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

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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,

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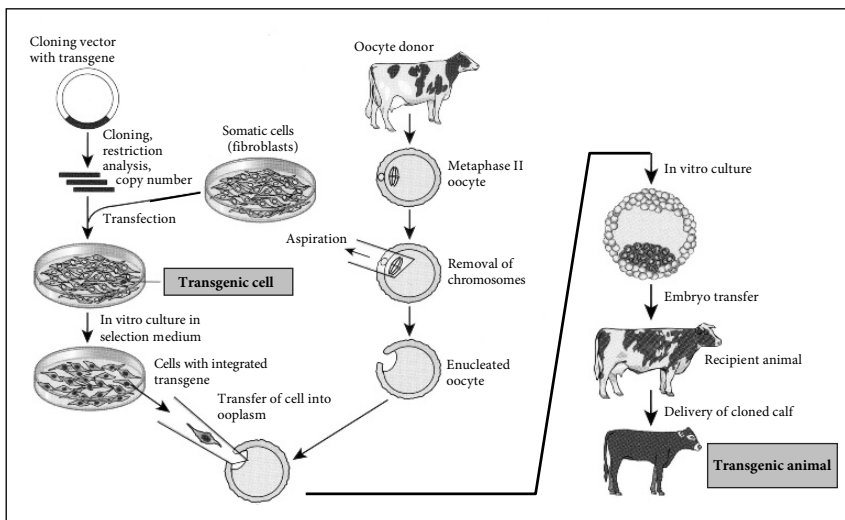
Figure 1: Scheme showing the production of transgenic farm animals by somatic cell nuclear transfer (SCNT)

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-lactalbumin	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	microinjection	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	microinjection	cattle	Platenburg 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.

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