader and Friederike Wütscher from the Europäische

Akademie for the organisation of the symposium and the editing of this proceeding.

The Federal Ministry of Education and Research (Bundesministerium für Bildung und Forschung, BMBF) and the Hermann und Elise geborene Heckmann Wentzel-Stiftung is hereby acknowledged for the funding of the symposium.

Bad Neuenahr-Ahrweiler, July 2008 Carl Friedrich Gethmann

Foreword

Last year, the first "animal pharming" product reached market approval: it is a recombinant human protein for medicinal use that is produced in the milk of transgenic goats. Products from other transgenic animals, including „environmentally friendly" pigs and faster-growing salmon, are awaiting regulatory approval. These are only some examples of upcoming applications of genetic engineering in animal species traditionally used as livestock, or farm animals. Other applications include enhancement of productivity (e.g., higher milk or meat yields), alterations of product quality (e.g., leaner meat), and disease resistance. Also, transgenic livestock may be used for xenotransplantation, where genetic engineering is hoped to improve the animals' suitability as organ donors for humans.

The possibility of using genetic engineering in agricultural animals to enhance production or to enable the development of novel products has been inspiring research right from the start of the age of genetic engineering. However, advances proved slower in large animals than in mice, which are nowadays genetically modified in numerous biomedical applications. In addition, low public acceptance of agricultural products from transgenic animals was expected on the basis of public reactions to genetically modified agricultural plants. Transgenic farm animal projects have, thus, been partly on hold. Yet there might be a new rise in the area: livestock genetic engineering is becoming increasingly feasible as the techniques to remove, modify, replace or add genes are being refined, and an increasing amount of genomic information about farm animals can be utilised.

While genetic engineering in livestock opens a huge range of possibilities, it also raises safety and justification concerns: does genetic engineering affect animal welfare? Is it safe and morally acceptable to apply genetic engineering to farm animals for the various purposes that are envisaged? It appears that after a bout of interest in the context of the media attention around "Dolly", the sheep, the topic of animal genetic engineering and cloning has moved into the background compared with genetic engineering in plants, possibly because the practical applications of transgenic animals have hitherto rarely left the laboratories.

It is against this background that the Europäische Akademie and the Berlin-Brandenburgische Akademie der Wissenschaften addressed the topic of transgenic farm animals in an interdisciplinary symposium in 2007. The aim of the symposium was to put the topic of genetically modified pro-

duction animals on the public agenda again and to start its interdisciplinary evaluation by bringing together some of the relevant scientific disciplines. We feel that this aim was met with the talks and the discussions at the symposium. In these proceedings we have assembled written contributions based on the majority of the talks: the state of the art of the technology and its applications (contributions of Niemann et al., Houdebine), the specific applications in the production of biopharmaceuticals (contribution by Schnieke on historical industrial development and technology, and by Walsh on the market for biopharmaceuticals), the ethical aspects (Kaiser), and animal welfare considerations (Van Reenen). We hope that this can provide readers with a useful introduction to the topic, and serve as a basis for further dialogue.

Bad Neuenahr-Ahrweiler and Berlin, July 2008

Margret Engelhard
Kristin Hagen
Mathias Boysen

List of Authors

Carnwath, Joseph W., Ph.D.; B.A. from Yale University, USA, 1966; M.Sc. in biology from Lehigh University, USA, 1976; Ph.D. from Cambridge University, England; thesis on intracellular electron probe microanalysis of Na/K, 1984. Post doctoral research on the mdx mouse model of Duchenne muscular dystrophy at Oxford University from 1983 to 1987. Post doctoral research on transgenic mouse models of liver carcinogenesis at the Fraunhofer Institute of Toxicology and Experimental Medicine in Hannover, Germany, from 1987 to 1991. Since 1991, senior researcher at the department of biotechnology, Institute of Farm Animal Genetics – Federal Ministry of Food, Agriculture and Consumer Protection (BMELV), Germany. Main research areas: transgenic animal production, gene expression patterns in oocytes and preimplantation embryos, epigenetic control of gene expression, stem cell production, xenotransplantation, cell therapy.
Address: Friedrich-Loeffler-Institute, Institute of Farm Animal Genetics, Mariensee, Höltystr. 10, 31535 Neustadt, Germany.

Houdebine, Louis-Marie, Professor Dr.; has studied mechanisms of animal gene expression and particularly of milk protein gene expression for forty years in the French National Institute of Research in Agronomy. He has developed biotechnology projects implying transgenic mice and rabbits for the generation of models to study human diseases and for the preparation of pharmaceutical proteins in milk. This led to the creation of a biotech company BioProtein Technology (www.bioprotein.com) which still collaborates with the INRA laboratory. Among the models are transgenic rabbits developed to study disorder of lipid metabolism and those expressing the green fluorescent protein which are being used for studying embryonic stem cells, cloning and tissue grafting. Houdebine has been an expert in several national biosafety committees for several years. He has also been expert for particular evaluations in WHO, FAO, EFSA, Codex Alimentarius and OECD. For six years he participated in a European Bioethic Course (BioTethics and BIOTETHED). He teaches biotechnology at universities and high schools in France. He also participates in public debates on GMOs and cloning specially in France.
Address: National Institute for Agronomic Research (INRA), 78352 Jouy en Josas, France.

Kaiser, Matthias, Professor Dr. phil.; Dr. phil. in philosophy at the Universität Frankfurt, after studies at the universities of München (Ludwig-Maximilians-Universität), Frankfurt a.M., Oslo and Stanford. Director of the National Committee for Research Ethics in Science and Technology (NENT), Norway, since 1991, a position including own research, with acknowledged competence as full professor in philosophy of science since 1996. Until 2006 he was also adjunct professor for philosophy of science in the doctoral education program at the College of Architecture (AHO) in Oslo. His main work and areas of expertise are in the fields of philosophy of science, ethics of science, and technology assessment. His areas of competence include social studies of science and technology, history of science, ethics, logic, and history of philosophy. For many years Kaiser's research activities have taken place in a multi- and inter-disciplinary environment. He is directly involved in matters of science and technology policy. Through detailed case studies he has considerable insights in diverse fields of science, such as the history of plate tectonics, aquaculture, and biotechnology. Another topic of detailed study is the Precautionary Principle, particularly in environmental science. His leading role in the conduct of three Norwegian consensus conferences has led to a special interest in participatory policy tools and their use in ethical debate. Kaiser is currently President of the European Society for Agricultural and Food Ethics (EurSafe).
Address: The National Committee for Research Ethics in Science and Technology in Norway, Prinsensgate 18, P.O. Box 522 Sentrum, 0105 Oslo, Norway.

Kues, Wilfried A., Dr.; Diploma in biology at the Universität Göttingen 1990. Ph.D. 1994 at the Universität Heidelberg on a thesis exploring gene regulation of ligand-gated ion channels in skeletal muscle. 1995 postdoc at the Universität Zürich, Switzerland, generating knock-out mice models for Alzheimer's disease. Since 1998 researcher at the department of biotechnology of the Institute of Farm Animal Genetics, Mariensee, Germany (Federal Ministry of Food, Agriculture and Consumer Protection, BMELV). Main research areas: stem cells, transgenesis of large animals, gene regulation, epigenetics and conditional gene regulation.
Address: Friedrich-Loeffler-Institute, Institute of Farm Animal Genetics, Mariensee, Höltystr 10, 31535 Neustadt, Germany.

Niemann, Heiner, Professor Dr.; Dr. med. vet. from the University of Veterinary Sciences on a thesis exploring fluorochromes to assess the viability of preimplantation embryos, Hannover, 1980; Habilitation from the same university, 1987; Professor for reproductive biology from the same university, 1994; head of department of biotechnology at the Institute of Farm Animal Genetics 1987 (Federal Ministry of Food, Agriculture and Consumer Protection, BMELV); Guest professorships at Monash University,

Melbourne, Australia, 2004, and Kinki University, Wakayama, Japan, 2007; member of the Board of Governors of the International Embryo Transfer Society (IETS) 1988–1990 and 1994–1996; IETS-President 1988/1989. Main research areas: regulation of oocyte and preimplantation development in livestock species, transgenic animals, somatic cloning, genetic diversity, epigenetics, stem cells.
Address: Friedrich-Loeffler-Institute, Institute of Farm Animal Genetics, Mariensee, Höltystr 10, 31535 Neustadt, Germany.

Schnieke, Angelika, Professor Dr.; Professor of livestock biotechnology at the Technische Universität München, Germany. She gained a diploma in bioengineering at the Fachhochschule Hamburg and her Ph.D. for a thesis entitled "Cell-mediated transgenesis in livestock" from the University of Edinburgh. Her research interests are the genetic manipulation of mammals to understand and combat human disease. Her early work was with Professor Rudolf Jaenisch, first at the Heinrich-Pette Institute, Hamburg and later at the Massachusetts Institute of Technology, and focussed primarily on retroviral vectors for gene therapy and insertional mutagenesis in mice. During this time she produced the first model of a human disease – a lethal disorder arising from collagen dysfunction and later an accurate model of human osteogenesis imperfecta type 1 (brittle bone disease) by a dominant negative mutant transgene. She subsequently joined Colorado State University where her research extended to the production of transgenic livestock. From 1992–2003 she worked with the biotechnology company PPL Therapeutics in Edinburgh, becoming Assistant Director of Research in 2001. Her research at PPL centered on the production of pharmaceutical proteins in the milk of transgenic large animals and generation of xenotransplantation donors. She developed key technologies, most notably somatic cell nuclear transfer – Dolly the sheep, in collaboration with Ian Wilmut of the Roslin Institute. In 1997 she reported the first transgenic animal produced by nuclear transfer – a sheep carrying human clotting factor IX, for which she was awarded 'paper of the year' by the journal "Science". This was followed shortly afterwards by the first gene-targeted large animal. Current research activities include the generation of large animal models of serious human disease and the development of novel techniques for genome engineering in livestock species: particularly animal stem cells, artificial chromosomes and RNA inhibition.
Address: Lehrstuhl für Biotechnologie der Nutztiere, Technische Universität München, WZW Weihenstephan, Hochfeldweg, 1, 85354 Freising, Germany.

Van Reenen, Cornelis G., Dr.; studied animal sciences at Wageningen University, The Netherlands, and graduated in 1988. Following military service, he became a civil servant in the Veterinary Service of the Dutch Ministry of

Agriculture. From 1991 until present he has been employed as a researcher at various agricultural research institutes. Between 1991 and 1996 he was involved in studies looking at the welfare of the first transgenic bull "Herman" (produced by the company Gene Pharming) and his offspring. At present, he is senior project leader at the Animal Sciences Group of Wageningen University and Research Centre (WUR) in Lelystad, The Netherlands, Cluster Animal Welfare. Current research topics include: the genetics of adaptive capacities in dairy cattle and poultry, feeding strategies and welfare in veal calves, and the development of systems for on-farm monitoring of farm animal welfare (part of the multinational European project Welfare Quality).
Address: Animal Sciences Group of WUR, Edelhertweg 15, P.O. Box 65, 8200 AB Lelystad, The Netherlands.

Walsh, Gary, Professor, Ph. Dr.; was awarded his Ph.D. degree from the National University of Ireland at Galway in 1989. He is currently Associate Professor of Biotechnology at the University of Limerick, Ireland. Prior to joining the University of Limerick he worked within industry for several years and was a visiting Fulbright Professor at the University of Georgia in the United States. His research interests span various aspects of pharmaceutical and enzyme biotechnology, and he has supervised (and continues to supervise) 20 post graduate and post doctoral students. He has also acted as scientific secretary and as a member of the board of governors of the European Association of Pharmaceutical Biotechnology. Walsh teaches various aspects of pharmaceutical biotechnology at both undergraduate and post graduate level and served as acting Dean, Teaching and Learning, at the University of Limerick in 2004. He is also a former recipient of both his University's excellence in teaching award and special achievement in research award.
Address: Department of Chemical and Environmental Sciences, University of Limerick, Limerick, Republic of Ireland.

Table of Contents

Preface VII

Foreword IX

List of Authors XI

Transgenic Farm Animals: Current Status and Perspectives for Agriculture and Biomedicine
Heiner Niemann, Wilfried Kues and Joseph W. Carnwath 1

Methods to Generate Transgenic Animals
Louis-Marie Houdebine 31

Animal Pharming: Past Experience and Future Prospects
Angelika Schnieke 49

Market Development of Biopharmaceuticals
Gary Walsh 69

Ethical Aspects of Livestock Genetic Engineering
Matthias Kaiser 91

Assessing the Welfare of Transgenic Farm Animals
Cornelis G. Van Reenen 119

Transgenic Farm Animals: Current Status and Perspectives for Agriculture and Biomedicine[1]

Heiner Niemann, Wilfried Kues, Joseph W. Carnwath

Summary

The first transgenic livestock were produced in 1985 by microinjection of foreign DNA into zygotic pronuclei. This was the method of choice for more than 20 years, but more efficient protocols are now available, based on somatic cell nuclear transfer (SCNT) which permits targeted genetic modifications. Although the efficiency of transgenic animal production by microinjection technology is low, many animals with agriculturally important transgenic traits were produced. Typical applications included improved carcass composition, lactational performance, and wool production as well as enhanced disease resistance and reduced environmental impact. Transgenic animal production for biomedical applications has found broad acceptance. In 2006 the European Medicines Agency (EMEA) approved the commercialization of the first recombinant protein drug produced by transgenic animals. Recombinant antithrombin III, produced in the mammary gland of transgenic goats, was launched as ATryn® for prophylactic treatment of patients with congenital antithrombin deficiency. Pigs expressing human immunomodulatory genes have contributed to significant progress in xenotransplantation research with survival periods of non-human primates receiving transgenic porcine hearts or kidneys approaching six months. Lentiviral vectors and small interfering ribonucleic acid (siRNA) technology are also emerging as important tools for transgenesis. As the genome sequencing projects for various farm animal species progress, it has become increasingly practical to target the removal or modification of individual genes. We anticipate that this approach to animal breeding will be instrumental in meeting global challenges in agricultural production in the future and will open new horizons in biomedicine.

1 Introduction: Transgenic Technologies for Farm Animals

The production of transgenic farm animals is extraordinarily labor and cost intensive and depends upon advanced techniques in molecular biology,

[1] This contribution is mainly based on the following reviews of the authors: Niemann et al. 2005; Niemann and Kues 2007.

cell culture, reproductive biology and biochemistry. The transfer of the foreign DNA is only one step in this process.

Critical steps involved in the production of transgenic farm animals are:

- Identification of the gene (genome analysis);
- Cloning of the gene;
- Production of a suitable gene construct;
- Transfer of the gene;
- Proof of integration of the foreign gene;
- Proof of expression (mRNA, protein);
- Demonstration of transmission (inheritance);
- Selective breeding.

Table 1: Milestones (live offspring) in transgenesis and somatic cloning in farm animals. Modified from Niemann et al. 2005.

Year	Milestone	Strategy	Reference
1985	First transgenic sheep and pigs	Microinjection of DNA into one pronucleus of a zygote	Hammer et al. 1985
1986	Embryonic cloning of sheep	Nuclear transfer using embryonic cells as donor cells	Willadsen 1986
1997	Cloning of sheep with somatic donor cells	Nuclear transfer using adult somatic donor cells	Wilmut et al. 1997
1997	Transgenic sheep produced by nuclear transfer	Random integration of the construct	Schnieke et al. 1997
1998	Transgenic cattle produced from fetal fibroblasts and nuclear transfer	Random integration of the construct	Cibelli et al. 1998
1998	Generation of transgenic cattle by MMLV injection	Infection of oocytes with helper viruses	Chan et al. 1998
2000	Gene targeting in sheep	Gene replacement and nuclear transfer	McCreath et al. 2000
2002	Trans-chromosomal cattle	Artificial chromosome	Kuroiwa et al. 2002
2002	Knockout in pigs	Heterozygous knock-out	Dai et al. 2002; Lai et al. 2002
2003	Homozygous gene knock-out in pigs	Homozygous knock-out	Phelps et al. 2003
2003	Transgenic pigs via lentiviral injection	Gene transfer into zygotes via lentiviruses	Hofmann et al. 2003
2006	Conditional transgene expression in pigs (tet-off)	Pronuclear DNA injection and crossbreeding	Kues et al. 2006

The first successful gene transfer method in animals (mouse) was based on the microinjection of foreign DNA into zygotic pronuclei. This was used to produce the first transgenic livestock more than 20 years ago (Hammer et al. 1985). Despite the inherent inefficiency of microinjection technology, a broad spectrum of genetically modified large animals has been generated since then for applications in agriculture and biomedicine, with the use of transgenic livestock for 'gene pharming' already at the level of commercial exploitation (Kues and Niemann 2004; Niemann and Kues 2007).

However, microinjection has several major shortcomings including low efficiency, random integration and variable expression patterns which mainly reflect the site of integration. Research has focused on the development of alternate methodologies for improving the efficiency and reducing the cost of generating transgenic livestock. These include sperm mediated DNA transfer (Lavitrano et al. 1989; Lavitrano et al. 2002; Chang et al. 2002), intracytoplasmic injection (ICSI) of sperm heads carrying foreign DNA (Perry et al. 1999; Perry et al. 2001), injection or infection of oocytes and/or embryos by different types of viral vectors (Haskell and Bowen 1995; Chan et al. 1998; Hofmann et al. 2004), RNA interference technology (RNAi) (Clark and Whitelaw 2003) and the use of somatic cell nuclear transfer (SCNT) (Schnieke et al. 1997; Cibelli et al. 1998; Baguisi et al. 1999; Dai et al. 2002; Lai et al. 2002; table 1). To date, somatic cell nuclear transfer, which has been successful in 13 species, holds the greatest promise for significant improvements in the generation of transgenic livestock (figure 1). The typical success rate (live births) of mammalian somatic nuclear transfer is low and usually is only 1–2% of the transferred embryos. Cattle seem

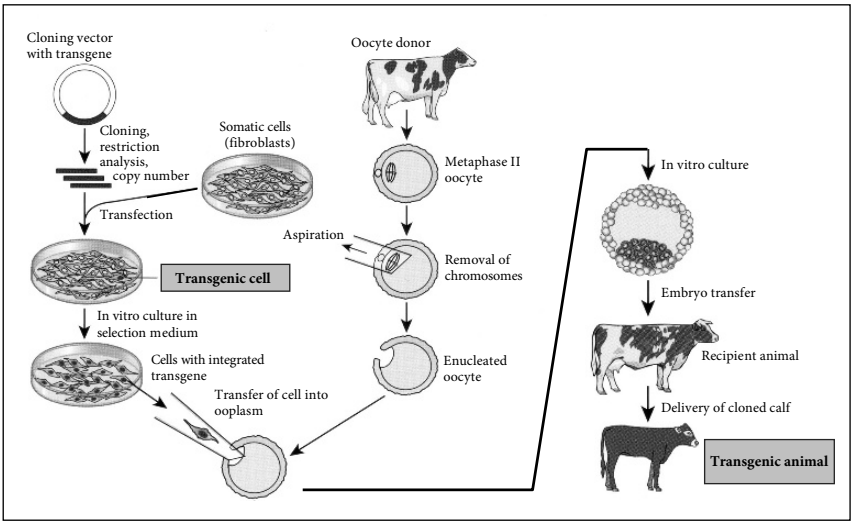


Figure 1: Scheme showing the production of transgenic farm animals by somatic cell nuclear transfer (SCNT)

to be an exception to this rule as levels of 15–20% can be reached (Kues and Niemann 2004). Recently, we have also obtained a significant improvement of porcine cloning efficiency by better selection and optimized treatment of the recipients, specifically by providing a 24h asynchrony between the pre-ovulatory oviducts of the recipients and the reconstructed embryos. Presumably, this gives the embryos additional time to achieve the necessary level of nuclear reprogramming. The improved protocol has resulted in pregnancy rates of ~80% and only a slightly reduced mean litter size (Petersen et al. 2007). These results show that the efficiency of SCNT is likely to be improved in the near future with significant impact on transgenic animal production.

Further qualitative improvements may be derived from technologies that allow precise modifications of the genome including targeted chromosomal integration by site-specific DNA recombinases, such as Cre or FLP, or methods that allow temporally and/or spatially controlled transgene expression (Capecchi 1989; Kilby et al. 1993). The genomes of farm animals (cattle, chicken, horse, dog, bee) have been sequenced and annotated in http://www.ensembl.org and http://www.ncbi.org (both July 2008). They, thus, provide new opportunities for selective breeding and transgenic animal production. After 12,000 years of domestic animal selection (Copley et al. 2003) based on the random mutations resulting from environmental factors such as radiation and oxidative injury, technology is now available to introduce or remove genes with known functions.

Here, we provide a comprehensive overview on the current status of transgenic animal production and look at future implications. We focus on large domestic species and do not cover recent developments in poultry breeding or in aquaculture.

2 Biomedical Applications of Transgenic Domestic Animals

2.1 Pharmaceutical Production in the Mammary Gland of Transgenic Animals

Gene 'pharming' entails the producti on of recombinant pharmaceutically active human proteins in the mammary gland or blood of transgenic animals. This technology overcomes the limitations of conventional and recombinant DNA based production systems (Meade et al. 1999; Rudolph 1999) and has advanced to the stage of commercial application (Ziomek 1998; Dyck et al. 2003; Schnieke this proceedings and Walsh this proceedings). The mammary gland is the preferred production site mainly because of the quantities of protein that can be produced in this organ using mammary gland specific promoter elements and established methods for extraction and purification of the respective protein (Meade et al. 1999; Rudolph 1999). Guidelines developed by the Food and Drug Administration (FDA)

of the USA require monitoring the animals' health in a specific pathogen free (SPF) facility, sequence validation of the gene construct, characterization of the isolated recombinant protein, and monitoring the genetic stability of the transgenic animals over several generations. This has necessitated, for example, the use of animals from scrapie free countries (New Zealand) and maintenance of production animals under strict hygienic conditions.

Several products derived from the mammary glands of transgenic goats and sheep have progressed to advanced clinical trials (Echelard et al. 2006). Phase III trials for antithrombin III (ATIII) (ATryn® from GTC-Biotherapeutics, USA), produced in the mammary gland of transgenic goats, have been completed and the recombinant product was approved as drug by the European Medicines Agency (EMEA) in August 2006. This protein is the first product from a transgenic farm animal to be accepted as a fully registered drug. ATryn® is registered for treatment of heparin resistant patients undergoing cardiopulmonary bypass procedures. GTC-Biotherapeutics has also expressed at least eleven other transgenic proteins in the mammary gland of transgenic goats at concentrations of more than one gram per liter. The enzyme α-glucosidase (Pharming BV) from the milk of transgenic rabbits has orphan drug status and has been successfully used for the treatment of Pompe's disease (van den Hout et al. 2001). Similarly, recombinant C1 inhibitor (Pharming BV), produced in the milk of transgenic rabbits, has completed phase III trials and is expected to receive registration in the near future. The overall global market for recombinant proteins from domestic animals is expected to exceed $ 1 billion in 2008 and to reach $ 18.6 billion in 2013.

An interesting new development is the production of recombinant proteins in the mammary gland of transgenic animals for use as antidotes against organophosphorus compounds used as insecticides in agriculture and chemical warfare. Butyrylcholinesterase is a potent prophylactic agent against these compounds. Recombinant butyrylcholinesterase has been produced at a concentration of 5g/liter in the mammary gland of transgenic mice and goats (Huang et al. 2007). The recombinant product was biologically active and had a half life in vitro which was sufficient to provide protection against organophophorus intoxication. Transgenic goats can produce sufficient butyrylcholinesterase to protect all humans at risk of organophosphorus poisoning.

Some gene constructs have failed to produce economically significant amounts of protein in the milk of transgenic animals indicating that the technology needs further refinement to insure consistent high-level expression. This is particularly true for genes having complex regulation, such as those coding for erythropoietin (EPO) or human clotting factor VIII (hFVIII) (Hyttinen et al. 1994; Massoud et al. 1996; Niemann et al. 1999). With the advent of transgenic plants that also produce pharmacologically active proteins, there is now an array of recombinant technologies that will

allow selection of an appropriate production system for each required protein (Ma et al. 2003). The use of somatic nuclear transfer will accelerate production of transgenic animals for mammary gland specific synthesis of recombinant proteins.

2.2 Antibody Production in Transgenic Animals

Numerous monoclonal antibodies are being produced in the mammary gland of transgenic goats (Meade et al. 1999) and cloned transgenic cattle have been created which produce a recombinant bi-specific antibody in their blood (Grosse-Hovest et al. 2004). When purified from serum, this antibody is stable and mediates target-cell restricted T cell stimulation and tumor cell killing. An interesting new development is the generation of trans-chromosomal animals. A human artificial chromosome (HAC), containing the complete sequences of the human immunoglobulin heavy and light chain loci, has been introduced into bovine fibroblasts, which were then used in nuclear transfer. Trans-chromosomal bovine offspring were obtained that expressed human immunoglobulin in their blood. This system is a significant step forward in the production of therapeutic human polyclonal antibodies (Kuroiwa et al. 2002). Follow-up studies showed that the HAC was maintained in most animals for several years in first generation cattle (Robl et al. 2007). How the HACs behave during meiotic cell divisions remains to be shown.

2.3 Blood Replacement

Functional human hemoglobin has been produced in transgenic swine. The transgenic protein could be purified from the porcine blood and showed oxygen binding characteristics similar to natural human hemoglobin. The main obstacle was that only a small proportion of porcine red blood cells contained the human form of hemoglobin (Swanson 1992). Alternate approaches to produce human blood substitutes have focused on linking hemoglobin to the superoxide-dismutase system (D'Agnillo and Chang 1998).

2.4 Xenotransplantation of Porcine Organs to Human Patients

Today more than 250,000 people are alive only because of a successful human organ transplantation (allotransplantation). Ironically, the success of organ transplantation technology has led to an acute shortage of appropriate organs, because cadaveric and live organ donation falls far short of meeting the demand in western societies. To close the growing gap between demand and availability of appropriate organs, transplant surgeons are now considering the use of xenografts from domesticated pigs (Platt and Lin 1998; Kues and Niemann 2004; Yang and Sykes 2007). Prerequisites for successful xenotransplantation are: (i) overcoming the immunological hurdles, (ii) preventing the transmission of pathogens from the donor animal to the human recipient, and (iii) compatibility of donor organs with human physiology.

With a discordant donor species such as the pig, it is necessary to overcome both hyperacute rejection (HAR) and acute vascular rejection (AVR). The two strategies that have been successfully explored for long term suppression of the HAR of porcine xenografts are: i) transgenic synthesis of human proteins regulating complement activity (RCAs) in the donor organ (Cozzi and White 1995; Bach 1998; Platt and Lin 1998) and ii) inactivation of the genes producing antigenic structures on the surface of the donor organ, e.g. the α-gal-epitope (Dai et al. 2002; Lai et al. 2002; Phelps et al. 2003). Prolonged survival of xenotransplanted porcine organs where the 1,3-α-galactosyltransferase (α-gal) gene has been knocked out has been demonstrated. Survival rates of up to six months have been achieved with transplanted porcine hearts (Kuwaki et al. 2005) and survival of up to three months has been obtained with kidneys transplanted from α-gal knockout pigs to baboons (Yamada et al. 2005).

The current approach to increasing survival time beyond six months is to create donor pigs with multiple transgenes that block a range of additional immunological barriers. To this end, we have recently produced triple transgenic pigs expressing either human thrombomodulin (hTM) or human heme oxygenase-1 (hHO-1) on top of one or two RCAs to suppress both HAR and the later stage coagulatory disorders observed in experimental porcine-to-primate xenotransplantation (Petersen et al. 2007; 2008). Reproducible survival of porcine xenografts for more than six months in non-human primate recipients is considered to be a necessary precondition to starting clinical trials with human patients. A particularly promising strategy for achieving long-term xenograft survival is to induce tolerance by creating permanent chimerism in the recipient by intraportal injection of embryonic stem cells (Fändrich et al. 2002) or by co-transplantation of vascularized thymic tissue (Yamada et al. 2005). Long term tolerance of HLA-mismatched kidneys has recently been demonstrated in humans (Kawai et al. 2008).

Extensive research has revealed that the risk of porcine endogenous retrovirus (PERV) transmission to human patients is low, opening the door for preclinical testing of xenografts (Switzer et al. 2001; Irgang et al. 2003). RNA interference (RNAi) is a promising method for knocking down the already low level of PERV expression in porcine somatic cells. Using RNAi mediated knockdown, PERV expression has been further reduced in porcine somatic cells for 4–6 months, these cells were successfully used in SCNT and gave normal piglets (Dieckhoff et al. 2007; Dieckhoff et al. 2008). RNAi mediated PERV expression knockdown provides an additional level of safety for porcine-to-human xenotransplantation.

Although additional refinements will always be possible, it is expected that appropriate lines of transgenic pigs will be available as organ donors within the next five to ten years. Transplantation of pancreatic islets from (transgenic) pigs may take place even earlier. Guidelines for the clinical

application of porcine xenotransplants already exist in the USA and are currently being developed in other countries. The worldwide consensus is that the technology is ethically acceptable provided that the individual's well-being does not compromise public health (e.g., the risk of PERV recombination). The improvement in quality of life for patients receiving conventional allotransplants is dramatic, but xenotransplantation is also economically attractive because the cost of maintaining patients with severe kidney disease on dialysis or long term treatment of patients with chronic heart disease can be greater than the cost of a successful transplantation. Preliminary functional data on porcine kidneys and hearts in non-human primates is promising although the long term effect of porcine organs on human physiology is to a great extent unexplored (Ibrahim et al. 2006).

2.5 Farm Animals as Models for Human Diseases

The physiology, anatomy, and life span of mice differ significantly from humans, making the rodent model inappropriate for many human diseases Farm animals, such as pigs, sheep or cattle, may be more appropriate models in which to study the treatment of human diseases such as artherosclerosis, non-insulin-dependent diabetes, cystic fibrosis, cancer and neuro-degenerative disorders, which require longer periods of observation than is possible with mice (Theuring et al. 1997; Palmarini and Fan 2001; Li and Engelhardt 2003; Hansen and Khanna 2004). Cardiovascular disease is increasing in ageing western societies where coronary artery diseases already account for the majority of deaths. Because genetically modified mice do not manifest myocardial infarction or stroke as a result of atherosclerosis, new animal models, such as pigs that exhibit similar pathologies, are needed to develop effective therapeutic strategies (Rapacz and Hasler-Rapacz 1989; Grunwald et al. 1999). An important porcine model has been developed for the rare human eye disease retinitis pigmentosa (PR) (Petters et al. 1997). Patients with PR suffer from night blindness early in life due to loss of photoreceptors. Transgenic pigs with a mutated rhodopsin gene have a phenotype quite similar to the human patients and effective treatments are being developed (Mahmoud et al. 2003).

An important aspect of SCNT derived large animal models of human diseases (and the development of regenerative therapies using these models) is that somatic cloning per se does not necessarily result in shortened telomeres as once feared and thus does not necessarily lead to premature ageing (Schätzlein and Rudolph 2005). Telomeres are the repetitive DNA sequences at the ends of the chromosomes and are crucial for their structural integrity and function and are thought to be related to lifespan. Telomere shortening is correlated with severe limitation of the regenerative capacity of cells, the onset of cancer, ageing and chronic dis-

ease with significant impact on human lifespan (Schätzlein and Rudolph 2005). Expression of telomerase, which is the enzyme primarily responsible for the formation and rebuilding of telomeres, is suppressed in most somatic tissues postnatally. However, recent studies have revealed that telomere length is (re-)established early in preimplantation development at the morula-blastocyst transition due to telomerase activity (Schätzlein et al. 2004).

3 Transgenic Animals in Agriculture

Agricultural exploitation of transgenic animal technology lags behind applications in biomedicine (Kues and Niemann 2004). Nevertheless, table 2 gives an overview of work in the production of animals transgenic with improved agricultural traits.

3.1 Carcass Composition

Transgenic pigs bearing a hMT-pGH construct (human metallothionein promoter driving the porcine growth hormone gene) showed significant improvement in economically important traits including growth rate, feed conversion and body composition (muscle/fat ratio) without the pathological phenotype seen with earlier GH constructs (Pursel et al. 1989; Nottle et al. 1999). Similarly, transgenic pigs carrying the human insulin-like growth factor-I gene (hIGF-I) had ~30% larger loin mass, ~10% more carcass lean tissue and ~20% less total carcass fat (Pursel et al. 1999). Unfortunately, commercialization of these pigs has been postponed due to the current lack of public acceptance of genetically modified foods.

An important step towards the production of more healthful pork products was made by creating pigs with a desaturase gene, derived either from spinach or from Caenorhabditis elegans, which increases the non-saturated fatty acid content in the skeletal muscles of these animals. The higher ratio of unsaturated to saturated fatty acids means more healthful pork, since it is well known that a diet rich in non-saturated fatty acids is associated with a reduced risk of stroke and coronary diseases in humans (Niemann 2004; Saeki et al. 2004; Lai et al. 2006).

3.2 Lactation

The physicochemical properties of milk are mainly due to the ratio of casein variants, making these a prime target for the improvement of milk composition. Dairy production is an attractive field for targeted genetic modification (Yom and Bremel 1993; Karatzas and Turner 1997) and it is possible to produce milk with a modified lipid composition by modulation of the enzymes involved in lipid metabolism and to increase curd and cheese yield by enhancing expression of the casein gene family in the mammary gland. The bovine casein ratio has already been altered by

Table 2: Approaches to generated transgenic livestock for agricultural production

Transgenic trait	Key molecule	Construct	Gene transfer method	Species	Reference
Increased growth rate, less body fat	Growth hormone (GH)	hMT-pGH	micro-injection	pig	Nottle et al. 1999
Increased growth rate, less body fat	Insulin-like growth factor-1 (IGF-1)	mMT-hIGF-1	micro-injection	pig	Pursel et al. 1999
Increased level of poly-un-saturated fatty acids in pork	Desaturase (from spinach)	maP2-FAD2	micro-injection	pig	Saeki et al. 2004
Increased level of poly-un-saturated fatty acids in pork	Desaturase (from C. elegans)	CAGGS-hfat-1	somatic cloning	pig	Lai et al. 2006
Phosphate metabolism	Phytase	PSP-APPA	micro-injection	pig	Golovan et al. 2001
Milk composition (lactose increase)	α-lactalbumin	genomic bovine α-lactal-bumin	micro-injection	pig	Wheeler et al. 2001
Influenza resistance	Mx protein	mMx1-Mx	micro-injection	pig	Müller et al. 1992
Enhanced disease resistance	IgA	α,K-α,K	micro-injection	pig, sheep	Lo et al. 1991
Wool growth	Insulin-like growth factor-1 (IGF-1)	Ker-IGF-1	micro-injection	sheep	Damak et al. 1996a,b
Visna Virus resistance	Visna virus envelope	visna LTR-env	micro-injection	sheep	Clements et al. 1994
Ovine prion locus	Prion protein (PRNP)	targeting vector (homologous recombination)	somatic cloning	sheep	Denning et al. 2001 (animals dead shortly after birth)
Milk fat composition	Stearoyl desaturase	β-lactogl-SCD	microin-jection	goat	Reh et al. 2004
Milk composition (increase of whey proteins)	β-casein/ κ-casein	genomic CSN2 CSN-CSN-3	somatic cloning	cattle	Brophy et al. 2003
Milk composition (increase of lactoferrin)	human lactoferrin	α-s2cas-mLF	microin-jection	cattle	Platen-burg et al. 1994
Staphylococcus aureus mastitis resistance	Lysostaphin	ovine β-lactogl-lysostaphin	somatic cloning	cattle	Wall et al. 2005

From Niemann and Kues 2007.

the over-expression of beta- and kappa-casein, demonstrating the potential of transgenic technology for improving the economic value of bovine milk (Brophy et al. 2003). It should also be possible to create 'hypoallergenic' milk by knocking out or knocking down the β-lactoglobulin gene. One could envision the production of enhanced 'infant milk' containing human lactoferrin or the production of milk which resists bacterial contamination by expressing lysozyme, the antibacterial component of egg white and human tears. To generate lactose-free milk, a knockout or knockdown at the α-lactalbumin locus would suppress this key step in milk sugar synthesis. Lactose reduced or lactose-free milk would render dairy products suitable for consumption by the large proportion of the world's adult population who do not produce an active intestinal lactase. Lactose is the major osmotically active substance in milk and its absence might be expected to interfere with milk secretion. However, a lactase construct has been tested in the mammary gland of transgenic mice and in hemizygous mice; this reduced lactose content by 50–85% without altering milk secretion (Jost et al. 1999). On the other hand, experimental transgenic mice with a homozygous knockout for α-lactalbumin could not nurse their offspring because of the high viscosity of their milk (Stinnakre et al. 1994). In the pig, increased transgenic expression of a bovine lactalbumin construct in the mammary gland resulted in increased lactose content and increased milk production which resulted in improved survival and development of the piglets (Wheeler et al. 2001). Increased survival of piglets at weaning would provide significant commercial benefits to the producer and improved animal welfare. These findings demonstrate the feasibility of producing significant alterations in milk composition by application of an appropriate transgenic strategy.

3.3 Wool Production

Transgenic sheep carrying a keratin-IGF-I construct expressed in their skin produced 6.2% more clear fleece than non-transgenic controls and no adverse effects on health or reproduction were observed (Damak et al. 1996a, b). Similar efforts to alter wool production by transgenic modification of the cystein pathway have met with more limited success, although it is known that cystein is the rate limiting biochemical factor for wool growth (Ward 2000).

3.4 Environmentally Friendly Farm Animals

Phytase transgenic pigs have been developed to address the problem of manure-related environmental pollution. These pigs carry a bacterial phytase gene under transcriptional control of a salivary gland specific promoter, which allows the pigs to digest plant phytate. Without the bacterial enzyme, phytate passes through the animal undigested and pollutes the environment with phosphorus if uncontained. With the bacterial enzyme,

fecal phosphorus output was reduced up to 75% (Golovan et al. 2001). These environmentally friendly pigs may be used for commercial production in Canada within the next few years.

3.5 Transgenic Animals and Disease Resistance

3.5.1 Transgenic Strategies to Increase Disease Resistance

In most cases, susceptibility to pathogens represent the interplay of numerous genes, i.e. the trait is polygenic in nature. However, some genetic loci are known to confer resistance against specific diseases. Transgenic strategies to enhance disease resistance include the transfer of major histocompatibility-complex (MHC) genes, T cell receptor genes, immunoglobulin genes, genes that affect lymphokines, or specific disease resistance genes (Müller and Brem 1991). A prominent example for a specific disease resistance gene is the murine Mx-gene. Production of the Mx1-protein is induced by interferon. This was discovered in inbred mouse strains that were resistant to influenza viruses (Staeheli 1991). Microinjection of an interferon- and virus-inducible Mx-construct into porcine zygotes resulted in two transgenic pig lines which expressed the Mx-mRNA; but no Mx protein was detected (Müller et al. 1992). The bovine MxI gene was identified and shown to confer antiviral activity when transfected into in Vero cells (Baise et al. 2004).

Transgenic constructs bearing the immunoglobulin-A (IgA) gene have been successfully introduced into pigs, sheep and mice in an attempt to increase resistance against infections (Lo et al. 1991). Expression of the murine IgA gene was successful in two transgenic pig lines but only the light chains could be detected and the IgA-molecules showed only marginal binding to phosphorylcholine (Lo et al. 1991). On the other hand, high levels of monoclonal murine antibodies with a high binding affinity for their specific antigen have been produced in transgenic pigs (Weidle et al. 1991).

Attempts to increase ovine resistance to Visna virus infection by transgenic production of Visna envelope protein have been reported (Clements et al. 1994). The transgenic sheep developed normally and expressed the viral gene without pathological side effects. However, the transgene was not expressed in monocytes, the target cells of the viral infection, and antibodies were detected after artificial infection of the transgenic animals (Clements et al. 1994).

Passive immunity has been induced against an economically important porcine disease in a transgenic mouse model (Castilla et al. 1998). These transgenic mice secrete a recombinant antibody in their milk that neutralized the corona virus responsible for transmissible gastroenteritis (TGEV) and this conferred resistance to TGEV. Strong mammary gland specific expression was achieved over the entire period of lactation. Extension of this work to pigs is promising.

Knockout of the prion protein is the only secure way to prevent infection and transmission of spongiform encephalopathies including scrapie and BSE (Weissmann et al. 2002). It was possible to knock out the ovine prion locus; however, the cloned lambs carrying the knockout locus died shortly after birth (Denning et al. 2001). On the other hand, cloned cattle with a knockout for the prion locus have been successfully produced and indeed show clear evidence of resistance to BSE infection (Richt et al. 2007). Transgenic animals with modified prion genes will be an appropriate model for studying the development of spongiform encephalopathies in humans and are crucial for developing strategies for the elimination of prion carriers from the farm animal population. This work is a prerequisite for the future production of recombinant proteins for human medicine in the blood or the mammary glands of transgenic cattle.

3.5.2 Transgenic Approaches to Increased Disease Resistance in the Mammary Gland

The level of anti-microbial peptides (lysozyme and lactoferrin) in human milk is many times higher than in bovine milk and transgenic expression of the human lysozyme gene in mice causes a significant reduction in bacterial contamination and a reduced frequency of mammary gland infections (Maga et al. 1995; Maga and Murray 1995). Lactoferrin has bactericidal and bacteriostatic effects in addition to being the main source of iron in milk. These properties make an increase in lactoferrin levels in the bovine mammary gland and are a practical way to improve milk quality. Human lactoferrin has, in fact, been expressed at high levels in the milk of transgenic mice and cattle (Krimpenfort et al. 1991; Platenburg et al. 1994) and was associated with an increased resistance against mammary gland diseases (van Berkel et al. 2002). Similarly, lysostaphin was shown to confer specific resistance against mastitis caused by Staphylococcus aureus. Mastitis resistant cows have been produced by expressing a lysostaphin gene construct in the mammary gland (Wall et al. 2005).

4 Transgenic Pets

As discussed above, most work in transgenic technology has focussed on livestock species either for biomedical or agricultural purposes. However, the methodology is becoming routine and recent applications include the development of new varieties of ornamental fish. For example, fluorescent green transgenic medaka (Oryzias latipes, rice fish) have been produced and approved for sale in Taiwan (Chou et al. 2001). The fluorescent medaka is currently marketed by the Taiwanese company Taikong. The "GloFish®" is a trademarked transgenic zebra fish (Danio rerio) expressing red fluorescent protein from a sea anemone under the transcriptional control of a muscle-specific promoter (Gong et al. 2003). Green and yellow fluores-

cent proteins have also been introduced into the zebra fish to give different fluorescence colors. Yorktown Technologies (www.glofish.com, July 2007) initiated commercial sales of the transgenic zebrafish in the United States with retail prices of approximately $ 5,00 each. The GloFish® thus became the first transgenic animal freely distributed throughout the USA. A recent report of the FDA contained no evidence that GloFish® represents a risk (US FDA, 2003). Commercialization of fluorescent fish has gone forward in several countries other than the USA, including Taiwan, Malaysia, and Hongkong, whereas marketing in Australia, Canada and European Union is currently prohibited.

5 Emerging Transgenic Technologies

Transgenic applications will undoubtedly become more widespread if even more efficient gene transfer methods will be developed and specific genetic traits can be targeted. Some of the emerging technologies are described below.

5.1 Lentiviral Mediated Transgenesis

Lentiviruses have proven to be efficient vectors for the delivery of genes into oocytes and zygotes. For example, transgenic cattle have been produced by injecting lentiviral vectors into the perivitelline space of oocytes (Hofmann et al. 2004) and injection of lentiviruses into the perivitelline space of porcine zygotes resulted in a high proportion of transgene expressing founder animals from which several lines of transgenic pigs were established (Hofmann et al. 2003; Whitelaw et al. 2004). Lentiviral mediated gene transfer in livestock has generated unprecedented high yields of transgenic animals due to multiple integration events. Unfortunately, multiple integration brings the disadvantage that here is an increased probability of unwanted side effects caused by oncogene activation or insertional mutagenesis. Additional problems which have been identified are gene silencing due to the presence of viral sequences (Hofmann et al. 2006) and a high frequency of mosaicism in founder animals.

5.2 Conditional Transgenesis in Farm Animals

Transgenic mice and farm animals harbouring the first-generation of conditional promoter elements showed expression in response to heavy metals or steroid hormones but suffered from high basal expression levels and pleiotropic effects (Lee et al. 1981; Mayo et al. 1982; Miller et al. 1989; Pursel et al. 1989). Newer, binary expression systems based on prokaryotic control elements are responsive to exogenous IPTG (Isopropyl-β-D-thiogalactopyranosid), RU-486, ecdysone or tetracycline derivatives and give more tightly controlled expression (Lewandowski 2001; Corbel and Rossi 2002). The first tetracycline system that was successfully used in mice

required two DNA constructs. One was for doxycycline controlled expression of a transactivator and the other contained regulatory elements which conferred transactivator dependent expression of the target gene. These DNA constructs were typically integrated into two different lines of transgenic mice. After crossbreeding the two lines of transgenic mice, their offspring expressed the target gene only after stimulation with doxycycline (Furth et al. 1994). Unfortunately, the long generation intervals make this approach unfeasible in livestock species (Niemann and Kues 2003).

Recently, we reported on a tetracycline-responsive bicistronic expression cassette (NTA) in which expression was amplified by transactivator mediated positive feedback (Kues et al. 2006). This was used to produce the first tetracycline controlled transgenic expression in a farm animal. The auto-regulatory cassette was integrated at a single chromosomal site in the pig genome after pronuclear DNA injection (Kues et al. 2006) and was designed to give ubiquitous expression of a human regulator of complement activation (RCA) independent of cellular transcription cofactors. Expression from this construct could be inhibited reversibly by feeding the animals doxycycline (tet-off system). In ten transgenic pig lines in which only one copy of the NTA cassette was integrated, the transgene was silenced in all tissues with the exception of skeletal muscle where expression was limited to a small number of discrete muscle fibers (Niemann and Kues 2003). However, crossbreeding of lines to produce animals with two NTA cassettes resulted in reactivation of the cassettes and strong, tissue-independent, tetracycline-sensitive RCA expression. It seems that crossing the transgenic pig lines, which doubled the level of transactivator, was able to overcome epigenetic silencing. Transgene expression in the double transgenic pigs was inversely correlated with the level of NTA cassette DNA methylation (Kues et al. 2006). This approach highlights the importance of understanding epigenetic (trans)-gene regulation in the pig.

5.3 Use of Pluripotent Cell Lines

Pluripotent embryonic stem (ES) cells have the ability to participate in organ and even germ cell development following injection into blastocysts or aggregation with morulae (Rossant 2001). True ES cells (i.e. those able to contribute to the germ line) are currently only available from inbred mouse strains (Kues et al. 2005a). These murine ES cells have become an important tool for gene knock-out and knock-in experiments and to study large chromosomal rearrangements (Downing and Battey 2004). ES-like cells and primordial germ cell cultures have been reported for several farm animal species, and ES-like cells which can produce chimeric animals albeit without germ line contribution have been reported in swine (Anderson 1999; Shim et al. 1997; Wheeler 1994) and cattle (Cibelli et al. 1998). Recent data indicate that somatic stem cells have a much broader developmental potential than previously assumed (Jiang et al. 2002; Kues et al. 2005b).

Whether these cells will improve the efficiency of chimera generation or somatic nuclear transfer in farm animals has yet to be shown conclusively (Kues et al. 2005b; Hornen et al 2007). Pluripotent cells are a valuable tool for improved production of animals with targeted genetic modification.

A revolutionary breakthrough in direct nuclear reprogramming of mouse somatic cells was recently reported (Takahashi and Yamanaka 2006; Okita et al. 2007; Wernig et al. 2007). Cells transfected with constructs expressing Oct4, Sox2, Myc and Klf4, carried in retroviral vectors, were reprogrammed to a totipotent state and were indistinguishable from ES cells generated from fertilized embryos with regard to differentiation potential and morphology. These induced pluripotent stem cells (iPS), derived from somatic cells, were able to populate the germ line upon injection into blastocysts and after transfer into recipient mice, clearly indicating complete reprogramming (Okita et al. 2007; Wernig et al. 2007; Maherali et al. 2007). The same genes have recently been found to be effective in reprogramming human fibroblasts and other human somatic cells into cells with pluripotent properties (Takahashi et al. 2007). This affords a new approach to the generation of pluripotent cells from farm animal species.

5.4 Spermatogonial Transgenesis

Transplantation of transgenic primordial germ cells into the testes is potentially an alternative approach to the generation of transgenic animals. Initial experiments in mice showed that the depletion of endogenous spermatogonial stem cells by treatment with busulfan prior to transplantation is effective and permits re-colonization of the testes by donor cells. Transmission of the donor haplotype to the next generation after germ-cell transplantation has been achieved in goats (Honaramooz et al. 2003). Current major obstacles of this strategy are the lack of efficient in vitro culture methods for primordial germ/prospermatogonial cells and the lack of efficient gene transfer techniques for these cells. Recently adeno-associated virus (AAV) was found to be suitable for delivering transgenes to infect a mal germ cell and germline tansmission was reported in goats and mice (Honaramooz et al. 2007). The efficiency of this approach and putative silencing of the AAV introduced transgenes requires further investigation.

5.5 RNA Interference Mediated Gene Knock Down

RNA interference (RNAi) is a conserved post-transcriptional gene regulatory process found in most biological systems including fungi, plants and animals. The common element is double stranded RNA which is cleaved to form small interfering RNA (siRNA) molecules 19-27 base pairs in length. A single strand of these small RNA molecules is incorporated in an RNA-induced silencing complex (RISC) which specifically binds to the complementary sequence of its target mRNA causing endonuclease mediated degradation. The result is that no protein is produced from that mRNA

transcript (Plasterk 2002). Natural RNA interference is involved in gene regulation, specifically to suppress the translation of mRNAs from endogenous and exogenous viral elements, so this can be exploited for therapeutic purposes (Dallas and Vlassow 2006).

For transient gene knockdown, synthetic siRNAs can be transfected into cells or early embryos (Clark and Whitelaw 2003; Iqbal et al. 2007). For stable gene repression, the siRNA sequences must be incorporated into a gene construct and constitutively expressed. The combination of siRNA with lentiviral vector technology is now a highly effective tool in this respect. RNAi knockdown of porcine endogenous retrovirus (PERV) has been demonstrated in porcine primary cells (Dieckhoff et al. 2007) and in cloned piglets (Dieckhoff et al. 2008). SiRNA mediated knockdown of the prion protein (PRNP) gene has been accomplished in bovine embryos (Golding et al. 2006). The modification appears to be permanent as lentiviral delivered siRNA has been shown to persist for three generations in rats (Tenenhaus Dann et al. 2006). The combination of siRNA and lentiviral vector technology provides a method for highly efficient targeted gene knockdown for functional genetic analysis in farm animals and could easily be integrated into existing breeding programs.

6 Health and Welfare of Transgenic Farm Animals

Concerns have been raised about the health of transgenic farm animals because it is known that insertional mutagenesis and other undesirable side effects can be caused by the integration and expression of recombinant gene constructs (Van Reenen et al. 2001, Van Reenen this proceedings). The health of all transgenic animals is closely monitored because of the time and money invested in their creation and because all work is basic research. A small number of studies have systematically investigated the health effects of transgenesis. A study of the effects of human growth hormone expression in pigs and sheep identified specific pathological phenotypes related to their accelerated growth rate. These problems were eliminated in subsequent transgenic animals by modifications to the gene constructs (Nottle et al. 1999). In pigs, transgenic for human DAF and maintained under qualified pathogen free conditions, haematological parameters and blood chemistry were similar to non-transgenic controls (Tucker et al. 2002). With the exception of slightly accelerated growth rates, no deviations were found. A detailed pathomorphological examination of nine lines of hemizygous pigs expressing human RCAs revealed no adverse effects related to transgene expression (Deppenmeier et al. 2006), providing clear evidence that transgenesis per se does not compromise animal health and welfare. Investigation of animals carrying the NTA bicistronic expression cassette, driving hCD59 and a tetracycline regulated transactivator (Kues et al. 2006)

revealed that multi-transgenic animals display a normal health status (Deppenmeier et al. 2006). The hemizygous lines were fertile and produced normal litter sizes.

Transgenesis based on SCNT is increasingly used for farm animals. In cloned animals, both pre- and postnatal development can be compromised and a proportion of SCNT offspring in both ruminants and mice exhibit increased perinatal mortality. The list of developmental abnormalities includes: extended gestation length, oversized offspring, aberrant placental function, cardiovascular problems, respiratory defects, immunological deficiencies, problems with tendons, adult obesity, kidney or hepatic malfunctions, behavioral changes and higher susceptibility to neonatal diseases, all of which are aspects of what has been called the "Large Offspring Syndrome" (LOS) (Renard et al. 1999; Tamashiro et al. 2000; Ogonuki et al. 2002; Rhind et al. 2003). The incidence of LOS is stochastic and has not been linked to aberrant expression of any single genes or to any specific pathophysiology. The general assumption is that the underlying cause of LOS is faulty epigenetic reprogramming of the transferred somatic cell nucleus.

Despite these problems, critical surveys of the published literature have revealed that most cloned animals are healthy and develop normally (Cibelli et al. 2002; Panarace et al. 2007). This demonstrates that mammalian development can tolerate minor epigenetic aberrations and subtle variations in gene expression without affecting survival of cloned animals (Humpherys et al. 2001). Six months old cloned cattle do not differ from age-matched controls with regard to biochemical blood and urine parameters (Lanza et al. 2001; Chavatte-Palmer et al. 2002), immune status (Lanza et al. 2001), body score (Lanza et al. 2001), somatotrophic axis (Govoni et al. 2002), reproductive parameters (Enright et al. 2002), or milk yield and composition (Pace et al. 2002). No differences were found in the meat or milk composition of bovine clones compared to age matched counterparts (Tian et al. 2005; Yang et al. 2007; Miller 2007). Similar findings were reported for cloned pigs (Carter et al. 2002; Archer et al. 2003).

Regulatory agencies around the world have agreed that food derived from cloned animals is safe and there is no scientific basis for questioning this (c.f. National Academy of Sciences, Committee on Defining Science-Based Concerns Associated with Products of Animal Biotechnology, (National Academy of Sciences 2002). Expert committee from the Japanese Ministry of Agriculture, Forestry and Fisheries (MAFF; Kumugai 2002), the FDA (Rudenko et al. 2007; Food and Drug Administration 2008) and EFSA (European Food Safety Agency, 2007). Since somatic cloning has only been used since 1997 and the lifespan of domestic animals is relatively long, the specific effects of cloning on longevity and senescence have not yet been fully assessed; however, preliminary data indicate no cumulative pathology even after serial cloning of mice and cattle (Wakayama et al. 2000; Kubota et al. 2004).

There are still insufficient numbers of transgenic farm animals produced by the newest technologies including viral vectors and spermatogonial transgenesis to reveal subtle effects on animal health and welfare.

7 Safety Aspects and Outlook

Biological products from any animal source are unique and must be handled differently than chemically synthesized drugs to assure their safety, purity and potency. Proteins are heat labile, subject to microbial contamination, can be damaged by shear forces and can be immunogenic and allergenic. In the United States, the FDA has developed guidelines to assure safe commercial exploitation of recombinant biological products. A crucial consideration with animal derived products is the prevention of transmission of pathogens from animals to humans (Kues and Niemann 2004). This requires sensitive and reliable diagnostic and screening methods for various pathogenic organisms. Furthermore, transgenic farm animal based applications require strict standards of quality control. MALDI-TOF-spectometry is an important tool in this context (Hughes et al. 2000; Templin et al. 2002). Meanwhile, improvements in RNA isolation and in unbiased global amplification of picogram amounts of mRNA enable researchers to analyse RNA from single embryos (Brambrink et al. 2002; Niemann et al. 2007). One can now monitor the entire transcriptome of a transgenic organ or organism to ensure the absence of unwanted effects (Hughes et al. 2000; Templin et al. 2002).

Detailed genomic information and new genetic engineering tools will accelerate and improve transgenic animal production in the future. Genetic technology presents not only a major opportunity to improve agricultural production but also offers exciting prospects for medical research by exploiting large animals as models of human health and disease. Progress in animal genomics has broadly followed the route pioneered by the human genome project in terms of the assembly, publication and utilization of the data. This is evident in the advanced drafts of the bovine, porcine, horse, canine, chicken, and honeybee genomic maps. The ability to engineer the genome is new and the advent of new molecular tools and breeding technologies is benefiting this field. However, full realization of this exciting potential is handicapped by our currently limited understanding of epigenetic controls and the role of natural siRNA and microRNA in regulating gene expression.

The convergence of recent advances in reproductive technology with the tools of molecular biology opens a new dimension for animal breeding. Major goals are the continued refinement of reproductive biotechnology and a rapid completion of the various genome sequencing and annotation projects. Induced pluripotent stem cell (iPS) (Takahashi and Yamanaka

2006) research will play a critical role in understanding epigenetic controls. Despite continued efforts, no ES-cell lines with germ line potential have been established from mammals other than the mouse although ES-like cells have been reported in several species and have been maintained in culture from 13 weeks to three years (Gjorret and Maddox-Hyttel 2005). True germ line competent ES cell lines from farm animal species will permit exploitation of the full power of recombinant DNA technology in animal breeding. This is critical for the development of sustainable and diversified animal production systems for the future. We anticipate that in the near future genetically modified animals will play a significant role in the biomedical field but that agricultural applications will develop more slowly due to the complexity of many economically important traits and to current resistance to the concept of engineered farm animals.

References

Anderson GB (1999) Embryonic stem cells in agricultural species. In: Murray JD, Anderson GB, Oberbauer AM, McGloughlin MM (eds) Transgenic Animals in Agriculture. CABI Publ., New York, USA, pp 57–66

Archer GC, Dindot S, Friend TH, Walker S, Zaunbrecher G, Lawborn B, Piedrahita JA (2003) Hierarchical phenotype and epigenetic variation in cloned swine. Biol Reprod 69:430–436

Baguisi A, Behboodi E, Melican D, Pollock JS, Destrempes MM, Cammuso C, Williams JL, Nims SD, Porter CA, Midura P, Palacios MJ, Ayres SL, Denniston RS, Hayes ML, Ziomek CA, Meade HM, Godke RA, Gavin WG, Overström EW, Echelard Y (1999) Production of goats by somatic cell nuclear transfer. Nat Biotech 17(5):456–461

Baise E, Pire G, Leroy M, Gerardin J, Goris N, De Clercq K, Kerkhofs P, Desmecht D, (2004) Conditional expression of type I interferon-induced bovine Mx1 GTPase in a stable transgenic vero cell line interferes with replication of vesicular stomatitis virus. J Interferon & Cytokine Res 24(9):513–521

Brambrink T, Wabnitz P, Halter R, Klocke R, Carnwath JW, Kues WA, Wrenzycki C, Paul D, Niemann H (2002) Application of cDNA arrays to monitor mRNA profiles in single preimplantation mouse embryos. Biotechniques 33(2):376–378

Brophy B, Smolenski G, Wheeler T, Wells D, L'Huillier P, Laible G (2003) Cloned transgenic cattle produce milk with higher levels of b-casein and k-casein. Nat Biotechnol 21(2):157–162

Capecchi MR (1989) The new mouse genetics: Altering the genome by gene targeting. Trends Genet 5(3):70–76

Carter BD, Lai L, Park KW, Samuel M, Lattimer JC, Jordan K R, Estes DM, Besch-Williford C, Prather R (2002) Phenotyping of transgenic cloned pigs. Cloning Stem Cells 4:131–14

Castilla J, Pintado B, Sola I, Sáncez-Morgado JM, Enjuanes L (1998) Engineering passive immunity in transgenic mice secreting virus-neutralizing antibodies in milk. Nat Biotechnol 16(4):349–354

Chan AW, Homan EJ, Ballou LU, Burns JC, Bremel RD (1998) Transgenic cattle produced by reverse-transcribed gene transfer in oocytes. Proc Natl Acad Sci USA 95(24):14028–14033

Chang K, Qian J, Jiang M, Liu YH, Wu MC, Chen CD, Lai CK, Lo HL, Hsiao CT, Brown L, Bolen J Jr, Huang HI, Ho PY, Shih PY, Yao CW, Lin WJ, Chen CH, Wu FY, Lin YJ, Xu J, Wang K (2002) Effective generation of transgenic pigs and mice by linker based sperm-mediated gene transfer. BMC Biotechnol 2(1):5

Chavatte-Palmer P, Heyman Y, Richard C, Monget P, LeBourhis D, Kann G, Chilliard Y, Vignon X, Renard JP (2002) Clinical, hormonal, and hematologic characteristics of bovine calves derived from nuclei from somatic cells. Biol Reprod 66:1596–1603

Chou CY, Horng LS, Tsai HJ (2001) Uniform GFP-expression in transgenic medaka (Oryzias latipes) at the F0 generation. Transgenic Res 10:303–315

Cibelli JB, Stice SL, Golueke PL, Kane JJ, Jerry J, Blackwell C, Ponce de Leon FA, Robl JM (1998) Transgenic bovine chimeric offspring produced from somatic cell-derived stem like cells. Nat Biotech 16(7):642–646

Cibelli JB, Campbell KH, Seidel GE, West MD, Lanza RP (2002) The health profile of cloned animals. Nat Biotechnol 20:13–14

Clark J, Whitelaw B (2003) A future for transgenic livestock. Nat. Rev Genet 4(10):825–833

Clements JE, Wall RJ, Narayan O, Hauer D, Schoborg R, Sheffer D, Powell A, Carruth LM, Zink MC, Rexroad CE (1994) Development of transgenic sheep that express the Visna virus envelope gene. Virology 200(2):370–380

Copley MS, Berstan R, Dudd SN, Docherty G, Mukherjee AJ, Straker V, Payne S, Evershed RP (2003) Direct chemical evidence for widespread dairying in prehistoric Britain. Proc Natl Acad Sci USA 100(4):1524–1529

Corbel YC, Rossi F (2002) Latest developments and in vivo use of the Tet system: ex vivo and in vivo delivery of tetracycline-regulated genes. Curr Opin Biotechnol 13:448–452

Cozzi E, White DJG (1995) The generation of transgenic pigs as potential organ donors for humans. Nat Med 1:964–966

D'Agnillo F, Chang TM (1998) Polyhemoglobin-superoxide dismutase-catalase as a blood substitute with antioxidant properties. Nat Biotechnol 16(7):667–671

Dai Y, Vaught TD, Boone J, Chen SH, Phelps CJ, Ball S, Monahan JA, Jobst PM, McCreath KJ, Lamborn AE, Cowell-Lucero JL, Wells KD, Colman A, Polejaeva IA, Ayares DL (2002) Targeted disruption of the α1,3-galactosyltransferase gene in cloned pigs. Nat Biotechnol 20(3):251–255

Dallas A, Vlassow A (2006) RNAi: A novel antisense technology and its therapeutic potential. Med Sci Monit 12:RA67–RA74

Damak S, Su H, Jay NP, Bullock DW (1996a) Improved wool production in transgenic sheep expressing insulin-like growth factor 1. Biotechnology 14(2):185–188

Damak S, Jay NP, Barrell GK, Bullock DW (1996b) Targeting gene expression to the wool follicle in transgenic sheep. Biotechnology 14(2):181–184

Denning C, Burl S, Ainslie A, Bracken J, Dinnyes A, Fletcher J, King T, Ritchie M, Ritchie WA, Rollo M, de Sousa P., Travers A, Wilmut I, Clark AJ (2001) Deletion of the alpha(1,3)galactosyltransferase (GGTA1) and the prion protein (PrP) gene in sheep. Nat Biotechnol 19(6):559–562

Deppenmeier S, Bock O, Mengel M, Niemann H, Kues W, Lemme E, Wirth D, Wonigeit K, Kreipe H (2006) Health status of transgenic pig lines expressing human complement regulator protein CD59. Xenotransplantation 13:345–356

Dieckhoff B, Karlas A, Hofmann A, Kues WA, Petersen B, Pfeifer A, Niemann H (2007) Inhibition of porcine endogenous retroviruses (PERV) in primary porcine cells by RNA interference using lentiviral vectors. Archives of Virology 152:629–34

Dieckhoff B, Petersen B, Kues WA, Kurth R, Niemann H, Denner J (2008) Knockdown of porcine endogenous retrovirus (PERV) expression by PERV-specific shRNA in transgenic pigs. Xenotransplantation 15:36–45

Downing GJ, Battey JF Jr (2004) Technical assessment of the first 20 years of research using mouse embryonic stem cell lines. Stem Cells 22(7):1168–1180

Dyck MK, Lacroix D, Pothier F, Sirard MA (2003) Making recombinant proteins in animals – different systems, different applications. Trends Biotechnol 21(9):394–399

Echelard Y, Ziome C, Meade H (2006) Production of recombinant therapeutic proteins in the milk of transgenic animals. BioPharm Intern 2(8):1–6 (http://www.biopharminternational.com/biopharm/content, July 2008)

Enright BP, Taneja M, Schreiber D, Riesen J, Tian XC, Fortune JE, Yang X (2002) Reproductive characteristics of cloned heifers derived from adult somatic cells. Biol Reprod 66:291–296

European Food Safety Agency (2007) (http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178676923092.htm, July 2007)

Fändrich F, Lin X, Chai G., Schulze M, Ganten D, Bader M, Holle J, Huang DS, Parwaresch R, Zavazava N, Binas B (2002) Preimplantation-stage stem cells induce longterm allogeneic graft acceptance without supplementary host conditioning. Nat Med 8(2):171–178

Food and Drug Administration USA (2003) FDA statement regarding Glofish (http://www.fda.gov/bbs/topics/NEWS/2003/NEW00994.html, July 2007)

Food and Drug Administration (2008) Animal cloning: a risk assessment – final. Rockville, MD: United States Food and Drug Administration (http://www.fda.gov/cvm/CloneRiskAssessment_Final.htm, July 2007)
Furth PA, St Onge L, Boger H, Gruss P, Gossen M, Kistner A, Bujard H, Hennighausen L (1994) Temporal control of gene expression in transgenic mice by a tetracycline-responsive promoter. Proc Natl Acad Sci USA 91:9302–9306
Gjorret JO, Maddox-Hyttel P (2005) Attempts towards derivation and establishment of bovine embryonic stem cell-like cultures. Reprod Fert Dev 17:113–124
Golding MC, Long CR, Carmell MA, Hannon GJ, Westhusin ME (2006) Suppression of prion protein in livestock by RNA interference. Proc Natl Acad Sci USA 103:5285–5290
Golovan SP, Meidinger RG, Ajakaiye A, Cottrill M, Wiederkehr MZ, Barney DJ, Plante C, Pollard JW, Fan MZ, Hayes MA, Laursen J, Hjorth JP, Hacker RR, Phillips JP, Forsberg CW (2001) Pigs expressing salivary phytase produce low-phosphorus manure. Nat Biotechnol 19(8):741–745
Gong W, Wan H, Tay TL, Wang H, Chen M, Yan T (2003) Development of transgenic fish for ornamental and bioreactor by strong expression of fluorescent proteins in the skeletal muscle. Biochemical and Biophysical Research Communications 308:58–63
Govoni K, Tian XC, Kazmer GW, Taneja M, Enright BP, Rivard AL, Yang X, Zinn SA (2002) Age-related changes of the somatotropic axis in cloned Holstein calves. Biol Reprod 66:1293–1298
Grosse-Hovest L, Muller S, Minoia R, Wolf E, Zakhartchenko V, Wenigerkind H, Lassnig C, Besenfelder U, Müller M, Lytton SD, Jung G, Brem G (2004) Cloned transgenic farm animals produce a bispecific antibody for T cell-mediated tumor cell killing. Proc Natl Acad Sci USA. 101(18):6858–6863
Grunwald KA, Schueler K, Uelmen PJ, Lipton BA, Kaiser M, Buhman K, Attie AD (1999) Identification of a novel Arg->Cys mutation in the LDL receptor that contributes to spontaneous hypercholesterolemia in pigs. J Lipid Res 40(3):475–485
Hammer RE, Pursel VG, Rexroad CE Jr, Wall RJ, Bolt DJ, Ebert KM, Palmiter RD, Brinster RL (1985) Production of transgenic rabbits, sheep and pigs by microinjection. Nature 315(6021):680–683
Hansen K, Khanna C (2004) Spontaneous and genetically engineered animal models; use in preclinical cancer drug development. Eur J Cancer 40(6):858–880
Haskell RE, Bowen R (1995) Efficient production of transgenic cattle by retroviral infection of early embryos. Mol Reprod Dev 40(3):386–390
Hofmann A, Kessler B, Ewerling S, Weppert M, Vogg B, Ludwig H, Stojkovic M, Boelhauve M, Brem G, Wolf E, Pfeifer A (2003) Efficient transgenesis in farm animals by lentiviral vectors. EMBO Rep 4(11):1054–1060
Hofmann A, Zakhartchenko V, Weppert M, Sebald H, Wenigerkind H, Brem G, Wolf E, Pfeifer A (2004) Generation of transgenic cattle by lentiviral gene transfer into oocytes. Biol Reprod 71(2):405–409
Hofmann A, Kessler B, Ewerling S, Kabermann A, Brem G, Wolf E, Pfeifer A (2006) Epigenetic regulation of lentiviral transgene vectors in a large animal model. Mol Ther 13:59–66
Honaramooz A, Behboodi E, Megee SO, Overton SA, Galantino-Homer H, Echelard Y, Dobrinski I (2003) Fertility and germline transmission of donor haplotype following germ cell transplantation in immunocompetent goats. Biol Reprod 69:1260–1264
Honaramooz A, Megee S, Zeng W, Destrempes MM, Overton SA, Luo J, Galantino-Homer H, Modelski M, Chen F, Blash, S, Melican DT, Gavin WG, Ayres S, Yang F, Wang PJ, Echelard Y, Dobrinski I (2007) Adeno-associated virus (AAV)-mediated transduction of male germ line stem cells results in transgene transmission after germ cell transplantation. FASEB Journal fj.07-8935

Hornen N, Kues WA, Carnwath JW, Lucas-Hahn A, Petersen B, Hassel B, Niemann H (2007) Production of viable pigs from fetal somatic stem cells. Cloning Stem Cells 9:364–373

Huang YJ, Huang Y, Baldassarre H, Wang B, Lazaris A, Leduc M, Bilodeau AS, Bellemare A, Cote M, Herskovits P, Touati M, Turcotte C, Valeanu L, Lemee N, Wilgus H, Begin I, Bhatia B, Rao K, Neveu N, Brochu E, Pierson J, Hockley DC, Cerasoli DM, Lenz DE, Karatzas CN, Langermann S (2007) Recombinant human butyrylcholinesterase from milk of transgenic animals to protect against organophosphate poisoning. Proc Natl Acad Sci USA 104 (34):13603–13608

Hughes TR, Roberts CJ, Dai H, Jones AR, Meyer MR, Slade D, Burchard J, Dow S, Ward TR, Kidd MJ, Friend SH, Marton MJ (2000) Widespread aneuploidy revealed by DNA microarray expression profiling. Nat Genet 25(3):333–337

Humpherys D, Eggan K, Akutsu H, Hochedlinger K, Rideout WM 3rd, Biniszkiewicz D, Yanagimachi R, Jaenisch R (2001) Epigenetic instability in ES cells and cloned mice. Science 293:95–97

Hyttinen JM, Peura T, Tolvanen M, Aalto J, Alhonen L, Sinervirta R, Halmekyto M, Myohanen S, Janne J (1994) Generation of transgenic dairy cattle from transgene-analyzed and sexed embryos produced in vitro. Biotechnology 12(6):606–608

Ibrahim Z, Busch J, Awwad M, Wagner R, Wells K, Cooper DKC (2006) Selected physiologic compatibilities and incompatibilities between human and porcine organ systems. Xenotransplantation 13:488–499

Iqbal K, Kues WA, Niemann H (2007) Parent-of-origin dependent gene-specific knock down in mouse embryos. Biochemical and Biophysical Research Communications 358:727–32

Irgang M, Sauer IM, Karlas A, Zeilinger K, Gerlach J, Kurth R, Neuhaus P, Denner J (2003) Porcine endogenous retroviruses: no infection in patients treated with a bioreactor based on porcine liver cells. J Clin Virology 28:141–154

Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418(6893):41–49

Jost B, Vilotte JL, Duluc I, Rodeau JL, Freund JN (1999) Production of low-lactose milk by ectopic expression of intestinal lactase in the mouse mammary gland. Nat Biotechnol 17(2):160–164

Karatzas CN, Turner JD (1997) Toward altering milk composition by genetic manipulation: current status and challenges. J Dairy Sci 80(9):2225–2232

Kawai T, Cosimi AB, Spitzer TR, Tolkoff-Rubin N, Suthanthiran M, Saidman SL, Shaffer J, Preffer FI, Ding R, Sharma V, Fishman JA, Dey B, Ko DSC, Hertl M, Goes NB, Wong W, Williams WW Jr, Colvin RB, Sykes M, Sachs DH (2008) HLA-Mismatched Renal Transplantation without Maintenance Immunosuppression. NEJM 358(4):353–361

Kilby NJ, Snaith MR, Murray JAH (1993) Site-specific recombinases: tools for genome engineering. Trends Genet 9(12):413–421

Krimpenfort P, Rademakers A, Eyestone W, van der Schans A, van den Broek S, Kooiman P, Kootwijk E, Platenburg G, Pieper F, Strijker R, de Boer H (1991) Generation of transgenic dairy cattle using in vitro embryo production. Biotechnology 9(9):844–847

Kubota C, Tian XC, Yang X (2004) Serial bull cloning by somatic cell nuclear transfer. Nature Biotechnology 22:693–694

Kues WA, Niemann H (2004) The contribution of farm animals to human health. Trends Biotechnol 22(6):286–294

Kues WA, Carnwath JW, Niemann H (2005a) From fibroblasts to stem cells: implications for cell therapies and somatic cloning. Reprod Fertil Dev 17(1,2):125–134

Kues WA, Petersen B, Mysegades W, Carnwath JW, Niemann H (2005b) Isolation of murine and porcine fetal stem cells from somatic tissue. Biol Reprod 72(4):1020–1028

Kues WA, Schwinzer R, Wirth D, Verhoeyen E, Lemme E, Herrmann D, Barg-Kues B, Hauser H, Wonigeit H, Niemann H (2006) Epigenetic silencing and tissue independent expression of a novel tetracycline inducible system in double-transgenic pigs. FASEB Journal Express doi: 10.1096/fj.05-5415fje; printed short version: FASEB J 20:1200–1202

Kumugai S (2002) Safety of animal foods that utilize cloning technology. Report to the Japanese Ministry for Agriculture, Forestry and Fishery (MAFF)

Kuroiwa Y, Kasinathan P, Choi YJ, Naeem R, Tomizuka K, Sullivan EJ, Knott JG, Duteau A, Goldsby RA, Osborne BA, Ishida I, Robl JM (2002) Cloned transchromosomic calves producing human immunoglobulin. Nat Biotechnol 20(9):889–894

Kuwaki K, Tseng YL, Dor FJ, Shimizu A, House SL, Sanderson TM, Lanceros CJ, Rabharasuth DD, Cheng J, Moran K, Hisashi Y, Mueller N, Yamadoa K, Greenstein JL, Hawley RJ, Patience C, Awwad M, Fishman JA, Robson SC, Schuurman HJ, Sachs DH, Cooper DK. (2005) Heart transplantation in baboons usion a1,3-glactosyltransferase knock out pigs as donors: initial experiments. Nat Med 11:29–31

Lai L, Kang JX, Li R, Wang J, Witt WT, Yong HY, Hao Y, Wax DM, Murphy CN, Rieke A, Samuel M, Linville ML, Korte SW, Evans RW, Starzl TE, Prather RS, Dai Y (2006) Generation of cloned transgenic pigs rich in omega-3 fatty acids. Nat Biotechnol 24(4):435–436

Lai L, Kolber-Simonds D, Park KW, Cheong HT, Greenstein JL, Im GS, Samuel M, Bonk A, Rieke A, Day BN, Murphy CN, Carter DB, Hawley RJ, Prather RS (2002) Production of α1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science 295(5557):1089–1092

Lanza RP, Cibelli JB, Faber D, Sweeney RW, Henderson B, Nevala W, West MD, Wettstein PJ (2001) Cloned cattle can be healthy and normal. Science 294:1893–1894

Lavitrano M, Camaioni A, Fazio VM, Dolci S, Farace MG, Spadafora C (1989) Sperm cells as vectors for introducing foreign DNA into eggs: genetic transformation of mice. Cell 57(5):717–723

Lavitrano M, Bacci ML, Forni M, Lazzereschi D, Di Stefano C, Fioretti D, Giancotti P, Marfe G, Pucci L, Renzi L, Wang H, Stoppacciaro A, Stassi G, Sargiacomo M, Sinibaldi P, Turchi V, Giovannoni R, Della Casa G, Seren E, Rossi G (2002) Efficient production by sperm-mediated gene transfer of human decay accelerating factor (hDAF) transgenic pigs for xenotransplantation. Proc Nat Acad Sci USA 99(22):14230–14235

Lee F, Mulligan R, Berg P, Ringold G (1981) Glucocorticoids regulate expression of dihydrofolate reductase cDNA in mouse mammary tumour virus chimaeric plasmids. Nature 294:228–232

Lewandowski M (2001) Conditional control of gene expression in the mouse. Nature Reviews 2:743–755

Li Z, Engelhardt JF (2003) Progress toward generating a ferret model of cystic fibrosis by somatic cell nuclear transfer. Reprod Biol Endocrinol 1(1):83

Lo D, Pursel V, Linton PJ, Sandgren E, Behringer R, Rexroad C, Palmiter RD, Brinster RL (1991) Expression of mouse IgA transgenic mice, pigs and sheep. Eur J Immunol 21(4):1001–1006

Ma JK, Drake PM, Christou P (2003) The production of recombinant pharmaceutical proteins in plants. Nat Rev Genet 4(10):794–805

Maga EA, Anderson GB, Murray JD (1995) The effects of mammary gland expression of human lysozyme on the properties of milk from transgenic mice. J Dairy Sci 78(12):2645–2652

Maga EA, Murray JD (1995) Mammary gland expression of transgenes and the potential for altering the properties of milk. Biotechnology 13(13):1452–1457

Maherali N, Sridharan R, Xie W, Utikal J, Eminli S, Arnold K, Stadtfeld M, Yacheko RJ, Janenisch R, Hochedlinger K (2007) Directly reprogrammed fibroblasts show epigenetic remodeling and widespread tissue contribution. Cell Stem Cell 1:55–70

Mahmoud TH, McCuen BW, Hao Y, Moon SJ, Tatebayashi M, Stinnett S, Petters RM, Wong F (2003) Lensectomy and vitrectomy decrease the rate of photoreceptor loss in rhodopsin P347L transgenic pigs. Graefes Arch Clin Exp Ophtalmol 241(4):298–308

Massoud M, Attal J, Thepot D, Pointu H, Stinnakre MG, Theron MC, Lopez C, Houdebine LM (1996) The deleterious effects of human erythropoietin gene driven by the rabbit whey acidic protein gene promoter in transgenic rabbits. Reprod Nutr Dev 36(5):555–563

Mayo KE, Warren R, Palmiter RD (1982) The mouse metallothionein-I gene is transcriptionally regulated by cadmium following transfection into human or mouse cells. Cell 29:99–108

Meade HM, Echelard Y, Ziomek CA, Young JM, Harvery M, Cole ES, Groet S, Curling JM (1999) Expression of recombinant proteins in the milk of transgenic animals. In: Fernandez JM, Hoeffler JP (eds) Gene Expression Systems, Academic Press San Diego, USA, pp 399–427

Miller HI (2007) Food from cloned animals is part of our brave old world. Trends Biotechnol 25:201–203

Miller KF, Bolt DJ, Pursel VG, Hammer RE, Pinkert CA, Palmiter RD, Brinster RL (1989) Expression of human or bovine growth hormone gene with a mouse metallothionein-1 promoter in transgenic swine alters the secretion of porcine growth hormone and insulin-like growth factor-I. J Endocrinol 120:481–488

Müller M, Brem G (1991) Disease resistance in farm animals. Experientia 47(9):923–934

Müller M, Brenig B, Winnacker EL, Brem G (1992) Transgenic pigs carrying cDNA copies encoding the murine Mx1 protein which confers resistance to influenza virus infection. Gene 121(2):263–270

National Academy of Sciences of the USA (2002) Safety of genetically engineered foods: Approaches to assessing unintended health effects. Sub-report on methods and mechanisms of genetic manipulation and cloning of animals. The National Academic Press. Washingthon DC, 217–235

Niemann H (2004) Transgenic pigs expressing plant genes. Proc Natl Acad Sci USA 101(19):7211–7212

Niemann H, Halter R, Carnwath JW, Herrmann D, Lemme E, Paul D (1999) Expression of human blood clotting factor VIII in the mammary gland of transgenic sheep. Transgenic Res 8:237–247

Niemann H, Kues WA (2003) Application of transgenesis in livestock for agriculture and biomedicine. Anim Reprod Sci 79:291–317

Niemann H, Kues W, Carnwath JW (2005) Transgenic farm animals: present and future. Rev sci tech Off int Epiz 24(1):285–298

Niemann H, Kues, WA (2007) Transgenic farm animals: an update. Reproduction, Fertility and Development 19:762–770

Niemann H, Carnwath JW, Kues WA (2007) Application of DNA array technology to mammalian embryos. Theriogenology 68 (Suppl 1):165–177

Nottle MB, Nagashima H, Verma PJ, Du ZT, Grupen CG, Mellfatrick SM, Ashman RJ, Harding MP, Giannakis C, Wigley PL, Lyons IG, Harrison DT, Luxford BG, Campbell RG, Crawford RJ, Robins AJ (1999) Production and analysis of transgenic pigs containing a metallothionein porcine growth hormone gene construct. In: Murray JD, Oberbauer AM, McGloughlin MM (eds) Transgenic Animals in Agriculture CABI Publ, New York USA, pp 145–156

Ogonuki N, Inoue K, Yamamoto Y, Noguchi Y, Tanemur, K, Suzuki O, Nakayama H, Doi K, Ohtomo Y, Satoh M, Nishida A, Ogura A (2002) Early death of mice cloned from somatic cells. Nat Genet 30:253–254

Okita K, Ichisaka T, Yamanaka S (2007) Generation of germline-competent induced pluripotent stem cells. Nature 448:260–262

Pace MM, Augenstein ML, Betthauser JM, Childs LA, Eilertsen KJ, Enos JM, Forsberg EJ, Golueke PJ, Graber DF, Kemper JC, Koppang RW, Lange G, Lesmeister TL, Mallon KS, Mell GD, Misica PM, Pfister-Genskow M, Strelchenko NS, Voelker GR, Watt SR, Bishop MD (2002) Ontogeny of cloned cattle to lactation. Biol Reprod 67:334–339

Palmarini M, Fan H (2001) Retrovirus-induced ovine pulmonary adenocarcinoma, an animal model for lung cancer. J Natl Cancer Inst 93(21):1603–1614

Panarace M, Agüero JI, Garrote M, Jauregui G, Segovia A, Cané L (2007) How healthy are clones and their progeny: 5 years of field experience. Theriogenology 67:142–151

Perry ACF, Wakayama T, Kishikawa H, Kasai T, Okabe M, Toyoda Y, Yanagimachi R (1999) Mammalian transgenesis by intracytoplasmic sperm injection. Science 284(5417):1180–1183

Perry AC, Rothman A, de las Heras JI, Feinstein P, Mombaerts P, Cooke HJ, Wakayama T (2001) Efficient metaphase II transgenesis with different transgene archetypes. Nat Biotechnol 19(11):1071–1073

Petersen B, Lucas-Hahn A, Lemme E, Hornen N, Hassel P, Niemann H (2007) Preovulatory embryo transfer increases cloning efficiencies in pigs. Reprod Fertil Dev 19:155 (abstract)

Petersen B, Lucas-Hahn A, Herrmann D, Kues WA, Ramackers W, Bergmann S, Carnwath JW, Winkler M, Niemann H (2008) Production of pigs transgenic for human hemeoxygenase-I by somatic nuclear transfer. ReprodFertil Dev 20:234 (abstract)

Petters RM, Alexander CA, Wells KD, Collins EB, Sommer JR, Blanton MR, Rojas G, Hao Y, Flowers WL, Banin E, Cideciyan AV, Jacobson SG, Wong F (1997) Genetically engineered large animal model for studying cone photoreceptor survival and degeneration in retinitis pigmentosa. Nat Biotechnol 15(10):965–970

Phelps CJ, Koike C, Vaught TD, Boone J, Wells KD, Chen SH, Ball S, Specht SM, Polejaeva IA, Monahan JA, Jobst PM, Sharma SB, Lamborn AE, Garst AS, Moore M, Demetris AJ, Rudert WA, Bottino R, Bertera S, Trucco M, Starzl TE, Dai Y, Ayares DL (2003) Production of alpha 1,3-galactosyltransferasedeficient pigs. Science 299:411–414

Plasterk RH (2002) RNA silencing: the genomes immune system. Science 296:1263–1265

Platenburg GJ, Kootwijk EAP, Kooiman PM, Woloshuk SL, Nuijens JH, Krimpenfort PJA, Pieper FR, de Boer HA, Strijker R (1994) Expression of human lactoferrin in milk of transgenic mice. Transgenic Res 3(2):99–108

Platt JL, Lin SS (1998) The future promises of xenotransplantation. Ann NY Acad Sci 862:5–18

Pursel VG, Pinkert CA, Miller KF, Bolt DJ, Campbell RG, Palmiter RD, Brinster RL, Hammer RE (1989) Genetic engineering of livestock. Science 244(4910):1281–1288

Pursel VG, Wall RJ, Mitchell AD, Elsasser TH, Solomon MB, Coleman ME, Mayo F, Schwartz RJ (1999) Expression of insulin-like growth factor-I in skeletal muscle of transgenic pigs. In: Murray JD, Oberbauer AM, McGloughlin MM (eds) Transgenic Animals in Agriculture. CABI Publ, New York, USA, pp 131–144

Rapacz J, Hasler-Rapacz J (1989) Animal models: the pig. In: Sparkes RS, Lusis AJ (eds) Genetic Factors in Atherosclerosis: model systems. Karger, Basel, Switzerland, pp 139–169

Reh WA, Maga EA, Collette NM, Moyer A, Conrad-Brink JS, Taylor SJ (2004) Hot topic: using a stearoyl-CoA desaturase transgene to alter milk fatty acid composition. Journal of Dairy Science 87:3510–3514

Renard JP, Chastant S, Chesne P, Richard C, Marchal J, Cordonnier N, Chavatte P, Vignon X (1999) Lymphoid hypoplasia and somatic cloning. Lancet 353:1489–1491

Rhind SM, King TJ, Harkness LM, Bellamy C, Wallace W, DeSousa P, Wilmut, I (2003) Cloned lambs--lessons from pathology. Nat Biotechnol 21:744–745

Richt JA, Kasinathan P, Hamir AN, Castilla J, Sathiyaseelan T, Vargas F (2007) Production of cattle lacking prion protein. Nature Biotechnology 25:132–138

Robl JM, Wang Z, Kasinathan P, Kuroiwa Y (2007) Transgenic animal production and animal biotechnology. Theriogenology 67:127–133 (epub ahead of print, doi: 10.1016/j.theriogenology.2006.09.034)

Rudenko L, Matheson JC, Sundlof SF (2007) Animal Cloning and the FDA: the risk assessment paradigm under public scrutiny. Nature Biotechnology 25:39–43

Rossant J (2001) Stem cells from the mammalian blastocyst. Stem Cells 19(6):477–482

Rudolph NS (1999) Biopharmaceutical production in transgenic livestock. Trends Biotechnol 17(9):367–374

Saeki K, Matsumoto K, Kinoshita M, Suzuki I, Tasaka Y, Kano K, Taguchi Y, Mikami K, Hirabayashi M, Kashiwazaki N, Hosoi Y, Murata N, Iritani A (2004) Functional expression of a Delta12 fatty acid desaturase gene from spinach in transgenic pigs. Proc Natl Acad Sci USA 101(17):6361–6366

Schätzlein S, Lucas-Hahn A, Lemme E, Kues WA, Dorsch M, Manns MP, Niemann H, Rudolph KL (2004) Telomere length is reset during early mammalian embryogenesis. Proc Natl Acad Sci USA 101(21):8034–8038

Schätzlein S, Rudolph KL (2005) Telomere length regulation during cloning, embryogenesis and aging. Reprod Fertil Dev 17(1,2):85–96

Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scott AR, Ritchie M, Wilmut I, Colman A, Campbell KH (1997) Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science 278(5346):2130–2133

Shim H, Gutierrez-Adan A, Chen LR, BonDurant RH, Behboodi E, Anderson GB (1997) Isolation of pluripotent stem cells from cultured porcine primordial germ cells. Biol Reprod 57(5):1089–1095

Staeheli P (1991) Intracellular immunization: a new strategy for producing disease-resistant transgenic livestock? Trends Biotechnol 9(3):71–72

Stinnakre MG, Vilotte JL, Soulier S, Mercier JC (1994) Creation and phenotypic analysis of α-lactalbumin-deficient mice. Proc Natl Acad Sci USA 91(14):6544–6548

Swanson ME, Martin MJ, O'Donnell JK, Hoover K, Lago W, Huntress V, Parsons CT, Pinkert CA, Pilder S, Logan JS (1992) Production of functional human hemoglobin in transgenic swine. Biotechnology 10(5):557–559

Switzer WM, Michler RE, Shangmugam V, Matthews A, Hussain AI, Wright A, Sandstrom P, Chapman L, Weber C, Safley S, Denny RD, Navarro A, Evans V, Norin AJ, Kwiatkowski P, Heneine W (2001) Lack of cross-species transmission of porcine endogenous retrovirus infection to nonhuman primate recipients of porcine cells, tissues and organs. Transplantation 71:959–965

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–76

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872

Tamashiro KL, Wakayama T, Blanchard RJ, Blanchard DC, Yanagimachi R (2000) Postnatal growth and behavioral development of mice cloned from adult cumulus cells. Biol Reprod 63:328–234

Templin MF, Stoll D, Schrenk M, Traub PC, Vohringer CF, Joos TO (2002) Protein microarray technology. Trends Biotechnol 20(4):160–166

Tenenhaus Dann C, Alvarado AL, Hammer RE, Garbers DL (2006) Heritable and stable gene knockdown in rats. Proc Natl Acad Sci USA 103:11246–11251

Theuring F, Thunecke M, Kosciessa U, Turner JD (1997) Transgenic animals as models of neurodegenerative disease in humans. Trends Biotechnol 15(8):320–325

Tian XC, Kubota C, Sakashita K, Izaike Y, Okano R, Tabara N, Curchoe C, Jacob L, Zhang Y, Smith S, Bormann C, Xu, J, Sato M, Andrew S, Yang X (2005) Meat and milk compositions of bovine clones. Proceedings National Academy of Sciences USA 102:6261–6266

Tucker A, Belcher C, Moloo B, Bell J, Mazzulli T, Humar Y, Hughes A, McArdle P, Talbot A (2002) The production of transgenic pigs for potential use in clinical xenotransplantation: baseline clinical pathology and organ size studies. Xenotransplantation 9:203–208

van Berkel PH, Welling MM, Geerts M, van Veen HA, Ravensbergen B, Salaheddine M, Pauwels EK, Pieper F, Nuijens JH, Nibbering PH (2002) Large scale production of recombinant human lactoferrin in the mik of trangenic cows. Nat Biotechnol 20(5):484–487

Van den Hout JM, Reuser AJ, de Klerk JB, Arts WF, Smeitink JA, Van der Ploeg AT (2001) Enzyme therapy for Pompe disease with recombinant human α-glucosidase from rabbit milk. J Inherit Metab Dis 24(2):266–274

Van Reenen CG, Meuwissen THE, Hopster H, Oldenbroek K, Kruip TH, Blokhuis HJ (2001) Transgenesis may affect farm animal welfare: a case for systemic risk assessment. J Anim Sci 79:1763–1769

Wakayama T, Shinkai Y, Tamashiro KL, Niida H, Blanchard DC, Blanchard RJ (2000) Cloning of mice to six generations. Nature 407:318–319

Wall RJ, Powell A, Paape MJ, Kerr DE, Bannermann DD, Pursel VG, Wells KD, Talbot N, Hawk H (2005) Genetically enhanced cows resist intramammary Staphylococcus aureus infection. Nat Biotechnol 23(4):445–451

Ward KA (2000) Transgene-mediated modifications to animal biochemistry. Trends Biotechnol 18(3):99–102

Weidle UH, Lenz H, Brem G (1991) Genes encoding a mouse monoclonal antibody are expressed in transgenic mice, rabbits and pigs. Gene 98(2):185–191

Weissmann C, Enari M, Klohn PC, Rossi D, Flechsig E (2002) Transmission of prions. Proc Natl Acad Sci USA 99(Suppl 4):16378–16383

Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, Bernstein BE, Jaenisch R. (2007) In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature 448:318–324

Wheeler MB (1994) Development and validation of swine embryonic stem cells: a review. Reprod Fertil Dev 6(5):563–568

Wheeler MB, Bleck GT, Donovan SM (2001) Transgenic alteration of sow milk to improve piglet growth and health. Reproduction Suppl 58:313–324

Whitelaw CB, Radcliffe PA, Ritchie WA, Carlisle A, Ellard FM, Pena RN, Rowe J, Clark AJ, King TJ, Mitrophanous KA (2004) Efficient generation of transgenic pigs using equine infectious anaemia virus (EIAV) derived vector. FEBS Lett 571:233–236

Willadsen SM (1986) Nuclear transplantation in sheep embryos. Nature 320:63–65

Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH (1997) Viable offspring derived from fetal and adult mammalian cells. Nature 385:810–813

Yang YG and Sykes M (2007) Xenotransplantation: current status and a perspective on the future. Nat Rev Immunol 7(7):519–531

Yang X, Tian C, Kubota C, Page R, Xu J, Cibelli J (2007) Risk assessment of meat and milk from cloned animals. Nature Biotechnology 25:77–83

Yamada K, Yazawa K, Shimizu A, Iwanaga T, Hisashi Y, Nuhn M, O'Malley P, Nobori S, Vagefi PA, Patience C, Fishman J, Cooper DK, Hawley RJ, Greenstein J, Schuurman HJ, Awwad M, Sykes M, Sachs DH (2005) Marked prolongation of porcine renal xenograft survival in baboons through the use of α1,3-galactosyltransferase gene-knockout donors and the cotransplantation of vascularized thymic tissue. Nat Med 11(1):32–34

Yom HC, Bremel RD (1993) Genetic engineering of milk composition: modification of milk components in lactating transgenic animals. Am J Clin Nutr 58 (Suppl 2):299–306

Ziomek CA (1998) Commercialization of proteins produced in the mammary gland. Theriogenology 49(1):139–144

Methods to Generate Transgenic Animals

Louis-Marie Houdebine

1 Introduction

Living organisms are by essence in permanent evolution. This phenomenon is relatively slow and it was probably not perceived by humans until they invented agriculture and breeding. The control of plant and animal reproduction made possible the empirical genetic selection which provided to human communities essentially all their food products, pets and ornamental plants. This led to the generation of profoundly genetically modified organisms. Carrots, tomatoes, silk worm, some dogs, etc., are unable to survive without the assistance of humans. The discovery of the Mendel laws allowed an improvement of the genetic selection. Yet, this selection remained based on spontaneous, thus random and unknown, mutations. During the first half of the last century, it appeared necessary and possible to increase the number of random mutations to enlarge the choice of genetically modified organisms corresponding to the expectations of experimenters, farmers and breeders. This was achieved by using chemical mutagens and by generating multiple intra- and interspecies hybrids. One of the most impressive examples is the creation of a new cereal, triticale, which results from an artificial crossing between wheat and rye. This new plant is currently a source of food for farm animals. All these methods are imprecise as they induce multiple unknown mutations in addition to those which are expected. Yet, these approaches were globally highly beneficial for humans. They show that the plasticity of living organisms is high and that humans have empirically learned to manipulate them successfully with limited undesirable side effects. The discovery of DNA and genes opened wide avenues for research and biotechnological applications. Indeed, the manipulation of isolated and known genes makes possible more diverse and better controlled genetic modifications.

The introduction of isolated genes into cells became a common practise in the 1970s, soon after the emergence of the genetic engineering techniques. It represented a great progress for the understanding of gene function and mechanisms of action. This technique is still widely used and it started being complemented in 1980 and 1983 by gene transfer into animals and plants respectively to generate lines of genetically modified organisms, also known as transgenic animals and plants. The first transgenic animals, mice, were obtained by microinjecting the genes into one on the nuclei

(pronuclei) of one day old embryos. This method could be extrapolated successfully to other mammals in 1985 but it soon appeared that other methods had to be used for some species. Another problem emerged rapidly. The transgenes worked and they were able to induce some phenotypic modifications in animals. The first example was the giant mice overexpressing growth hormone genes. It also appeared that the expression of the transgenes was not satisfactory and not easily controlled in all cases.

Two decades later, very significant improvements of the transgenesis methods have been obtained. Yet, the efficiency of gene transfer and the control of transgene expression remain limiting factors for the use of transgenic animals for research as well as for biotechnological applications. The generation and use of transgenic animals are not neutral as they imply the sacrifice and in some cases the suffering of animals. This paper aims at reviewing the different techniques of transgenesis and some of their possible interference with animal welfare.

2 Techniques of Gene Transfer

A transgenic organism results from an inheritable genetic modification induced by the artificial transfer of an exogenous DNA fragment. This implies that the introduced foreign gene is present in the gametes and is integrated into a chromosome to be transmitted to progeny as a host gene. To reach this goal, the foreign gene may be introduced in embryos at the first cell stage by a direct microinjection or via gametes. Alternatively, the foreign gene may be introduced in cells capable of participating to the complete development of the animal in which gametes contain the foreign gene. These different methods have been described in previous publications (Houdebine 2003, 2005) and they are summarized in figure 1.

2.1 DNA Microinjection

About 1,000 copies of the isolated foreign gene contained in 1–2 pl may be injected into one of the pronuclei of one day old mammalian embryos. This method implies a superovulation of the females followed by a mating with a male. The resulting embryos are collected the next day and microinjected with DNA. The embryos are then transferred to hormonally prepared recipient females using surgery operations. The yield of this method in mice is of 1–2 of transgenics for 100 microinjected and transferred embryos. It is lower in all the other mammalian species and very low in ruminants.

In non mammalian species, the pronuclei cannot be visualized and DNA must be injected into the cytoplasm of the one day old embryos. This relatively simple technique is efficient in most fish species but highly inefficient in chicken, in Xenopus, in some fish and in insects. For unknown reasons, the integration of the foreign DNA, thus, does occur in some species.

2.2 Use of Transposons

Transposons are short genomic DNA regions which are replicated and randomly integrated into the same genome. The number of a given transposon is thus increasing until the cell blocks this phenomenon to protect itself from a degradation of its genes. Foreign genes can be introduced into trans-

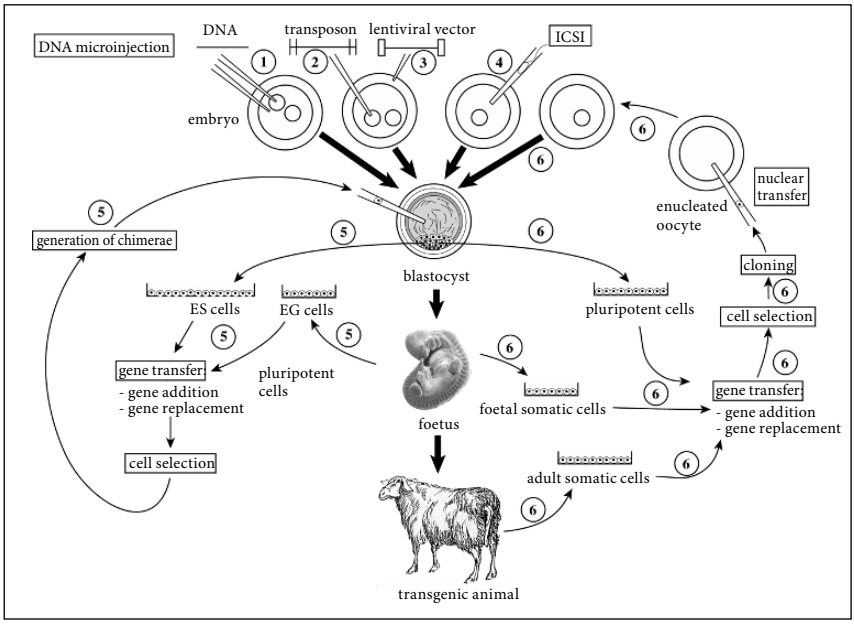


Figure 1: Different methods to generate transgenic animals: (1) DNA transfer via direct microinjection into pronucleus or cytoplasm of embryo; (2) DNA transfer via a transposon: the foreign gene is introduced in the transposon which is injected into a pronucleus; (3) DNA transfer via a lentiviral vector: the gene of interest in a lentiviral vector is injected between the zona pellucida and membrane of the oocyte or embryo; (4) DNA transfer via sperm: sperm is incubated with the foreign gene and injected into the oocyte cytoplasm for fertilization by ICSI (intracytoplasmic sperm injection); (5) DNA transfer via pluripotent cells: the foreign gene is introduced into pluripotent cell lines (ES: embryonic stem cells: lines established from early embryo, EG: embryonic gonad cells: lines established from primordial germ cells of foetal gonads); the pluripotent cells containing the foreign gene are injected into an early embryo to generate chimeric animals harbouring the foreign gene DNA; (6) DNA transfer via cloning: the foreign gene is transferred into a somatic cell, the nucleus of which is introduced into the cytoplasm of an enucleated oocyte to generate a transgenic clone. Methods 1, 2, 3 and 4 allow random gene addition whereas methods 5 and 6 allow random gene addition and targeted gene integration via homologous recombination for gene addition or gene replacement including gene knockout and knockin.

posons in vitro. The recombinant transposons may then be microinjected into one day old embryos. The foreign gene becomes integrated into the embryos with a yield of about 1%. All the transgenic insects are being generated by using transposons as vectors. Transposons also proved to be efficient to generate transgenic fish, chicken and mammals (Ding et al. 2005). Transposons are efficient tools but they can harbour no more than 2–3 kb of foreign DNA.

2.3 Use of Lentiviral Vectors

Retroviruses do not have the capacity to autoreplicate and they have to be integrated stably in the genome of the cells they infected to replicate. This explains why up to 1% of animal genomes contain degenerated retroviral genes. This property of retroviruses is being implemented to integrate foreign genes. For this purpose, the genes are removed from the genome of lentiviruses (a category of retroviruses) and replaced by the genes of interest. Viral particles are then prepared and used to transfer the foreign genes into oocytes or one-cell embryos. Safe experimental conditions have been defined to use the lentiviral vectors. This method has proved highly efficient in several species including mammals (Pfeifer 2006) and birds (Lillico et al. 2007).

2.4 Use of ICSI

More than a decade ago, it was shown that sperm, incubated in the presence of DNA before being used for fertilization, was able to transfer the foreign gene into the oocyte and generate transgenic mice. This method appeared difficult to use due to a frequent degradation of DNA (Smith and Spadafora 2005). Transgenic mice and rabbits were obtained by incubating sperm with DNA in the presence of DMSO (dimethylsuphoxide) and by using conventionnal in vitro fertilization (Shen et al. 2006). The method has been greatly improved, mainly by using ICSI (Intracytoplasmic Sperm Injection). This technique, which consists of injecting sperm into the cytoplasm of oocytes, is currently used for in vitro fertilization in humans. To transfer genes, sperms from which plasma membrane has been damaged by freezing and thawing were incubated in the presence of the gene of interest and further used for fertilization by ICSI. This method has proved efficient in mice (Moreira et al. 2007; Shinoara et al. 2007) and pigs (Yong et al. 2006). Transposon use and ICSI may be combined to increase the yield of transgenesis (Shinoara et al. 2007).

ICSI is therefore an excellent method to generate transgenic animals on condition that ICSI is possible in the considered species. One advantage of ICSI is that long fragments of DNA may be used to transfer the gene of interest. Another advantage is that foreign DNA is integrated at the first cell stage of embryos. This reduces the number of animals being mosaic for the transgene.

Another possibility consists of using sperm precursors. These cells can be collected from testes, in vitro cultured, genetically modified and reintroduced into recipient testes. This complex approach needs further studies to become utilisable.

2.5 Use of Episomal Vectors

The methods described above to transfer foreign genes rely on the integration of the DNA into the host genome. Another possibility may theoretically be to use episomal vectors capable of autoreplicating in host cells and transferring them to daughter cells. Fragments of chromosomes are being used for particular projects requiring the transfer of very long DNA fragments. These chromosomal vectors are not of an easy use and they carry a number of genes in addition of the gene of interest. These extra genes may interfere with the transgene or with the whole organism of the host.

Another possibility consists of using vectors which derive from viruses having the capacity to replicate in animal cells and to be transferred into daughter cells. Herpes viruses are naturally stably maintained as autonomous circular minichromosomes at a low copy number in animal cells. Foreign genes can be introduced into Herpes viral vectors and be maintained during cell division. This kind of vectors is generally species specific. This greatly reduces their potential use as well-known Herpes viruses are not available for all animal species.

However, episomal vectors not based on the use of viral elements are available. Such a vector proved highly efficient to transfer foreign genes into pig embryo using ICSI (Manzini et al. 2006). This vector is maintained without any selection pressure in the cells of the developing embryos but seemingly not later. This kind of vectors is therefore excellent tools to study transgene effect during early embryo development. Hence, until now, only the integration of foreign DNA into the host genome makes possible the generation of stable lines of transgenic animals.

2.6 Use of Pluripotent Cells

In some situations, the efficiency of the genetic modification is too low to be achieved by the methods described above. This is particularly the case for gene targeting (see 2.8). One possibility is to do the genetic modification in pluripotent cells further used to participate in the development of living organisms. Pluripotent cells have the capacity to participate in the development of all the organs but they cannot – such as totipotent cells are able to – each give birth to living animals. Pluripotent cells exist in the early embryos (morula and blastocysts) known as ES cells (embryonic stem cells) and in the primordial gonads known as EG cells (embryonic gonad cells). The pluripotent cells can be cultured, genetically modified, selected and transferred into morula or blastocysts. These cells participate to the development of the embryo to give birth to chimeric animals (figure 1). This

means that the organs of the animals, including sexual cells, derive from the genetically modified cells or from the recipient embryo. The offspring of these chimeric animals will harbour the genetic modification if they derive from the transplanted cells. This method is extensively used essentially in mice to inactivate (knockout) genes specifically (see 2.8).

For unknown reasons, ES cell lines have been established and used only in two mouse lines. In other lines and species, the ES loose their pluripotency and can no more give birth to chimeric animals transmitting the genetic modification to their offspring. Recent experiments have shown that the transfer of four genes into somatic cells, normally expressed in pluripotent cells, can differentiate these organ cells into pluripotent cells (Takahasha et al. 2007; Wernig et al. 2007, Nakagawa et al. 2008). These experiments open avenues for cell and gene therapy. The approach known as therapeutic cloning may no longer be necessary and pluripotent cells can potentially be obtained in different species by this method.

Recent experiments showed that the culture conditions to maintain pluripotent chicken EG cells have been found. This has greatly simplified the generation of transgenic chicken (Van de Lavoir et al. 2006; Han 2008).

2.7 Use of Cloning

The birth of Dolly the sheep demonstrated that the genome of somatic cells can be reprogrammed after being introduced into an enucleated oocyte. This generates a pseudo-embryo with a relatively low yield, capable to give birth to clones of the cell donor. This technique was initially designed to improve transgenesis efficiency in farm animals. This approach is likely to be used to accelerate genetic selection but its only real application presently is transgenesis (Robl et al. 2007). The principle of this method is described in figure 1. Genes are transferred into somatic cells which are then used to generate transgenic clones. This method has become the one most frequently used for large farm animals.

2.8 Targeted Gene Integration

All the techniques described above lead to uncontrolled but not strictly random gene integration. Foreign DNA is preferentially integrated in gene rich genome regions and its location can be precisely identified. A foreign DNA fragment can recombine very precisely with a genomic DNA region containing a similar sequence. This natural mechanism known as homologous recombination makes the precise replacement of a gene by another possible (figure 2). An active gene may thus be replaced by an inactive version leading precisely to an inactivation of the targeted gene (gene knockout). The targeted gene may also be replaced by an active gene (gene knockin). This technique therefore allows for a better controlled transgenesis reducing possible damage of the genomic DNA at the integration site and frequent side effects of the genes located in the vicinity of the transgene on the expression

of the transgene (see 3.1). Yet, this approach remains limited by the fact that the homologous recombination required for gene targeting is a rare event. The targeted integrations by homologous recombination of a foreign DNA represent 0.1–1% of the total integrations. The cells in which targeted integration has occurred must be selected and used to generate a transgenic animal. The formation of chimeric embryos using pluripotent cells (see 2.6) or the cloning technique (2.7) is required to obtain a targeted integration.

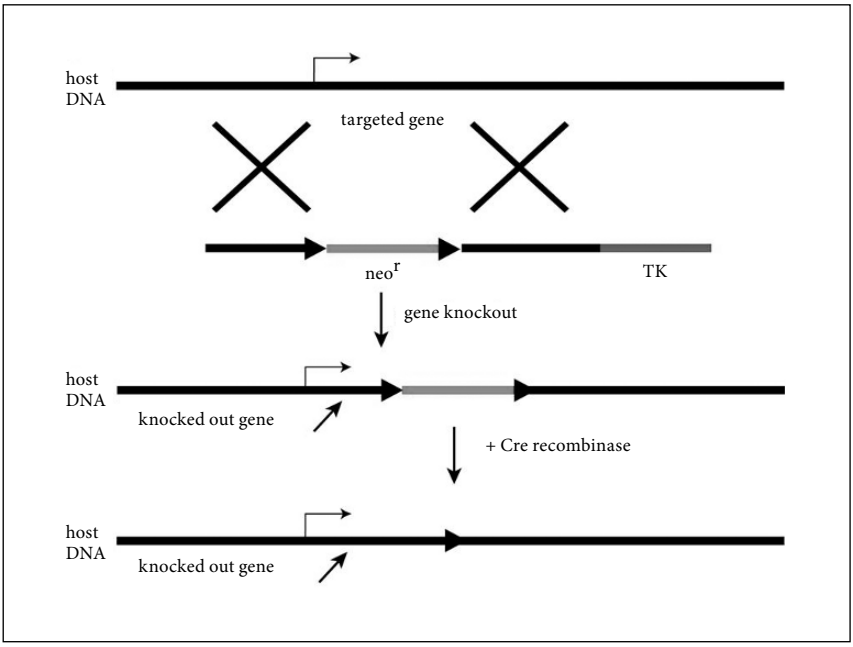


Figure 2: Targeted gene transfer via homologous recombination. A vector containing a gene for positive selection (neo R), a gene for negative selection (TK) and two sequences identical to those targeted in the genome is transferred into a cell (pluripotent or somatic cells). The homologous sequences recombine, leading to a precise replacement of the targeted genomic regions by the sequences of the vector. The neoR gene is then integrated into the targeted gene which is then inactivated (gene knockout). Cells, in which the targeted integration occurred, are selected by the addition of neomycine to the culture medium whereas the cells, in which a random integration occurred, possess the TK gene which induced a destruction of these cells in the presence of ganciclovir. The selected cells are then used to generate chimeric animals (ES or EG cells) or to generate a clone (somatic cells). If the neoR gene has been previously bordered by LoxP sequences, it may be selectively deleted by adding the Cre recombinase. The genomic region has then lost a chosen region and a LoxP sequence (about 30 bp) is the only residue of the vector. The LoxP sequence is sufficient to knock out the targeted gene.

The efficiency of homologous recombination can be markedly increased (at least 100 times) by a local break of the two DNA strands in the targeted site of integration. This can be achieved by using special restriction enzymes known as meganucleases. These enzymes, mainly found in yeast, have the capacity to cut DNA at sites which are longer than those of the classical restriction enzymes and which are usually not present in animal genomes, avoiding the degradation of DNA. The DNA sequences recognized by meganucleases must then be added to the genome of animals either at targeted sites by homologous recombination or at random sites. In the latter case, the integration sites must be validated for its capacity to allow a good gene expression before targeting the gene of interest at the meganuclease site. In practise, the recombination vector containing the gene to be transferred bordered by two DNA sequences similar to that of the genomic DNA, is introduced in the cell with the meganuclease. Engineered meganucleases capable of recognizing natural genomic DNA sequences make gene targeting at multiple sites of the genome possible (Porteus and Caroll 2005). This method, which is being developed to improve the efficiency and the precision of gene therapy, can be applied to target the integration of foreign genes into experimental animals.

In the same line, the bacterial enzyme phiC31, which is an integrase, recognizes several sites in various animal genomes and allows the efficient integration of foreign genes at the targeted sites (Rao 2008).

Several other recombination systems rely on the use of integrases such as Cre and Flp which recognize specific sites of about 30 nucleotides (LoxP and FRT respectively) which must be added to the animal genome (Baer and Bode 2001). These systems are more often used to delete a DNA region previously bordered by the LoxP or the FRT sequences (see 3.3).

3 Methods to Control Gene Expression

3.1 Use of Long Genomic DNA Fragment

The majority of DNA in somatic cells of animals is methylated on the C of CpG motifs. This results in the specific inactivation of the corresponding genes. Mammalian genomes contain about 25,000 genes and only 2,000 are active in somatic cells. About 1,000 genes, known as housekeeping genes, are active in all cell types whereas the other 1,000 genes are specifically active in given cell types to support their differentiated state. During gametogenesis and the early embryo development, DNA is heavily demethylated to blunt the gene expression programme of gonad cells from which gametes derive. After embryo implantation, DNA is progressively and selectively methylated to define which gene in the different cell types will have to be active or not in foetuses, newborns and adults. The mechanisms which control this programme of the gene expression are only partly known. It implies the contribution of multiple DNA regulatory sequences, some of them being

located far upstream or downstream of the genes. The low expression of many transgenes containing only the transcribed regions with a promoter, proximal enhancers, at least one intron and a transcription terminator, has revealed that remote regulatory regions must be involved in the control of gene expression. In a limited but significant number of cases, using long genomic DNA regions (up to 200 kb) surrounding the gene of interest greatly increases the proportion of active transgenes and also often the level of their expression (Long and Miano 2007). Interestingly and expectedly, association of classical plasmid expression vectors with long DNA fragments markedly enhances the functioning of the transgenes. This point is examplified by a milk protein gene, the WAP (whey acidic protein gene) gene. A 30 kb genomic fragment containing the pig WAP gene expressed the WAP gene in transgenic mice very poorly whereas fragments of 80 kb or 145 kb allowed a high expression of the gene, although not as a function of the integrated copy number and at various levels according to species (Saidi et al. 2007). Interestingly, coding sequences added after the WAP gene promoter in the 145 kb BAC (bacterial artificial chromosome) were expressed at a much higher level than when they were under the dependency of the only WAP gene promoter (Soler et al., unpublished data).

3.2 Specific Inhibition of Gene Expression

Transgenesis is mostly used to add foreign genetic information into an animal. Inactivating a gene in an animal is also essential, particularly for the identification of the function of the gene. Indeed, inactivating a gene may have a much stronger impact than adding a gene to an animal and thus reveal the function of the gene.

A gene is usually activated to eliminate the corresponding protein in the animal. This can be achieved by different techniques and at different levels of the protein synthesis process.

3.2.1 At the Gene Level

The data reported in section 2.8 indicate that gene knockout based on homologous recombination is a very efficient but laborious method with a major limit. The gene knockout is currently performed early in development and this event is irreversible. Experimenters may wish to prevent the expression of a gene reversibly, in a given cell type only and at chosen periods of the animal's life. Available methods make the gene knockout possible in a given cell type at a chosen moment. This technique, which has been used successfully for more than one decade, leads to an irreversible inactivation of the gene.

3.2.2 At the mRNA Level

It is possible to inhibit a mRNA specifically by adding to the cells short synthetic oligonucleotides, having a sequence complementary to that of the targeted mRNA. These oligonucleotides contain some analogues of natural

deoxyribonucleotides to enhance their stability in vivo. The binding of the oligonucleotides to the corresponding mRNA induces a degradation of the mRNA. This tool is currently used to inhibit the expression of genes in cultured cells. Oligonucleotides are also administered to patients to tentatively inhibit viral genes in order to block infections. This approach has met limited success so far.

Another possibility to inhibit a mRNA consists of introducing in a cell an anti-sense RNA generated by the transcription of the non-coding strand of the gene. The anti-sense RNA forms a double strand RNA with the mRNA which cannot be translated anymore. In practise, this method is rarely efficient, as the mRNA and the anti-sense RNA each form multiple short double strands with their own sequences. These RNA are also more or less associated with proteins. These two events prevent the easy formation of a double strand mRNA-anti-sense RNA. Hence, anti-sense RNAs meet success at most when a single region strand of the anti-sense is complementary to a single strand region of the targeted mRNA.

Ribozymes are short RNAs capable of cleaving a complementary RNA. This natural mechanism does not imply the contribution of proteins as ribozymes possess an intrinsic RNase activity. The use of ribozymes has proved disappointing in practise as, like antisense RNAs, they do not easily reach their target RNA sequence.

The discovery of interfering RNA one decade ago has profoundly improved the situation. It was unexpectedly found that a long double strand RNA can induce the degradation of a RNA having a complementary structure. Soon after, it was shown that the long double strand RNAs are randomly cut into 19–21 nucleotide fragments known as siRNA (small interfering RNA). One of the two strands of the siRNA is kept and targeted to a mRNA having a complementary sequence. This induces the degradation of the mRNA. It was also demonstrated that synthetic siRNAs transfected into cells had an RNAi effect. Soon after, the use of promoters directed by RNA polymerase III could synthesize siRNAs. In practise, a synthetic gene, containing the targeted 19–21 nucleotide sequence, followed a short random sequence and by the targeted sequence in the opposite orientation is linked to a promoter acting with RNA polymerase III (usually U6 or H1 gene promoters). The RNAs synthesized by such vectors form a 19–21 nucleotide double strand RNA separated by a loop containing the random sequence. These RNAs known as shRNAs (short hairpin RNA) are processed in cells to generate active siRNAs.

The recent discovery of the role of microRNAs has increased the possibility to use interfering RNAs. Indeed, microRNAs (miRNAs) are present in all eukaryotic cells. They are encoded by short genes expressed under the control of RNA polymerase II promoters. The primary products of these genes are 135 nucleotides RNAs which are processed and transferred to the cytoplasm where they are transformed into siRNAs. The mature miRNAs

which are fully complementary to the targeted mRNA induce a degradation of this mRNA. The miRNAs, which are only partially complementary to the targeted mRNA and which recognize a sequence located in the 3'UTR (3'untranslated region) of the mRNA, inhibit translation of this mRNA without inducing its degradation. A mammalian genome contains several hundreds of miRNA genes. Their function is essential as they control the expression of a large proportion of the genes by modulating translation of the mRNAs. On the contrary, the siRNAs described above appear more as a defence mechanism degrading exogenous double strand RNAs (e.g. some viral RNAs) as well as badly shaped cellular RNAs.

The possibility to generate transgenic animals expressing siRNAs preventing specifically the expression of a gene by degrading the corresponding mRNA or inhibiting its translation has opened avenues for the control of gene expression in vivo. This approach has met brilliant success in plants. A rapidly increasing number of transgenic plants expressing genes coding for siRNAs resistant to pathogens or having a metabolic pathway specifically blocked are available. The application of the siRNA approach is not so easy in animals for several reasons. Long double strand RNAs induce interferons and some unspecific immune reactions (Sioud 2006) and they cannot be easily used. The siRNAs are auto-amplified in plants in lower invertebrates but not in vertebrates. Moreover, the reliable expression of transgenes coding for siRNAs is more difficult in animals than in plants, as promoters directed by RNA polymerase III are generally poorly active in transgenic animals unless they are inserted into lentiviral vectors (Tiscornia et al. 2003) or associated with vectors directed by RNA polymerase II (Sawafta et al., unpublished data). It remains that the use of RNA polymerase II promoters to direct the synthesis of siRNAs offers unprecedented possibilities for experimenters and biotechnologists to modulate specific gene expression in a potent and subtle manner.

3.2.3 At the Protein Level

In most cases, the expression of a gene is achieved to suppress or inactivate the corresponding protein in cells or whole living organisms. This goal can be reached directly using different techniques. One of them consists of expressing a gene coding for an antibody specifically recognizing and inactivating the targeted protein. This kind of antibodies, known as intrabodies, must not be secreted and can even be targeted to some cell compartments to reach the intracellular proteins.

Another possibility relies on the overexpression of a gene coding for an inactive analogue of the targeted protein. The protein encoded by the gene can suppress or greatly attenuate the activity of the protein of interest by playing the role of a decoy. This was exemplified more than one decade ago. A gene coding for an inactive analogue of an insulin receptor still capable of binding the hormone allowed the generation of transgenic mice that were

no more sensitive to insulin and thus suffering from type II diabetes. This strategy is currently used in nature. This is namely the case for some hormone receptors and transcription factors which exist under different forms synthesized in the same cells in a controlled manner.

3.3. Control of Transgene Expression by Exogenous Factors

All the vectors described above and used to express transgenes contain promoters which are naturally active in the cells of the transgenic animals. This implies that the transgenes are regulated by the natural inducers of the host genes. In some cases, artificial promoters containing natural or mutated regulatory elements associated in a non natural manner are used to direct transgene expression. Transgene expressed under the direction of injected oestrogens is one example among many others. This approach is fundamentally limited. The oestrogenic inducers will stimulate or inhibit not only the transgene but a number of host genes, leading to complex and unknown side effects in the animals.

To circumvent these problems, artificial promoters, containing regulatory elements from both animal genes and bacterial genes, have been designed. The resulting promoters are active in animal cells but controlled by substances active in bacteria but not in animals. The most popular system is based on the use of the bacterial tetracycline repressor gene. In practise, the transgene becomes reversibly activated only when tetracycline is administered to the animals. A number of similar systems are available and currently used in transgenic animals with good success (Malphettes and Fussenegger 2006). These tools virtually offer the possibility to express a transgene precisely in a given cell type and at a given moment.

3.4 Deletion of Transgenes or Genomic DNA Regions

Deletion of genomic DNA regions is required in some circumstances. Conventional homologous recombination makes gene deletion, known as knockout, possible (see 2.8). Another possibility consists of using the Cre-LoxP or Flp-FRT sytems. A LoxP sequence must first be added on both ends of the fragment to delete. The presence of the Cre recombinase will then recombine the two LoxP sites leading to a deletion of the DNA fragment located between the LoxP regions. The Cre recombinase may be synthesized by the corresponding gene under the direction of a cell specific promoter. The Cre recombinase will be present and the deletion of the DNA fragment will take place only in the cells in which the promoter is active (figure 3). This process can be controlled by still more sophisticated tools. The promoter directing the Cre gene expression can be under the dependency of tetracycline. The deletion of the DNA fragment will then occur only in the chosen cell type and at the chosen moment. Another level of control can be obtained by using an engineered Cre recombinase which becomes reversibly active in the presence of an oestrogen analogue, 4-hydroxy tamoxifen.

This offers the advantage of having the active Cre recombinase for short periods of time. This prevents the non-specific action of the Cre recombinase which can recognize cryptic sites in the host genome and induce illegitimate recombinations damaging the integrity of the host DNA. The steps to use of 4-hydroxy tamoxifen-dependent Cre recombinase are depicted in figure 3.

The Cre-LoxP approach may be implemented to withdraw a given gene or a regulatory region from a genome. In the first case, the operation is known as conditional knockout. The same technique may also be used to activate a gene conditionally. In this case, a DNA sequence having an inhibitory action on transcription and bordered by two LoxP sequences may be added between the promoter of a gene and its transcription start site. The

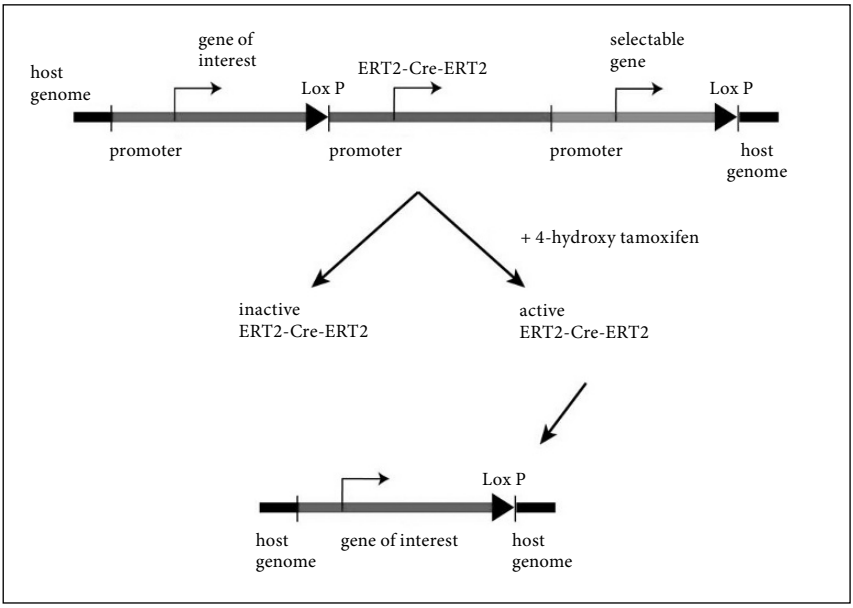


Figure 3: Elimination of the marker and selectable genes. The vector for homologous recombination (not shown here) allowed a gene knockout. The genomic targeted gene was interrupted by a DNA sequence containing a selectable gene but also the gene for a form of Cre recombinase (ERT2-Cre-ERT2 active only in the presence of 4-hydroxy tamoxifen) and two LoxP sequences. After the gene knockout, even at the next animal generation, 4-hydroxy tamoxifen may be added to embryos. This activates the Cre recombinase which recombines the two LoxP sequences leading to the elimination of the selectable gene and of the Cre recombinase gene. The remaining LoxP sequence is sufficient to knockout the targeted gene. This approach allows the elimination of the DNA sequences not necessary for the inactivation of the targeted gene and it avoids the toxic effects of overexpressed Cre recombinase.

gene will remain silent until the inhibitory sequence is deleted by the action of the Cre recombinase. Another application of this tool is the elimination of marker genes. Some of the techniques described in section 3 imply the use of marker genes or selection genes. This is the case when pluripotent cells or the cloning techniques are implemented (Houdebine 2007a). The marker genes and selection genes are not required for the action of the transgene. Their presence in the animals may have no effects, yet their elimination has been recommended by FAO/WHO and Codex Alimentarius (FAO/WHO 2007).

4 Conclusions and Perspectives

The methods to generate transgenic animals and to control transgene expression have made very significant progress during the last few years. This greatly contributes to facilitate basic research and biotechnological applications, even if the efficiency of the transgenesis techniques remains a limiting point.

The main uses of transgenic animals are presently the following ones. More than 90% of transgenic animals are used to study gene function and mechanisms of action. Many transgenic models are also generated specifically to study human diseases and to validate new medicaments (Houdebine 2007b). The possibility of grafting pig organs to humans requires transgenesis for both studying rejection mechanisms and to generate the pig donors in future (Petersen et al. 2008; Niemann, this issue). Milk from transgenic mammals and chicken egg white has started being the source of pharmaceutical proteins (Van de Lavoir et al. 2006; Houdebine 2008). A number of projects aiming at improving animal breeding are in course (Laible 2008). The most advanced project concerns salmon farming. Faster growing salmons have been obtained by overexpressing salmon growth hormone genes. This project waits for industrial development until the confinement, either physical or physiological, of these fish will become a reality (Kaputchinsky et al. 2007).

The use of transgenic animals raises specific biosafety and ethical problems. A number of research projects imply the use of dangerous animals, essentially when pathogenic organisms are being studied. These experiments are performed in appropriate confined areas and no accident due to this kind of research has been reported so far. Environmental problems are less numerous than those raised by some transgenic plants. Indeed, most farm animals are kept in confined areas and most of them have no wild partners in their neighbourhood. The consumption of products from transgenic animals is likely to become a reality in the coming years. Specific guidelines have been defined for this purpose and are ready to be adopted by Codex Alimentarius. The tests applied to transgenic plants have been

extrapolated to animals. Animals offer similar and distinct biosafety problems. Healthy animals and mainly mammals have very little chance to contain toxic substances generated by the presence of a transgene as they are themselves the first target of such substances. Compared with plants, some pathogens might proliferate more easily on some transgenic animals and be transmitted to humans.

Some problems of welfare are clearly specific of transgenic animals. Transgenesis per se may reduce animal welfare in some cases as it includes oocyte collection and embryo transfer. In some cases, the transgene can induce a specific suffering. The use of transgenesis is very diverse and the ethical problems are also diverse. It is important to note that the improvement of transgenesis techniques contributes to a diminuition of animal suffering. Indeed, an increase in transgenesis efficiency reduces the number of animals to be used for experimentation. A well controlled expression of the transgenes may also reduce their side effects. A number of projects aimed at preventing animal diseases are in course. They may contribute to the reduction of animal suffering. It may be useful to classify the different uses of transgenic animals. In a first class there are the experimental animals used for research. These animals are not very numerous in each experiment, they are not produced to make money and the effects of transgenes cannot be predicted in all cases. A second class may include animals being the source of products for the treatment of human diseases. Animals producing pharmaceutical proteins or organs for patients belong to this class. In these cases, the suffering of animals is known and a reality for a number of them. These animals may contribute to patient treatment but they may also bring substantial benefit to companies. The third class may include farm animals. Their use virtually implies a large number of animals having a known and transmittable suffering if any, with a limited impact on human survival. Tolerance towards suffering may then be the highest for the use of the first class animals, on a case by case basis for the second class animals and totally non-existent for the third class animals.

A specific problem of animal transgenesis is its possible extrapolation to humans. This extrapolation is very likely possible, especially by using gene transfer techniques such as those implementing ICSI or lentiviral vectors. It is worth noting that, as far as we know, these techniques of gene transfer have never been used in humans. Important technical problems remain to be solved to envisage transgenesis in humans. One of these problems is the control of gene integration. It must be noted that gene targeting is potentially feasible using engineered meganucleases. The major problem remains the actions of the transgenes. Many genes have complex and multiple functions in mammals. The side effects generated by a transgene cannot be fully predicted and this bottleneck may remain for a long time. Whatever happens, the major potentially acceptable application of transgenesis in humans is expected to be the protection against diseases. This implies

that the genes responsible for the diseases are known. If this is the case, it appears simpler, safer and more ethical to eliminate the embryos harbouring the faulty alleles than trying to modify them.

References

Baer A, Bode J (2001) Coping with kinetic and thermodynamic barriers: RMCE, an efficient strategy for the targeted integration of transgenes. Curr Opin Biotechnol 12:473–480

Ding S, Wu X, Li G, Han M, Zhuang Y, Xu T (2005) Efficient transposition of the piggyBac (PB) transposon in mammalian cells and mice. Cell 122:473–483

FAO/WHO (2007) expert consultation on safety assessment of foods derived from recombinant-DNA animals (for Codex Alimentarius)

Han JY (2008) Germ cells and transgenic chicken. Comp Immunol Microbiol Infect Dis (in press)

Houdebine LM (2005) Use of transgenic animals to improve human health and animal production. Reprod Dom Anim 40:269–281

Houdebine LM (2003) Animal Transgenesis and Cloning. Wiley and Sons, London

Houdebine LM (2007a) Latest developments in relation to the use of reporter and selectable genes in animal biotechnology. FAO/WHO expert consultation on safety assessment of foods derived from recombinant-DNA animals (for Codex Alimentarius)

Houdebine LM (2007b) Transgenic animal models and target validation. Methods in Molecular Biology 360:163–202

Houdebine LM (2008) Production of pharmaceutical proteins by transgenic animals. Comp Immunol Microbiol Infects Dis (in press)

Kaputchinsky AR, Hayes KR, Li S, Dana G (eds) Environmental risk assessment of genetically modified organisms. Methodologies for transgenic fish, vol 3 (2007) CAB International Publisher

Laible G (2008) Enhancing livestock through genetic engineering – recent advances and future prospects. Comp Immunol Microbiol Infect Dis (in press)

Lillico SG, Sherman A, McGrew MJ, Robertson CD, Smith J, Haslam C, Barnard P, Radcliffe PA, Mitrophanous A, Elliot EA, Sang HM (2007) Oviduct-specific expression of two therapeutic proteins in transgenic hens. Proc Natl Acad Sci 104: 1771–1776

Long X, Miano JM (2007) Remote control of gene expression. J Biol Chem 282:15941–15945

Malphettes L, Fussenegger M (2006) Improved transgene expression fine-tuning in mammalian cells using a novel transcription-translation network. J Biotechnol 124:732–746

Manzini S, Vargiolu A, Stehle IM, Bacci ML, Cerrito MG, Giovannoni R, Zannoni A, Bianco MR, Forni M, Donini P, Papa M, Lipps HJ, Lavitrano M (2006) Genetically modified pigs produced with a nonviral episomal vector. Proc Natl Acad Sci USA 103:17672–17677

Moreira PN, Pozueta J, Pérez-Crespo M, Valdivieso F, Gutiérrez-Adán A, Montoliu L (2007) Improving the generation of genomic-type transgenic mice by ICSI. Transgenic Res 16:163–168

Nakagawa M, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T, Okita K, Mochiduki Y, Takizawa N, Yamanaka S (2008) Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat Biotechnol 26:101–106

Petersen B, Carnwath JW, Niemann H (2008) The perspectives for porcine- to-human-xenografts. Comp Immunol Microbiol Infect Dis (in press)

Pfeifer A (2006) Lentiviral transgenesis – a versatile tool for basic research and gene therapy. Curr Gene Ther 6:535–542

Porteus MF Carroll D (2005) Gene targeting using zinc finger nucleases. Nat Biotechnol 23:967–973

Rao M (2007) Scalable human ES culture for therapeutic use: propagation, differentiation, genetic modification and regulatory issues. Gene Therapy 15:82–88
Robl JM, Wang Z, Kasinathan P, Kuroiwa Y (2007) Transgenic animal production and animal biotechnology. Theriogenology 67:127–133
Saidi S, Rival-Gervier S, Daniel-Carlier N, Thépot D, Morgenthaler C , Viglietta C, Prince S, Passet B, Houdebine LM, Jolivet G (2007) Distal control of the pig whey acidic protein (WAP) locus in transgenic mice. Gene 401:97–107
Shen W, Li L, Pan Q, Min L, Dong H, Deng J (2006) Efficient and simple production of transgenic mice and rabbits using the new DMSO-sperm mediated exogenous DNA transfer method. Mol Reprod Dev 73:589–594
Shinohara ET, Kaminski JM, Segal DJ, Pelczar P, Kolhe R, Ryan T, Coates CJ, Fraser MJ, Handler AM, Yanagimachi R, Moisyadi S (2007) Active integration: new strategies for transgenesis. Transgenic Res 16:333–339
Sioud M (2006) Innate sensing of self and non-self RNAs by Toll-like receptors. Trends Mol Med 12:167–176
Smith K, Spadafora C (2005) Sperm mediated gene transfer: applications and implications. BioEssays 27:551–562
Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872
Tiscornia G, Singer O, Ikawa M, Verma IM (2003) A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. Proc Natl Acad Sci USA 100:1844–1848
Van de Lavoir MC, Diamond JH, Leighton PA, Mather-Love C, Heyer BS, Bradshaw R, Kerchner A, Hooi LT, Gessara TM, Swanberg SE, Delany ME, Etches RJ (2006) Germline transmission of genetically modified primordial germ cells. Nature 441:766–769
Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, Bernstein BE, Jaenisch R (2007) In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature 448:318–324
Yong HY, Hao Y, Lai L, Li R, Murphy CN, Rieke A, Wax D, Samuel M, Prather RS (2006) Production of a transgenic piglet by a sperm injection technique in which no chemical or physical treatments were used for oocytes or sperm. Mol Reprod Dev 73:595–599

Animal Pharming: Past Experience and Future Prospects

Angelika Schnieke

Summary

'Pharming' can be defined as the use of transgenic animals or plants for the production of pharmaceutical proteins or peptides. Since the 1980s it has been proclaimed as an efficient and cost-effective method for the production of biopharmaceuticals. In 2006, the first therapeutic product produced in the milk of transgenic livestock gained approval, ATryn®, a recombinant form of human antithrombin III, produced by GTC Biotherapeutics. This was an important milestone but a long time coming, too long for some biotechnology companies. The near future will show if pharming can regain investor confidence, and whether society and the pharmaceutical industry will accept transgenic livestock as an alternative to more established production methods. There is cause for optimism for biopharmaceuticals represent a considerable and growing market opportunity and animal pharming has made considerable strides. In two decades a novel production platform has been established, new and groundbreaking technologies developed and the necessary regulatory framework put in place. This article highlights some of the obstacles pharming has faced and what the near future might bring.

Introduction

In plants the term 'pharming', or more commonly 'molecular farming' or 'plant made pharmaceuticals', encompasses both production in whole plants and cultured cells. Animal pharming is conventionally restricted to whole animals. One reason for this difference is that biomanufacturing using mammalian cell culture is an established and successful industry that does not wish to become associated with the socio-economic, legal and ethical issues raised by transgenic animals, as for example discussed in several chapters of this book. Nevertheless, the systems have a great deal in common at a technical level. The markedly different circumstances of the cell culture and pharming industries seem more a result of history rather than a reflection of their relative merits. The questions therefore arise whether there are markets for both, what products are best suited to production in animals, what are the rational safety concerns, what are the effects of

intellectual property rights and what are the requirements for regulatory approval. We review how these questions affect the pharming industry and its prospects.

Principles and Technology

The basic scheme for producing pharmaceutical proteins in transgenic animals is illustrated in figure 1. DNA, which encodes the desired protein, is isolated and incorporated into an expression construct with a gene promoter and regulatory regions that determine the pattern of expression. This is introduced as a stable transgene into animals which are propagated normally. Body fluid such as milk is collected, the protein purified and formulated for human use. Protein manufacture in cell culture follows the same scheme, with immortalised cells in bulk culture vessels substituting for animals.

Three important considerations inform the design of a transgenic expression construct. Choice of tissue: secretion of protein into body fluids or into the white of chicken eggs are favoured approaches because these are naturally renewed and can be harvested without excessive invasion. Specificity of expression: strictly restricting the expression of a foreign protein to a particular tissue may be important to avoid ill effects in the producing ani-

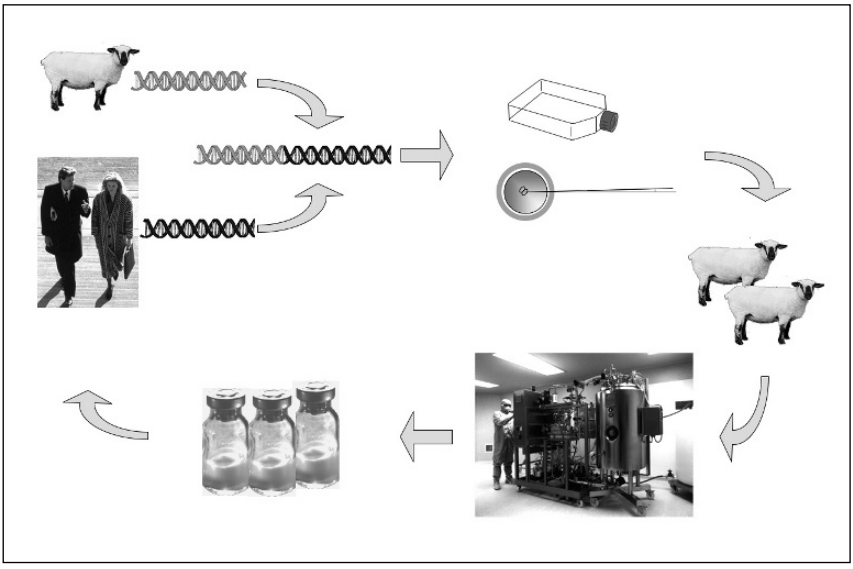


Figure 1: Overview of recombinant protein production in transgenic animals. Recombinant expression constructs are generated and transgenic animals are produced by either DNA microinjection or somatic cell nuclear transfer. Animals are bred, fluid such as collected milk, protein purified and formulated.

mal. Abundance of expression: high levels of expression are desirable to aid protein recovery and the economic viability of the project.

In mammals, expression in the lactating mammary gland and secretion into milk has been by far the most common method (Clark 1998). Gene promoters and regulatory regions of the major milk proteins β-casein, whey acidic protein and β-lactoglobulin have all been recruited for transgene expression. Secretion into blood has been used less widely but will probably increase significantly for the production of antibodies. Urine and seminal fluid are alternatives which have not been widely investigated for commercial production, but their proponents assert that they may be useful for bioactive proteins, because products secreted into the urinary or seminal tracts are sequestered from the rest of the body. Pharming in birds has focused on chicken egg white, with transgene expression directed from the ovalbumin promoter (Lillico 2005).

Techniques of producing transgenic animals are dealt with in more detail in the papers in this book by Niemann et al. and Houdebine. In mammals, DNA microinjection was the predominant method for almost two decades. Retroviral transduction is considerably more efficient in terms of the percentage transgenic animals obtained (Hofmann et al. 2003; Whitelaw et al. 2004), but severely limits the size of DNA that can be introduced. Viral vectors are therefore rarely used for mammalian pharming, but are the method of choice for birds. Cell-mediated transgenesis by somatic cell nuclear transfer (SCNT) has now become the preferred method for producing transgenic livestock. Cultured primary cells are transfected, selected and used as nuclear donors. Although more labour intensive than direct transgenesis, in vitro culture offers the advantage that the presence, location and structure of the integrated transgene can be analysed in individual cell clones and the most suitable chosen to produce an animal. DNA microinjection in contrast is a lottery. In some circumstances it may also be possible to gain predictive information from cultured cells about likely RNA or protein expression in animals. Cell-mediated transgenesis also allows precise genetic modifications to be engineered by gene targeting.

SCNT requires fewer experimental animals than DNA microinjection, which reduces costs and benefits animal welfare. Techniques are continuing to improve. For example, bovine nuclear transfer data from 1998–1999 shows an efficiency of 6.3% calves born per reconstructed embryo, while in 2003–2004 this increased to 15% (Heyman 2005). Also, the need for live donors to obtain fertilised oocytes has now been entirely superseded by in vitro production using slaughterhouse-derived ovaries.

Market Opportunities for Both Cell Culture and Pharming?

Animal cell culture commenced in 1951 with the derivation of the HeLa cell line, and modern bulk culture dates from 1958 when Theodore Puck first

derived and propagated the now ubiquitous Chinese hamster ovary (CHO) cell line. After around fifty years of development, manufacturing in cultured cells is now an outstanding success. According to a 2007 report (Lawrence 2007) approximately one sixth of "blockbuster" drugs were biotechnology products. Nine of the top ten biotechnology drugs, ranked by U.S. sales, are produced in mammalian cell culture and at least 23 protein therapeutics have sales exceeding $1 billion. Many more are in late stage development.

Many earlier products were straightforward protein replacements such as erythropoietin and the blood clotting factors VIII and IX. This was followed by a second generation of derivatives with improved properties such as longer half-life, e.g. darbepoetin (Aranesp®). Recombinant antibodies, particularly with applications in oncology or oncology support, are rapidly becoming more important and can reasonably be expected to provide highly successful products in the near future. However the quantities required to supply the world market are often huge, hundreds of kilograms, exceeding the current and likely future production capacity.

Proponents of animal pharming have long stressed the advantages of transgenic animals as an alternative cost-effective means of production, capable of fulfilling this need. The most significant gains arise from the low-tech nature of the actual production system, animals in a field rather than complex culture vessels. Production herds can be expanded or reduced by normal animal husbandry in a way that bulk culture simply cannot. The choice of species depends on the amount of protein required, with large animals (cattle) being most suitable for the largest quantities; and also on time lines for product development, where small animals (chicken or rabbit) with short gestation times and large reproductive capacity may be more suitable. Comparative data for recombinant protein production in milk and chicken eggs are summarised in table 1.

Table 1: Basic data for protein production in milk and eggs

Species	Gestation period (months)	Age at sexual maturity (months)	Number of offspring	Age at first lactation (months)	Recombinant protein production (kg/individual/yr)
Cattle	9	16	1	33	40–80
Goat	5	8	1–2	18	4
Pig	4	6	~10	16	1.5
Sheep	5	8	1–3	18	2.5
Rabbit	1	5	~8	7	0.02
Chicken	21	5	1 egg per day	n.a.	up to 400µg/egg

The yield of recombinant protein produced in milk compares reasonably well with cell culture, at anywhere between 1–20g/L depending on the protein. CHO cell expression typically produces 0.5g/L or less. Some companies, such as Wyeth, are developing culture regimes which yield up to 5g/L, but it remains to be seen whether this can be maintained in large scale routine production.

The single most important expense in cell based production is the cell culture faculty. Commitment to a large facility necessarily falls at an early stage of product development, at phase 1 clinical trials or even during preclinical investigations, because production capacity must be sufficient to deliver material for mass clinical trials and a subsequent market launch. A cell culture production facility requires investment of approximately €600–900 million and 4–5 years for construction, validation, and licensing. All of this is exposed to the risk that the drug may fail trials and have to be either abandoned or radically modified, although this can be mitigated by using contract manufacturing facilities. Subsequent scale-up requires additional culture tanks, associated facilities and running costs.

By comparison, the cost structure of production with animals is more flexible. Until recently the cost of generating a transgenic animal suitable for founding a production herd or flock was approximately €50–150,000 depending on species. Advances in producing early mammalian embryos by in vitro maturation and fertilisation of slaughterhouse derived oocytes have reduced this to approximately €30–50,000. All of which can be achieved with relatively modest facilities. The time and costs involved in generating production herds from founder animals depend on the species, but are basically those required for conventional animal husbandry, plus costs of biocontainment and good manufacturing practice (GMP) compliance. Further details of the economics of transgenic animal production are provided in the paper by Walsh in this proceedings.

The purification and formulation stages of cell culture and animal derived products are basically the same, and facilities require similar investment for construction, validation and licensing.

Products

The main reason to express a particular protein in either animals or cultured animal cells, rather than bacteria, plants or yeast, is because appropriate post-translational processing is required for bioactivity. This category of proteins includes many important biomedical products, such as blood clotting factors and antibodies and there are currently no alternative means of expression. Efforts are underway to enhance the capacity of yeast to process complex proteins, but it is unknown if and when this would offer a practical substitute.

Evidence available indicates that almost any protein currently produced in mammalian cell culture could also be produced successfully in milk. Exceptions are highly bioactive proteins, such as erythropoietin, that can damage animal health if even a minute amount is expressed ectopically or leaks into general circulation. Alternatives to milk where products are strictly sequestered from the body, e.g. urine or avian eggs, may overcome this limitation in future. However, because pharming companies were founded on a means of production rather than product discovery, access to potential products has been a considerable problem. Most proteins with biomedical indications are protected by patents or proprietary information, and this has forced reliance on industrial partnerships. This has sometimes made pharming companies vulnerable to third-party decisions, including the termination of successful large animal studies. It also meant that only those products which had performed poorly or failed in cell culture (e.g. fibrinogen and other coagulation factors) were tried in animals. It is therefore difficult to make a fair comparison, but the successful synthesis, processing and assembly of several highly complex proteins, suggests the probable superiority of the mammary gland over CHO cells.

At the time of writing, the two major players in the field, Pharming (www.pharming.com, July 2008) and GTC Biotherapeutics (www.transgenics.com, July 2008) list a total of twelve proteins as under active development. Seven of these (α1-antitrypsin, human serum albumin, type I collagen, fibrinogen and clotting factors VII, VIII and IX) were previously investigated by the Edinburgh company PPL Therapeutics during the 1990s. This limited range does not reflect a shortage of products technically suitable for pharming, but rather restrictions imposed by the current commercial environment. However, many early products are now coming off patent and this will bring new product opportunities.

Intellectual Property and Pharming Technology

In addition to restricting access to potential products, intellectual property rights have also had a major impact on the access to key technologies. DNA microinjection, nuclear transfer, stem cells, gene targeting, promoters and other elements required for successful gene expression have all been covered by granted or pending patents. It is noteworthy that at the outset, the number of patents corresponding to useful milk promoters effectively determined the number of companies which could pursue transgenic mammary gland expression.

However the current patent system is widely viewed as poorly suited to the realities of modern biology and biotechnology, and practices such as the granting of overbroad claims criticised as protectionist and stifling innovation. For example, a patent granted within the US to GTC Biotherapeu-

tics claims "any therapeutic protein in the milk of any transgenic mammal" (www.transgenics.com, July 2008) making it somewhat difficult for other companies to compete. While patent rights can be disputed, the financial costs are high. Such a dispute was largely responsible for the bankruptcy of the Dutch company Pharming in 2002. However, as with products, many patents covering valuable tools such as milk gene promoters are due to expire in the near future, which should ease restrictions on new ventures.

Product Safety and Quality

An important motivation for the production of proteins in animals and animal cells has been to provide safe replacements for products derived from human tissues. These carry a risk of associated pathogens if not properly screened and this is dangerously exacerbated when material from multiple donors is pooled. There have been numerous tragic illustrations of this hazardous practice. In France, several thousand haemophilia patients were infected with HIV following treatment with contaminated blood products during the 1980s (Anderson 1991), and more than two hundred children treated with human brain derived growth hormone contracted Creutzfeldt-Jakob disease (Brown 2000). In Japan, more than ten thousand people are estimated to have been infected with hepatitis C from blood derived fibrinogen used during surgery between 1964 and 1988 (Yasunaga 2007). Large scale contaminations have also been documented in several other countries. Recombinant proteins dramatically reduce these dangers, an advantage that has not always been communicated effectively to the public.

Production in animal systems does however incur new risks: possible zoonotic disease, contamination with animal proteins and DNA, and alterations in the structure of recombinant protein. The magnitude of each of these risks has had to be ascertained during the development of the industry. Transgenic technology is relatively new and has therefore been scrutinised very intensely.

The possibility of transmitting zoonotic disease, such as BSE (Bovine spongiform encephalopathy) or avian flu, is well recognised. Measures against both known and unknown pathogens must therefore taken during both production and protein purification. For example, animal health is regularly monitored and strict precautions taken to prevent contact with other domestic or wild animals and to exclude people and equipment in recent contact with either. Concerns regarding transmission of prion diseases (BSE, scrapie) also mean that land used as animal pasture should not have seen contact with other farm animals for several years. For this reason, some companies have chosen to raise animals in countries free of prion diseases, for example New Zealand. Alternatively, some animals such as pigs and rabbits can be raised indoors in specific pathogen free facilities.

Large scale recombinant protein purification has so far only been developed for milk. This is a multi-step process that combines standard methods developed for the dairy industry with procedures developed for purification of recombinant proteins produced in cell culture. The intention is to remove animal proteins, DNA and any micro-organisms, bacteria, viruses or prions. The acceptable amount of contaminating animal protein present in a particular product is determined by its ultimate application. For example a product intended for regular intravenous injection over long periods would require extremely high levels of purity, while more animal protein would be acceptable in a product to be applied topically or ingested as a nutraceutical.

The specifics of purification depend on the properties of each protein, but here is an outline. Most recombinant proteins are present in the whey fraction, therefore the first steps are removal of fat and suspended caseins. This is followed by chromatography to isolate the recombinant protein away from remaining milk proteins. Final clean-up steps might include ultrafiltration and possibly heat treatment to prepare a pharmaceutical-grade therapeutic product. Current experience indicates a final yield of purified product of between 40–60% of the amount in milk, depending on the nature of the protein and the required purification procedure.

Production and purification of products from transgenic animals must comply with current US Food and Drug Administration (FDA) or European Medicines Agency (EMEA) guidelines and GMP. GMP encompasses training of personnel, validation of procedures, equipment, materials and facilities as well as standard operating procedures. Production criteria must be defined at the outset, such as acceptance criteria for source material, product pooling, batch size and product quality and purity required at various stages of purification to ensure product consistency. Throughout, documentation is essential and meticulous records must be kept of all activities, from the production of the DNA construct to the final product.

The concept of the "well-characterised biologic" has been central to the standards required by the FDA for both cell culture and pharming products. This was first defined in 1995 as "a chemical entity whose identity, purity, impurities, potency, and quantity can be determined and controlled" (Schultz 1995). Meeting this standard is however considerably more difficult than for chemically produced products. Biopharmaceuticals of all types have encountered problems and there have been calls for a revised definition. Although appropriate standards for purity, potency and quantity can readily be established for a product intended for a particular application, the concept of "identity" is not straightforward. The molecular structure of a recombinant product can be characterised extensively by in vitro analysis, but both recombinant and native proteins sometimes exist as a mixture of subtly different forms. Whether this represents uncertain and unacceptable identity is open to debate. Furthermore, a protein produced in a heter-

ologous species and cell type will rarely be an exact replica of the native (e.g., human) form, but could nevertheless function as a good "bio-equivalent". Safety and efficacy as determined by pre-clinical and clinical trials are clearly the most important criteria by which to judge potential products.

Protein glycosylation has been a particular concern. The patterns of oligosaccharide chains cross-linked to asparagine residues in the protein chain (N-linked glycosylation) vary between mammalian species, cell types and even metabolic states of the same cell. Notably, humans differ from the majority of other mammals in the type of sialic acids present. Most mammals have a mixture of two principle forms, N-acetylneuraminic acid and N-glycolylneuraminic acid. Humans have a mutation in the enzyme responsible for producing N-glycolylneuraminic acid and therefore lack this form. Proteins with non-human patterns of glycosylation may have altered pharmacological properties in patients, for example being cleared from the body at an increased rate, and could also be immunogenic especially if administered intravenously over long periods. These issues are similar for both cell culture and transgenic animal production. Whether glycosylation constitutes an actual, rather than a perceived problem for pharming has yet to be determined, because the number of trials is still too low. Trial results from GTC Biotherapeutics' lead product, recombinant human antithrombin III, are so far encouraging with no immune reactions detected in more than 200 patients treated.

From Concept to Product

The birth of the first transgenic large animal was reported in 1985 (Hammer et al. 1985) and within three years several companies had been formed to produce pharmaceutical proteins in milk. Only in the following years were the first pharming animals actually born: a sheep "Tracey" from Pharmaceutical Proteins Ltd, a bull "Hermann" from Pharming, and a goat from Genzyme Transgenics. There was therefore little experience available at the time, and these pioneering companies faced a formidable task establishing a new production platform for the most exacting of markets. Not only did the technology have to be evaluated and improved, but legal guidelines were lacking and had to be worked out in parallel. This was carried out in the face of high investor expectations and scepticism, even hostility, from the public.

Production and breeding of transgenic livestock necessarily takes time, so several years were required to fully evaluate and refine the technology, recognise problems and address shortcomings. This included improvements in the design of transgene vectors to provide abundant and specific expression, as described in the paper by Houdebine in this proceedings. The processing and post-translational modification capability of the mammary gland also had to be assessed and compared between species. For exam-

ple, γ-carboxylation of glutamate residues, essential for bioactivity of several blood coagulation factors, is carried out poorly by CHO cells (Sinha et al. 1994). The lactating mammary gland provided a considerable improvement in γ-carboxylation, but significant differences were discovered in the proportion of fully γ-carboxylated protein obtained from the species investigated: pigs, sheep and rabbits, with rabbits proving to be the best.

Because there was, and still is, no practical method of stably culturing lactating mammary cells for long periods, transgenes destined for expression in milk are generally first tested in transgenic mice to gain some predictive information. Mice can provide an indication of the overall expression level, bioactivity of the protein and any effects on animal health. However, many issues only emerged once transgenic livestock had been generated and studied in detail. These include species-specific differences in RNA processing leading to aberrant splicing, and species-specific protein interactions. As studies were carried out over generations, it became apparent that transgene mosaicism was more frequent in microinjected livestock than mice, an important factor when breeding from founder animals. Another observation rarely reported in mice, despite the large numbers being produced, was instability of the transgene locus due to inverted repeats. Although transgene rearrangements had been reported earlier (Covarrubias et al. 1986) the first proper description of the phenomenon in mice was in 1996 (Collick et al. 1996). In contrast, inverted repeats were observed several times in transgenic livestock despite their far smaller numbers.

In 1995, the United States Food and Drug Administration (FDA) produced guidelines for pre-market data submission for potential products from transgenic sources. Amongst other specifications, these require that the structure and expression pattern of the integrated transgene construct be characterised in the founder animal and demonstrated as reliable through subsequent generations.

The production of recombinant fibrinogen in the milk of sheep was a project carried out by PPL Therapeutics between 1993 and 2004 and is useful in illustrating the range of problems that can be encountered with regard to transgene, RNA and protein stability. It also demonstrates that a complex multimeric protein, which had repeatedly failed in tissue culture, can be successfully expressed in the mammary gland and provide a valuable product.

Case Study: Fibrinogen

Fibrinogen is a vital part of the blood coagulation system. It circulates as a soluble protein in blood plasma until activated by thrombin and stabilised by factor XIII in the final stage of the coagulation cascade. Activated fibrinogen converts to insoluble fibrin, which forms the structural compo-

nent of the blood clot until it is eventually degraded by plasmin. Fibrinogen is a complex molecule composed of six polypeptide chains, two each of α, β and γ chains cross-linked by multiple disulphide bonds. Extensive post-translational modification is required for correct assembly of the hexamer.

The most important biomedical applications of fibrinogen are in surgery, for example as a tissue glue, haemostat, tissue sealant and anti-adhesive. It can be derived from donated human blood with the attendant risks, or alternatively prepared from a patient's own blood if circumstances allow several weeks notice before surgery, clearly not always possible. A safe readily available recombinant product is therefore highly desirable. Attempts to express functional fibrinogen in cultured cells have so far been unsuccessful, the main problem being failure of β chain expression. Fibrinogen is therefore an attractive candidate for production in animals.

Three protein chains require three genes. Three independent expression constructs were produced, each driven by the β-lactoglobulin promoter and coupled to a 3' untranslated region of the β-lactoglobulin gene. Triple transgenic sheep were generated by co-injecting an equimolar mixture of each. Twelve of the resultant animals were transgenic (6%) and nine, four females and five males, contained all three transgenes. Two of these expressed relatively high levels of recombinant fibrinogen in their milk (between 2.5–5g/L) and were chosen for production.

However, analysis of the transgene locus over subsequent generations of these two lines revealed that the copy number varied between animals. This was traced to an unstable inverted repeat within a tandem array of α, β and γ transgene copies in the founders. Indeed, individual sheep were found to be mosaic for different transgene structures, indicating that recombination also occurred in somatic cells. Stable genetics is a fundamental requirement for any transgenic product, so these lines could not be used in production. Conceivably they could have been bred over several generations until the transgene array resolved to a stable configuration, but a far better approach was to generate new transgenic lines by SCNT.

Analysis of fibrinogen RNA expression also showed a high proportion of unexpectedly large α and β mRNA species. These were found to be splice variants retaining an intron from the 3' β-lactoglobulin region and had not been observed from the same constructs in mice. Fortunately these variants did not affect the protein expressed as all were outside the translated region, but they illustrate the difficulty in obtaining reliable predictive information.

Protein analysis revealed degradation products due to proteolysis by plasmin in milk. In retrospect this was foreseeable as plasmin is the major protease in milk. PPL could solve this by crossing fibrinogen transgenic sheep with another line which expressed the protease inhibitor AAT (α1-antitrypsin). Analysis of fibrinogen/AAT milk showed good quality undegraded fibrinogen chains. Even though AAT is not a particularly

strong inhibitor of plasmin, co-expression at high level efficiently blocked fibrinogen proteolysis.

The first concept for a fibrinogen product was to reproduce the natural coagulation process in vitro using recombinant thrombin produced in CHO cells and factor XIII from yeast. This produced a fibrin mesh with mechanical strength equivalent to a natural clot. However, approaches to the FDA revealed that such a triple recombinant product would encounter significant difficulties gaining approval. For example although recombinant thrombin was produced in cell culture under specific pathogen free conditions using serum-free media with no exposure to other animal products, however it was activated using venom obtained from snakes fed on rodents. After lengthy explorations whether all stages in the process could be controlled sufficiently to ensure safety for human use, it was decided that a product based on such diverse origins was unlikely be approved.

This forced a return to the development stage and a search for an alternative, simpler product formulation. It was decided to avoid thrombin and factor XIII altogether but rather use batroxobin, a snake venom available as a recombinant product which had already gained regulatory approval. Batroxobin induces partial activation of fibrinogen by releasing the fibrinopeptide from the α fibrinogen chain only. This is sufficient to induce fibril formation. The resulting product was easy to use and highly effective as a tissue sealant and anti-adhesive in preclinical animal studies. However the delay involved in generating new transgenic lines could not be sustained by the financial resources available to PPL. Following withdrawal of the principal industrial partner for it's lead product AAT, PPL declared bankruptcy in 2004. Fibrinogen development is now being pursued by Pharming.

New Technologies and Novel Opportunities

The fibrinogen study was an example of transgenesis using pronuclear DNA microinjection. A technique now recognised as suffering several disadvantages: the random location of transgene integration, the inability to analyse transgene loci until after animals are born, and the inability to carry out sophisticated genetic alterations such as gene inactivation and transgene placement by gene targeting. In mice, gene targeting in embryonic stem (ES) cells has been phenomenally productive, but ES (embryonic stem) cells have still not been derived from livestock mammals. Here too the pharming industry played an important role, collaborating with academic researchers to circumvent this deficiency. The development of somatic cell nuclear transfer (SCNT) often loosely termed "cloning" was motivated largely by the need for an alternative to DNA microinjection as a means of producing transgenic large animals (Schnieke et al. 1997), in particular a method

that would enable transgene analysis before animals are produced and also facilitate gene targeting (McCreath et al. 2000). SCNT opened several new opportunities in biomedicine and has now become the method of choice for producing transgenic large animals, including those behind Pharming's fibrinogen and GTC's approved product ATryn®.

SCNT and new transgene vectors such as artificial chromosomes are now instrumental in bringing about new developments in pharming. The prospect of producing human polyclonal antibodies is particularly exciting as it will transform transgenic animals from their current role as passive vehicles for the production of pre-existing proteins to a means of generating totally new pharmaceutical products. This has the potential to propel animal pharming to a more prominent role in the pharmaceutical industry and is the subject of the next two sections.

Therapeutic Antibodies, Current Status

Antibodies for human therapy are the fastest growing set of new biopharmaceuticals, in terms of both number of products and market share. At the time of writing, over twenty antibody products have been approved for therapeutic use and several hundred are undergoing trials with potential applications in cancer therapy, autoimmune diseases, transplantation, antibiotic resistant infections, biodefense and immune deficiencies. Most of these are monoclonals produced by variations of the hybridoma technique originally developed by Köhler and Milstein in 1975 (Köhler 1975). Mice are immunised, B-cells extracted from the spleen and fused with myeloma cells to produce immortalised hybridoma cell clones, each secreting a particular antibody molecule. Hybridoma clones secreting an antibody with the desired specificity are chosen and expanded. Recombined immunoglobulin genes or cDNAs are now usually transferred from hybridomas into CHO cells to maximise antibody yield.

The first therapeutic monoclonal to gain regulatory approval was Ortho Biotech's orthoclone OKT3® (Muromonab) antibody to T-cell CD3 antigen for the suppression of kidney transplant rejection in June 1986. These first generation monoclonals, composed entirely of mouse immunoglobulins have generally been of limited use in human therapy, because patients often raise an immune response. This can neutralise antigen binding and cause rapid clearance of the antibody from the body. More seriously, repeated administration risks severe adverse reactions such as anaphylaxis that effectively rule out their use for chronic or recurring disease.

Immunogenicity can be reduced to some extent by using proteolytic fragments containing the antigen binding regions (Fab fragments) rather than the whole mouse antibody. Even so, such products are now mainly used in disease imaging rather than direct therapy. Examples are Immu-

nomedics' Sulesomab (Leukoscan®) and Arcitumomab (Cea-Scan®) used in the imaging of inflammatory conditions and various cancers, Centocor's Imciromab (Myoscint®) anti-myosin monoclonal used in the detection of heart muscle damage, and Boehringer Ingelheim's Nofetumamb (Verluma®) in imaging lung cancer.

Considerable efforts have been made to reduce the mouse content by genetic engineering. The first products were chimeric antibodies composed of human constant regions fused with mouse variable regions with about one third mouse and two thirds human protein. A Fab fragment from such a chimeric antibody was approved in 1994, Centocor's Abciximab (ReoPro®) to block platelet aggregation after cardiac angioplasty or stent insertion. The first whole chimeric antibody to be approved was from Biogen Idec and Genentech, Rituximab® (Rituxan) against B-cell CD20 antigen approved in 1997 for non-Hodgkin lymphoma. It is now also used to treat leukemia and rheumatoid arthritis and is the top selling monoclonal with US sales for 2006 estimated around $3.9 billion (Lawrence 2007). The well known monoclonal Herceptin® for epidermal growth factor receptor 2 positive breast cancer also belongs to this category. US sales of Herceptin® in 2006 are estimated around $3.1 billion (Lawrence 2007).

The mouse component of an antibody can be further reduced by CDR (complimentary determining regions) grafting, often loosely termed "humanisation", in which all but the mouse complimentarity determining regions are substituted with human sequences. Protein Design Lab's Daclizumab (Zenapax®) antibody to the T-cell CD25 antigen was the first of this type to be approved in 1997 for organ transplantation.

Remodelling a mouse antibody, while maintaining antigen affinity is however difficult and time consuming, motivating a search for a more direct means of generating human antibodies. Although human hybridoma technology has been pursued since the 1980s, it has so far not been commercially viable, a major problem being the narrow range of B-cell specificities available. Phage display, an in vitro method which avoids the use of animals altogether, has been more successful. Abbot laboratories' Adalimumab (Humira®) antibody to tumour necrosis factor α was isolated from a large library of randomised combinations of synthetic human antibody variable regions in a bacteriophage expression vector. The product was approved for rheumatoid arthritis in 2002.

At present, considerable hopes are placed on animals engineered to replicate the human humoral immune response. This work was pioneered in the late 1980s by Marianne Brüggemann of Cambridge University (Brüggemann et al. 1989) and subsequently pursued by two companies, GenPharm (later acquired by Medarex) and Cell Genesys (which formed Abgenix, later acquired by Amgen). In 1994, both independently reported mice with immunoglobulin genes inactivated by gene targeting and carrying human immunoglobulin transgenes. These mice could be immunised and hybri-

domas derived that produced fully human monoclonal antibodies (Green et al. 1994; Lonberg et al. 1994). Subsequent work has sought to increase the repertoire of antibodies produced by providing more complete sets of human immunoglobulin variable and constant genes. Kirin Pharma have used human artificial chromosome vectors with very large DNA fragments to generate transchromosomic mice (Tomizuka et al. 2000).

Amgen have two upcoming products using their Xenomouse: Panitumumab (Vectibix®) against epidermal growth factor receptor, which received US approval for metastatic colorectal cancer in September 2006 and is awaiting approval in Europe; and Denosumab a monoclonal against NF-κB receptor activator ligand involved in bone resorption, that is undergoing clinical trials for various types of bone loss. Medarex have several monoclonals in trials using their HuMAb-mouse® and Kirin's transchromosomic mice™.

CHO cells remain by far the dominant means of producing monoclonals, but there are some signs that arguments for animal production are becoming persuasive, at least for the most successful products. GTC report the development of two monoclonal antibodies from goat milk (www.transgenics.com, July 2008). An anti-CD20 monoclonal equivalent to the hugely successful CHO derived Rituximab®, developed in partnership with LFB, a French blood fractionation company. Also a CDR-grafted anti-CD137 monoclonal, intended to stimulate immune rejection of solid tumours.

Polyclonal Antibodies: Future Blockbusters?

Therapeutic monoclonals from whatever source all share the feature that a single antibody binds only one epitope of an antigen. This contrasts with the normal vertebrate immune response, in which many B-cell clones generate a population of antibodies with a range of affinities against various epitopes. Monoclonals are therefore of limited effectiveness against pathogens which require neutralisation of multiple epitopes, pathogens with diverse strains or which mutate rapidly and venoms, e.g., from insects, snakes, spiders and scorpions, which may have tens or hundreds of toxic components.

Interest has therefore been increasing in the use of polyclonal antibodies, because they more closely mimic the natural response and provide a variety of binding capacities. Polyclonals are also more effective than monoclonals in the formation of immune complexes responsible for complement activation and the recruitment of neutrophils and macrophages. Passive polyclonal immunotherapy, or serum therapy, was successfully demonstrated in 1891 by Emil Behring as a treatment against diphtheria toxin, and so is hardly a new concept. Until recently, however, human polyclonal antisera could only be obtained from people, severely restricting the quantity and specificity of sera available.

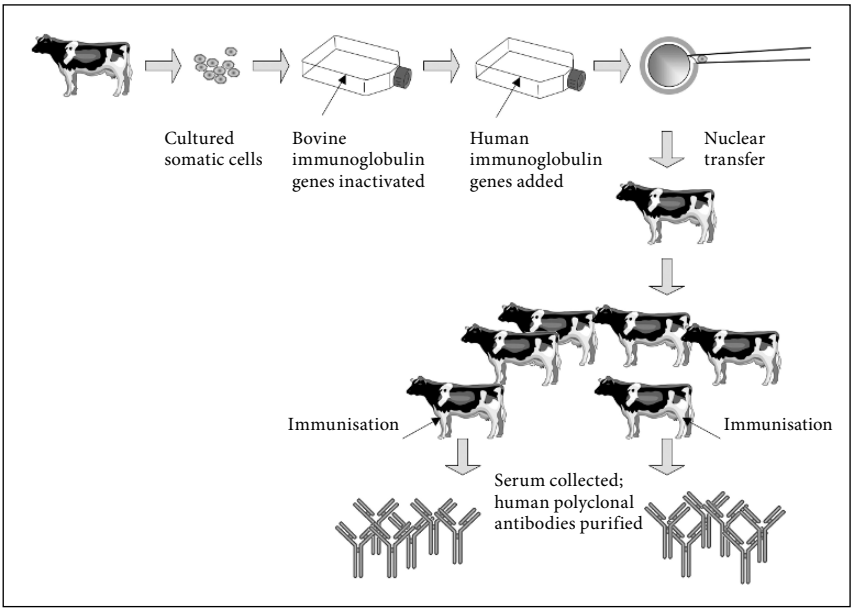


Figure 2: Production of human polyclonal antibodies in gene-targeted, transgenic or transchromosomic livestock

The production of mice capable of a human humoral immune response has radically transformed prospects for polyclonal antibodies. Mice are not a practical source of sera, as each adult contains only about 10mg total immunoglobulins. Cattle however have approximately 1kg total immunoglobulins and are eminently suitable, if the necessary genetic modifications can be carried out. Figure 2 provides an outline of the scheme.

This has not yet been accomplished, but significant progress has been made. Kirin Pharma have created transchromosomic cattle using a human artificial chromosome vector containing the entire un-rearranged sequences of the human immunoglobulin G heavy-chain and lambda light-chain loci (Kuroiwa et al. 2002). The loci were functional, but transmission of the artificial chromosome through the germ line and stable maintenance over generations may prove difficult. The same researchers have also reported targeted inactivation of the endogenous bovine immunoglobulin-μ heavy chain gene and the production of cattle by nuclear transfer (Kuroiwa et al. 2004).

It remains to be seen whether herds of large animals capable of producing human polyclonal antibodies can be raised and bred. But if this can be realised, the potential benefits for human health would be enormous. The range of new polyclonal sera that could be produced by immunising animals is effectively unlimited. Because the cost would be only a fraction of that required to develop a new monoclonal, many new applications would become economically feasible. Antisera could also be raised relatively

quickly, allowing a more rapid response to newly emerging pathogens. The ready availability of human antisera against an unlimited range of antigens could conceivably bring about a transformation of medicine comparable to the antibiotics revolution of the 1940s and 50s.

Concluding Statements

It is almost exactly twenty years since the first pharming companies were founded. Since then small companies have pioneered dramatic scientific and technical advances in the genetic manipulation of large animals and established novel methods of transgene expression. This period saw the establishment of guidelines and a coherent regulatory framework to cover production and management of transgenic herds and the purification, characterisation and administration of transgenic products.

Recent attitudes to pharming have been quite negative and many commentators have referred to the slow development of the field. However to be fair these are more reactions to initial over-hyped claims and consequently high investor expectations, than impartial judgements on the actual progress made. Certainly pharming has yet to fulfil its initial promise and make a significant impact on the pharmaceutical industry, but the first product has now come to market. GTC's ATryn®, recombinant human antithrombin III has been approved by EMEA, if only for the narrow indication of prophylaxis against venous thromboembolism during surgery in patients with congenital antithrombin deficiency. ATryn® is unlikely to become a blockbuster drug, but is nevertheless vitally important in countering the widespread scepticism that has grown around the technology. A commentary on ATryn® (Schmidt 2006) quotes a leading US equity analyst as saying "Before this decision, pessimists believed regulatory authorities would always find a way to shoot down transgenic proteins, … But now that we have an approval, that argument goes away".

The near future will likely see more milk-derived products coming to market and the establishment of new pharming companies, probably including some in developing economies such as China. However, while pharming was undergoing its gestation period, other technologies have made significant strides and the niche available for pharming may have been reduced. Cell culture is increasingly well established, and alternatives such as chemical synthesis of peptides and transgenic plants for bulk products are providing strong competition. To borrow the jargon of computer software development, pharming now needs a "killer application" to secure it's future. That is, a product or set of products with high biomedical value that can only be produced in transgenic animals. At the moment human antibodies offer tantalising possibilities, the next few years will reveal whether these will be realised.

References

Anderson C (1991) "AIDSgate" – a chronology. Nature 353:197–197

Brown P, Preece M, Brandel JP, Sato T, McShane L, Zerr I, Fletcher A, Will RG, Pocchiari M, Cashman NR, d'Aignaux JH, Cervenáková L, Fradkin J, Schonberger LB, Collins SJ (2000) Iatrogenic Creutzfeldt-Jakob disease at the millennium. Neurology 55:1075–1081

Brüggemann M, Caskey HM, Teale C, Waldmann H, Williams GT, Surani MA, Neuberger MS (1989) A repertoire of monoclonal antibodies with human heavy chains from transgenic mice. Proc Natl Acad Sci USA 86:6709–6713

Clark AJ (1998) The mammary gland as a bioreactor: expression, processing, and production of recombinant proteins. J Mammary Gland Biol Neoplasia 3:337–350

Collick A, Drew J, Penberth J, Bois P, Luckett J, Scaerou F, Jeffreys A, Reik W (1996) Instability of long inverted repeats within mouse transgenes. EMBO J 15:1163–1171

Covarrubias L, Nishida Y, Mintz B (1986) Early Postimplantation Embryo Lethality due to DNA Rearrangements in a Transgenic Mouse Strain. Proc Natl Acad Sci USA 83:6020–6024

Green LL, Hardy MC, Maynard-Currie CE, Tsuda H, Louie DM, Mendez MJ, Abderrahim H, Noguchi M, Smith DH, Zeng Y, David NE, Sasai H, Garza D, Brenner DG, Hales JF, McGuinness RP, Capon DJ, Klapholz S, Jakobovits A (1994) Antigen-specific human monoclonal antibodies from mice engineered with human Ig heavy and light chain YACs. Nat Genetics 7:13–21

Hammer RE, Pursel VG, Rexroad CE Jr, Wall RJ, Bolt DJ, Ebert KM, Palmiter RD, Brinster RL (1985) Production of transgenic rabbits, sheep and pigs by microinjection. Nature 315:680–683

Heyman Y (2005) Nuclear transfer: a new tool for reproductive biotechnology in cattle. Reprod Nutr Dev 45:353–361

Hofmann A, Kessler B, Ewerling S, Weppert M, Vogg B, Ludwig H, Stojkovic M, Boelhauve M, Brem G, Wolf E, Pfeifer A (2003) Efficient transgenesis in farm animals by lentiviral vectors. EMBO Rep 4:1054–1060

Köhler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495–497

Kuroiwa Y, Kasinathan P, Choi YJ, Naeem R, Tomizuka K, Sullivan EJ, Knott JG, Duteau A, Goldsby RA, Osborne BA, Ishida I, Robl JM (2002) Cloned transchromosomic calves producing human immunoglobulin. Nat Biotechnol 20: 889–894

Kuroiwa Y, Kasinathan P, Matsushita H, Sathiyaselan J, Sullivan EJ, Kakitani M, Tomizuka K, Ishida I, Robl JM (2004) Sequential targeting of the genes encoding immunoglobulin-mu and prion protein in cattle. Nat Genetics 36:775–780

Lawrence S (2007) Billion dollar babies – biotech drugs as blockbusters. Nat Biotechnology 25:380–382

Lillico SG, McGrew MJ, Sherman A, Sang HM (2005) Transgenic chickens as bioreactors for protein-based drugs. Drug Discov Today 10:191–196

Lonberg N, Taylor LD, Harding FA, Trounstine M, Higgins KM, Schramm SR, Kuo CC, Mashayekh R, Wymore K, McCabe JG, Munoz-O'Regan D, O'Donnell SL, Lapachet ESG, Bengoechea T, Fishwild DM, Carmack CE, Kay RM, Huszar D (1994) Antigen-specific human antibodies from mice comprising four distinct genetic modifications. Nature 368:856–859

McCreath KJ, Howcroft J, Campbell KHS, Colman A, Schnieke AE, Kind AJ (2000) Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. Nature 405:1066-1069

Schmidt C (2006) Belated approval of first recombinant protein from animal. Nature Biotechnology 24:877

Schultz WB (1995) Interim definition and elimination of lot-by-lot release for well-characterized therapeutic recombinant DNA-derived and monoclonal antibody biotechnology products. US Federal Register 60:63048–63049

Schnieke AE, Kind AJ, RitchieW A, Mycock K, Scott A R, Ritchie M, Wilmut I, Colman A, Campbell KHS (1997) Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science 278:2130–2133

Sinha U, Hancock TE, Nzerem JJ, Lin PH, Tomlinson JE, Wolf DL (1994) Effect of gamma carboxylation on prothrombinase inhibitory activity of catalytically inactive factor XA. Thromb Res 75:427–436

Tomizuka K, Shinohara T, Yoshida H, Uejima H, Ohguma A, Tanaka S, Sato K, Oshimura M, Ishida I (2000) Double trans-chromosomic mice: maintenance of two individual human chromosome fragments containing Ig heavy and kappa loci and expression of fully human antibodies. Proc Natl Acad Sci USA 97:722–727

Whitelaw CB, Radcliffe PA, Ritchie WA, Carlisle A, Ellard FM, Pena RN, Rowe J, Clark AJ, King TJ, Mitrophanous KA (2004) Efficient generation of transgenic pigs using equine infectious anaemia virus (EIAV) derived vector. FEBS Lett 571:233–236

Yasunaga H (2007) Risk of authoritarianism: fibrinogen-transmitted hepatitis C in Japan. Lancet 370:2063–2067

Market Development of Biopharmaceuticals

Gary Walsh

Products of pharmaceutical biotechnology represent the fastest growing, the most technically complex and in many ways, the most exciting sector within the pharmaceutical industry. Within this chapter we firstly consider what exactly a biopharmaceutical is. An overall snapshot of the current status of the biopharmaceutical sector is then presented, followed by a consideration of technical trends currently characteristic of the industry. The chapter concludes by considering some of the likely significances of biopharmaceuticals within the broader pharmaceutical sector for the future.

1 Biopharmaceuticals

'Biopharmaceutical' has now become an accepted, commonly used word in the pharmaceutical vocabulary. The term originated in the 1980s, when a general consensus evolved that it represented a class of therapeutic product produced by modern biotechnological techniques. These incorporated, protein-based products are produced by genetic engineering or, in the case of monoclonal antibodies, produced by hybridoma technology. During the 1990s the concept of nucleic acid-based drugs for use in gene therapy and antisense technology came to the fore. Such products are also considered to be biopharmaceuticals. On that basis biopharmaceuticals may be defined – or at least described – as proteins or nucleic acid based pharmaceuticals, used for therapeutic or in vivo diagnostic purposes, and produced by means other than direct extraction from a non-engineered biological source. The vast majority of biopharmaceuticals therefore are therapeutic proteins produced via recombinant DNA technology (genetic engineering), and it is upon these products we focus upon for the remainder of this chapter.

2 The Global Biotechnology Sector

Globally there are over 4,000 biotech companies in existence. The vast majority of these companies focus specifically upon medical/healthcare biotech – mainly developing diagnostic and therapeutic biotech products. Figures from 2006 estimate that there are in excess of 1,400 biotech companies within Europe with just over 1,100 companies based in the United

States. The Asia pacific area represents the third largest region, being home to some 600 biotechnology concerns (Lawrence 2007). Some are publicly quoted companies (an estimated 710) but the majority are privately owned and are relatively small, often employing less than 100 people.

Collectively these companies generated revenues of $73 billion in 2006, an increase of 14% over the previous year. The vast majority are research intensive, spending an average of 28% of revenues on research and development, an increase of some 33% when compared to the 2005 figures (Lawrence, 2007). While overall revenues grow steadily each year, the sector as a whole remains a loss making one, with net losses for 2006 estimated to be over $5 billion.

3 Biopharmaceutical Industry 0verview

The biopharmaceutical sector represents the backbone of the global biotech industry. This industry can trace its roots back to the late 1970s and early 1980s. The advent of genetic engineering and monoclonal antibody technology underpinned the establishment of hundreds of start-up biopharmaceutical companies, mainly in the United States, with smaller numbers of start-ups emanating from Europe and other world regions.

Many of these initial companies were founded by academics or researchers in the area of recombinant DNA technology/antibody technology who sought to take commercial advantage of developments in these areas. Such start up companies were largely financed by speculative monies attracted by the hype associated with the establishment of the modern biotech era. While most of these early companies displayed significant technical expertise, the vast majority lacked experience in the practicalities of the drug development process. Most of the well established large pharmaceutical companies on the other hand were slow to invest heavily in biotech research and development. However, as the actual and potential therapeutic significance of biopharmaceuticals became evident, many traditional pharmaceutical companies diversified into this area. Most of them either purchased small, established biopharmaceutical concerns, or formed strategic alliances with them. One example was the long-term alliance formed by Genentech and Eli Lilly. Humulin® (recombinant human insulin, produced in Escherichia coli and developed by Genentech in collaboration with Eli Lilly) was the first biopharmaceutical ever to come on the market. Lilly received marketing authorization in the USA for the product in 1982. This marked the true beginning of the biopharmaceutical industry.

The merger of biotech capability with pharmaceutical experience helped accelerate development of the biopharmaceutical sector. Many of the earlier dedicated biopharmaceutical companies no longer exist, failing for either technical or financial reasons. The promise and hype of biotechnol-

ogy sometimes exceeded its ability to actually deliver a final product. Some biopharmaceutical substances showed little efficacy in treating their target condition, and/or exhibited unacceptable side effects. Mergers and acquisitions also led to the disappearance of several biopharmaceutical concerns. Examples of successful dedicated biotech companies formed during pioneering era of the biopharmaceutical sector include Genentech, Amgen and Biogen.

Amgen now employs over 9,000 people worldwide making it one of the largest dedicated biotechnology companies in existence. Its headquarters are situated in Thousand Oaks, California, although it has research, manufacturing, distribution and sales facilities worldwide. Company activities focus upon developing novel (mainly protein) therapeutics for application in oncology, inflammation, bone disease, neurology, metabolism and nephrology. By mid-2006, seven of its recombinant products had been approved for general medical use (the erythropoietin-based products, 'Aranesp®' and 'Epogen®', the colony stimulating factor-based products, 'Neupogen®' and 'Neulasta®', as well as the interleukin-1 receptor antagonist, 'Kineret®', the anti-rheumatoid arthritis fusion protein, 'Enbrel®' and the keratinocyte growth factor 'Kepivance®', indicated for the treatment of severe oral mucositis). Total product sales reached US$9.9 billion by mid-decade. In July 2002, Amgen acquired Immunex Corporation, another dedicated biopharmaceutical company founded in Seattle in the early 1980s.

Biogen was founded in Geneva, Switzerland in 1978 by a group of leading molecular biologists. Currently, its global headquarters are located in Cambridge, MA, and it employs in excess of 2,000 people worldwide. The company developed and directly markets the interferon-based product, 'Avonex®', but also generates revenues from sales of other Biogen-discovered products which are licensed to various other pharmaceutical companies. These include Schering Plough's 'Intron A®' as well as a number of hepatitis B based vaccines sold by SmithKline Beecham (SKB) and Merck and Tysabri, which it jointly markets with Elan.

Genentech was founded in 1976 by scientist Herbert Boyer and the venture capitalist, Robert Swanson. Headquartered in San Francisco, it employs almost 5,000 staff worldwide. Recombinant protein-based products it now has on the market include human growth hormones (Nutropin®), the antibody-based products 'Herceptin®' and 'Rituxan®', and the thrombolytic agents 'Activase®' and 'TNKase®'.

Table 1 lists the major (bio)pharmaceutical companies who now manufacture/market biopharmaceuticals approved for general medical use.

3.1 Current Status

Currently some 180 biopharmaceuticals have gained approval for general human use in the EU and/or USA and some 325 million people worldwide have been treated to date with these drugs. Summary profiles of those

Table 1: The major pharmaceutical companies who manufacture and/or market biopharmaceutical products which have been approved for general medical use in the USA and EU

Baxter	Pfizer
Eyetech	Elan
BioMarin	Insmed
Genetics Institute	Hoechst AG
Bayer	Aventis Pharmaceuticals
Novo Nordisk	Genzyme
Centeon	Schwartz Pharma
Genentech	Pharmacia and Upjohn
Centocor	Bio-Technology General
Boehringer Manheim	Serono
Galenus Manheim	Organon
Eli Lilly	Amgen
Ortho Biotech	Dompe Biotec
Schering Plough	Immunex
Hoffman-la-Roche	Bedex Laboratories
Luitpold pharmaceuticals	BioMimetic
Leopharma	Nycomed
Biopartners	Alexion
ImClone	Bristol-Myers Squibb
Chiron	Merck
Biogen	GlaxoSmithKline
Pasteur Merieux MSD	Medeva Pharma
Immunomedics	Cytogen
Novartis	Med Immune
Abbott	Roche
Wyeth	Isis Pharmaceuticals
Unigene	Sanofi-Synthelabo

approved in the USA and/or the EU are available in various publications (e.g., Walsh 2006; Raider 2007).

The major sub-categories of biopharmaceuticals thus far approved include:

– 39 hormone-based products (e.g., insulins, glucagon, gonadotropins and human growth hormone);

- 32 antibody-based products;
- 20 vaccine products containing a recombinant antigenic component;
- 19 recombinant interferons and interleukins;
- 14 haematopoietic growth factors (erythropoietins and colony stimulating factors);
- 8 recombinant blood factors (factors VIIa, VIII and IX);
- 6 thrombolytics (tissue plasminogen activator based products.

The major categories of product indications are as one might expect; mirroring major killers in the western world, including various forms of cancer and heart attacks.

A summary of individual products approved more recently (within the last five years) are provided in table 2.

Table 2: Biopharmaceuticals (defined as recombinant proteins, monoclonal antibody and nucleic acid-based products) approved in the USA or European Union from January 2003–December 2007

Abbreviations: r = recombinant, rh = recombinant human, CHO = Chinese hamster ovary, KGF = keratinocyte growth factor, VEGF = vascular endothelial growth factor, IGF-1 = insulin like growth factor 1, IGFBP-3 = insulin like growth factor binding protein, 3.hGH = human growth hormone, TNF = tumour necrosis factor, EGF = Epidermal growth factor, LFA-1 = lymphocyte function associated antigen-1, LH = leutinizing hormone, EPO = erythropoietin, FSH = follicle stimulating hormone, PDGF-BB = platelet derived growth factor BB.

Product	Company	Therapeutic Indication	Approved
2003			
Advate (octocog-α, r human factor VIII produced in a CHO cell line).	Baxter	Haemophilia	2003 (USA) 2004 (EU)
Aldurazyme (laronidase; rh alfa-L-iduronidase produced in an engineered CHO cell line)	Genzyme	Long term enzyme replacement therapy in patients suffering from Mucopolysaccharidosis I	2003 (EU)
Amevive (Alefacept; fusion protein consisting of the extracellular human leukocyte functional antigen 3 domain linked to an IgG fragment, produced in CHO cells).	Biogen, Inc.	Chronic plaque psoriasis	2003 (USA)
Bexxar (tositumomab; murine monoclonal raised against CD20 surface antigen, found on the surface of B-lymphocytes, produced in a mammalian cell line)	Corixa and GlaxoSmithKline	Non-hodgkin's lymphoma	2003 (USA)

Continued on next page

Product	Company	Therapeutic Indication	Approved
Forteo (Forsteo in EU; teriparatide; r shortened form of human parathyroid hormone, produced in Escherichia coli).	Eli Lilly	Treatment of osteoporosis in selected postmenopausal women	2003 (EU) *(Available in USA since 2002)*
Humira (EU & USA; also sold as Trudexa in EU) (adalimumab; r (anti-TNF) human monoclonal antibody created using phage display technology	Cambridge Antibody Technologies & Abbott (USA) Abbott (EU)	Rheumatoid arthritis	2003 (EU) *(Available in USA since 2002)*
Raptiva (efalizumab; humanized antibody, binds to LFA-1, which is expressed on all leukocytes. Produced in a CHO cell line)	Genentech (USA) Serono (EU)	Treatment of adult patients with chronic moderate to severe plaque psoriasis	2003 (USA) 2004 (EU)
Somavert (pegvisomant; r engineered hGH analogue (antagonist), produced in Escherichia coli)	Pharmacia	Treatment of selected patients suffering from acromegaly	2003 (USA) *(Available in EU since 2002)*
Xolair (omalizumab; humanized monoclonal which binds immunoglobulin E at the site of high affinity IgE receptor binding)	Genentech/ Novartis/ Tanox/ Sankyo	Treatment of adults/adolescents with moderate to severe persistant asthma	2003 (USA) 2005 (EU)
2004			
Apidra (insulin glulisine; a rapid acting insulin analogue produced in Escherichia coli).	Aventis	Diabetes Mellitus	2004 (USA & EU)
Avastin (bevacizumb; a humanized monoclonal raised againstVEGF) expressed in a CHO cell line.	Genentech (USA) Roche (EU)	Carcinoma of the colon or rectum	2004 (USA) 2005 (EU)
Dukoral (oral cholera vaccine; vibrio cholerae and recombinant cholera toxin B subunit)	SBL Vaccin AB	Active immunization against disease caused by Vibrio cholerae serogroup O 1	2004 (EU)
Erbitux (cetuximab; a chimaeric antibody raised against human EGF receptor, produced in a murine myeloma cell line)	ImClone Systems and Bristol-Myers Squibb (USA) Merck (EU)	Treatment of EGF receptor-expressing metastatic colorectal cancer	2004 (USA & EU)
Kepivance (palifermin, a rKGF produced in Escherichia coli)	Amgen	Treatment of severe oral mucositis in selected patients with hematologic malignancies	2004 (USA)

Continued on next page

Product	Company	Therapeutic Indication	Approved
Levemir (insulin detemir; a long acting rh Insulin analogue produced in Saccharomyces cerevisiae)	Novo Nordisk	Diabetes Mellitus	2004 (EU)
Macugen (pegaptanib sodium injection, a synthetic pegylated oligonucleotide which specifically binds VEGF)	Eyetech/ Pfizer	Treatment of neovascular, age-related macular degeneration	2004 (USA) 2006 (EU)
NeutroSpec (contains fanolesomab, a murine monoclonal antibody raised against CD15 surface antigen of selected leukocytes, produced via hybridoma technology)	Palatin Technologies/ Mallinckrodt Inc.	Imaging of equivocal appendicitis	2004 (USA)
Tysabri (natalizumab; a humanized monoclonal antibody raised against selected leukocyte integrins, expressed in a murine myeloma cell line)	Biogen Idec/ Elan	Treatment of patients with relapsing forms of multiple sclerosis	USA: approved 2004, suspended 2005, resumed 2006. EU: 2006
Zevalin (ibritumomab tiuxetan; a murine monoclonal antibody produced in a CHO cell line, targeted against the CD20 antigen)	IDEC Pharmaceuticals (USA) Schering AG (EU)	Treatment of patients with relapsing forms of multiple sclerosis	2004 (EU) *(Available in USA since 2002)*
2005			
Fortical (r salmon calcitonin produced in Escherichia coli)	Upsher-Smith Laboratories/ Unigene	Postmenopausal osteoporosis	2005 (USA)
GEM 21S (growth factor enhanced matrix; contains rhPDGF-BB produced in Saccharomyces cerevisiae, in addition to tricalcium phosphate)	Luitpold Pharmaceuticals, BioMimetic Pharmaceuticals	Periodontally related defects	2005 (USA)
Hylenex (rh hyaluronidase produced in CHO cells)	Baxter/ Halozyme Therapeutics	Adjuvant to increase absorption and dispersion of other drugs	2005 (USA)
Increlex (mecasermin, rh IGF-1 produced in Escherichia coli)	Tercica/ Baxter	Long term treatment of growth failure in children with severe primary IGF-1 deficiency or with growth hormone gene deletion	2005 (USA) 2007 (EU)

Continued on next page

Product	Company	Therapeutic Indication	Approved
IPLEX (mecasermin rinfabate, a complex of rh IGF-1 and rh IGFBP-3 produced separately in Escherichia coli)	Insmed	Long term treatment of growth failure in children with severe primary IGF-1 deficiency or with growth hormone gene deletion	2005 (USA)
Naglazyme (galsulfase, rh N-acetylgalactosamine 4 sulfatase produced in a CHO cell line)	BioMarin	Long tern enzyme replacement therapy in patients suffering from Mucopolysaccharidosis VI	2005 (USA) 2006 (EU)
Orencia (abatacept, soluble fusion protein produced in a mammalian cell line)	Bristol-Myers Squibb	Rheumatoid arthritis	2005 (USA) 2007 (EU)
2006			
Atryn (rh antithrombin, from milk of transgenic goats)	Genzyme/ Leopharma	Hereditary antithrombin deficiency	2006 (EU)
Elaprase (rh Iduronate-2-sulfatase, produced in a human cell line)	Shire Human Genetic Therapies Inc.	Mucopolysaccharidosis II (Hunter's syndrome)	2006 (USA) 2007 (EU)
Exubera (rh Insulin, produced in Escherichia coli)	Pfizer	Diabetes	2006 (USA & EU)
Gardasil (human papillomavirus vaccine, type 6, 11, 16, 18, recombinant, produced in Saccharomyces cerevisiae) Also marketed in EU as **Silgard**	Merck (USA) Sanofi Pasteur (EU) Merch Sharp & Dome (EU under tradename Silgard)	Vaccine against cervical cancer & related conditions caused by HPV	2006 (USA & EU)
Lucentis (ranibizumab, a humanized IgG fragment produced in Escherichia coli. Binds & inactivates VEGF-A)	Genentech	Neovascular (wet) age-related macular degeneration	2006 (USA) 2007 (EU)
Myozyme (rh acid-α-glucosidase, produced in a CHO cell line)	Genzyme	Pompe disease (glycogen storage disease type II)	2006 (EU & USA)
Omnitrope (rhGH, produced in Escherichia coli)	Sandoz	GH deficiency/ growth failure	2006 (EU & USA)

Continued on next page

Product	Company	Therapeutic Indication	Approved
Preotach (rh parathyroid hormone, produced in Escherichia coli)	Nycomed	Osteoporosis	2006 (EU)
Valtropin (rhGH produced in Saccharomyces cerevisiae)	Biopartners	Growth failure/ GH deficiency	2006 (EU) 2007 (USA)
Vectibix (panitumumab, a rh Mab that binds to hEGFR, produced in a CHO cell line)	Amgen	EGFR-expressing colorectal carcinoma	2006 (USA) 2007 (EU)
2007			
Abseamed (recombinant human erythropoietin alfa, a rhEPO produced in a CHO cell line)	Medice Arzneimittel Putter	Anaemia associated with chronic renal failure	2007 (EU)
Binocrit (recombinant human erythropoietin alfa, a rhEPO produced in a CHO cell line)	Sandoz	Anaemia associated with chronic renal failure	2007 (EU)
Cervarix (r, C-terminally truncated major caspid L 1 proteins from human papillomavirus types 16 and 18 produced in a baculovirus-based expression system)	GlaxoSmith-Kline	Prevention of cervical cancer	2007 (EU)
Epoetin alfa Hexal (recombinant human erythropoietin alfa, a rhEPO produced in a CHO cell line)	Hexal Biotech	Anaemia associated with chronic renal failure	2007 (EU)
Mircera (methoxy polyethylene glycol-epoetin beta, PEGylated rhEPO produced in a CHO cell line)	Roche	Anaemia associated with chronic kidney disease	2007 (EU & USA)
Pergoveris (follitropin alfa/ lutropin alfa; combination product containing rhFSH and rhLH, both produced in a CHO cell line)	Serono	Stimulation of follicular development in women with severe LH and FSH deficiency	2007 (EU)
Retacrit (epoetin zeta; a rhEPO produced in a CHO cell line)	Hospira Enterprises	Anaemia associated with chronic renal failure	2007 (EU)
Silapo (epoetin zeta; a rhEPO produced in a CHO cell line)	Stada Arzneimittel	Anaemia associated with chronic renal failure	2007 (EU)
Soliris (eculizumab, a humanized IgG that binds human C5 complement protein, produced in a murine myeloma cell line)	Alexion	Paroxysmal nocturnal hemoglobinuria	2007 (EU & USA)

Products of this table are registered trademarks.

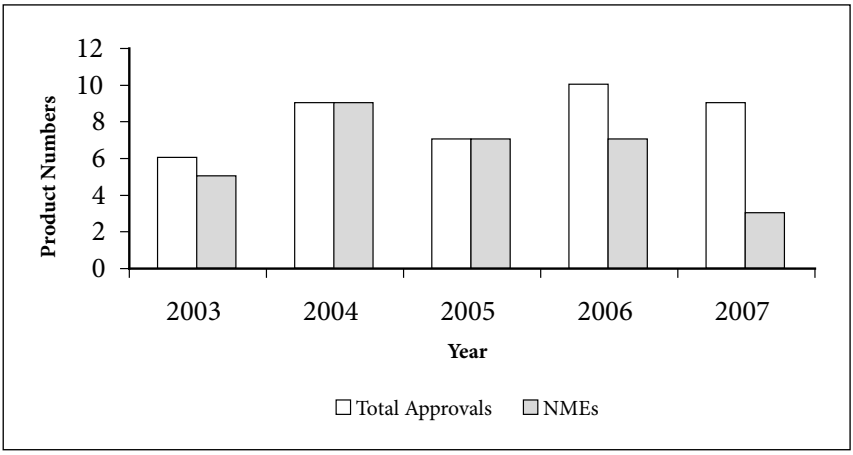


Figure 1: Product numbers approved in either the EU and/or the USA, 2003–2007

Notes: (a) Each product is included only once. Thus, if a product was approved in the EU in 2003 and subsequently in the US in 2004 it would contribute to the 2003 statistics alone. (b) In addition to total approvals, the number of truly new molecular (biopharmaceutical) entities are included for each year. Biosimiliar products are not considered to be new molecular entities (NMEs).

Currently in the region of one in four of all genuinely new drugs coming on to the market are biopharmaceuticals and typically eight to ten new biopharmaceuticals are approved for general medical use each year. Product numbers approved over the last five years are provided in Figure 1. The majority of biopharmaceuticals coming on the market in any one year are truly novel products (i.e. are new biotechnological/molecular entities). However, a proportion of 'new' approvals in some cases contain an active biotech ingredient which is not truly novel. This is particularly evident in the case of products approved in 2007 where only three of the nine products approved were genuinely new. The other six approvals included biosimiliar erythropoietins (see later) as well as one product (Pergoveris™, table 2), consisting of a combination of two active ingredients – a recombinant human follicle stimulating hormone and a recombinant human luteinizing hormone (FSH and LH). Both of these molecular entities had been previously approved as stand alone, single active ingredient medicinal products.

The revenues generated in the biopharmaceutical sector are estimated to be in excess of $30 billion in 2004 and will approximately double its global value in 1999 (Lawrence 2004). Continued strong growth is expected, with the industry projected to reach $70 billion by the end of the decade (Pavlou and Belsey 2005; Pavlou and Reichert 2004).

The top ten biopharmaceutical products (by revenues generated) are presented in table 3. From this it can be seen that recombinant erythropoietin-based products represent the single most lucrative biopharmaceu-

Table 3: The ten best selling biopharmaceuticals in 2006. Data sourced from company annual reports (abbreviations: see table 2, p. 73)

Rank	Product	Company	Indication	Year approved	Revenues ($ billion)
1	**Enbrel** (rTNFR-IgG fragment fusion protein produced in CHO cells)	Amgen/ Wyeth	Rheumatoid arthritis	1998 (USA) 2000 (EU)	$4.4
2	**Aranesp** (darbepoetin alfa; long acting rEPO analogue produced in CHO cells)	Amgen	Treatment of anaemia	2001 (EU & USA)	$4.1
3	**Rituxan/Mabthera** (Rituximab chimaeric Mab directed against CD20 antigen found on the surface of B-lymphocytes)	Biogen/ Genentech/ Roche	Non-Hodgkin's Lymphoma	1997 (USA) 1998 (EU)	$3.9
4	**Remicade** (Infliximab, chimaeric Mab directed against TNF α)	J&J/ Schering plough	Treatment of Crohn's disease	1998 (USA) 1999 (EU)	$3.6
5	**Procrit/Eprex** (rhEPO produced in a mammalian cell line)	J&J	Treatment of anaemia	1990 (USA)	$3.2
6	**Herceptin** (Trastuzumab, Humanized antibody directed against HER 2, i.e. human epidermal growth factor receptor 2)	Genentech/ Roche	Treatment of metastatic breast cancer if tumor overexpresses HER2 protein	1998 (USA) 2000 (EU)	$3.1
7	**Epogen** (rhEPO produced in a mammalian cell line)	Amgen	Treatment of anaemia	1989 (USA)	$2.9
8	**Neulasta** (pegfilgrastim, r pegylated filgrastim) Also marketed in EU as **Neupopeg**	Amgen	Neutropenia	2002 (USA & EU)	$2.7
9	Human insulins	Novo	Diabetes	Various	$2.5
10	**Avastin** (humanized monoclonal raised against vascular endothelial growth factor)	Genentech/Roche	Carcinoma of the colon or rectum	2004 (USA) 2005 (EU)	$2.4

Products of this table are registered trademarks.

tical on the market, with cumulative sales surpassing the $10 billion mark. The table also illustrates another point, namely that the biopharmaceutical sector is dominated by the United States. With the exception of Novo and Roche, the other companies listed are all headquartered in the USA.

3.2 China – an Emerging Market

With a population of $1,3 billion China represents a huge, and as yet largely untapped market in the context of biopharmaceuticals. Although commencing from a very modest baseline, it is estimated that China's biopharmaceutical sector grew by some 30% annually from 2000 to 2005, reaching a $3 billion market value (Frew et al. 2008). During the same period China's pharmaceutical industry as a whole grew by a strong but more modest 19% annually. In an overall context, biotech products are believed to represent less than 8% of China's total pharmaceutical market (Hu et al. 2006). These products are mainly biogenerics, with less than 10% estimated to be novel products. There are some 15 products approved,

Table 4: Some of China's major biopharmaceutical companies and a summary overview of the products they market or have in development

Company	Products marketed/in development
Amoytop Biotech	Market a recombinant granulocyte-macrophage colony stimulating factor (GM-CSF), a granulocyte colony stimulating factor (G-CSF) and an interleukin 11. Have additional recombinant interleukins, interferons and a human growth hormone in development.
Bio-Bridge science	Developing an oral HIV vaccine as well as colon cancer vaccine.
GeneScience pharmaceuticals	Markets recombinant human growth hormone and recombinant colony stimulating factors, developing recombinant follicule stimulating hormone (FSH) and a PEGylated human growth hormone.
Shanghai Fudan-Yueda BioTech	Markets recombinant viral subunit vaccines with additional subunit vaccines in development.
Shanghai United Cell Biotech	Markets a recombinant human growth hormone and a recombinant oral Cholera toxin B-subunit vaccine. Developing recombinant human parathyroid hormone.
Shenzhen Belke Biotechnologies	Markets and developing a number of stem cell injections for the treatment of Alzheimer's and other mainly neurodegenerative conditions.
SiBiono GeneTech	Markets the gene therapy product Gendicine® (see main text) and developing a range of both viral and non-viral-based gene delivery systems for gene therapy.
Tianjin SinoBiotech	Developing a recombinant interferon as well as a range of engineered tumour-killing viruses.

with in the region of 60 additional products in the clinical pipeline (Louet 2004). Some of China's major biopharmaceutical companies are summarized in table 4. Most of these companies were founded within the last decade or so and, when compared to typical western biopharmaceutical concerns, are as yet relatively small. Most appear to generate revenues in the multi million and at most several tens of millions US dollars per annum and typically employ 50–500 people. Most, however, display a relatively strong research and development focus and will doubtlessly continue to grow very rapidly in line with China's continuance of an overall strong economic expansion.

Perhaps the best-known (and for some the only known) biopharmaceutical product is that of Gendicine® (Peng 2004). Developed and produced by SiBono GeneTech, Shenzhen, Gendicine® was the first and, thus, far the only gene therapy based product to be approved anywhere in the world. It gained approval for use in the treatment of head and neck squamous cell carcinoma from China's state food and drug administration (SFDA) in 2003. The product is a replication-incompetent human serotype 5 adenovirus engineered to contain the human wild type p53 tumor suppressor gene. Direct intratumoral injection is believed to trigger vector uptake and expression of p53, leading to cell cycle arrest and apoptosis (Peng 2005). Company data showed complete regression of tumours in 64% of patients treated with Gendicine® in combination with radiation therapy with few associated side effects. The product is believed to have been administered to some 50,000 patients and is in late stage clinical trials for various other cancers (Wilson 2005).

4 Technical Trends within the Industry

Scientific trends within the industry will, of course, influence the market profile of these drugs into the future. As such, some of these trends are reviewed below.

4.1 Trends in Product Types Gaining Approval

Some interesting trends become apparent when product types, approved year by year, are reviewed. Interferons, interleukins, recombinant subunit vaccines thrombolytics and other blood-related products, including anticoagulants represent major overall categories of biopharmaceuticals approved. However, very few such products have gained approval over the last five years (see table 2). Proportionately, on the other hand, increasing numbers of growth factors, monoclonal antibody-based products and enzymes were approved in this latter period. These trends more likely reflect commercial rather than technical considerations; new interferons, thrombolytics or blood factors, for example, would face stiff competition from the large

number of such products already on the market. Blockbusters are well represented on the market but it is interesting to note that at least eight of the new approvals have designated orphan status.

Another trend, evident in recent years, relates to the proportion of approvals that have been engineered in some way. Of the 45 products listed in table 2, 16 (i.e., 35%) have been engineered, displaying either an altered amino acid sequence or an addition of a chemical group such as polyethylene glycol.

A particularly noteworthy engineering approach is exemplified by Novo's long acting insulin analogue, Levemir®. The primary engineering strategy entailed the covalent attachment (acylation) of the insulin with myristic acid (a 14 carbon saturated fatty acid). Human serum albumin harbours 3 high affinity fatty acid binding sites and as a result binds the insulin analogue tightly, both at the site of injection and in systemic circulation. The overall effect is to ensure a constant and prolonged release of free insulin molecules, bestowing on the product an extended duration of action of up to 24 hours.

Insulin in many ways remains the prototypic biopharmaceutical. It was the first recombinant protein ever approved. It was one of the first biopharmaceuticals to be engineered, with the approval of seven fast and long acting engineered analogues over the past decade. It has now also become the first biopharmaceutical approved for delivery via the pulmonary route (discussed later).

4.2 Trends in Production Systems

Escherichia coli and mammalian cell lines such as CHO and BHK cell lines still represent the workhorses of biopharmaceutical production. Twelve of the 45 therapeutic proteins approved within the last five years are produced in Escherichia coli. Four are produced in Saccharomyces cerevisiae, and one each produced using transgenic goats, and a baculoviral-based system. The remaining products are produced by various mammalian cell lines (table 2).

Mammalian cell culture is technically complex, slow and expensive, yet these cell lines thus far command an effective monopoly in terms of producing large therapeutic proteins that require post translational modification, in particular glycosylation. O-linked glycosylation (sugar side chains attached via serine/threonine residues), but most especially N-linked glycosylation (sugar side chains attached via asparagine residues) can influence protein stability, ligand binding, immunogenicity and serum half life, and is significant in the context of the efficacy and sometimes the safety of a wide range of biopharmaceuticals, including antibodies, blood factors and some hormones/cytokines (Walsh and Jefferis 2006).

Alternative production systems capable of undertaking glycosylation include yeast, insect and plant cells. When compared to mammalian cell lines these typically grow to higher cell densities in shorter fermentation

cycles and in less expensive and more chemically defined media, and should display reduced risk of transmission of mammalian pathogens. Despite such technical and economic advantages no glycosylated biopharmaceutical produced using such alternatives has yet gained approval for human use, mainly because the exact glycosylation patterns characteristic of human proteins are not adequately reproduced by these systems, with consequent functional and safety/immogenicity implications.

Significant technical progress has been reported in terms of 'humanizing' the glycosylation profile characteristic of these alternative production systems, particularly yeast (Gomord et al. 2005; Chen et al. 2005; Wildt and Gerngross 2005). Glycosylation is a multi enzyme-based mechanism and the engineering process pursued generally entails elimination of non-human glycosylation reactions, and/or concurrent introduction of characteristic human ones.

Advances in this area are exemplified by the successful production of a human IgG displaying a humanized N-glycan structure in glycoengineered yeast (a Pichia pastoris strain; Li et al. 2006). Antibody glycosylation influences ability to interact with various immune effector cells, triggering antibody-dependant cell cytotoxicity (ADCC; important for effective therapeutic functioning).

Glycoengineered yeast may represent an attractive alternative to mammalian cells for producing therapeutic glycoproteins. Yeasts have been long used for the production of both recombinant and native industrial enzymes, upstream processing spans hours/days rather than days/weeks and expression levels several fold higher have been reported (up to 15 g/l) Werten et al. 1999). Additionally, engineered yeast have been developed which perform essentially uniform glycosylation, facilitating attainment of better batch to batch product consistency (Wildt and Gerngross 2005).

Despite the promise of these alternative production systems animal cells will continue to represent the major production vehicle for glycosylated therapeutic proteins for the foreseeable future. Moreover, advances continue which underpin more efficient mammalian cell culture technology (Butler 2005). A combination of improved expression constructs as well as increased understanding of animal cell metabolism and physiology has underpinned continuous improvements in product yield. Recombinant protein levels approaching 5 g/l are now possible, probably ten fold higher than some years ago. Also of note is the successful development of serum-free and indeed animal component-free media for several cell lines. Ongoing research avenues include exploring approaches based both on media manipulation and genetic engineering to prevent/retard apoptosis (in order to prolong protein production) and manipulation of process parameters to minimize glycosylation heterogeneity.

A notable milestone in the context of biopharmaceutical production systems was the approval within Europe in 2007 of Cervarix®, the first bio-

pharmaceutical approved for general medical use in humans produced via a baculovirus-based expression system (table 2). Infections with human papillomavirus (HPV) are the major casual factor for the development of cervical cancer, with various strains of HPV (most commonly the oncolytic strain numbers 16 and 18) being associated with some 98% of all cervical carcinomas. The Cervarix® vaccine product contains recombinant, C-terminally truncated major capsid L1 proteins of HPV types 16 and 18 as active ingredients. These recombinant viral caspid proteins are expressed separately using a recombinant Baculovirus expression system and an insect cell line derived from Trichoplusia ni. Again, these recombinant proteins are not post translationally modified.

Transgenic Plant-Based Systems. Potential production advantages associated with the expression of therapeutic proteins in transgenic plants are well documented in the literature (e.g., Gomord et al. 2005). Major advantages include ease of scalability, and reduced production costs are most often cited. Farid (2007) estimated the cost of therapeutic protein production per gram (at a 100kg/year production scale level) to be $300–3000 in the case of CHO cells, $105 for transgenic goats and only $50 in the case of transgenic corn.

Despite such potential advantages, considerable technical barriers remain to be overcome before injectable proteins, particularly glycoproteins, produced in transgenic plants are likely to gain regulatory approval for human use. Structural differences between plant and mammalian N-linked glycans represent a formidable hurdle, particularly as some plant-derived sugar motifs are highly immunogenic in man. Engineering strategies to humanize plant-based glycosylation profiles are simply not sufficiently advanced to support as yet an acceptable glycosylation pattern on biopharmaceuticals destined for human parenteral use. Immunological concerns would of course be all but eliminated if the products were to be administered orally or topically. This likely explains why lead products produced in plant-based systems are destined for administration in this way. Two leading plant-produced products in development are 'CaroRX™'and 'Merispase®'. CaroRX™ is an antibody capable of binding Streptococcus mutans, a major causative agent of bacterial tooth decay. Binding prevents bacterial adherence to teeth and the product is intended for regular topical application. Merispase® is a recombinant mammalian gastric lipase produced in transgenic corn. It is aimed at countering lipid malabsorption related to exocrine pancreatic insufficiency caused by cystic fibrosis and chronic pancreatitis, and is obviously destined for oral administration.

Transgenic Animal-Based Systems. The production of therapeutic proteins in transgenic animals, and in particular in the milk of transgenic animals, is a goal that has been in development for well over a decade. A notable milestone in this context was reached in 2006, with the approval of ATryn®

(table 2), a recombinant form of human antithrombin (AT) produced by transgenic goats in their milk. The European authorities approved ATryn® under exceptional circumstances as the rarity of the condition prevented the generation of comprehensive clinical data. The product is an anti-clotting agent and is indicated for the prophylaxis of venous thromboembolism in surgery of patients with congenital antithrombin deficiency. Like the native human molecule, ATryn® is glycosylated. It harbours 4 N-linked glycosylation sites (and 3 disulfide linkages). The recombinant molecule displays significant differences in its glycosylation profile as compared to native human AT, both in terms of monosaccharide composition (it is for example fucosylated and less sialylated) and overall side chain structure. This influences the heparin binding affinity and pharmacokinetics of the product. Glycosylation patterns characteristic of proteins produced in transgenic milk of higher mammals differ somewhat from those characteristic of native human proteins, though the therapeutic significance of such differences, if any, must be evaluated on a case by case basis.

An additional novel system currently being evaluated as a production method is that of the transgenic hen egg. (Ivarie 2006). A fully human antibody has been expressed in this system (Zhu et al. 2005). When compared to the identical antibody produced in a CHO cell line a number of differences in oligosaccharide detail were noted (e.g., chicken-derived one had less galactose and no fucose in the antibody's glycocomponent) but the antigen-binding capability of both antibodies were identical and the in vitro ADCC activity was actually enhanced. Also noted was the fact that the plasma half-life of the egg-derived antibody was only half that of the CHO derived product. Therefore, clearly more detailed investigation is required before this system can be seriously considered as a potential production platform for therapeutic antibodies.

The approval of ATryn® finally proves that such a production approach is acceptable in principle to the regulatory authorities. It remains, however, to be seen how widespread the use of transgenic based systems for the production of therapeutic proteins will be, particularly given the significant advances in optimizing mammalian cell-based systems, as discussed earlier.

4.3 Alternatives to Non-Parenteral Delivery of Therapeutic Proteins

Parenteral administration of drugs can suffer from a number of disadvantages, including reduced patient compliance and potential complications for use in a non-clinical setting. The administration of labile, large and hydrophilic biopharmaceuticals via non-parenteral means represents a considerable technical challenge. Despite such challenges development of oral, nasal, pulmonary, transmucosal and transdermal based biopharmaceutical delivery systems remains an active area of research (Brown et al. 2006; Chen et al. 2006; Hamman et al. 2005; Orive et al. 2003) and the

approval of two products administered by non parenteral means (Fortical® in 2005 and subsequently Exubera® in 2006; table 2) represent very notable milestones in this regard.

Fortical® (recombinant salmon calcitonin) is administered as a nasal spray. The protein is adsorbed rapidly from the nasal mucosa but displays significantly lower bioavailability compared to when it is administered via intramuscular injection. The low bioavailability is likely a consequence of ciliary clearance, the presence of proteases and low uptake of larger molecules. Successful absorption of peptides/proteins across the nasal mucosa is significantly influenced by molecular mass. While the 32 amino acid, 3,5 kDa Fortical® crosses with relative ease, most therapeutic proteins, having molecular masses in the 20–150 kDa range, will not. This somewhat limits the practical chances of intranasal administration becoming a mainline method of therapeutic protein administration, at least in the near to intermediate future.

A major recent milestone in the context of non-parenteral delivery of biopharmaceuticals was marked by the approval of the insulin product Exubera® in 2006 (table 2). Moreover, several additional insulin based products destined for pulmonary delivery are in advanced clinical trials. Adsorption of macromolecules up to and indeed exceeding 100 kDa from the deep lung is possible (Patton 1999). This potentially makes the pulmonary route more generally applicable to biopharmaceuticals than the nasal route. However, technical and scientific advances do not automatically translate into market advantage. This is exemplified by Exubera®, which was withdrawn from the market in 2007, one year after approval – apparently due to disappointing sales.

4.4 *The Approval of Biosimiliars*

The development of generic versions of biotech products gone off patent has and remains a particularly contentious issue in the USA and the EU. Quite a number of biotech products – many of them blockbusters – have already come off patent protection (table 5) and it is estimated that by the end of this decade some $10 billion worth of biopharmaceuticals will have lost such patent protection (Ben-Maimon and Garnick 2006). As many as 75 currently approved therapeutic proteins may eventually become targets for 'generic' production (Diliberti 2006).

One of the main factors of contention within the 'biogenerics' debate relates to the fact that the exact molecular structure of 'generic' versions of many biopharmaceuticals cannot be guaranteed to be identical to the original product. This is true mainly for recombinant proteins with post translational modifications – in particular those that are glycosylated. Glycosylation is normally heterogeneous and expression of a 'generic' version of a glycoprotein using a production system other than used for the production of the original product would automatically result in at least some differences in glycosylation profile. The core question then becomes: Do such

Table 5: Some biopharmaceuticals which have come off patent

Protein	Indication	Patent expiration
Glucocerebrosidase	Gaucher disease	2001
Interferons	Cancer, viral infections	2001+
Human insulins	Diabetes	2002+
Human Growth Hormone	hGH deficiency	2003+
Erythropoietin	Anaemia	2004+
Tissue plasminogen activator	Myocardial infarction, stroke, embolism	2005+
Granulocyte colony stimulating factor	Neutropenia	2006

often seemingly minor structural differences influence product safety and/or efficacy? This question remains a focus of contention within the industry and within regulatory authorities. The European regulatory authority (European Medicines Agency, EMEA) have made provision for the evaluation and approval of such 'follow on' products, calling them 'biosimiliars' to reflect potential differences in the exact molecular structure compared with the originator product. The European approval system for such products demands the biosimiliar to undergo limited clinical studies in order to compare its safety and efficacy with the originator product (the 'reference' medicine). The first biosimiliars (Omnitrope® and Valtropin®, table 2) were approved within Europe in 2006, followed by the approval of several others in 2007. Regulatory requirements for prospective biosimiliars within the US remain unclear, and no defined regulatory pathway has been developed yet.

5 The Future

Market and technical trends aside, what does the future likely hold in overview for the biopharmaceutical sector? As already outlined, some 20–25% of all new drugs coming on the market since 2000 are biopharmaceuticals. Annual biopharmaceutical research and development expenditure has stood at $19–$20 billion over the last number of years (Lawrence 2005) and globally some 130,000 R&D staff are employed within the biotech sector (Lawrence 2007b). The continued high level of R&D investment is obviously paying off as biopharmaceuticals are forming an increasingly significant portion of the drug discovery pipeline (Lawrence 2006). For example, while 24–25% of all drugs, in advanced phase three clinical trials or that have completed phase 3 trials, are biopharmaceuticals, some 53% of drugs in the very early discovery phase are biopharmaceuticals. This bodes well for the future of the industry.

References

Ben-Maimon C, Garnick R (2006) Biogenerics at the crossroads. Nature Biotechnology 24:268–269
Brown M, Martin G, Jones S, Akomeah F (2006) Dermal and transdermal drug delivery systems: current and future prospects. Drug Delivery 13:175–187
Butler M (2005) Animal cell cultures: recent achievements and perspectives in the production of biopharmaceuticals. Applied Microbiology and Biotechnology 68:283–291
Chen Y, Shen Y, Guo X, Zhang C, Yang W et al. (2006) Transdermal protein delivery by a coadministered peptide identified via phage display technology. Nature Biotechnology 24:455–460
Chen M, Liu X, Wang Z, Song J, Qi Q, Wang P (2005) Modification of plant N-glycan processing: The future of producing therapeutic protein by transgenic plants. Medical Research Reviews 25:343–360
Diliberti C (2006) The best targets for biogenerics. BioPharm International 19:50–64
Farid S (2007) Process economics of industrial monoclonal antibody manufacture. J Chromatogr B 848:8–18
Frew S, Sammut S, Shore A, et al. (2008) Chinese health biotech and the billion patient market. Nature Biotechnology 26:37–53
Gomord W, Chamberlain P, Jefferis R, Faye L (2005) Biopharmaceutical production in plants: problems, solutions and opportunities. Trends in Biotechnology 23:559–565
Grengross T (2004) Advances in the production of human therapeutic proteins in yeasts and filamentous fungi. Nature Biotechnology 22:1409–1414
Hamman J et al. (2005) Oral delivery of peptide drugs – barriers and developments. Biodrugs 19:165–177
Hu X, Ma Q, Zhang S (2006) Biopharmaceuticals in China. Biotechnology Journal 1:1215–1224
Ivarie R (2006) Competitive bioreactor hens on the horizon. Trends in Biotechnology 24:99–101
Li H, Sethuraman N, Stadheim TA, Zha D, Prinz B, Ballew N, Bobrowicz P, Choi B-K, Cook WJ, Cukan M, Houston-Cummings NR, Davidson R, Gong B, Hamilton SR, Hoopes JP, Jiang Y, Kim N, Mansfield R, Nett JH, Rios S, Strawbridge R, Wildt S, Gerngross TU (2006) Optimization of humanized IgGs in glyocoengineered Pichia pastoris. Nature Biotechnology 24:210–215
Lawrence S (2005) Biotech drug market steadily expands. Nature Biotechnology 23:1466
Lawrence S (2006) Biotech blockbusters consolidate markets. Nature Biotechnology 24(12):1466
Lawrence S (2007) State of the biotech sector – 2006. Nature Biotechnology 25:706
Lawrence S (2007b) Biotech as an employer. Nature Biotechnology 25:12
Louet S (2004) Can China bring its own pipeline to the market? Nature Biotechnology 22:1497–1499
Orive G, Bukar J, Nagarajan S (2003) Drug delivery in biotechnology: present and future. Current Opinion in Biotechnology 14:659–664
Patton J, Hernandez R, Gascon A, Dominquez-Gil A, Pedraz, J (1999) Inhaled insulin, Advanced Drug Devivery Reviews 35:235–247
Pavlou A, Belsey M (2005) The therapeutic antibody market to 2008. European Journal of Pharmaceutics and Biopharmaceutics 59:389–396
Pavlou A, Reichert J (2004) Recombinant protein therapeutics – success rates, market trends and values to 2010. Nature Biotechnology 22:1513–1519
Peng Z (2004) The genesis of Gendicine. BioPharm International 17:42–49

Peng Z (2005) Current status of Gendicine in China: recombinant human Ad-p53 agent for the treatment of cancers. Human Gene Therapy 16:1016–1027
Rader R (2007) Biopharmaceutical products in the US and the European markets, sixth edition. Bioplan associates, Rockville, MD
Walsh G (2006) Biopharmaceutical benchmarks 2006, Nature Biotechnology 24:769–776
Walsh G, Jefferis R (2006) Post translational modifications in the context of therapeutic proteins. Nature Biotechnology 24:1241–1252
Werten M, Van de Bosch T, Wind R, Mooibroek H, De Wolf F (1999) High-yield secretion of recombinant gelatins by Pichia pastoris. Yeast 15:1087–1096
Wilson J (2005) Gendicine, the first commercial gene therapy product. Human Gene Therapy 16:1014
Wildt S, Gerngross T (2005) The humanization of N-glycosylation pathways in yeast. Nature Reviews Microbiology 3:119–128
Zhu L, Van de Lavoir M-C, Albanese J, Beenhouwer DO, Cardarelli PM, Cuison S, Deng DF, Deshpande S, Diamond JH, Green L, Halk EL, Heyer BS, Kay RM, Kerchner A, Leighton PA, Mather CM, Morrison SL, Nikolov ZL, Passmore DB, Pradas-Monne A, Preston BT, Rangan VS, Shi M, Srinivasan M, White SG, Winters-Digiacinto P, Wong S, Zhou W, Etches RJ (2005) Production of human monoclonal antibody in eggs of chimeric chickens. Nature Biotechnology 23:1159–1169

Ethical Aspects of Livestock Genetic Engineering[1]

Matthias Kaiser

Science and technology has brought us to the point where transgenic animals have become a real possibility for production purposes. We are no longer discussing mere future scenarios, given purely hypothetical scientific advances, but we are faced with concrete choices about how livestock production can meet a variety of needs today. The discussion about transgenic animals has for many years been accompanied by a certain "yuk-factor" in the eyes of the general public. To what degree this has changed significantly is still an open question. However, the potential benefits that may arise in some areas of our lives may be too significant to simply base our policies on these largely emotional reactions. We need to consider our technological options rationally and comprehensively. When it comes to evaluating the ethics of livestock genetic engineering, science and technology alone cannot provide the answers, and neither can surveys that simply mirror emotional reactions of sectors of the public. We need a broad societal debate about the paths that we choose, in particular a debate that pays respect to ethical arguments and is informed by science. This paper purports to contribute to such a broad societal debate, even though it should not be seen as anything else but the considered judgements of its author.

Introduction

Transgenic animals have been a reality for a long time. They function as animal models in many important areas of research, and their use has facilitated many insights in modern medical research. The use of these animals is never to be taken lightly; there are ethical issues that need to be resolved ahead of each single experiment. Our consciousness about the ethics of animal use in research has improved considerably since the first publication of the classic "3 R's" (cf. Russell et al. 1992; see also @ltweb: http://altweb.jhsph.edu/publications/humane_exp/het-toc.htm, July 2008). However, a

[1] This is an extended version of an oral presentation given at the symposium arranged by the Europäische Akademie in Berlin 21–22 September 2007 on "New applications of genetic engineering in livestock". Some sections of this paper have been published earlier in (Kaiser 2005). Some other sections have been included in a project proposal on "Value isobars" under EC FP7.

total replacement of all such experiments is not yet in sight, and a weighing of the benefits against the suffering of the animals thus often comes out in favour of such animal experiments. In some countries, notably the UK and the USA, activist groups take a strong and sometimes violent stand against such uses of animals, but large parts of society seem to acknowledge the principal need of them (cf. a recent study from Sweden: Vetenskapsrådet 2008).

The development of transgenic animals for normal livestock production has been slower. This is due to a number of factors, among others the fact that the genetic outfit of these animal species normally is more complex than for animal models like mice, and that the demands on stability and performance are higher. Some developments, however, are in the pipeline, e.g. in regard to pigs and sheep. So far no such animal has been marketed anywhere. Interesting developments include the Enviropig™, developed in Canada to generate pigs with low-phosphorus manure (cf. Enviropig™ 1, 2, 3). Since pigs lack the enzyme phytase which is essential in the digestion of phosphorus in the feed, the Enviropig™ produces phytase and thus reduces the phosphorus emissions to the environment. Significant about this particular development is that the main benefit does not reside with the producer, but that in this case the environment, the commons, is the beneficiary.

Closest to the potential entry into markets is assumedly a gm (genetically modified) salmon, developed by Aqua Bounty Technologies, currently waiting for approval of the American Food and Drug Agency (FDA). While many experts assumed that market approval will be a matter of routine, it turned out that the FDA saw a need for a much more comprehensive risk assessment than usual, including among others the Environmental Protection Agency (EPA). A transgenic tilapia, developed in Cuba, has temporarily been withdrawn from any attempts of marketing due to environmental concerns. However, judging from the available information on current developments, it seems that genetically engineered fish for aquaculture production will be the first area where genetically engineered animals reach the markets.

Recently, the use of antithrombin from transgenic goats, developed by GTC Biotherapeutics, were approved for market use in the EU. In their milk they produce a recombinant human protein for medical use against the disorder of inherited antithrombin deficiency. Experts seem to agree that this is only the first of a long row of coming "zoopharming" products that will enter the markets.

Other medical uses of transgenic animals include xenotransplantation. This is a field where companies have invested large sums of money for many years, since the potential benefits and earnings are assumed to be tremendous. Particular attention has been on transgenic pigs since their physiology is rather well known and also several other factors make them more attractive as source organism than, e.g., primates. In practice, progress has been

much slower than expected and controversies about the positive potentials of xenotransplantation persist. One reason for worry is the possibility of zoonosis, i.e, the possibility to spread infectious material from donor animals to humans where it could cause new epidemics. European research in this field has partly been hampered by unethical practices of some companies in their animal experiments (cf. http://www.xenodiaries.org/summary.htm, July 2008).

In sum, the current development gives the impression that some areas of genetically engineered livestock for production purposes has been accompanied by a hype where promises of immediate gains were made without sufficient basis in the status of the science and technology itself. Yet, it would be wrong to conclude that this holds true for the whole field. Rather it seems reasonable to conclude that at least some applications of genetic engineering of animals will be ready for market approval in the near future.

This raises the question of the role of ethics. Large parts of the public opinion seem to agree that these applications of genetechnology should be subjected to a thorough ethical assessment (cf. Eurobarometer 2005), and many people also assume that such an assessment will raise serious ethical problems of such genetically engineered livestock. We shall therefore have a closer look at some ethical aspects of this development in the remainder of the paper.

The Concept of Ethics and Some Knowledge Gaps

This paper is based on a non-classical conception of what it means to discuss ethics in relation to science and technology (hereafter: S&T). While it is common to regard ethics as the theoretical foundation of moral, i.e. normative, attitudes, that set clear and principled limits for acceptable development and action, our conception of ethics pays more attention to practical ethics and the positive function of ethics to provide value-based development goals. The author has contributed to matters of practical ethics in various previous papers (see, e.g., Kaiser 2004, 2005, 2006; Kaiser et al. 2007). The underlying idea is that practical ethics in relation to S&T refers to the platforms of dialogue between the main societal actors where social values are brought forward that are perceived either as threatened or as strengthened by S&T, and where the involved actors seek to resolve implied dilemmas by open and rational discourse. It is noteworthy that none of the involved actors (groups, stakeholders, etc.) can claim any privileged access to supremacy over, or special insight into these values that are disputed. That is why ethics as value discourse is structurally opposed to any purely expert-based ethical assessments, and needs to realize these platforms in an open and democratic manner. While practical ethics does not rest on the relativist assumption that one ethical assessment is as good as any other, it does emb-

race the idea that due respect should be paid to the plurality of values that are brought to the fore, and that the resulting dilemmas of practice need to be resolved by a broad and inclusive discourse (Kaiser et al. 2007). Thus, this conception of ethics is not so much in the spirit of "applied ethics", but rather a version of largely theory-independent normative discourse (cf., e.g., von Schomberg 2007; Clarke and Simpson 1989; Darwall et al. 1997; Kaiser 2005). See Figure 1 as illustration.

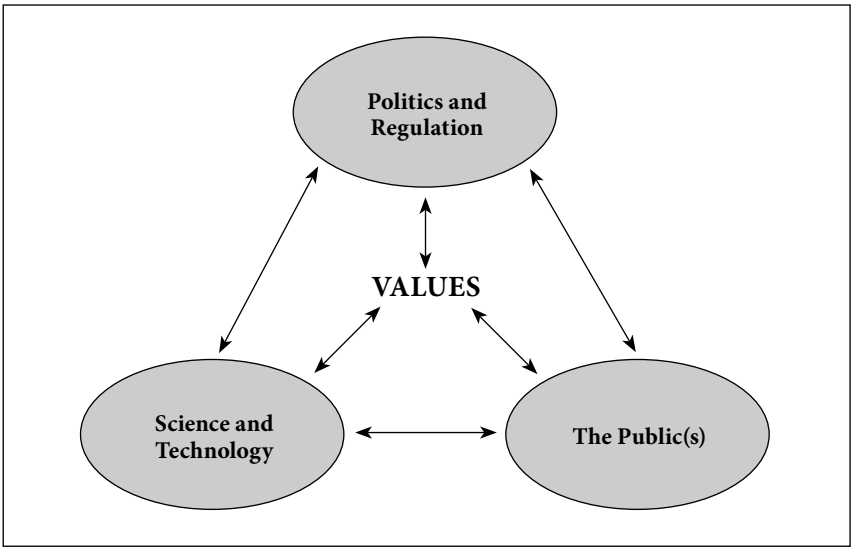


Figure 1: The platform of dialogue between relevant groups, focussed on and driven by the debate about common values. "Ethics" signifies this focus, and delineates both the conceptions of the (moral) limits and constraints of scientific inquiry, and the positive conceptions of the ultimate ends to which science and technology are expected to contribute.

As some people define ethics as pro-social attitudes, this same notion might in particular apply to ethical values. But the very concept of values is far from clear in spite of these sweeping characterizations. Obviously there is nowadays a lot of talk about values, both within academia and in the public sphere, but the concept remains largely elusive. There is no theory of values that can claim epistemic superiority.

The notion of "value" is usually characterized as "an ambiguous concept that governs human behaviour. A set of values may be placed into the notion of a value system. Values are considered subjective and vary across people and cultures. Types of values include "ethical/moral values, doctrinal/ideological (political, religious) values, social values, and aesthetic values" (http://en.wikipedia.org/wiki/Value_(personal_and_cultural), July 2008).

The use of the concept "value" outside an economic context first became common around the end of the 19th century. Even within economy the usage of the concept of "value" dates back only to the late 17th century (Nicholas Barbon, John Locke). The use of the concept "value" outside an economic context first became common around the end of the 19th century (Joas 2000). The "ethics of value" ("Wertethik") is rooted in German philosophy of the late 19th/early 20th century (Hermann Lotze, Max Scheler, Nicolai Hartmann, all of which do not appear in current ethical debates in a prominent place). It is worth noticing that the development of application-oriented ethics from the 1970s has led to a re-discovery of "values" both in the scholarly discourse and in public debates. Large parts of the debate on moral values can nowadays rather be related to utilitarian thought.

In utilitarian ethics the fundamental assumption is that the best/right course of action is the one which provides for the greatest happiness for the greatest number of people. In other words, the right action will invariably maximize the aggregated utility function of all outcomes in a given community. Whether happiness is achieved by money or other goods does not really count for utilitarian theory. The theory simply presupposes that there is something like a subjective value function among the people that then can be maximized. The theory of values is thus exogenous to utilitarian ethics. Utilitarians, and along with them many economic theorists, tend to identify the values of a person with the preferences that the person has.

The economist and Nobel laureate Amartya Sen (in his Ethics and Economics, 1987) is among those who has criticized the traditional economists' picture of economic rationality and thus their neglect of ethics. One important criticism concerns the traditional picture of economists – including utilitarians – that economic actors are typically motivated by self-interest. The subjective preferences, the utilities, may differ in degree but there is no qualitative difference attached to them. However, in fact it seems that each pleasure is qualitatively unique. The pleasure of eating is one thing, the pleasure of listening to music another, etc. This would then imply a variety of moral values that are not interchangeable with each other. Sen argues that people indeed are motivated by a great variety of non-self-interested motives, and some of them could be characterized as ethical motives. This criticism points to an important shortcoming in economic theory and in utilitarianism. It also points into the direction of a more sophisticated theory of values where moral values are an important part.

The sociologist Hans Joas (2000) has delivered one of the most thorough attempts to get to the core of the nature of values, in particular the question of the genesis of values. His central insight is: "[…] values originate in experiences of self-formation and self-transcendence […]" (ibid:164). Joas conceives of his theory as one that integrates a theory of the genesis of values with a universalistic account of morality, notably a pragmatist, i.e. discourse-based, theory of ethics. The basic process of the formation of the self

is through dialogical experiences. Individuals are conceived of as beings who in the meeting with the Other develop an understanding of the Self as a part of a larger community. This is seen as a condition for forming relationships both to oneself and to the Others. As Joas (2000:160) says:

> Identity, not in the sense of stable features, but of a communicative and constructive relationship of the person to himself and to that which does not belong to the self, is the precondition for creative intercourse with the Other and for an ethos of difference.

While qualitative research (Grove-White et al. 2000) and quantitative survey research such as the Eurobarometers on the 'Public Understanding of Science' and on the 'Life Sciences' (Gaskell et al. 2003) have already provided indications of the publics' attitudes to some selected developments of S&T, the underlying forces behind the dynamics of these attitudes remain largely unexplored. One can make the claim that values are one of the key and unexplored parameters responsible for changing attitudes on S&T. Values provide common identity and visions, they stimulate positive or negative attitudes, and their conflict is experienced as major obstacle to action. Paradoxically, it is this landscape of European values of which we still are largely ignorant.

The above deliberations point to the need of a better understanding of the theoretical structure of the very notion of value, further exploring the landscape of people's values and to develop empirical tools to get a truly informative picture of them. They also point to the need to utilize innovative participatory schemes to engage segments of the public in a rational discourse about ethics related to S&T development. Ultimately, practical ethics and the governance of science need to engage much more directly with people's values.

However, all of this does not belittle the significance of contributions based on knowledge and competence within different philosophical traditions and normative disciplines. Indeed, detailed ethical argument need to be an input to the wider ethical societal deliberations. In our experience such a contribution is widely appreciated as a stimulus to normative change and critical testing of one's values. Ethical arguments thus provide a cornerstone in our conception of ethics as a value-based public discourse.

Some Pitfalls

One of the shortcomings of existing societal debates about S&T is the often held assumption that most people will readily accept a "progress narrative" on science and technology. This narrative is based on the conception that advances of knowledge invariably bring about a social good that we need to pursue because of its future benefits. In addition, this narrative often also includes the assumption that we are part of a global competitive market

where others will pursue a certain development if we do not, and that we will loose out economically in this competition unless we act quickly and decisively. One has seen several indications that this narrative has lost its grip on large parts of the populace, e.g., in relation to biotechnology. One has seen that alternative narratives are simultaneously present in the minds of people and form a powerful counterweight to the assumed one-dimensional scientific progress. Fictional narratives such as the Frankenstein storyline or the Brave New World of Aldous Huxley are very much present in the minds of people when scientific advances are debated in the media. In this way, a fundamental positive outlook on the role of science is balanced against a view on the limits of scientific and technical expertise (cf. CEC 2007).

Several factors play into this view and add to the complexities of our views on the role of science. Firstly, many people have realized that quite a number of the problems that science is set to solve were produced by other applications of science in the first place. This applies, e.g., to matters of pollution and technological risks. Secondly, people are also generally aware of the fact that large-scale new technologies effectively turn the whole society into a laboratory. The effects of these technologies will not be known before they have actually been applied on larger scales in our societies (see also, e.g., Latour 1987). Thirdly, people are also to some degree aware of the fact that at least parts of the techno-scientific community are dependent on outside interest-based funding, e.g. by industry. This reflects negatively on the assumed independence of science and may affect the ideal of objectivity. It also undermines trust. Fourthly, those with some first hand knowledge of the societal debates of risk also appreciate that risks are basically a social construct (e.g., see Bayerische Rück 1993), and that risk concerns are fundamentally a political expression that invites pro and con argumentation. Fifth, some people who are well acquainted with, e.g., debates about biotechnology, have come to realize that "more science" is not necessarily the answer to our quest to have prognostic knowledge. New knowledge also produces new uncertainties, and many areas of our surrounding world display a degree of complexity that severely impairs our predictive capacities. Finally, it is more and more recognized that even the best science is infected with value judgements that cannot easily be separated out of the science. But value judgements are no domain in which science holds a supremacy over other, alternative value judgements.

It is the negligence of these factors that explains why scientific contributions often fail to make an impact on the societal debates that they purport to address. The so-called "deficiency view" (i.e. the view that if the publics had the knowledge and understanding provided by science, then they would readily accept certain technologies) is still common among scientists since the above factors do not sufficiently inform their contributions. This is also the reason why many scientific contributions to the risk discussions

commit what some people have called "the type 3 fallacy": excellent science, but altogether addressing the wrong problem.

Thus, arguing for an extended public debate about ethical values connected to new technologies, such as genetically engineered animals, presupposes a mutually shared framework of dialogue that is not one-sided in its overriding narratives and that recognizes the complexities of factors that play into the conceptions of science. It is the task of philosophers and social scientists to work towards such a common, shared framework for public debate.

Scientists in particular need to realize that ethical debates cannot be limited to evaluating the possible benefits of a given technology. A benefit alone just will not do. Evaluating the ethical aspects of a new (risky) technology should be based on a comprehensive assessment that includes a number of critical questions that people want answers to. A simple checklist could for instance look like this:

- What is the core of the problem that needs to be solved in the first place?
- Who is actually in a position to formulate the problem adequately, i.e. who is a potentially affected party?
- Which aspects of the problem would be solved by the proposed technical solution?
- Could the problem alternatively be solved by simple changes of practice and behaviour?
- Are there any other, technical or non-technical solutions that also would solve at least some aspects of the problem? And what are the pros and cons of these other solutions?
- What would be the costs/risks of non-action?
- What counts as a measure of the quality of the proposed solutions?
- What are the side-effects and the long-term effects of the proposed technical solution?
- What are the risks, uncertainties, and areas of ignorance that accompany the technical solutions?
- Who takes on responsibility and who carries the costs if harm should occur along the way?
- Who will be consulted and what is the proper decision procedure in order to decide on the applicability of a technical solution and its access to markets?
- Are all possible regulatory mechanisms, spanning from unconditional approval, via approval under certain conditions, via non-approval unless certain special conditions obtain, to unconditional non-approval, included in the list of options?
- What are the relevant ethical values, principles and arguments that would obtain in regard to the different technical solutions?

– How are these values, principles and arguments perceived and specified when seen from the different perspectives of stakeholders involved?

All these issues will have to be addressed on a case-by-case basis. Thus, practical ethics will not hold in store generic ethical positions on generic technologies. This applies in particular also to the question of genetic engineered livestock. Specific ethical assessments need to be performed in order to do justice to the involved complexities. Before addressing the last two points of the checklist in some more detail, I shall first comment on the institutional and regulatory settings.

Regulatory Settings

Ethics is usually not an element in regulatory legal frameworks for new technologies, including biotechnologies. Traditionally, it is implicitly assumed that a legal framework as such already reflects ethical positions and basic value perspectives. Parliaments in functioning democracies are the proper platform for value debates that result in laws and regulations. However, since science and new technologies within the realm of genetic engineering have been met with public scepticism, criticism and opposition, many have come to realize that traditional forms of government and representative democracy are not that well adjusted to changed societal conditions, and that governance in a wide and inclusive sense is indicated. Within the EU the matter of good governance is a high priority of S&T policy (CEC 2007).

There is a remarkable difference between Europe and the USA in this respect. It is often noted that relevant regulatory frameworks within the US do not accept ethics as an element of assessments of new technologies. In fact, many of the political differences between Europe and the US hinge on the fact that the American side wants to restrict all relevant considerations to what they deem as strictly scientific facts. Thus the Precautionary Principle and other ethical considerations would fall outside the sphere of relevant arguments recognized by the US. This does, of course, not signify that the USA would readily accept unethical technologies or scientific applications. Also within the USA, laws on such issues as, e.g., animal protection or environmental protection reflect basic values and ethical commitments. Arguably, in some areas they may even be stronger than similar legal frameworks in Europe. However, to the best of my knowledge within none of these US regulatory frameworks do ethics figure as an independent, relevant factor that needs to be specially assessed case-by-case.

In rough outline, this situation is not so different within the EU either. Also within the EU the assessments that have to precede the introduction of a new technology are largely scientific risk assessments focussing on safety. However, in some areas and to some extent there are some significant open-

ings for ethical assessments. For instance, the EC Directive on deliberate release, 2001/18, mentions in its Preamble, consideration no 9, the possibility of including ethical principles in the assessment of GMOs. This may in particular refer to the protection clause in article 23 relating to health and environment. One should also note that article 29 opens for the possibility of consultations of the EGE or other ethical bodies.

A similar point applies to the regulation of medicinal products within the EU where ethics is also a significant consideration. More indirectly, the EU position on the Precautionary Principle implies a further distance to the purely science-based evidence in traditional risk assessments (see, e.g., CEC 2000, endorsed in the 2001 Treaty of Nice, article 174, section 2, and especially mentioned in the Presidency conclusions of the Nice European Council Meeting 2000, Resolutions annex III). Furthermore, the endorsement of the Aarhus convention (1998) implies public consultations on all matters that affect the common environment.

Opinions of the European Food Safety Agency (EFSA) do not generally enter into ethical considerations. However, one should note that, for instance, the EFSA opinion on cloned animals is supplemented by an opinion of the European Group of Ethics in Science and New Technologies to the European Commission (EGE) on the same matter, and that both assessments together provide the advisory input to European policy in this field (EGE 2008). This may be seen as an indication that European governance of S&T is ready to supplement safety assessments with ethical argumentation.

In sum, it seems to me that there are significant openings to let ethics play an important role in European policy decisions. Ethical assessments are still not a matter of routine and many aspects of them remain unclear. However, given the public scepticism on new technologies, there is a real chance that even the few openings for ethical considerations might prove important in the near future.

Considering Ethical Arguments

Ethical concerns about genetic engineering of animals are often divided into two basic types. Genetic engineering of a living organism may for a variety of reasons be thought of as being morally problematic in itself, i.e. due to its mode of production or to its source of genetic material be perceived as wrong or morally at least dubious. In that case we shall talk of intrinsic concerns. But genetic engineering may also be thought of as morally problematic because of its consequences. In that case we shall talk of extrinsic concerns. When discussing the ethical issues of genetically engineered livestock it is important to keep these two categories apart. Risk considerations, for instance, have no force whatsoever if fundamental intrinsic worries are brought to the fore.

Comstock (2002) argues that all variants of intrinsic arguments against (animal) biotechnology could be summarized in the following claim:

It is unnatural to genetically engineer plants, animals and foods.

The commonly most well-known argument of this sort is the so-called "Playing God-argument" (cf., e.g., Chadwick 1989, Häyry and Häyry 1998). Some people hold that it is ethically questionable to transfer genes from one species to another species. This attitude is sometimes grounded in a religious belief that it is not up to humankind to violate boundaries that are set by God. Any design of nature through the insertion of new genes is, according to this argument, morally unacceptable. The argument does not occur in the Bible (in fact one may cite places to the opposite), but is based on an interpretation of God's will.

If one would accept the Playing God-argument, it would amount to an unconditional rejection of the genetic modification of all livestock, disregarding what species we deal with and what the purposes and benefits of the modification are.

The basic assumptions of the argument are the following: God has drawn up invisible boundaries between the realm of God and the realm of humans. Those that transcend this boundary are guilty of hybris, i.e. excessive pride. Obviously any such argument would also be dependent on the more specific assumptions of a religion concerning the relation of God, humans and animals. Typically the argument is proposed by adherents of a Christian religion. The problem is to know where this boundary is.

The only safe assumption would be not to interfere with the course of nature at all. But this would arguably exclude all systematic breeding efforts, i.e. all agriculture, particularly all selective breeding of animals. If one, however, wants to allow for breeding, then one assumedly regards some changes of genetic outfit of species as ethically acceptable. In this regard the results of selective breeding and the results of recombinant DNA-techniques are on a par. They differ in only two main respects: first in the way offspring is conceived, and second, in the case of recombinant DNA techniques the source of the inserted genetic information is not necessarily taken from the same species. Are these differences ethically relevant for the Playing God-argument? If the first differences were ethically relevant, then it would equally rule out artificial insemination or test-tube offspring. Since most people, including strongly religious people apparently accept both, this line of reasoning would not affect transgenic animals specifically. In fact, artificial insemination of livestock is the rule rather than the exception for most livestock production.

The second difference is somewhat more tricky. However, one would then have to clarify what the ethically relevant difference is between genetic information obtained from a distant species and genetic information that occurs through natural mutation. Both are nothing but a certain sequence

of adenin (A), thymin (T), cytosin (C), and guanin (G) in the DNA molecule. The mutant genetic information is, when expressed in an individual, an important tool for selective breeding. In principle, the results of recombinant DNA-techniques could equally well be the results of mutation, given sufficient time. After all, according to evolutionary theory, all species have developed from a common core. The dimension of time would become a morally relevant feature for ethical considerations. While time may indeed play a crucial role in regard to the risks (i.e. extrinsic viewpoint) involved in genetic modification (e.g., concerning the "disrupting" effects of the spread of a genetic modification to the environment), it is difficult to conceive of time as an intrinsic ethical consideration. Therefore we do not believe that we could construe this difference as ethically relevant without a number of questionable ad hoc assumptions, or without rejecting the most basic assumptions of evolutionary theory.

In sum, we do not believe that the Playing God-argumention can be backed by weighty rational argument. It would imply that, for instance, breeding programmes in agriculture or introductions of new plant species in a given ecosystem are morally unacceptable. The domestication of animals and their "design" for human production purposes is celebrated as a cultural achievement. The proponents of this argument should then be equally critical to traditional agricultural practices. Thus it seems incoherent to restrict such an objection to gene technology. We therefore suspect that the argument is based on ad hoc reasoning.

Alternatively, proponents of the Playing God-argument could reject the demand of rational justification. Instead they could rely on personal feelings. One would then accept the basic argument and add that one somehow intuitively feels that the dividing line should be drawn precisely at the point when it comes to transgenic animals. As a consequence, the argument is then restricted to explaining one's personal preferences, without attempt to convince others. Empirically speaking, this may be precisely what explains the scepticism towards gene technology that is common among certain (but not all) groups of people in European societies (cf. Hviid Nielsen 1997). If the argument is framed in this way, the proponents retreat from rational social debate with groups that hold different opinions. This does, however, not imply that these sceptics be disregarded in an attempt at building up social consensus. Their attitude should still demand respect from those who disagree. As a consequence, the ethically correct way to deal with those sceptics would be to provide for informed consumer choices and participatory decision-making, i.e. to stress procedural ethics rather than purely substantive ethics.

There are other variants of the Playing God-argument. One version holds that it is morally wrong to break down naturally occurring boundaries between different species, and another holds that it is morally wrong to commodify living nature. We shall not pursue these arguments in detail

here. Let it suffice to say that similar counter arguments as to the Playing God-argument – mutatis mutandis – apply to them as well.

It seems there is no really convincing intrinsic argument against animal biotechnology. The proposed intrinsic arguments seem apparently to point in the direction of some important ethical considerations, but in the end they fail to establish their case. Yet there are two important provisos here. First, the strong initial intuitive appeal of some of these arguments should count as sufficient reason not to disregard them out of hand, but to re-evaluate their relevance in every qualitatively new case. Perhaps an unrestricted and extensive use of animal biotechnology at some point indeed crosses borders that are unprecedented and thus let us forget the basic respect for life that is essential to our moral nature. Second, the mere fact that some of these intrinsic arguments are apparently strongly felt by many people, i.e. represent what sometimes is called their gut-feelings, should count as sufficient reason to not force upon them products that they find morally unacceptable. Even if we are unable to detect sufficient rationality in these reasons to accept them for ourselves, we should, out of respect for other humans – humans that apparently operate with a different sense of rationality –, provide them with choices that make a moral life possible for them. This would mean two things: there should be alternatives to food from animal biotechnology on the market, and there should be clear labelling of products from animal biotechnology.

This leaves us to consider the extrinsic arguments against animal biotechnology, i.e. arguments that relate to the consequences rather than the nature of our technology. One such line of argument is animal welfare considerations, another one is environmental considerations. All extrinsic considerations call for a case-by-case assessment including among others the risks, costs and benefits of the proposed technology. Typically such an assessment will provide for making trade-offs between several aspects. We shall argue that ethical assessments can – and should – in principle be included, provided they are done in a systematic, methodical manner that allows for transparency and quality assurance.

Remarks on Extrinsic Ethical Concerns

Animal Welfare

There are different schools in regard to animal welfare, some being more restrictive than others. Some take as their only reference point the animal's own feelings, so that the ability to feel pain becomes the crucial property (e.g., Duncan 1996). Others look at animal health and physiological function (e.g., Broom 1996) as crucial anchor for defining animal welfare. Some other people (e.g., those propagating biodynamic farming practices) regard the conditions of the animal's natural living environment as the foundation

of animal welfare, so that the ability to follow natural instincts in a biologically preferred environment becomes crucial. These differences between competing conceptions are only partly based on science. To a considerable extent the differences are due to different philosophical outlook, and they may not be reconcilable with each other. This is not completely surprising, since the consideration of animal welfare obviously is dependent on a variety of implicit value assumptions.

In Europe, the UK report an animal welfare from 1965 that became known as the Brambell report, was highly influential. The Brambell report (1965) included the well known "five freedoms":

1. Freedom from hunger and thirst by ready access to fresh water and a diet to maintain full health and vigour.
2. Freedom from discomfort by providing an appropriate environment including shelter and a comfortable resting area.
3. Freedom from pain, injury or disease by prevention or rapid diagnosis and treatment.
4. Freedom to express normal behaviour by providing sufficient space, proper facilities and company of the animal's own kind.
5. Freedom from fear and distress by ensuring conditions and treatment which avoid mental suffering.

While it would be certainly wrong to claim that these freedoms have actually been translated into national law, one might still hold that they set a soft-law standard that is often referred to and that some countries have aimed at including in their national regulations for farm animals. As all such general principles, these freedoms are not very precise and they leave room for different interpretations. Therefore, many activities are aimed at specifying what such clauses as "sufficient space" could mean for specific species in animal production, e.g. for battery hens. It is not too far fetched to say that these animal freedoms function in much the same way as the Charter of Human Rights does for humans. They define a prima facie ethical standard that inform our judgements of particular cases.

It is difficult to say anything specific about animal welfare of transgenic animals in general. In principle it may be better or worse than the welfare of the original, non-transgenic animal. Obviously, a lot will depend on the kind of modification that has been made, i.e. which genes have been modified. Thus one will need research to document the effects of such modifications. One difficulty regarding livestock production is that even the choice of comparator is not unproblematic. If one takes as standard the welfare conditions in industrial intensive production systems to which the transgenic animal is then compared, then obviously one may be reinforcing a practice that is already considered ethically problematic by some people. This only underlines the fact that in all such assessments, including animal welfare, the choice of standard is not beyond discussion.

Even though the principal responsibility to document welfare effects may rest with the company that seeks to market the product, one may assume that the availability of independent research scrutinizing the effects will be crucial. This will be a consequence of the lack of trust that the public now expresses towards industrial research.

However, there are already some experiences and data available in regard to transgenic fish that may provide an indication of welfare effects in transgenic animals in general. It has been claimed that unexpected phenotypical disadvantageous changes are the rule rather than the exception in the genetic modification of fish (Royal Society of Canada 2001). More specifically, deformities of the head and other parts of the body have been documented for transgenic salmon (Devlin et al. 1995). The documented deformities have obviously serious welfare implications for the fish. Also morphological changes and changed allometry have been documented for the same fish, leading to reduced swimming abilities (Ostenfeld et al. 1998; Farrel et al. 1997; McLean et al. 1997). Furthermore, it seems that transgenic salmon shows deviant behaviour in the sense that there is an increased level of activity in regard to feed-intake and swimming (Abrahams and Sutterlin, 1999; Devlin et al. 1999). Thus, one can claim that the scientific literature documents significant changes in morphology, physiology and behaviour in transgenic salmon.

On the background of these experiences one may claim that the welfare aspects of biotechnology in animal production deserve a close case-by-case and step-by-step evaluation in order to avoid negative impacts of the technology. There are still many unknowns in our use of genetic biotechnology, and without more research on these issues one seems well advised to opt for a precautionary practice that would give the animal the benefit of the doubt. Indeed, a precautionary approach to the welfare issue in animal biotechnology would seem to be justified if not implied by our assumed ethical responsibility for the stewardship of production animals. I shall deal with this point a bit further down in the paper.

Environmental Concerns

Some animal biotechnology may have negative effects on the environment and biodiversity. As a rule of thumb one may hold that the larger the animal and the better the containment, the lesser the danger of unintended environmental effects. This has to do with our ability of control. Since ecological interaction of species may be quite complex and not always easily or quickly detectable, such a rule cannot be totally general.

Leaving aside other environmental effects of feeding and containing the animals in appropriate surroundings – effects that may not be specific to animal genetic biotechnology – the main concern today is the spread of the

genetic material to wild relatives. This is particularly relevant for those species whose escape into the environment cannot easily be controlled. Disturbances in our ecosystem raise ethical concerns, especially when some of the affected species and populations may already be threatened by extinction. Again, the main studies of these effects have been done in regard to transgenic fish. One way to approach the possible gene flow from one population to another is to assess the net fitness of a specific type of fish (Pew Initiative on Food and Biotechnology 2003). Muir and Howard (2001, 2002) provide six traits for determining the net fitness of any animal, including a transgenic fish:

- juvenile viability (chances of surviving to sexual maturity);
- adult viability (chances of surviving to procreate);
- fecundity (number of eggs produced by a female);
- fertility (percent of eggs successfully fertilized by male sperm);
- mating success (success at securing mates); and
- age at sexual maturity.

Instead of regarding these as independent or associated factors, scientists now develop mathematical models to combine these parameters of the organisms' net fitness that could say something about the genetic consequences of escapes of a transgenic organism into the environment. Three possible scenarios or hypotheses can be discerned (Pew Initiative on Food and Biotechnology 2003).

The Purge scenario: When the net fitness of a transgenic organism is lower than that of its wild relatives, natural selection will work to purge any transgenes that the wild relatives may inherit (Hedrick 2001). Some transgenic fish may indeed fall under this scenario, depending on the kind of modification that has been done. It is claimed, though, that this scenario need not be without any long term effects. In small populations even temporary declines in net fitness are said to potentially threat the survival of the population.

The Spread scenario: "When the net fitness of a transgenic fish is equal to or higher than the net fitness of a wild mate, gene flow is likely to occur and the genes of the transgenic fish will spread through the wild population." (Pew Initiative on Food and Biotechnology 2003). Some transgenic fish, e.g. growth enhanced coho salmon, reach sexual maturity earlier than their wild counterparts, and this has a large effect on net fitness. In this scenario there is a lasting genetic effect of the invasion by transgenic fish on the wild counterparts.

The Trojan gene scenario: In this model it is suggested that enhanced mating success coupled to reduced adult viability would result in a rapid decline of the wild population. The transgenes would introduce their genetic material into the wild population rapidly, while the lower viability would reduce population sizes. Thus, interbreeding of transgenic organisms with wild relatives would cause a rapid decline in total population.

Again one faces a situation with a number of significant uncertainties relating to the effects on the environment and biodiversity. Obviously, no general and no definite answer is possible at this stage of our knowledge. One may point out that, e.g., the introduction of transgenic species with sterility could change the potential environmental threats significantly. Yet, even in these cases instability and uncertainty are factors to be taken seriously in our assessments. While transgenic fish may pose an environmental threat of serious proportions due to our lack of control of escapes, the principal points made here apply to all animal biotechnology. The exotic species experiences can serve as a rough model for some of the potential threats one may have to face.

It should be pointed out, though, that some of the risks mentioned here are equally posed by organisms obtained from traditional selective breeding. It would thus be wrong to restrict these considerations one-sidedly to genetic biotechnology. As we are talking from an extrinsic point of view, it is the consequences that matter, and not the technology that brings them about. Therefore, one consequence of ethical clarification on these issues would be to argue for a stricter regime of control and license for all new breeds that are brought to production and market, including the non-transgenic variants.

There is now a widespread agreement that the environment deserves our moral consideration. Several different positions have been propagated in environmental ethics, ranging from more moderate anthropocentric positions (Shrader-Frechette 1981), to biocentric, ecocentric and deep ecology positions (Pojman 2001). The discussion on these issues is currently characterized by a very basic and assumedly unavoidable pluralism. Yet, in spite of this there is recognition that some moral concern and value has to be assigned to environmental factors, regardless of how we argue for it, and how far we are willing to go with it. Even with a minimalist anthropocentric account one cannot morally defend many of the environmental harms that result from our technologies, given that the natural resources turn out to be scarcer than we thought. Therefore, the ethical assessment of animal biotechnology needs to take account of this, and include these considerations explicitly. Current practices seem to indicate that mankind falls short on any ethical measure that one may apply to environmental concerns. The inclusion of these concerns in our ethical assessments thus appears as a moral imperative.

The above considerations on animal welfare and environment will prove relevant when evaluating the ethical aspects of some genetically engineered animals. We shall not go into all the details here, but mention just two cases: production animals for zoopharming and xenotransplantation.

In general, public acceptance of using animals for zoopharming seems widespread. Surveys such as various Eurobarometers on biotechnology reveal little principled objections against this technology due to a percep-

tion of important benefits. The envisaged benefits mostly relate to human health, though they might also include animal health. People are ready to accept the genetic engineering of animals if it is an effective – if not the only – way of securing important medicinal products. Behind such attitude is what is commonly called a gradualistic view of moral standing. Even if one is not accepting a fully anthropocentric view of moral standing, one is often inclined to differentiate among various species to the extent that some "higher" species are assumed to be more worthy our moral concern than others. This can, e.g., be based on our conception of sentience. If one, however, endorses a strong biocentric viewpoint, basically assuming equal moral standing of all living beings, then one may still have objections against this technology. Yet, in all European countries our agricultural practice is built upon the view that demonstrated important human uses override animal welfare and other ethical concerns. Legal regulations ensure that animal welfare and environmental concerns are taken care of in due measure.

Similar considerations apply to the area of xenotransplantation. As with zoopharming, the important envisaged medical uses provide for weighty arguments in favour of this technology, especially since the availability of donor organs is significantly less than the perceived demand in most countries. However, in this case the ethical dilemmas are significantly more complex. It is not only the animal welfare considerations of the donor animals and the considered risks to the recipient patient that have to be included in our ethical assessment. Now we also have to consider the risks to public health as well. This is due to the fact that all xenotransplantation will carry a certain risk of xenosis, i.e. a special case of zoonosis – a transferral of infectious material from animals to humans. A focus has been on so called porcine endogene retrovirus (PERV) in the case of using pig organs, e.g. hearts, for implantation into humans. PERVs are unproblematic for pigs, but we do not know how they or some mutations of them might behave in a human organism. The worst case scenario would be when PERVs become virulent with easy transferral of infections between humans through, e.g. drop infections, with a long period of latency and eventual fatal outcome. Now one has to deal with a situation where patient risks and benefits also have to be weighed against societal risks. Furthermore, attempts to minimize societal risks will typically include measures and control regimes that seriously infringe on people's autonomy and privacy, for instance through demands on monitoring sex-partners after surgery (cf. Kaiser 2004).

In sum, while the ethical discussions about zoopharming are mainly dependant on whether or not one is willing to defend a strong biocentric viewpoint, the ethical issues in xenotransplantation are much more complex and far from even minimal societal consensus. The question is whether scientific advances will be able to significantly reduce the involved uncertainties. Many proponents of xenotransplantation believe that this can realistically be achieved. However, many would argue to the opposite, namely

that the total eradication of all scientific uncertainty is an impossibility on philosophical and methodological grounds. The occurrence of scientific uncertainty raises in any case a particular challenge for the ethical assessment of a technology.

Dealing with Scientific Uncertainty

All assessments of new technologies look at possible outcomes and consequences of the uses of the technology in question. Thus the availability of prognostic knowledge is crucial for any assessment of risks, benefits or otherwise. Yet, it is well known that fully reliable prognostic knowledge is rarely available, given the complexities of a technology in use. There is always a certain degree of uncertainty concerning possible outcomes. Given extensive testing over long periods of time some uncertainties will diminish, but they may not totally disappear. For instance, proponents of gm crops would argue that the scientific uncertainties relating to the safety risks of gm Bt-maize, gm soya and gm herbicide tolerant oilseed rape have been significantly reduced after intensive testing and cultivation in various countries (Sandvido et al. 2007; ACRE 2007). However, even then one may conclude that the assessments have been somewhat incomplete since the evaluation of the benefits, falling outside the range of purely scientific criteria, still contains significant uncertainties (De Melo-Martin and Meghani 2008).

From a decision-maker's point of view, uncertainties are as important as scientific certainties. Ethically speaking, managing the uncertainties of a given technology directly relates to the concepts of responsibility and fairness, i.e. distribution of benefits and risks. A decision-maker will be held responsible for judging the available information at the time of the decision with respect to the balance of evidence, including unknowns and the degrees of confidence related to evidence. Thus, it is vitally important for decision-makers to be provided not only with information on what is definitely known, but also with what is still uncertain even though it may be a possible, realistic outcome.

Typically, scientists are good in communicating what they know with a reasonable degree of confidence, but they are not as good in communicating those aspects that are still uncertain, nor how uncertain they might be. Since it is totally unrealistic to strive for zero-risk technologies (in spite of some political rhetoric to the opposite), the real challenge is to improve our communication of complex risks and scientific uncertainties. Efforts to work out indicators and communication schemes for scientific uncertainty have been intensified since the groundbreaking work of Silvio Funtovicz and Jerome Ravetz (1990) and have led to a variety of interesting contributions (cf. especially Walker et al. 2003; see also Cooke 1991; Lemons 1996; van der Sluijs 1997; van Asselt 2000; Fjelland 2002; Krayer von Krauss et

al. 2008). Many of the mentioned scholars note that uncertainty is a lasting feature of scientific knowledge, so that hopes of eliminating all uncertainties through more research are essentially in vain. More knowledge and research will typically also generate new uncertainties, though some specific uncertainties may disappear.

Communicating uncertainties is crucial also because they play a decisive role in evaluating whether or not a precautionary approach (i.e. the application of the Precautionary Principle; hereafter referred to as PP) is indicated. While there has been an extensive discussion about precaution, in Europe at least there is a widespread consensus that the PP is important (cf. CEC 2000). There is, however, an ongoing debate about how to formulate the PP in terms that are suitable for risk management.

The classic definition stems from the Rio Declaration of 1992, §15:

> In order to protect the environment, the precautionary approach shall be widely applied by States according to their capabilities. Where there are threats of serious or irreversible damage, lack of full scientific certainty shall not be used as a reason for postponing cost-effective measures to prevent environmental degradation.

The shortcomings of this formulation are apparent. Among others it contains a triple negation: Not having knowledge is not a reason for not acting. Various other attempts have been made to rectify these shortcomings later (for a more comprehensive discussion see UNESCO COMEST 2005).

Personally I think the new definition endorsed by UNESCO is a significant advance:

> When human activities may lead to morally unacceptable harm that is scientifically plausible but uncertain, actions shall be taken to avoid or diminish that harm.
> Morally unacceptable harm refers to harm to humans or the environment that is
> - threatening to human life or health, or
> - serious and effectively irreversible, or
> - inequitable to present or future generations, or
> - imposed without adequate consideration of the human rights of those affected.
>
> The judgment of plausibility should be grounded in scientific analysis. Analysis should be ongoing so that chosen actions are subject to review.
> Uncertainty may apply to, but need not be limited to, causality or the bounds of the possible harm.
> Actions are interventions that are undertaken before harm occurs that seek to avoid or diminish the harm. Actions should be chosen that are proportional to the seriousness of the potential harm, with consideration of their positive and negative consequences, and with an assessment of the moral implications of both action and inaction. The choice of action should be the result of a participatory process. (UNESCO COMEST 2005)

Whatever version of the PP one adheres to, the occurring of scientific uncertainty is crucial for all such application of precaution. In our context it is important to note that the PP has an ethical underpinning, relating to the concept of culpable ignorance that makes it part and parcel of an ethical approach to the ethics of livestock engineering. The PP, though still

the object of heated discussions between the USA and the EU, is widely interpreted as an ethically responsible way to deal with an uncertain future where great harms may be lurking. The PP is essentially designed to manage scientific uncertainties in such a way that one rather errs on the side of nature than on the side of expected benefits.

A Proposal For Ethical Assessments

The above deliberations are meant to show that an ethical assessment of livestock genetic engineering needs to consider a variety of viewpoints and value perspectives. In a pluralistic society, different values are brought to the fore, and it is supposedly seldom that one may be in a position to take a stand on purely intrinsic grounds. Thus, assessments need to be made on a case-by-case and supposedly also on a step-by-step basis. We have argued that different values need to be respected and that ethical considerations need to be clarified for each new animal product. Risks and benefits are only part of this assessment. Other considerations, e.g., justice and equity and dignity of the creature, need to be included. The challenge is to make such an ethical assessment in a transparent, systematic and methodical manner that is adapted to an open democratic debate. Several such possibilities were explored in an EC-funded project (see http://www.ethicaltools.info/, July 2008).

One such method for ethical evaluations originates in principle-based ethics. It starts not with ethical theory, but with a selection of principles that can find a broad degree of support from different ethical theories or cultural beliefs. The principles are selected in such manner that they can be seen as roughly representative of whole families of values. They capture the relevant plurality of ethical concerns that are brought to the fore. The origin of this method is Beauchamps and Childress' (1994) approach in medical ethics. Inspired by medical ethics, Ben Mepham from the University of Nottingham was the first to transfer Beauchamps and Childress' principles from medical ethics to a practical approach for addressing broader policy related problems, modifying them slightly (Mepham 1996). This was then termed an "ethical matrix".

The challenge with this approach consists in moving from the general level of the principles down to the level of practical questions. The first stage of the method is thus the setting up of a two-dimensional ethical matrix where chosen ethical principles are specified in regard to stakeholders' interest-constellation. Who the relevant stakeholders are needs to be determined beforehand. In general all those groups who are affected one way or the other by the new technology count as candidates for stakeholders. One way to capture the relevant ethical principles would mirror the 4-principle approach known from medical bioethics: Risks, Benefits, Dignity, and Fair-

ness (Justice). The principle of respect for dignity seems more appropriate than the alternative principle autonomy when dealing with animal stakeholders or the environment, or at least I would claim so (cf. Kaiser 2003; see also FAO/WHO Report 2004).

There are competing conceptions of how the list of basic ethical principles is introduced and justified. Mepham tends to refer to what he calls common morality for justifying his list of principles. Others see the principles and the resulting matrix as being informed by some version of pragmatist philosophy (Forsberg 2007). For our current purposes we believe it suffices to view the ethical matrix as a rough framework for ethical assessment that needs to prove its usefulness in practical contexts and that eventually may be improved upon as concrete experience accumulates.

It must then, in the next step, be determined how the technology at stake, in our example the genetic modification of animals, will affect the values described in the ethical matrix. This state of affairs can be structured in a consequence matrix, which will consist of the same cells as the ethical matrix. The consequence matrix gives a brief description of the assumed or possible consequences of a decision upon every affected cell as specified in the ethical matrix. With an ethical matrix and a consequence matrix filled in, it is possible to see whether the different consequences amount to a violation (expressed, e.g., as a minus) of certain specified norms or whether they seem to be in accordance (expressed, e.g., as a plus) with the values. These relations of consequences to specified norms are noted in an evaluation matrix. An evaluation matrix is thus a matrix that provides an overall picture of the ethical status of the issue at stake, where individual may assign special weights to particular cells, i.e. considerations. There is no deduction of the correct ethical answer from the evaluation matrix since weighing is essential; the best ethical solution is essentially a moral judgment on the totality of considerations expressed in the matrix.

Assume we want to assess the ethical aspects of a certain genetic modification of an animal species for food production in a region. Following the ethical matrix approach one would first address the issue of who the relevant stakeholders are, for example small scale producers and consumers. We also need to agree on potentially affected organisms and their components of the environment. A proper set of ethical principles then needs to be established, as, e.g., justice/fairness, dignity/autonomy, and the obligation to avoid risks and produce benefits. Once a common understanding of these principles is ensured, it is important that the principles are specified for each interest perspective. The result is an ethical matrix that represents the starting point of the ethical assessment. It is clear that some of the cells in table 1 [here marked in dark grey] directly relate to the scientific description in the safety and benefit assessments of gm animals. Thus, there is an overlap between the ethical assessment and the risk assessment and management. Scientific data will provide information on how the technology

relates to the qualities described in the various cells of the matrix. This is termed the consequence matrix. One can now mark whether specific consequences of the new technology promote, inhibit or remain neutral to each of the specifications in the cells. At the same time we need to mark the significant uncertainties in our matrix. In the end one is prepared to issue recommendations that are informed by the totality of ethical considerations and conflicts. Table 1 below depicts one such tentative basic matrix. The next step would then be to evaluate how the consequences of a specific product would affect the considerations expressed in the different cells.

Table 1: Simplified Ethical Matrix for the hypothetical case of gm-fish

Ethical matrix for gm-fish	**Risk**	**Benefit**	**Dignity**	**Justice / fairness**
Small producers	Dependencies on nature and corporations	Adequate income and work security	Freedom to adopt or not to adopt	Fair treatment in trade
Consumers	Safe food	Nutritional quality; price	Respect for consumer choice (labelling)	General affordability of food product; access
Treated fish	Proper animal welfare	Improved disease resistance	Behavioural freedom	Respect for natural capacities (telos)
Biota	Pollution and strain on natural resources; Reduction of biodiversity	Increasing sustainability; Maintaining threatened biodiversity	Respect for ecological systems interactions	No additional strain on regional natural resources; equal value for future generations

Several applications of this method have been conducted and analyzed by several research groups (see: http://www.ethicaltools.info/, July 2008). Once one acknowledges the need for a tool or framework to assist the ethical evaluation in decision making, it seems indicated to work with a practical framework of the kind presented here. The absence of such formalized ethical decision frameworks invites accusations of ad hoc-ness or bias in the ethical evaluation, hinders transparency of the involved process, and makes international comparison and mutual learning difficult. The ethical matrix is a possible tool to facilitate quality assurance in practical ethics. In particular in the decision-making context addressed here, i.e. a context where a decision-maker is faced with competing value claims in society and a plurality of ethical theories appealed to by various groups, several con-

siderations enter the picture. The main properties of good ethical frameworks, as the one suggested here, are, I believe, the following (cf. Kaiser et al. 2007):

- Inclusion of all values at stake;
- Transparency;
- Multiplicity of viewpoints;
- Exposition of case-relevant and ethically-relevant facts ;
- Inclusion of ethical arguments.

When ethical assessments need to be made in a practical setting, an explicit framework or tool to assist this task should be employed in order to allow for greater transparency and quality control. In this article we have attempted to show that explicit ethical and welfare assessments in regard to animal biotechnology are a necessity, and in spite of their value-dimensions they are far from only being delegated to the realm of the mere subjective and idiosyncratic. Science and ethics go together in making this task practical and transparent. Hopefully this can pave the way for ethics and animal welfare aspects to become solid and permanent features of our regulatory systems and integrated in routine quality assessments of animal biotechnology for farm production.

References

Aarhus Convention (1998) Convention on Access to Information, Public Participation in Decision-Making and Access to Justice in Environmental Matters, United Nations Economic Commission for Europe (UNECE) (http://www.unece.org/env/pp/documents/cep43e.pdf, July 2008 and general information at: http://www.unece.org/env/pp/, July 2008)

Advisory Committee on Releases to the Environment (ACRE) (2007) (http://www.defra.gov.uk/environment/acre/fsewiderissues/pdf/acre-wi-final.pdf, July 2008)

Abrahams MV, Sutterlin A (1999) The foraging and antipredator behavior of growth-enhanced transgenic Atlantic salmon. Animal Behaviour 58:933–942

@ltweb: The global clearinghouse for information on alternatives on animal testing (http://altweb.jhsph.edu/publications/humane_exp/het-toc.htm, July 2008)

Bayerische Rück (ed) (1993) Risiko ist ein Konstrukt. Knesebeck, München

Beauchamp TL, Childress JF (2002) Principles of biomedical ethics (5th ed) Oxford University Press, Oxford

Brambell committee (1965) Report of the technical committee to enquire into the welfare of animals kept under intensive livestock husbandry systems. Command Report 2836, Her Majesty's Stationery Office, London

Broom D (1996) Animal welfare defined in terms of attempts to cope with the environment. Acta Agric Scand, Sect A Animal Sci (Suppl 27):22–28

Clarke SG, Simpson E (eds) (1989) Anti-Theory in Ethics and Moral Conservatism. State University of New York Press, Albany

Chadwick R (1989) Playing God. Cogito 3:189–193

Comstock G (2002) Ethics and Genetically Modified Foods. In: Ruse M, Castle D (eds) Genetically Modified Foods. Debating Biotechnology. Prometheus Books, New York

Cooke RM (1991) Experts in Uncertainty: Opinion and Subjective Probability in Science. Oxford University Press, Oxford

CEC (2007) Taking European Knowledge Society Seriously. Report of the Expert Group on Science and Governance to the Science, Economy and Society Directorate, Directorate-general for Research, EC, Brussels

Darwall S, Gibbard A, Railton P (eds) (1997) Moral Discourse and Practice. Oxford University Press, New York, Oxford

De Melo-Martin I, Meghani Z (2008) Beyond risk. European Molecular Biology Organization reports vol 9(4):302–306

Duncan, IJH (1996) Animal welfare defined in terms of feelings. Acta Agric Scand, Anim Sci Suppl 27:29–35

Devlin RH, Yesaki TY, Donaldson EM, Hew C-L (1995) Transmission and phenotypic effects of an antifreeze/GH gene construct in coho salmon (Oncorhynchus kisutch). Aquaculture 137:161–169

Devlin RH, Johnsson JI, Smailus DE, Biagi CA, Jönsson EB, Björnsson BT (1999) Increased ability to compete for food by growth hormone-transgenic coho salmon Oncoryhnchus kisutch (Walbaum). Aqua Res 30:479–82

European Group on Ethics of science and new technologies (EGE) (2008) Ethical aspects of animal cloning for food supply. The European Group of Ethics in Science and New Technologies to the European Commission. Opinion no 23. 16th January 2008 (http://ec.europa.eu/european_group_ethics/activities/docs/opinion23_en.pdf, July 2008)

Enviropig™ 1 (http://www.uoguelph.ca/enviropig/, July 2008)

Enviropig™ 2 (http://en.wikipedia.org/wiki/Enviropig, July 2008)

Enviropig™ 3 (http://www.bioethics.iastate.edu/classroom/docs/enviropig_letter.html, July 2008)

Eurobarometer (2005) Social Values, Science and technology. June 2005. Brussels (http://ec.europa.eu/public_opinion/archives/ebs/ebs_225_report_en.pdf, July 2008)
FAO/WHO Report (2004) Expert Consultation on Safety Assessment of Foods Derived from Genetically Modified Animals including Fish. November 2003 (ftp://ftp.fao.org/docrep/fao/006/y5316E/y5316E00.pdf, July 2008)
Farrell AP, Bennet W, Devlin RH (1997) Growth-enhanced transgenic salmon can be inferior swimmers. Can J Zool 75:335–37
Fjelland R (2002) Facing the Problem of Uncertainty. Journal of Agricultural and Environmental Ethics 15(2):155–169
Forsberg E-M (2007) A Deliberative Ethical Matrix Method – Justification of Moral Advice on Genetic Engineering in Food Production. Dr.art. dissertation. Faculty of Humanities, University of Oslo
Funtowicz SO, Ravetz JR (1990) Uncertainty and quality in science for policy. Theory and decision library. Series A. Philosophy and methodology of the social sciences (vol 15) Kluwer Academic Publishers, Dordrecht
Gaskell G, Allum N, Stares S (2003) Europeans and Biotechnology in 2002 (www.europa.eu.int/comm/public_opinion/archives/eb/ebs_177_en.pdf, July 2008)
Grove-White R, MacNaghten P, Wynne B (2000) Wising up: the public and new technologies. Centre for the Study of Environmental Change. Lancaster University
Hedrick PW (2001) Invasion of transgenes from salmon or other genetically modified organisms into natural populations. Can J Fish Aquatic Sci 58:841–844
Häyry M, Häyry H (1989) Genetic Engineering. Encyclopedia of Applied Ethics (vol 2):407–417
Hviid Nielsen T (1997) Behind the color code of 'no'. Nature Biotechnology 15:1320–1321
Joas, H (2000) The Genesis of Values. The University of Chicago Press, Chicago
Kaiser M (2003) Ethical issues surrounding the gm-animals/gm-fish production. Expert paper for the FAO/WHO Expert Consultation on Safety Assessment of Foods Derived from Genetically Modified Animals including Fish 2003 (http://www.fao.org/ag/agn/food/risk_biotech_animal_en.stm, July 2008)
Kaiser M (2004) Xenotransplantation – Ethical Considerations based on Human and Societal Perspectives. In: Farstad W, Andresen Ø, Nyberg O, Christensen B (eds) Acta Veterinaria Scandinavica. Proceedings of the 16th Internordic Symposium of the Nordic Committee for Veterinary Scientific Cooperation on Animal Organs to Save Human Lives. Suppl 99, pp 65–73
Kaiser M (2005) Assessing ethics and animal welfare in animal biotechnology for farm production. OiE. Scientific and Technical Review of the World Organisation for Animal Health, Rev Sci Tech Off Int Epiz 24(1):75–87
Kaiser M (2006) Practical ethics in search of a toolbox: Ethics of science and technology at the crossroads. In: Gunning J, Holm S (eds) (2006) Ethics, Law and Society. Vol II. Ashgate Publishing Ltd, Cardiff, pp 35–44
Kaiser M, Millar K, Thorstensen E, Tomkins S (2007) Developing the ethical matrix as a decision support framework: GM fish as a case study. Journal of Agricultural and Environmental Ethics 20:65–80
Krayer von Krauss MP, Kaiser M, Almaas V, van der Sluijs J, Kloprogge P (2008) Diagnosing and prioritizing uncertainties according to their relevance for policy: The case of transgene silencing. Science of the Total Environment 390:23–34
Latour B (1987) Science in Action. Harvard University Press, Cambridge, MA
Lemons J (ed) (1996) Scientific Uncertainty and environmental problem solving. Blackwell Science Cambridge, MA

McLean E, Devlin RH, Byatt JC, Clarke WC, Donaldson EM (1997) Impact of a controlled release formulation of recombinant growth hormone upon growth and seawater adaptation in coho (Oncorhynchus kisutch) and chinook (Oncorhynchus tschawytscha) salmon. Aquaculture 156:113–28

Mepham B (1996) Ethical analysis of food biotechnologies: An evaluative framework. In: Mepham B (ed) Food Ethics. Routlede, London, pp 101–119

Muir WM, Howard RD (2001) Fitness component and ecological risk of transgenic release; A model using Japanese medaka (Oryzius latipes). American Nature 159:1–16

Muir WM, Howard RD (2002) Assessment of possible ecological risks and hazards of transgenic fish with implications for other sexually reproductive organisms. Transgenic Res 11:101–114

Ostenfeld TH, McLean E, Devlin RH (1998) Transgenesis changes body and head shape in Pacific salmon. J Fish Biol 52:850–54

Pew Initiative on Food and Biotechnology (2003) Future fish: issues in science and regulation of transgenic fish. Washington, Pew Initiative on Food and Biotechnology.
(http://pewagbiotech.org/research/fish/fish.pdf, July 2008)

Pojman LP (ed) (2001) Environmental Ethics. Readings in theory and application. Wadsworth, Belmont, CA

Royal Society of Canada (2001) Elements of Precaution: Recommendations for the Regulation of Food Biotechnology in Canada. The Ottawa, Ontario Canada (http://www.rsc.ca//files/publications/expert_panels/foodbiotechnology/GMreportEN.pdf, July 2008)

Russell WMS, Burch RL, Hume CW (1992) The Principles of Humane Experimental Technique. New edition (original 1959). Universities Federation for Animal Welfare (UFAW)

Sanvido O, Romeis J, Bigler F (2007) Ecological impacts of genetically modified crops: ten years of field research and commercial cultivation. Adv Biochem Engin/Biotechnol 107:235–278

Sen A (1987) On Ethics & Economics. Blackwell, Oxford

Shrader-Frechette KS (1991) Environmental Ethics. 2nd ed. The Boxwood Press, Pacific Grove, CA

van Asselt MBA (2000) Perspectives on Uncertainty and Risk. Kluwer Academic Publishers, Boston, Dordrecht, London

van der Sluijs JP (2007) Uncertainty and Precaution in Environmental Management: Insights from the UPEM conference. Environmental Modelling and Software 22(5):590–598

von Schomberg R (2007) From the Ethics of Technology towards an Ethics of Knowledge Policy and Knowledge Assessment. A working document from the European Commission Services, January 2007
(http://ec.europa.eu/research/science-society/pdf/ethicsofknowledgepolicy_en.pdf, July 2008)

UNESCO/COMEST (2005) The Precautionary Principle. A report of an ad hoc working group, endorsed by COMEST/UNESCO April 2005. UNESCO, Paris (http://unesdoc.unesco.org/images/0013/001395/139578e.pdf, July 2008)

Vetenskapsrådet (2008) Public Opinion in Sweden on the Use of Animals in Research, Vetenskapsrådets Rapportserie 8:2008, Stockholm
(http://www.v-a.se/downloads/rapport-8-2008.pdf, July 2008)

Walker WE, Harremoës P, Rotmans J, Van der Sluijs JP, van Asselt MBA, Janssen P, Krayer von Krauss MP (2003) Defining Uncertainty. A Conceptual Basis for Uncertainty Management in Model-Based Decision Support. Integrated Assessment. Vol. 4(1) Vancouver, 5–17

Xenodiaries. Diaries of despair: The secret history of pig-to-primate organ transplants (http://www.xenodiaries.org/summary.htm, July 2008)

Assessing the Welfare of Transgenic Farm Animals

Cornelis G. Van Reenen

1 Introduction

The application of transgenic technologies in (farm) animals has raised a number of concerns, both within the scientific community and the larger public (e.g., Mench 1999; Rhind et al. 2003b; Einsiedel 2005; Gjerris and Sandøe 2007). An important concern is related to the welfare of transgenic animals. For one thing, unless any negative effects on animal welfare outweigh the benefits of the technology – for example, in terms of human health – animal transgenesis would not be acceptable from an ethical point of view (Olson and Sandøe 2004; Lassen et al. 2006). In addition to a moral dimension, animal welfare concerns also encompass more practical matters such as, for example, the impact of animal welfare on animal health and viability, and, thereby, on the efficiency and feasibility of a transgenic technology (Wilmut 2002; Rhind et al. 2003b; Campbell 2007). Thus, assessing the welfare of transgenic farm animals is a matter of great relevance. Indeed, animal health and welfare considerations play a central role in risk assessments of animal cloning (a technology also used in farm animal transgenesis) that were recently carried out in the United States (FDA 2006; Rudenko et al. 2007) and the European Union (EFSA 2007). However, not in the least due to the relative novelty of the technology, there is a lack of systematic and comprehensive studies addressing animal welfare effects of transgenesis. In the current paper I will revisit and update the main assumptions and principles underlying the evaluation of the welfare of transgenic farm animals, as previously reported (Van Reenen et al. 2001). Next, in the light of these principles, I will briefly review some existing studies associated with the assessment of risks of transgenic technologies.

2 Technologies and Applications

In order to appreciate the animal welfare risks involved in transgenesis, it is necessary to briefly address the main experimental methods that are used to create transgenic farm animals and to summarize the most important applications of farm animal transgenesis. More extensive and detailed information is provided elsewhere (e.g., these proceedings; Clark and Whitelow 2003; Vajta and Gjerris 2006; Niemann and Kues 2007; Robl et al. 2007).

2.1 Technologies

For the production of transgenic farm animals the currently most common technologies are pronuclear microinjection and somatic cell nuclear transfer (SCNT). With pronuclear microinjection, multiple copies of a foreign gene (transgene or DNA construct) are introduced into the pronucleus of in vitro fertilized eggs. Subsequent experimental steps prior to the birth of offspring include in vitro embryo culture and the transfer of embryos to the uterus of recipients. Pronuclear microinjection is now increasingly being replaced by SCNT, because it has the disadvantage of a low transgenic efficiency and it does not allow for gene targeting by homologous recombination. SCNT-mediated transgenesis involves the transfection of cultured somatic (often fetal) cells with a DNA construct by electroporation, the isolation, selection and propagation of transgenic cells, the introduction of the nucleus of a transgenic cell into an enucleated oocyte, the fusion of recipient cytoplasm and donor nucleus, and the activation of the reconstructed embryo with the use of various mechanical, electrical or chemical agents. Similar to microinjected embryos, embryos reconstructed in this manner are transferred to a recipient after a period of in vitro embryo culture. In addition to electroporation, lentiviral transduction could be an effective method for transfecting cultured cells prior to SCNT (Dieckhoff et al. 2008). With SCNT, it has been shown that it is possible to generate for example transgenic sheep, pigs and cattle that carry the same genetic modification, including a selective deletion of genes (gene knockout), at a predetermined site of the genome (McCreath et al. 2000; Dai et al. 2002; Kuroiwa et al. 2004).

Transgenic founder animals resulting from the actual manipulation of early embryos (via microinjection or SCNT) generally carry exogenous DNA at a single site in one of the host chromosomes, giving so-called hemizygous transgenic individuals. According to recently proposed guidelines on the nomenclature of transgenic rodents (FELASA Working Group 2007), hemizygous transgenic animals should be denoted in general as +/Tg or +/tm, in case of non-homologous (i.e., random) or homologous recombination, respectively. The '+' sign indicates either the corresponding wild type allele or the unaffected (wild type) insertion locus of the partner chromosome. Similarly, hemizygous transgenic founders carrying a gene knockout are denoted as +/-, where the '-' sign refers to the deficient allele.

2.2 Applications

There are several (potential) applications of transgenesis in farm animals, described in more detail by Niemann (these proceedings). One application aims at the production of recombinant human pharmaceutical proteins in transgenic animals – transgenic animals as bioreactors (see Schnieke, these proceedings). For example, by using promoter sequences of milk protein genes, transgene expression can be targeted to the mammary gland, with

the foreign protein being expressed in the milk. Harvesting the desired proteins involves the collection and subsequent purification of the milk. So far, a wide range of recombinant proteins have been successfully produced in the mammary gland of transgenic sheep, goat, cattle, pigs and rabbits.

Transgenic animals may also be used as donors of xenografts. Xenografts from domestic pigs are considered a potentially useful source of transplants (e.g. hearts or kidneys) for humans. To try to overcome the rejection of a porcine xenograft resulting from the activation of complement factors belonging to the human immune system, transgenic pigs have been generated that either express transgenes encoding for human complement inhibitory factors, or lack genes that are responsible for the presence of antigenic structures on the porcine organ that cause the transplant rejection response.

Transgenic approaches to improve production traits in farm animals have remained largely experimental so far. Traits of interest include growth and body composition, quality of wool production, and disease resistance. Suggested strategies are based on the introduction into the germline of specific disease resistance genes, or genes that encode for growth promoting substances, biochemical substrates critical in the synthesis of wool, and various factors modulating the immune response. Following an approach similar to that used in generating transgenic animals as bioreactors, transgenic dairy cows were produced with a substantially altered milk casein composition (Brophy et al. 2003), or expressing recombinant lysostaphin (an antibacterial agent) in their milk, which protected them to some extent against Staphylococcus aureus mastitis (Wall et al. 2005).

3 Factors That May Affect the Welfare of Transgenic Farm Animals

Three separate sets of factors may, potentially, affect the welfare of transgenic farm animals (table 1).

3.1 Insertional Mutations

Common gene transfer techniques, including pronuclear microinjection, electroporation and lentiviral transduction, result in the random insertion of the transgene (Robl et al. 2007). Random transgene integration may take place within or close to an endogenous gene, creating a so-called insertional mutation with a resultant loss of host gene function (Woychik and Alagramam 1998). Depending on the function of the disturbed gene, this may harm animal welfare. Phenotypic abnormalities associated with insertional mutations frequently express themselves in homozygous transgenic (Tg/Tg) individuals only, which points to a recessive trait (e.g., Ballester et al. 2004; Mukai et al. 2006).

Table 1: Factors that may affect the welfare of transgenic farm animals (modified from Van Reenen et al. 2001)

Factor	Actual or potential welfare hazard
Insertional mutations	Random transgene insertion
	Loss of host gene function
Transgene expression	Lack of control of transgene expression (level, site, developmental control, temporal control)
	Exposure of host to biologically active transgene-derived proteins
	Perturbation of homeostasis
In vitro technologies	Disturbances of regulation of early gene expression
	Incomplete or aberrant epigenetic reprogramming
	Inappropriate gene expression and developmental abnormalities
	Large offspring syndrome (LOS), abnormal offspring syndrome, cloning syndrome

3.2 Transgene Expression

The expression of a transgene could be harmful in at least two (interdependent) ways. First, transgene regulation can be inappropriate because the recombinant regulatory elements do not provide sufficient control over the site (cell-type, tissue), level and time of expression of the transgene. This problem can be exacerbated by so-called 'position effects', when regulatory elements of neighbouring endogenous genes override those situated within the transgene itself (Wells and Wall 1999). Second, transgene-derived proteins may have biological properties per se that pose a risk to animal welfare, particularly when they enter the general circulation in relatively high or even supra-physiological levels. Salient examples of these include several powerful growth-promoting or immune-stimulating factors which, in the absence of rigorous transgene regulation, were the cause of deleterious side-effects (e.g., Fattori et al. 1994; Pinkert et al. 1994; Massoud et al. 1996; Park et al. 2006). It should be noted that, similar to milk proteins (McFadden et al. 1988; Stelwagen et al. 1997), recombinant proteins secreted into milk may enter the general circulation (e.g., Carver et al. 1992). Thus, transgenic dairy animals as bioreactors may be exposed to transgene-derived proteins irrespective of proper transgene regulation, and plasma levels of a recombinant protein synthesized in the mammary gland could become unacceptably high beyond a certain intensity of transgene expression.

3.3 In Vitro Technologies

In vitro technologies employed in the process of generating transgenic farm animals (e.g., in vitro embryo culture and SCNT) have been associated with a host of deleterious side-effects, including placental abnormalities, high incidences of abortion throughout pregnancy, prolonged gestation, increased birth weights, and high rates of stillbirth, congenital malformation and perinatal and pre-weaning mortality, accompanied by a range of pathological conditions and abnormalities such as neonatal weakness, respiratory failure, incomplete development of the vascular and the urogenital tract, malformations in liver and brain, immune and digestive dysfunction, anemia, and an increased proneness to infections (Kruip and den Daas 1997; Cibelli et al. 2002; Rhind et al. 2003a; McEvoy et al. 2006). Some of these characteristics have been collectively referred to as the large offspring syndrome (LOS), although symptoms are unpredictable (Young et al. 1998; Cibelli et al. 2002). To account for this, alternative terms such as 'cloning syndrome' (Wells et al. 2004) or 'abnormal offspring syndrome' (Farin et al. 2006) have been proposed.

There is now increasing evidence that LOS symptoms result from environmentally induced disturbances in the regulation of early gene expression. More specifically, in vitro manipulations are believed to interfere with epigenetic modifications (e.g., demethylation and de novo remethylation of DNA) of developmentally important genes during early embryonic development (Young and Fairburn 2000; Farin et al. 2006). Reconstructed embryos generated by SCNT may be particularly liable to epigenetic errors since somatic donor nuclei introduced into enucleated oocytes are required to go through the sensitive process of complete genetic reprogramming (Dean et al. 2001; Wilmut et al. 2002; Rhind et al. 2003b; Dindot et al. 2004). More recently, in addition to epigenetic errors, aberrant nuclear-mitochondrial interactions have also been implicated as a possible factor underlying LOS symptoms following SCNT (St John et al. 2004; Hiendleder et al. 2005; Lloyd et al. 2006).

4 Assessment of Effects of Transgenesis on Animal Welfare

Given the potential risks outlined above, it seems appropriate to devote a reasonable effort to the study of welfare of transgenic farm animals. In this respect, I strongly advocate an evidence-based approach ultimately aimed at the unequivocal identification of risk factors for animal welfare through scientifically valid and meaningful studies (e.g., Whay 2007). Such an approach may, for example, complement prospective and tightly controlled experiments that were suggested to disentangle the effects of embryo manipulation from specific effects of SCNT on genetic and physiologic mechanisms essential for (pre- and postnatal) survival (Wilmut 2002; Rhind et

al. 2003b). Basic assumptions are, first, that research efforts are adjusted to the objectives and stage of a transgenic program (i.e., a research program aimed at generating transgenic farm animals) and, second, that effects of transgenes should be distinguished or separated as much as possible from effects of in vitro technologies (e.g. in vitro embryo culture, SCNT).

4.1 Welfare Evaluation in Successive Stages of a Transgenic Program

Successive and partially overlapping stages in a transgenic program are listed in table 2, together with corresponding animal welfare research efforts. Each stage in this sequential process poses its own set of conditions and resources available for research into animal welfare and, correspondingly, its own scope of answers that can be provided. The main objectives of the first stage of a transgenic program are, first, to innovate and validate experimental procedures and, second, to demonstrate that the production of an improved or novel type of transgenic farm animal is technically feasible. At this stage, the number of transgenic animals is generally low, and the assessment of animal welfare risks should, therefore, be primarily based on descriptive and anecdotal data with regard to both animal health and abnormalities as well as various molecular biological determinants of transgenesis, including site of integration, integrity of the transgene, transgene expression (level, temporal pattern and tissue specificity) and characteristics of the transgene product (e.g., Kuipers et al. 1997; Brink et al.

Table 2: Risk assessment and welfare evaluation in successive stages of a transgenic program (modified from Van Reenen et al. 2001)

Stage		Objectives	Transgenic animals	Basis for risk assessment and welfare evaluation
1.	First-time generation of transgenic founders	Innovate experimental procedures and test feasibility	Low numbers of (unique) founders	Desk study Treatment with recombinant proteins Descriptive animal data and anecdotal information
2.	Increasing numbers of animals	Determination of transgene expression and phenotypic properties	Groups of clones or transgenic offspring	Quantitative research and accurate comparisons
3.	Establishment of production herds	Utilization of desired animals or harvesting desired (gene) product	Many animals	Epidemiological surveillance and meta-analyses

2000; Kues and Niemann 2004). For some transgenic approaches, the treatment of non-transgenic animals with the (homologous analogue of the) recombinant protein could be important for the early detection of possibly deleterious side-effects of the intended gene-product. For example, problems observed in transgenic pigs or fish expressing growth hormone transgenes could be induced in normal animals by the administration of exogenous growth hormones (Pinkert and Murray 1999; Hallerman et al. 2007). Finally, animal welfare risks could be initially assessed even in the absence of actual animals by performing a desk study where information of similar transgenic models in other animals is evaluated (e.g., Dahl et al. 2003).

In the second stage of a transgenic program a dependable protocol to generate transgenic animals has been established, and, thus, increasing numbers of transgenic animals may become available, both founders and transgenic offspring produced by mating founders with non-transgenic animals. This is the appropriate stage to commence comprehensive and quantitative research into the health and welfare of transgenic animals.

In the final stage of a transgenic program, production herds of transgenic farm animals would have been established, and the animals and/or their desired organs or (gene) products would be utilized. Until now, completion of this stage has rarely been achieved. Theoretically, large numbers of transgenic animals may become available, allowing for epidemiological surveillance and meta-analyses concerning the effect of transgenesis on farm animal welfare.

4.2 Breeding and Testing

For the efficient and unbiased estimation of the effects on animal welfare of those potential risk factors that are relevant in the context of farm animal transgensis, the following breeding and testing schemes might be appropriate.

4.2.1 Effect of Transgenesis

To reliably assess the effects of transgenesis, some essential characteristics of transgenic animals have to be taken into consideration (table 3). Random transgene insertion (with regard to both the number of integrated transgene copies and the site of integration), as occurs with microinjection and electroporation (or lentiviral transduction), has a most crucial methodological implication: each of the resulting transgenic founders (following microinjection) or group of identical founders (following electroporation/SCNT) is unique in terms of its genetic makeup and the expression pattern of the transgene and, consequently, in terms of the nature of any defects that may arise from transgenesis. Therefore, possible deleterious effects of transgenesis must be evaluated for each transgenic founder or group of identical founders separately, case by case. Gene targeting, on the other hand, is expected to result in similar transgene expression patterns among inde-

Table 3: Essential characteristics of transgenic founder animals when evaluating effect of transgenesis on animal welfare (modified from Van Reenen et al. 2001)

Technology	Transgene insertion	Phenotypic properties	Evaluation of welfare
Microinjection	Random	Unique in each founder	Case by case for each unique founder
CNT (Cell nuclear transfer)	Random	Each cycle of SCNT-mediated transgenesis creates unique group of founders	Case by case for each unique group of founders
Gene targeting	Specific site	Each cycle of gene targeting creates similar group of founders	In both sexes and in different genetic backgrounds

pendently generated transgenic founders and their offspring. Here, the welfare assessment in one group of founders may have predictive value for the welfare assessment in another group of founders carrying the same modification at the same site of the genome. However, in view of the likelihood of profound interactions between transgenic status, gender and genetic background or strain (e.g., Doetschmann 1999), effects of targeted transgenesis should be evaluated in both sexes and in different genotypes.

Steps in the breeding and testing of transgenic farm animals generated by microinjection or electroporation/SCNT are outlined in table 4. Using the same transgene (Tg), unique hemizygous founders (+/Tg_1 to +/Tg_k) or groups of founders (n_1+/Tg_1 to n_k+/Tg_k) are generated at generation 0 (G0). Each individual founder (+/Tg_i), or one of multiple founders from each SCNT-derived group of clones, is mated with non-transgenic (+/+) animals, to produce first-generation (G1) non-transgenic (+/+) and hemizygous (+/Tg_i) offspring, in equal proportion on the assumption of a Mendelian inheritance of the transgene. The comparison between hemizygous transgenic half-sibs, each carrying the same genetic modification on the same site of the genome, with their non-transgenic counterparts provides an unbiased estimate of the effect of the transgene. When a transgenic program would aim at the production of purebreeding homozygous transgenic stock, the detection of reduced viability or lethality among homozygous transgenic animals (e.g., because of a deleterious recessive insertional mutation) becomes pertinent. Homozygous transgenic animals (Tg/Tg) can be produced by intercrossing of hemizygous ones. The number of transgenic animals can be increased, and the level of inbreeding can be reduced before the intercrossing, by several generations of backcrossing of hemizygous

Table 4: Steps in the breeding and testing of transgenic farm animals produced by microinjection or electroporation/SCNT (modified from Van Reenen et al. 2001)

Step	Generation	Creation of founders and breeding[a]		Testing
		Microinjection	SCNT	
1	G0	+/Tg_1, +/Tg_2, ... , +/Tg_k Mating +/Tg_i x +/+	n_1+/Tg_1, n_2+/Tg_2, ... , n_k+/Tg_k Mating +/Tg_i x +/+	Identify characteristics in each unique individual founder +/Tg_i or group of founders n_i+/Tg_i
2	G1	50% +/Tg_i and 50% +/+ Mating +/Tg_i x +/+		Comparison between +/Tg_i and +/+
3	G2	50% +/Tg_i and 50% +/+ Interbreeding +/Tg_i x +/Tg_i		Comparison between +/Tg_i and +/+
4	G3	25% Tg_i/Tg_i, 50% +/Tg_i and 25% +/+		Comparisons between Tg_i/Tg_i and +/Tg_i, and Tg_i/Tg_i and +/+

[a] A single hemizygous transgenic founder animal produced by microinjection is indicated by +/Tg_i, and a single group of identical hemizygous transgenic founder animals, created in the same nuclear transfer trial, is indicated by n_i+/Tg_i, with n_i the number of individuals (clones). T_i represents the transgene inserted at a unique site in one of the host chromosomes. Non-transgenic animals are indicated by +/+. Mating the transgenic founder +/Tg_i with non-transgenic animals produced non-transgenic (+/+) and hemizygous (+/Tg_i) in equal proportions. Homozygous transgenic animals, harbouring the same transgene at the same site of both chromosomes within a pair, are indicated by Tg_i/Tg_i and are produced by intercrossing of hemizygous transgenic animals.

transgenic to non-transgenic animals (e.g. for two generations, see table 4). Comparisons between homozygous and hemizygous animals, and between homozygous and non-transgenic ones, provide the estimation of the homozygous transgene effects.

Steps in the breeding and testing of transgenic farm animals produced by gene targeting are given in table 5. Application of the same targeted event during independent nuclear transfer trials is assumed to result in the generation of multiple hemizygous male or female transgenic founders that either carry the same transgene at the same site of the genome (+/tm), or are deficient in one of two alleles of the same endogenous gene (+/-). In addition to those steps described for transgenic animals produced by microinjection or electroporation/SCNT (table 4), gene targeting offers the possibility to create lines of homozygous transgenic animals (tm/tm or -/-) at the earliest possible occasion while completely avoiding inbreeding by crossing independent male and female groups of identically tar-

Table 5: Steps in the breeding and testing of transgenic farm animals produced by microinjection or electroporation/SCNT (modified from Van Reenen et al. 2001)

Step	Generation	Creation of founders and breeding[a]		Testing
		Targeted insertion	Gene knockout	
1	G0	n_i+/tm ♀ and n_j+/tm ♂ Mating +/tm ♀ x +/tm ♂	n_k+/- ♀ and n_l+/- ♂ Mating +/- ♀ x +/- ♂	Identify characteristics in each group of identical male or female founders (clones) +/tm and +/-
2	G1	25% tm/tm, 50% +/tm and 25% +/+	25% -/-, 50% +/- and 25% +/+	Comparisons between tm/tm and +/tm, and tm/tm and +/+, or between -/- and +/-, and -/- and +/+

[a] Multiple (n_i, n_j, n_k, n_l) identical hemizygous transgenic male and female founder animals produced by gene targeting either carry the same transgene on the same site of the genome, denoted +/tm, or are deficient in one of the two alleles of the same endogenous gene, denoted +/-. Homozygous transgenic animals are indicated by tm/tm and -/-, in case of targeted transgene insertion and selective gene knockout, respectively.

geted hemizygous transgenic animals (see table 5, step 1). Comparisons at G1 between homozygous transgenic, hemizygous transgenic and non-transgenic animals provide an unbiased estimation of hemizygous and homozygous transgene effects.

4.2.2 Effects of In Vitro Reproductive Technologies

The effects on animal welfare of in vitro reproductive technologies that are employed in the process of generating transgenic farm animals (e.g., SCNT) are most efficiently studied in separate experiments, i.e. without the application of transgenesis. To investigate the effect of nuclear transfer on animal welfare, SCNT-derived clones should be compared with in vivo-produced controls. A limitation in this respect is the fact that in a group of identical clones, derived from the same nuclear donor cell line, the effect of SCNT is completely confounded with donor genotype. A comparison between non-cloned animals and any number of identical clones will, therefore, merely indicate the extent to which that particular cloned group differs from normal controls, but will not provide insight into the risk of SCNT as a treatment. Notably, the genetic background of nuclear donor cells may markedly affect the outcome of SCNT in cattle (Batchelder et al. 2005; Poehland et al. 2007). The true replicate (or experimental unit) in a population of cloned individuals is represented by the average performance of a group of clones with the same genotype. Thus, an unbiased

Table 6: Steps in the creation of groups of clones to study side-effects of SCNT (modified from Van Reenen et al. 2001)

Step	Reproductive technology	Result
1	Natural mating, AI or MOET	♀, ♂ Fetuses F_1, F_2, ..., F_k
2	Cell culture	♀, ♂ Nuclear donor cell lines L_1, L_2, ..., L_k
3	SCNT	♀, ♂ Groups of clones n_1C_1, n_2C_2, ..., n_kC_k

AI: Artificial Insemination; MOET: Multiple Ovulation and Embryo Transfer

estimate of the effect of SCNT can only be obtained when groups of clones are generated from many independent nuclear donor cell lines. Steps in the creation of groups of clones to study side-effects of SCNT are given in table 6. Multiple groups, say k groups, of male and female clones (n_1C_1 to n_kC_k, with n_1 to n_k the respective numbers of identical clones generated in each group) could be derived from multiple lines of nuclear donor cells L_1 to L_k, which were supplied by multiple fetuses F_1 to F_k. Natural mating or AI could be used to produce many half-sib or unrelated fetuses F_1 to F_k. The appropriate in vivo counterparts of groups of clones would be half-sibs of fetuses F_1 to F_k, or a random sample of in vivo controls from the same population as the parents that provided the fetuses F_1 to F_k. Multiple fetuses F_1 to F_k could also be obtained from a limited number of females or mating pairs, using multiple ovulation and embryo transfer (MOET). In groups of clones derived from fetuses produced by the same mating pair, individuals across groups are full-sibs. Here, testing multiple groups of clones against control animals produced by the same females or mating pairs that generated the fetuses F_1 to F_k provides an unbiased estimate of the effect of nuclear transfer. The generation of adequate numbers of in vivo control animals per female or per mating pair (i.e., full-sibs of groups of clones) will also require the use of MOET. Although MOET involves some embryo manipulation, it lacks in vitro fertilization and in vitro embryo culture and has been shown to produce calf and calving characteristics that are highly similar to those obtained with AI (Van Wagtendonk-de Leeuw et al. 2000). In order to examine the possibility that side-effects of SCNT may be transmitted from parent to offspring (e.g., Roemer et al., 1997), observations on the welfare of SCNT-derived farm animals could be extended to multiple generations.

Prior to larger-scale experiments as described above, controlled studies within the same (potential) nuclear donor cell line could be performed, looking at various individual experimental factors involved in the process of SCNT, including, for example, cell culture conditions or agents intended to facilitate fusion between nucleus and recipient cytoplasm and to promote appropriate oocyte activation (Wilmut 2002; Rhind et al. 2003b; Batchelder et al. 2005; Poehland et al. 2007).

4.2.3 Welfare Protocol

In order to be truly effective, research into the welfare of transgenic farm animals should not only be based on sound and efficient breeding and testing procedures, but should also make use of an appropriate protocol in terms of the measures that are recorded, the stages (pre- or postnatal) of the life of a farm animal observations take place, and the number of animals (independent replicates) involved. Relevant measures of animal welfare include clinical symptoms of health and disease, measures of growth and (re)production, measures of immunocompetence and measures of behavior (Broom 1993; Crawley 1999, 2008; Mertens and Rülicke 1999; Van der Meer et al. 1999). Generally speaking, a basic welfare protocol may encompass a cross-section of these latter types of measures, the scope of which could then be adjusted, either extended or reduced, according to the stage of a transgenic program, the treatment under observation, or specific characteristics of the genetic modification (e.g., expected site of expression of the transgene, properties of transgene-derived proteins, biological function of endogenous gene inactivated after knockout, etc.). Protocols used for the evaluation of in vitro reproductive technologies should involve observations appropriate for detecting LOS symptoms, such as specific measures of neonatal vitality and viability, or ultrasound measurements to investigate disproportionate organ development (e.g., Garry et al. 1996; Van Wagtendonk-de Leeuw et al. 2000; Batchelder et al. 2007a,b; Buczinski et al. 2007). A comprehensive and detailed examination of animal welfare aspects, including the application of certain breeding procedures (e.g., tables 4 and 5), should be made particularly in offspring of those transgenic founders, or in those groups of transgenic clones, that are intended to serve as foundation stock for transgenic herds. The stages of life of a transgenic farm animal at which to monitor welfare aspects should at least include gestation and birth, the developmental phase from birth to puberty, and a representative period of adult life, including the stage of (re)productive performance. Most importantly, observations on the welfare of transgenic farm animals should be carried out during the period(s) when expression of a transgene or the exertion of the desired effect of the genetic modification are expected to occur. As far as the number of animals (or replicates) required for a welfare study is concerned, it will be relatively easy to identify genetic modifications or in vitro embryo manipulations with extremely unfavourable effects. Large numbers of animals will be needed to detect smaller, but biologically relevant, harmful effects of transgenesis (see Smith et al. 1987, Gama et al. 1992, and Blasco 2008, for quantitative considerations on sample sizes in the context of farm animal transgenesis).

5 Recent Experiments on the Assessment of Effects of Transgenic Technologies

In table 7 and table 8, I have attempted to provide an overview of papers published so far dealing with the effects on measures related to animal welfare of transgenesis and SCNT, respectively. It may be particularly worthwhile to briefly consider the experimental approaches taken, since this may permit the suggestion of improvements.

Table 7: Recent studies examining effects and risks of transgenesis (ANOVA = analysis of variance)

Reference	Species	Technology used to create transgenic animals	Animals and/or groups compared	Measures recorded	Statistical analysis
Damak et al. 1996	Sheep	Micro-injection	Offspring produced by AI of a single male transgenic founder. Comparison of transgenic (n = 22) with non-transgenic half-sibs (n = 30).	Wool characteristics, growth	ANOVA Unit: animal Effects: transgenic status, gender
Hughes et al. 1996	Sheep	Micro-injection	Female offspring produced by AI of a single male transgenic founder. Comparison of transgenic (n = 25) with non-transgenic half-sibs (n = 25).	Behaviour	ANOVA Unit: animal Effect: transgenic status
Baldassarre et al. 2008	Goats	Micro-injection	Female offspring produced by AI of a single G1 male hemizygous transgenic animal derived from a female founder. Comparison of transgenic (n = 3–50) with non-transgenic half-sibs (n = 3–50). Numbers of animals depending on the measure examined	Milk production, milk composition, milk somato cell count, histopathology of mammary gland wall	ANOVA Unit: animal Effect: transgenic status
Behboodi et al. 2005	Goats	SCNT	2 clones derived from cell line A, and 2 clones derived from cell line B, compared with 4 matched controls	Birth and weaning weight, blood chemistry, estrus	Students t-tests Unit: animal Effect: transgenic status
Van Reenen and Blokhuis 1997	Cattle	Micro-injection	Offspring of single male transgenic founder produced by AI. Comparison of transgenic (n = 23) with non-transgenic half-sibs (n = 32)	Birth weight, health status, growth, behaviour, clinical biochemical analytes in blood, measures of reproduction and immunocompetence	ANOVA Unit: animal Effect: transgenic status, gender

Continued on next page

Reference	Species	Technology used to create transgenic animals	Animals and/or groups compared	Measures recorded	Statistical analysis
Tucker et al. 2002	Pigs	Micro-injection	Transgenic offspring from different lines	Hematology and serum biochemistry, body weight, weights and sizes of organs	Descriptive Comparison with reference values
Carter et al. 2002	Pigs	SCNT	2 cloned transgenic litters derived from cell line A, and 1 cloned transgenic litter derived from cell line B (total of 10 cloned pigs), compared with matched controls	Birth weight, growth, hematology, serum chemistry, serum IgM, IgG, blood cell counts and differentiation, mortality, necropsy data	Descriptive as well as ANOVA Unit: animal Effect: transgenic status
Deppenmeier et al. 2006	Pigs	Micro-injection	Comparison of hemizygous transgenic offspring from 9 different lines (n = 19, 1–4 animals per line) with matched non-transgenic controls (n = 9)	Health status, measures of pathomorphological analysis	Descriptive
Park et al. 2006	Pigs	Micro-injection	Offspring of single male transgenic founder. Comparison of transgenic pigs (n = 7) produced by interbreeding of G1 hemizygous transgenic animals with non-transgenic controls (n = 2)	Health status, white blood cell count, hemoglobin, hematocrit, platelet count	Descriptive
Chrenek et al. 2007	Rabbits	Micro-injection	Offspring of four male transgenic founders obtained by breeding with normal non-transgenic animals. Comparison of hemizygous transgenic rabbits (n = 10) with non-transgenic matched controls (n = 5)	Ejaculate characteristics, reproductive capabilities, histological structure of testis	ANOVA Unit: animal Effect: transgenic status

Table 8: Recent studies examining risks of in SCNT

Reference	Species	Animals and/or groups compared	Measures recorded	Statistical analysis
Archer et al. 2003	Pigs	2 cloned litters derived from the same cell line, compared with 2 matched control litters	Behaviour	ANOVA Unit: animal Effect: cloning
Batch-elder et al. 2007a,b	Cattle	3 clones derived from cell line A (Hereford donor), and 5 clones derived from cell line B (Holstein donor), compared with 3 Hereford and 3 Holstein calves obtained after embryo transfer	Carbohydrate parameters in blood, clinical chemical measures of kidney, liver and muscle function, hematology, electrolytes, minerals, blood gases	ANOVA Unit: animal Effects: cloning, breed
Chavatte-Palmer et al. 2002	Cattle	6 clones derived from cell line A, 1 clone derived from cell line B, 14 clones from cell line C, compared with various numbers of Holstein controls, depending on the measure examined (total number of controls: 196, 20 produced after IVP, 176 by AI)	Body temperamture, hematology, clinical biochemistry, IGF's	ANOVA Unit: animal Effect: cloning
Constant et al. 2006	Cattle	18 clones, derived from four cell lines, compared with 16 controls (6 produced after IVP, 10 by AI)	Measures of placental development	ANOVA Unit: animal Effect: cloning
Coulon et al. 2007	Cattle	1 clone derived from cell line A, 1 clone derived from cell line B, and 3 clones derived from cell line C, compared with 5 matched controls	Behaviour	Kruskal Wallis test, Mann Whitney test Unit: animal Effect: cloning
Enright et al. 2002	Cattle	4 clones derived from the same cell line, compared with matched controls produced by AI (n = 4)	Reproductive characteristics	ANOVA Unit: animal Effect: cloning
Estrada et al. 2007	Pigs	23 cloned litters (143 piglets) generated from 5 cell lines, compared with 112 matched control litters (1,300 piglets) produced by AI	Birth and weaning weight, mortality rate	ANOVA Unit: animal or litter Effect: Cloning
Govoni et al. 2002	Cattle	4 clones derived from the same cell line compared with 4 matched controls	Measures of somatotropic axis	ANOVA Unit: animal Effect: cloning
Heyman et al. 2004	Cattle	8 female clones derived from cell line A, 6 female clones derived from cell line B, and 9 female clones derived from cell line C, compared with 20 matched controls. Three male clones derived from the same cell line, compared with nuclear donor. Offspring of one male clone (63 inseminations) and of 10 female clones	Growth, milk production and milk quality (female clones), semen quality (male clones), gestation length and birth weight (offspring of clones)	ANOVA (data female clones) and descriptive (data male clones, offspring of clones) Unit: animal Effect: cloning
Heyman et al. 2007a	Cattle	17–21 clones derived from 4 cell lines, compared with 17–19 contemporary controls produced by AI	Health status, measures of milk and meat quality	ANOVA Unit: animal Effect: cloning

Continued on next page

Reference	Species	Animals and/or groups compared	Measures recorded	Statistical analysis
Heyman et al. 2007b	Cattle	37 clones derived from 5 cell lines, compared with 38 contemporary controls produced by AI	Wide range of measures of health status, hematology, blood biochemestry, physiological status, milk production and milk quality, muscle characteristics	ANOVA Unit: animal Effect: cloning
Kasai et al. 2007	Cattle	1 clone derived from cell line A (male), 1 clone derived from cell line B (female), compared with 2 matched controls	Clinical health, hematology, blood biochemistry	Descriptive
Laible et al. 2007	Cattle	3 clones derived from cell line A (Holstein donor), 3 clones derived from cell line B (Holstein donor), and 3 clones derived from cell line C (Jersey donor), compared with 3 Holstein and 2 Jersey controls	Milk production, milk composition	Descriptive
Lanza et al. 2001	Cattle	Clones (n = 24) derived from unknown number of cell lines (110 initiated pregnancies)	Pre- and postnatal mortality rates, health status, necropsy data, physical examination, body condition score, behaviour, reproductive performance, blood chemistry, hematology, immunological measures	Descriptive
Ortegon et al. 2007	Cattle	Offspring of single cloned bull, produced after AI (n = 30), compared with a group of normal matched controls	Clinical and growth parameters, hematology, reproductive parameters	ANOVA Unit: animal Effect: cloning
Pace et al. 2002	Cattle	Clones derived from 34 cell lines (535 initiated pregnancies)	Pre- and postnatal mortality rates, health status, necropsy data	Descriptive
Panarace et al. 2007	Cattle	Clones derived from a range of cell lines, produced in three countries (calves born: n = 388)	Production and reproduction features	Descriptive
Savage et al. 2003	Cattle	4 clones derives from the same cell line, compared with 4 matched controls	Behaviour	ANOVA Unit: animal Effect: cloning
Shibata et al. 2006	Pigs	1 clone derived from cell line A, 4 clones derived from cell line B, and 1 clone derived from cell line C, compared with 50 matched controls. Offspring of 6 female clones (3 derived from cell line A, 3 derived from cell line B) compared with matched controls	Growth, reproductive performance, meat quality	T-tests Unit: animal Effect: cloning

Continued on next page

Reference	Species	Animals and/or groups compared	Measures recorded	Statistical analysis
Shiga et al. 2005	Cattle	4 clones (bulls) derived from the same cell line, compared with contemporary controls. Semen from 1 cloned bull and from donor sire was used for AI	Birth weight, growth, reproductive performance	ANOVA, chisquare Unit: animal or ejaculate Effect: cloning or animal
Tecirlioglu et al. 2006	Cattle	2 male clones derived from cell line A, and 1 male clone derived from cell line B, compared with nuclear donors of cell lines A and B	Reproductive characteristics	ANOVA Unit: ejaculate or embryo produced Effect: anima
Tian et al. 2005	Cattle	4 female clones derived from the same cell line, compared with 4 matched controls. 2 male clones derived from the same cell line, compared with 8 matched controls	Milk production and milk quality (females), meat quality (males)	ANOVA and descriptive Unit: animal Effect: cloning
Walker et al. 2007	Pigs	Offspring of 4 male clones (n = 242), compared with offspring of normal boars (n = 162)	Meat composition	Descriptive
Williams et al. 2006	Pigs	3 male clones derived from cell line A, and 1 male clone derived from cell line B, compared with nuclear donors of cell lines A and B, and 2 half-sibs of the nuclear donor of cell line B	Reproductive performance	ANOVA Unit: ejaculate or gilts bred Effect: animal
Yonai et al. 2005	SCNT	6 female clones derived from cell line A (Holstein donor) and 4 female clones derived from cell line B (Jersey donor)	Growth, reproductive and lactation characteristics	Descriptive

5.1 Effect of Transgenesis

Studies focused on the effects of transgenesis on measures of animal welfare are listed species-wise in table 7. In addition to the animal species involved, there are columns indicating the technology used to create transgenic animals, the animals that were studied and/or the groups that were compared (design), the measures that were recorded, and the statistical analysis that was performed. Information about the statistical analysis includes: the main statistical test, i.e. analysis of variance (ANOVA) or Students t-test, the experimental unit (replicate) used by the authors, and the effects (i.e., fixed effects or experimental factors) that were considered.

Four out of the total of 10 studies I was able to identify two in sheep (Damak et al. 1996; Hughes et al. 1996), one in goats (Baldasarre et al. 2008) and one in cattle (Van Reenen and Blokhuis 1997), report on a comparison of half-sibs produced from a transgenic founder generated with microinjection. This would be in line with the approach suggested above (see table 4, steps 1–3). From a methodological point of view the design employed by Park et al. (2006) mainly falls short in the lack of proper controls in the form of half-sibs of the transgenic pigs (see table 7). Moreover, in the sample

of transgenic pigs produced by interbreeding of hemizygous transgenic animals, both hemizygous and homozygous transgenic pigs may exist (see table 4, steps 3 and 4). However, consistent with a confounded design and a limited number of (transgenic) animals, only descriptive statistics were applied. Similarly, the descriptive comparisons made by Tucker et al. (2002) and by Deppenmeier et al. (2006) seem appropriate in view of the fact that transgenic offspring from different transgenic lines generated by microinjection were pooled and compared with matched controls rather than the appropriate half-sibs. The pooling of such hetereogenous data (i.e., offspring of essentially unique transgenic founders) may, in fact, resemble a clear case of comparing "apples and pears". For this same reason, I suggest that the outcome of the ANOVA performed by Chrenek et al. (2007) is biased and not very informative (see table 7).

The effect of a transgene incorporated into the genome with the use of SCNT was examined by Behboodi et al. (2005) in goats, and by Carter et al. (2002) in pigs. In both studies, the effects of transgenic status, SCNT and genotype of the donor cell line were confounded, and the numbers of nuclear donors and cloned transgenics were low (table 7). Thus, the results of ANOVA performed here do not refer to the unbiased estimation of a treatment effect.

5.2 Effect of SCNT

Table 8 summarizes, in alphabetical order, based on the name of the first author, the contents of recent studies looking into possible side-efects of SCNT. The prevailing experimental design (employed in 22 out of the total of 25 studies I was able to identify) seems to consist of the comparison between relatively small numbers of clones derived from a limited number of nuclear donor cell lines (between 1 and 5) and contemporary matched controls. None of these studies took into account the effect of genotype of the nuclear donor, and this introduces a pertinent element of pseudoreplication into the experimental design. An even higher level of pseudoreplication is obtained when repeated measures within the same cloned animal, such as ejaculates, embryos produced or gilts bred (Shiga et al. 2005; Tecirlioglu et al. 2006; Williams et al. 2006), are used as the experimental unit (see table 8).

In a study aiming at the assessment of risks of SCNT for animal welfare, the average performance of a group of identical clones (i.e. with the same genotype) rather than the individual animal constitutes the independent experimental unit (see above). Thus, on a critical note, the failure to observe this latter principle would render an experiment invalid or incomplete (see, for example, Shutler et al., 2005, for a critical discussion of the experiment by Archer et al. 2003, looking at cloned litters derived from a single nuclear donor cell line, see table 8). Some authors recognize this methodological limitation, and explicitly mention that their findings should be interpreted

cautiously (e.g., Tecirlioglu et al. 2006; Heyman et al. 2004, 2007b). Correspondingly, other authors primarily resort to descriptive rather than analytical statistics, including the comparison of various measures as observed in (groups of) cloned individuals with published reference values obtained in normal subjects (Lanza et al. 2001; Pace et al. 2002; Heyman et al. 2004; Yonai et al. 2005; Kasai et al. 2007; Laible et al. 2007; Panarace et al. 2007; Walker et al. 2007). This is a useful first approach to characterize particular groups of clones as to whether they are within the normal range. Sufficient and convincing evidence on the risks of SCNT only comes from studies involving large numbers of clones derived from many different donor cell lines (e.g., Pace et al. 2002; Panarace et al. 2007).

6 Concluding Remarks

The current paper intended to provide a structured approach for the assessment of animal welfare risks of transgenesis in farm animals. This approach, however, does not necessarily apply to any project, or any stage of a project, concerned with the generation of transgenic farm animals. It particularly refers to those situations where explicit claims are made, or should be made, about the safety of the technology for the animals. From the review of existing studies it is suggested that, at present, there is a definite lack of reliable and sufficiently comprehensive data on the effects of transgenic status and of technologies used in the process of generating transgenic farm animals, such as SCNT, on the welfare of the animals involved. Much of the current knowledge about the animal welfare risks of transgenesis in farm animals seems to be based on inconclusive and small-scale experiments. This might sound a cautionary note for those engaged in risk assessment in the field of farm animal transgenesis. Interestingly, whereas some authors emphasize the preliminary nature of reviewed data on possible side-effects of cloning (e.g., Chavatte-Palmer et al. 2004), others are prepared to draw more far-reaching conlusions (e.g., Yang et al. 2007).

Reiterating our previous conclusion (Van Reenen et al. 2001), the systematic research into the welfare of farm animals involved in transgenesis may facilitate the identification of safe experimental protocols as well as the selection and propagation of healthy animals and, thereby, enable technological progress that could be ethically justified.

References

Archer GS, Friend TH, Piedrahita J, Nevill CH, Walker S (2003) Behavioral variation among cloned pigs. Appl Anim Behav Sci 82:151–161

Baldassarre H, Hockley DK, Doré M, Brochu E, Hakier B, Zhao X, Bordignon V (2008) Lactation performance of transgenic goats expressing recombinant human butyryl-cholinesterase in milk. Transgenic Res 17:73–84

Ballester M, Molist J, Lopez-Bejar M, Sánchez A, Santaló J, Folch JM, Ibáñez E (2004) Disruption of the mouse phospholipase C-beta1 gene in a beta-lactoglobulin transgenic line affects viability, growth, and fertility in mice. Gene 341:279–289

Batchelder CA, Hoffert KA, Bertolini M, Moyer AL, Mason JB, Petkov SG, Famula TR, Anderson GB (2005) Effect of the nuclear-donor cell lineage, type, and cell donor on development of somatic cell nuclear transfer embryos in cattle. Cloning Stem Cells 7:238–254

Batchelder CA, Bertolini M, Mason JB, Moyer AL, Hoffert KA, Petkov SG, Famula TR, Angelos J, George LW, Anderson GB (2007a) Perinatal physiology in cloned and normal calves: physical and clinical characteristics. Cloning Stem Cells 9:63–82

Batchelder CA, Bertolini M, Mason JB, Moyer AL, Hoffert KA, Petkov SG, Famula TR, Angelos J, George LW, Anderson GB (2007b) Perinatal physiology in cloned and normal calves: hemotologic and biochemical profiles. Cloning Stem Cells 9:83–96

Behboodi E, Ayres SL, Memili E, O'Coin M, Chen LH, Reggio BC, Landry AM, Gavin WG, Meade HM, Godke RA, Echelard Y (2005) Health and reproductive profiles of malaria antigen-producing transgenic goats derived by somatic ceel nuclear transfer. Cloning Stem Cells 7:107–118

Blasco A (2008) The role of genetic engineering in livestock production. Livest Sci 113:191–201

Brink MF, Bishop MD, Pieper FR (2000) Developing efficient strategies for the generation of transgenic cattle which produce biopharmaceuticals in milk. Theriogenology 53:139–148

Broom DM (1993) Assessing the welfare of modified or treated animals. Livest Prod Sci 36:39–54

Brophy B, Smolenski G, Wheeler T, Wells D, L'Huillier P, Laible G (2003) Cloned transgenic cattle produce milk with higher levels of β-casein and κ-casein. Nat Biotechnol 21:157–162

Buczinski SM, Fecteau G, Lefebvre RC, Smith LC (2007) Fetal well-being assessement in bovine near-term gestations: Current knowledge and future perspectives arising from comparative medicine. Can Vet J 48:178–183

Campbell KH (2007) Ten years of cloning: questions answered and personal reflections. Cloning Stem Cells 9:8–11

Carter DB, Lai L, Park KW, Samuel M, Lattimer JC, Jordan KR, Estes DM, Besch-Williford C, Prather RS (2002) Phenotyping of transgenic cloned piglets. Cloning Stem Cells 4:131–145

Carver A, Wright G, Cottom D, Cooper J, Dalrymple M, Temperley S, Udell M, Reeves D, Percy J, Scott A, Barrass D, Gibson Y, Jeffrey Y, Samuel C, Colman A, Garner I (1992) Expression of human alpha 1 antitrypsin in transgenic sheep. Cytotechnology 9:77–84

Chavatte-Palmer P, Heyman Y, Richard C, Monget P, LeBourhis D, Kann G, Chilliard Y, Vignon X, Renard JP (2002) Clinical, hormonal and hematologic characteristics of bovine calves derived from nuclei from somatic cells. Biol Reprod 66:1596–1603

Chavatte-Palmer P, Remy D, Cordonnier N, Richard C, Issenman H, Laigre P, Heyman Y, Mialot JP (2004) Health status of cloned cattle at different ages. Cloning Stem Cells 6:94–100

Chrenek P, Trandzik J, Massanyi P, Makarevich A, Lukac N, Peskovicova D, Paleyanda R (2007) Effect of transgenesis on reproductive traits of rabbit males. Anim Reprod Sci 99:127–134

Cibelli JB, Campbell KH, Seidel GE, West MD, Lanza RP (2002) The health profile of cloned animals. Nat Biotechnol 20:13–14

Constant F, Guillomot M, Heyman Y, Vignon X, Laigre P, Servely JL, Renard JP, Chavatte-Palmer P (2006) Large offspring or large placenta syndrome? Morphometric analysis of late genstation bovine placentomas from somatic nuclear transfer pregnancies complicated by hydroallantois. Biol Reprod 75:122–130

Coulon M, Baudoin C, Depaulis-Carre M, Heyman Y, Renard JP, Richard C, Deputte BL (2007) Dairy cattle exploratory and social behaviors: Is there an effect of cloning? Theriogenology 68:1097–1103

Crawley JN (1998) Behavioral phenotyping of transgenic and knockout mice: experimental design and evaluation of general health, sensory functions, motor abilities, and specific behavioral tests. Brain Res 835:18–26

Crawley JN (2008) Behavioral phenotyping strategies for mutant mice. Neuron 57:809–18

Dahl K, Sandøe P, Johnsen PF, Lassen J, Kornerup-Hansen A (2003) Outline of a risk assessment: the welfare of future xeno-donor pigs. Anim Welfare 12:219–237

Dai Y, Vaught TD, Boone J, Chen SH, Phelps CJ, Ball S, Monahan JA, Jobst PM, McCreath KJ, Lamborn AE, Cowell-Lucero JL, Wells KD, Colman A, Polejaeva IA, Ayares DL (2002) Targeted disruption of the alpha1,3-galactosyltransferase gene in cloned pigs. Nature Biotech 20:251–255

Damak S, Su H, Jay NP, Bullock DW (1996) Improved wool production in transgenic sheep expressing an insulin-like growth factor 1. Biotechnology (NY) 14:185–188

Dean W, Santos F, Stojkovic M, Zakhartchenko V, Walter J, Wolf E, Reik W (2001) Conservation of methylation reprogramming in mammalian development: aberrant reprogramming in cloned embryos. Proc Natl Acad Sci USA 98:13734–13738

Deppenmeier S, Bock O, Mengel M, Niemann H, Kues W, Lemme E, Wirth D, Wonigeit K, Kreipe H (2006) Health status of transgenic pigs expressing the human complement regulatory protein CD59. Xenotransplantation 13:345–356

Dieckhoff B, Petersen B, Kues WA, Kurth R, Niemann H, Denner J. (2008) Knockdown of porcine endogenous retrovirus (PERV) expression by PERV-specific shRNA in transgenic pigs. Xenotransplantation 15:36–45

Dindot SV, Farin PW, Farin CE, Romano J, Walker S, Long C, Piedrahita JA (2004) Epigenetic and genomic imprinting analysis in nuclear transfer derived Bos Gaurus/Bos Taurus hybrid fetuses. Biol Reprod 71:470–478

Doetschman T (1999) Interpretation of phenotype in genetically engineered mice. Lab Anim Sci 49:137–143

EFSA (2007) Draft scientific opinion on food safety, animal health and welfare and environmental impact of animals derived from cloning by somatic cell nucleus transfer (SCNT) and their offspring and products obtained from those animals. Draft Sientific Opinion of the Scientific Committee. EFSA-Q-2007-092, 1–47

Einsiedel EF (2005) Public perceptions of transgenic animals. Rev Sci Tech OIE 24:149–157

Enright BP, Taneja M, Schreiber D, Riesen J, Tian XC, Fortune JE, Yang X (2002) Reproductive characteristics of cloned heifers derived from adult somatic cells. Biol Reprod 66:291–296

Estrada J, Sommer J, Collins B, Mir B, Martin A, York A, Petters RM, Piedrahita JA (2007) Swine generated by somatic cell nuclear transfer have increased incidence of intrauterine growth restriction. Cloning Stem Cells 9:229–236

Farin PW, Piedrahita JA, Farin CE (2006) Errors in development of fetuses and placentas from in-vitro produced bovine embryos. Theriogenology 65:178–191

Fattori E, Della Rocca C, Costa P, Giorgio M, Dente B, Pozzi L, Ciliberto G (1994) Development of progressive kidney damage and myeloma kidney in interleukin-6 transgenic mice. Blood 83:2570–2579

FDA (2006) Animal cloning: a draft risk assessment. Center for Veterinary Medicine. US Food and Drug Administration. Department of Health and Human Services, USA, 1–358

FELASA Working Group: Rülicke T, Montagutelli X, Pintado B, Thon R, Hedrich (2007) FELASA guidelines for the production and nomenclature of transgenic rodents. Lab Anim 41:301–311

Gama LT, Smith C, Gibson JP (1992) Transgene effects, introgression and testing schemes in pigs. Anim Prod 54:427–440

Garry FB, Adams R, McCann JP, Odde KG (1996) Postnatal characteristics of calves produced by nuclear transfer cloning. Theriogenology 45:141–152

Gjerris, M, Sandøe P (2007) Ethical concerns related to cloning of animals for agricultural purposes. In: Zollitsch W, Winckler C, Waiblinger S, Haslberger A (eds) Proceedings Sustainable food production and ethics: Preprints of the 7th Congress of the European Society for Agriculture and Food Ethics. EurSAFE 2007, Vienna, Austria, 13–15 September 2007, pp 455–460

Govoni KE, Tian XC, Kazmer GW, Taneja M, Enright BP, Rivard AL, Yang X, Zinn SA (2002) Age-related changes of the somatotropic axis in cloned Holstein calves. Biol Reprod 66:1293–1298

Hallerman EM, MacLean E, Fleming I (2006) Effects of growth hormone transgenes on the behavior and welfare of aquacultured fishes: A review identifying research needs. Appl Anim Behav Sci 104:265–294

Heyman Y, Chavatte-Palmer P, Berthelot V, Fromentin G, Hocquette JF, Martignat L, Renard JP (2007a) Assessing the quality of products from cloned cattle: An integrative approach. Theriogenology 67:134–141

Heyman Y, Chavatte-Palmer P, Fromentin G, Berthelot V, Jurie C, Bas P, Dubarry M, Mialot JP, Remy D, Richard C, Martignat L, Vignon X, Renard P (2007b) Quality and safety of bovine clones and their products. Animal 1:963–972

Heyman Y, Richard C, Rodriguez-Martinez H, Lazzari G, Chavatte-Palmer P, Vignon X, Galli C (2004) Zootechnical performance of cloned cattle and offspring: preliminary results. Cloning Stem Cells 6:111–120

Hiendleder S, Zakhartchenko V, Wolf E (2005) Mitochondria and the success of somatic cell nuclear transfer cloning: from nuclear-mitochondrial interactions to mitochondrial complementation and mitochondrial DNA recombination. Reprod Fertil Dev 17:69–83

Hughes BO, Hughes GS, Waddington D, Appleby MC (1996) Behavioural comparison of transgenic and control sheep; movement order, behaviour on pasture and in covered pens. Anim Sci 63:91–101

Kasai K, Sano F, Miyashita N, Watanabe S, Nagai T (2007) Comparison of the growth performances of offspring produced by a pair of cloned cattle and their nuclear donor animals. J Reprod Dev 53:135–142

Kruip, TAM, den Daas JHG (1997) In vitro produced and cloned embryos: Effects on pregnancy, parturition and offspring. Theriogenology 53:611–618

Kues WA, Niemann H (2004) The contribution of farm animals to human health. Trends Biotechnol 22:286–294

Kuipers HW, Langford GA, White DJ (1997) Analysis of transgene integration sites in transgenic pigs by fluorescence in situ hybridization. Transgenic Res 6:253–259

Kuroiwa Y, Kasinathan P, Choi YJ, Naeem R, Tomizuka K, Sullivan EJ, Knott JG, Duteau A, Goldsby RA, Osborne BA, Ishida I, Robl JM (2002) Cloned transchromosomic calves producing human immunoglobulins. Nat Biotechnol 20:889–894

Kuroiwa Y, Kasinathan P, Matsushita H, Sathiyaselan J, Sullivan EJ, Kakitani M, Tomizuka K, Ishida I, Robl JM (2004) Sequential targeting of genes encoding immunoglobulin-μ and prion protein in cattle. Nat Genet 36:775–780

Laible G, Brophy B, Knighton D, Wells DN (2007) Compositional analysis of dairy products derived from clones and cloned transgenic cattle. Theriogenology 67:166–177

Lanza RP, Cibelli JB, Faber D, Sweeney RW, Henderson B, Nevala W, West MD, Wettstein PJ (2001) Cloned cattle can be healthy and normal. Science 294:1893–1894

Lassen J, Gjerris M, Sandøe P (2006) After Dolly: ethical limits to the use of biotechnology on farm animals. Theriogenology 65:992–1004

Lloyd RE, Lee JH, Alberio R, Bowles EJ, Ramalho-Santos J, Campbell KH, St John JC (2006) Aberrant nucleo-cytoplasmic cross-talk results in donor cell mtDNA persistence in cloned embryos. Genetics 172:2515–2527

Massoud M, Attal J, Thépot D, Pointu H, Stinnakre MG, Théron MC, Lopez C, Houdebine LM (2006) The deleterious effects of human erythropoietin gene driven by the rabbit whey acidic protein gene promoter in transgenic rabbits. Reprod Nutr Dev 36:555–563

McCreath KJ, Howcroft J, Campbell HS, Colman A, Schnieke AE, Kind AJ (2000) Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. Nature 405:1066–1069

McEvoy TG, Alink FM, Moreira VC, Watt RG, Powell KA (2006) Embryo technologies and animal health – consequences for the animal following ovum pick-up, in vitro embryo production and somatic cell nuclear transfer. Theriogenology 65:926–942

McFadden TB, Akers RM, Capuco AV (1988) Relationship of milk proteins in blood with somatic cell counts in milk of dairy cows. J Dairy Sci 71:826–834

Mench JA (1999) Ethics, animal welfare and transgenic farm animals. In: Murray JD, Anderson GB, Oberbauer AM, McGloughlin MM (eds) Transgenic Animals in Agriculture. CAB International, Wallingford, Oxon, UK, pp 251–268

Mertens C, Rülicke T (1999) Score sheets for the monitoring of transgenic mice. Anim Welfare 8:433–438

Mukai HY, Motohashi H, Ohneda O, Suzuki N, Nagano M, Yamamoto M (2006) Transgene insertion in proximity to the c-myb gene disrupts erythroid-megakaryocytic lineage bifurcation. Mol Cell Biol 26:7953–7965

Niemann H, Kues WA (2007) Transgenic farm animals: an update. Reprod Fertil Dev 19:762–770

Olson IAS, Sandøe P (2004) Ethical decisions concerning animal biotechnology: what is the role of animal welfare science? Anim Welfare 13:S139–S144

Ortegon H, Betts DH, Lin L, Coppola G, Perrault SD, Blondin P, King WA (2007) Genomic stability and physiological assessment of live offspring sired by a bull clone, Starbuck II. Theriogenology 67:116–126

Pace MM, Augenstein ML, Betthauser JM, Childs LA, Eilertsen KJ, Enos JM, Forsberg EJ, Golueke PJ, Graber DF, Kemper JC, Koppang RW, Lange G, Lesmeister TL, Mallon KS, Mell GD, Misica PM, Pfister-Genskow M, Strelchenko NS, Voelker GR, Watt SR, Bishop MD (2002) Ontogeny of cloned cattle to lactation. Biol Reprod 67:334–339

Panarace M, Agüero JI, Garrote M, Jauregui G, Segovia A, Cané L, Gutiérrez J, Marfil M, Rigali F, Pugliese M, Young S, Lagioia J, Garnil C, Forte Pontes JE, Ereno Junio JC, Mower S, Medina M (2007) How healthy are clones and their progeny? Theriogenology 67:142–151

Park JK, Lee YK, Lee P, Chung HJ, Kim S, Lee HG, Seo MK, Han JH, Park CG, Kim HT, Kim YK, Min KS, Kim JH, Lee HT, Chang WK (2006) Recombinant human erythropoietin produced in milk of transgenic pigs. J Biotechnol 122:362–371

Pinkert CA, Galbreath EJ, Yang CW, Striker LJ (1994) Liver, renal and subcutaneous histopathology in PEPCK-bGH transgenic pigs. Transgenic Res 3:401–405

Pinkert CA, Murray JD (1999) Transgenic farm animals. In: Murray JD, Anderson GB, Oberbauer AM, McGloughlin MM (eds) Transgenic Animals in Agriculture. CAB International, Wallingford, Oxon, UK, pp 1–18

Poehland R, Al-Rostum F, Becker F, Viergutz T, Brunner RM, Kanitz W, Bhojwani S (2007) Donor cell lines considerably affect the outcome of somatic nuclear transfer in the case of bovines. J Reprod Dev 53:737–748

Rhind SM, King TJ, Harkness LM, Bellamy C, Wallace W, DeSousa P, Wilmut I (2003a) Cloned lambs – lessons from pathology. Nat Biotechnol 21:744–745

Rhind SM, Taylor JE, De Sousa PA, King TJ, McGarry M, Wilmut I (2003b) Human cloning: can it be made safe? Nat Rev Genet 4:855–64

Robl JM, Wang Z, Kasinathan P, Kuroiwa Y (2007) Transgenic animal production and animal biotechnology. Theriogenology 67:127–133

Roemer I, Reik W, Dean W, Klose J (1997) Epigenetic inheritance in the mouse. Curr Biol 7:277–280

Rudenko L, Matheson JC, Sundlof SF (2007) Animal cloning and the FDA – the risk assessment paradigm under public scrutiny. Nat Biotechnol 25:39–43

Savage AF, Maull J, Tian XC, Taneja M, Katz L, Darre M, Yang X (2003) Behavioral observations of adolescent Holstein heifers cloned from adult somatic cells. Theriogenology 60:1097–1110

Shibata M, Otake M, Tsuchiya S, Chikyu M, Horiuchi A, Kawarasaki T (2006) Reproductive and growth performance in Jin Hua pigs cloned from somatic cell nuclei and the meat quality of their offspring. J Reprod Dev 52:583–590

Shiga K, Umeki H, Shimura H, Fujita T, Watanabe S, Nagai T (2005) Growth and fertility of bulls cloned from the somatic cells of an aged and infertile bull. Theriogenology 64:334–343

Shutler D, McLellan N, Weary DM (2005). The clones need to return. A comment on Archer et al. (2003). Appl Anim Behav Sci 91:363–365

Smith C, Meuwissen THE, Gibson JP (1987) On the use of transgenes in livestock improvement. Anim Breed Abstr 55:1–10

St John JC, Lloyd RE, Bowles EJ, Thomas EC, El Shourbagy S (2004) The consequences of nuclear transfer for mammalian foetal development and offspring survival. A mitochondrial DNA perspective. Reproduction 127:631–641

Stelwagen K, Farr VC, McFadden HA, Prosser CG, Davis SR (1997) Time course of milk accumulation-induced opening of mammary tight junctions, and blood clearance of milk components. Am J Physiol 273:R379–R386

Tecirlioglu RT, Cooney MA, Lewis IM, Korfiatis NA, Hodgson R, Ruddock NT, Vajta G, Downie S, Trounson AO, Holland MK, French AJ (2006) Semen and reproductive profiles of genetically identical cloned bulls. Theriogenology 65:1783–1799

Tian XC, Kubota C, Sakashita K, Izaike Y, Okano R, Tabara N, Curchoe C, Jacob L, Zhang Y, Smith S, Bormann C, Xu J, Sato M, Andrew S, Yang X (2005) Meat and milk composition of bovine clones. Proc Natl Acad Sci USA 102:6261–6266

Tucker A, Belcher C, Moloo B, Bell J, Mazzulli T, Humar A, Hughes A, McArdle P, Talbot A (2002) The production of transgenic pigs for potential use in clinical xenotransplantation: baseline clinical pathology and organ size studies. Xenotransplantation 9:203–208

Vajta G, Gjerris M (2006) Science and technology of farm animal cloning: State of the art. Anim Reprod Sci 92:211–230

Van der Meer M, Costa P, Baumans V, Olivier B, van Zutphen B (1999) Welfare assessment of transgenic animals: Behavioural responses and morphological development of newborn mice. Alt Lab Anim 27:857–868

Van Reenen CG, Blokhuis HJ (1997) Evaluation of welfare of transgenic animals: lessons from a case study in cattle. In: Nilsson A (ed) Proceedings Transgenic Animals and Food Production. Workshop, Stockholm, Sweden, 22–24 May 2007. Special Issue of the Royal Swedish Academy of Agriculture and Forestry (Kungl Skogs och Lantbruksakademiens) 136, pp 99–105

Van Reenen CG, Meuwissen THE, Hopster H, Oldenbroek K, Kruip ThAM, Blokhuis HJ (2001) Transgenesis may affect farm animal welfare: a case for systematic risk assessment. J Anim Sci 79:1763–1779

Van Wagtendonk-de Leeuw AM, Mullaart E, de Roos AP, Merton JS, den Daas JH, Kemp B, de Ruigh L (2000) Effects of different reproduction techniques: AI MOET or IVP, on health and welfare of bovine offspring. Theriogenology 53:575–597

Walker SC, Christenson RK, Ruiz RP, Reeves DE, Pratt SL, Arenivas F, Williams NE, Bruner BL, Polejaeva IA (2007) Comparison of meat composition from offspring of cloned and conventionally produced boars. Theriogenology 67:178–184

Wall RJ, Powell AM, Paape MJ, Kerr DE, Bannerman DD, Pursel VG, Wells KD, Talbot N, Hawk HW (2005) Genetically enhanced cows resist intramammary Staphylococcus aureus infection. Nat Biotechnol 23:445–451

Wells DN, Forsyth JT, McMillan V, Oback B (2004) The health of somatic cell cloned cattle and their offspring. Cloning Stem Cells 6:101–110

Wells KD, Wall RJ (1999) One gene is not enough: Transgene detection, expression and control. In: Murray JD, Anderson GB, Oberbauer AM, McGloughlin MM (eds) Transgenic Animals in Agriculture. CAB International, Wallingford, Oxon, UK, pp 67–78

Whay HR (2007) The journey to animal welfare improvement. Anim Welfare 16:117–122

Williams NE, Walker SC, Reeves DE, Sherrer E, Galvin JM, Polejaeva I, Rampacek G, Benyshek L, Christenson RK, Graves WM, Pratt S (2006) A comparison of reproductive characteristics of boars generated by somatic cell nuclear transfer to highly related conventionally produced boars. Cloning Stem Cells 8:130–139

Wilmut I (2002) Are there any normal cloned animals? Nat Med 8:251–216

Wilmut I, Beaujean N, de Sousa PA, Dinnyes A, King TJ, Paterson LA, Wells DN, Young LE (2002) Somatic cell nuclear transfer. Nature 429:583–585

Woychik RP, Alagramam K (1998) Insertional mutagenesis in transgenic mice generated by the pronuclear microinjection procedure. Int J Dev Biol 42:1009–17

Yang X, Tian XC, Kubota C, Page R, Xu J, Cibelli J, Seidel G Jr (2007) Risk assessment of meat and milk from cloned animals. Nat Biotechnol 25:77–82

Yonai M, Kaneyama K, Miyashita N, Kobayashi S, Goto Y, Bettpu T, Nagai T (2005) Growth, reproduction and lactation in somatic cell cloned cows with short telomeres. J Dairy Sci 88:4097–4110

Young LE, Fairburn HR (2000) Improving the safety of embryo technologies: possible role of genomic imprinting. Theriogenology 53:627–648

Young LE, Sinclair KD, Wilmut I (1998) Large offspring syndrome in cattle and sheep. Rev Reprod 3:155–163