

The possibilities of practical application of transgenic mammalian species generated by somatic cell cloning in pharmacology, veterinary medicine and xenotransplantation

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Abstract

Cloning of genetically-modified mammals to produce: 1) novel animal bioreactors expressing human genes in rens, urinary bladder and the male accessory sex glands, as well as 2) porcine organs suitable in pig-to-human xenotransplantation, could offer new advantages for biomedical purposes. So too does the generation and/or multiplication of genetically-engineered cloned animals in order to produce: 3) physiologically-relevant animal models of serious monogenic human diseases and 4) prion disease-resistant small as well as large animals (i.e., rodents, ruminants). The basic purpose of this paper is to overview current knowledge deciphering the possibilities of using transgenic specimens created by somatic cell nuclear transfer in medical pharmacology, veterinary medicine, agriculture, transplantational medicine and immunology.

Key words: somatic cell nuclear transfer, transgenesis, kidney, urinary bladder, male accessory sex gland, veterinary medicine, xenotransplantation

Introduction

Genomic embryo engineering (i.e., transgenesis linked to somatic cell cloning) in mammals is a particularly important research field of assisted reproductive technologies (ARTs) due not only to generation of animal bioreactors providing biopharmaceuticals, but also to the increasing role of porcine organs in xenotransplantation. Among the transgenic farm livestock species used so far, cattle, goats, sheep, pigs and rabbits are good candidates for the pharmaceutical industry; their urine and seminal plasma (besides

milk) can be alternative sources of recombinant human proteins at a high scale (Fig. 1). Despite significant improvement in somatic cell nuclear transfer (SCNT) technique in farm livestock species, high early-, mid- and late-gestation mortality rates for nuclear-transferred embryos/fetuses, as well as numerous malformations of resultant cloned offspring, still often appear (Samiec et al. 2003, Skrzyszowska et al. 2008).

Generally, the main cause of low somatic cell cloning efficiency in different mammalian species, with many severe developmental anomalies (anatomo-

-histological disorders in fetal and extrafetal/placental tissues, immune dysfunction) leading to high pregnancy loss and neonatal death, may be incomplete reprogramming of transcriptional activity for donor cell-descended genes. The process of epigenomically dependent reprogramming is related to the stable erasure ("vanishing") of donor cell nuclear DNA-epigenetic status and turning back ("molecular zeroing/nulling") the somatogenic "transcriptional and translational clock". This contributes to recapitulation of a particular program of the embryonic genome expression, which is induced by the re-establishment of the embryo cell genome-associated methylation pattern. SCNT-linked problems are hypothesized to result from aberrant gene dedifferentiation of somatic cell nuclei at the levels of epigenomic, genomic and molecular memory. The mechanism underlying the dedifferentiation process of donor nuclei is the cessation of their own gene expression and reversal of the differentiated (specialized) somatic nucleus to a totipotent/pluripotent embryonic (undifferentiated/unspecialized) state within the host ooplasm and cytoplasm of cleavage descendant blastomeres of nuclear-transferred (NT) embryos. In turn, impaired restoration (re-establishment) of the totipotency/pluripotency of embryonic cell lines in the first phase of epigenetic reprogramming (i.e., gene dedifferentiation) during preimplantation development, by the blastocyst stage may trigger disadvantageous alterations in the second phase of donor nuclear reprogramming. These are connected with improper redifferentiation of somatic cell-inherited genes throughout postimplantation fetal/placental development. Additional work is needed to determine whether failures in the early-stage reprogramming are magnified downstream in development (Kang et al. 2001, Mann et al. 2003, Samiec 2004, Shi et al. 2004).

Epigenetic reprogramming of donor cell nuclei suggests that a new program for their transcriptional activity is loaded and reloaded immediately following reconstruction of enucleated oocytes. The success of SCNT may depend upon both genomic DNA-associated reprogramming of gene expression for dedifferentiation of the donor somatic cell nuclei during early development of cloned embryos and reprogramming of gene expression for somatogenic nuclear redifferentiation during tissue genesis and organogenesis in the later development of cloned conceptuses. It has been ascertained that somatic cell nuclei should undergo the wide DNA cytosine residue demethylation changes throughout the early development of NT embryos to erase and then reset their own overall epigenetic as well as parental genomic imprinting memory, which has been established by re-methylation of the nuclear genome within the framework of the specific pathway of somatic and germ cell lineage commitment and differentiation (Mann et al. 2003, Santos

and Dean 2004, Samiec 2005). Understanding of the molecular mechanisms of epigenetic transcriptional reprogramming of the donor nuclear genome (Shi et al. 2004) will be helpful in solving the problems resulting from mammalian SCNT and may open up new possibilities for common application of this technology in human biomedicine. An important milestone of gene engineering and reproductive biotechnology in livestock was the commercialisation of the first human therapeutic protein produced in the milk of transgenic cloned goats on the pharmaceutical markets of both the European Union and USA, in 2006 and 2009, respectively (Fig. 1). This genetically-transformed protein is recombinant human antithrombin III (rhAT), which has been launched/certified as Atryn® (Echelard et al. 2006, Melo et al. 2007). Atryn® has been recently used in the prophylactic treatment of patients with congenital antithrombin deficiency.

Systems for efficient targeted genetic transformations (knock-out; *locus*-specific mutagenesis) by homologous recombination in somatic nuclear donor cells are being developed and the adaptation of sophisticated molecular genetics/epigenomics tools, already explored in mice, for transgenic cloned livestock production is underway. The availability of technologies involving gene targeting and nuclear/mitochondrial DNA analyses is essential to maintain genetic security and to ensure absence of unwanted side effects of transcriptional and translational activity for fusion genes in tissues and organs of transgenic animals created via SCNT. The safety of genetic engineering products can also be preserved and maintained by standardisation of procedures, and monitored by polymerase chain reaction (PCR) and microarray technology. As sequence information and genomic maps of farm animals are refined, it becomes increasingly practical to remove or modify individual genes. This approach to animal breeding will be instrumental in meeting global challenges in agricultural production in the future (Niemann and Kues 2007, Kind and Schnieke 2008).

Production of transgenic cloned livestock provides a method of rapidly introducing "new" genes into cattle, sheep, goats and pigs without crossbreeding. It is a more extreme methodology, but in essence not really different from crossbreeding or genetic selection in its result. There are many potential applications for technology involving transgenesis and SCNT to develop new and improved strains of livestock. Practical applications of transgenesis and somatic cell cloning in livestock production include enhanced prolificacy and reproductive performance, increased feed utilization and growth rate, improved carcass composition, improved milk production and/or composition and increased disease [e.g., transmissible spongiform encephalopathy (TSE) forms] resistance (Fig. 1). Additionally, frequent generation of transgenic

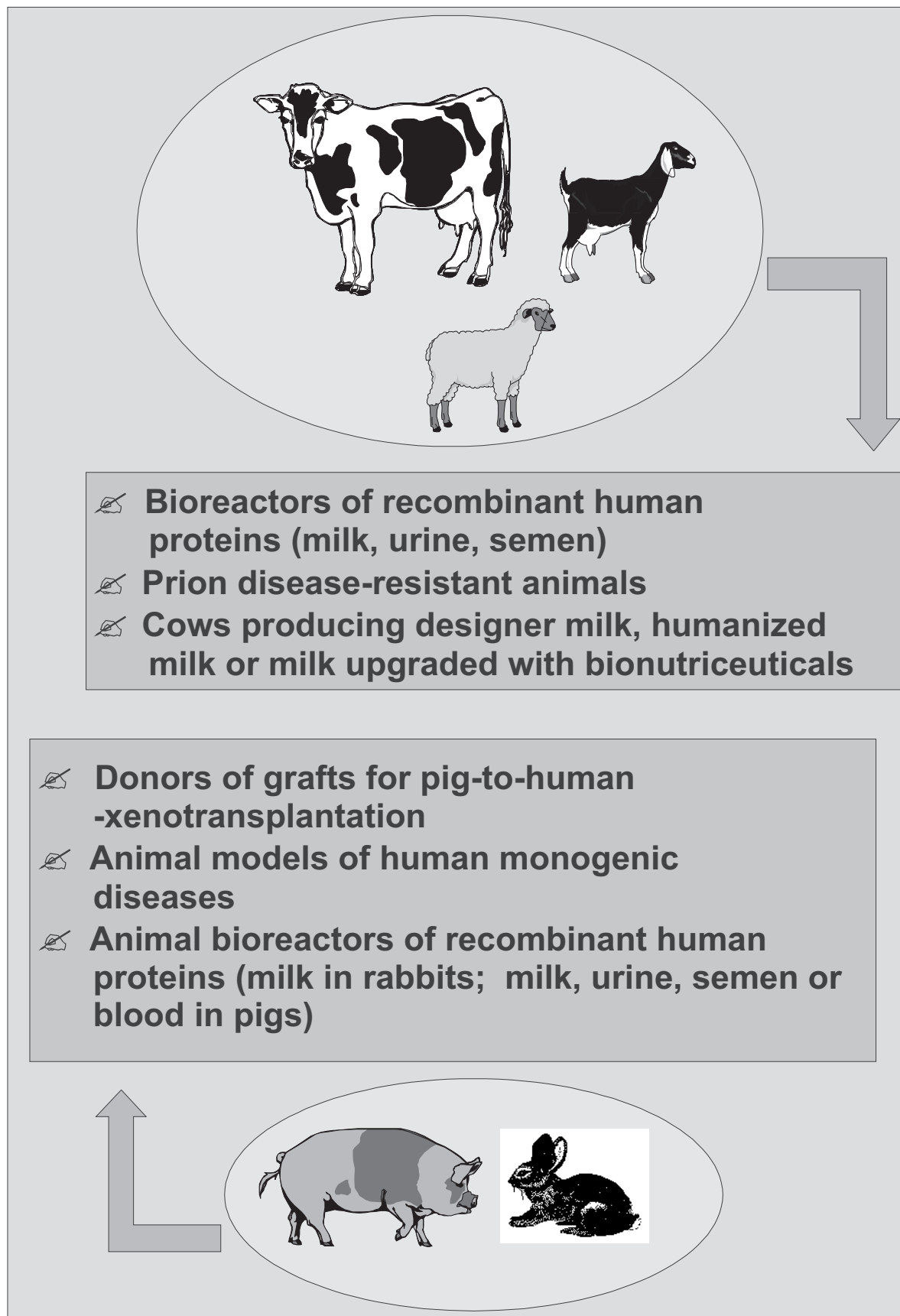


Fig. 1. Generation of different species transgenic cloned livestock for various biomedical, veterinary and agricultural purposes.

cloned farm animals will allow more flexibility in direct genetic manipulation of livestock (Wang and Zhou 2003, Melo et al. 2007, Kind and Schnieke 2008).

Genetically-engineered urinary bladder, kidneys and male accessory sex glands as animal bioreactors of recombinant human proteins

The production of genetically-transformed females of farm livestock species seems to be much more attractive than multiplication of such males, considering its higher productive potential of heterologous therapeutic proteins. However, the place of synthesis for xenogeneic biopharmaceuticals is not necessarily only the mammary gland. Kerr et al. (1998) and Zhu et al. (2003a,b) revealed that an alternative source of bioreactors can also be the urinary bladder or kidney *epithelium* (in the thick ascending limbs/TALs of Henle's nephronic loop or distal convoluted tubules) both of male and female individuals (Meade and Ziomek 1998) (Fig. 1). The authors obtained mice with genetically-modified *urothelium* cells or renal tubule epithelial cells expressing human growth hormone (*hGH*) gene under the promoters of murine uroplakin II or Tamm-Horsfall glycoprotein (*THP*/uromodulin) genes, respectively. The concentration of the recombinant human somatotropin in one milliliter of urine of such mice ranged from 100 ng up to 500 ng. The high expression profile of recombinant human growth hormone (rhGH) in the urine of genetically-modified mice was maintained at a relatively constant level for more than 8 months (Kerr et al. 1998, Zhu et al. 2003a).

Uroplakins (UPs) are a group of integral membrane proteins that have been identified as the major proteins of urothelial plaques. These plaques cover a large portion of the apical surface of mammalian *urothelium* and prevent the urothelial luminal surface from rupturing during bladder distention. Furthermore, it has been shown that mammalian urothelial plaques contain four major integral membrane proteins: UP-Ia (27 kDa), UP-Ib (28 kDa), UPII (15 kDa), and UPIII (47 kDa) (Zhang et al. 2002). Immunohistochemical survey of various tissues found UPs to be mammalian *urothelium* specific proteins that represent excellent markers for an advanced stage of urothelial differentiation (Lin et al. 1995, Zhu et al. 2004).

Uroplakin II (*UPII*) gene expression is highly tissue- and cell-specific, with mRNA present in the suprabasal cell layers of the bladder and urethra. Although many reports have described the mouse *UPII* (*mUPII*) promoter as primarily urothelium selective so far, ectopic (most often negligible) expression of

a transgene under the 3.6 kb *mUPII* promoter was also detected in the brain, kidneys and testis of some transgenic mouse lines and murine cell lines (Lin et al. 1995, Kerr et al. 1998, Zhang et al. 2002, Zhu et al. 2004, Kwon et al. 2006). Kwon et al. (2006) cloned an 8.8 kb pig *UPII* (*pUPII*) promoter region and investigated which cells within the bladder and urethra express a transgene consisting of the *pUPII* promoter fused to recombinant human erythropoietin (*rhEPO*) or a luciferase gene. The *pUPII*-luciferase expression vectors with various deletions of the promoter region were introduced into mouse fibroblast (NIH3T3), Chinese hamster ovary (CHO), and human bladder transitional cell carcinoma (TCC) RT4 cell lines. A 2.1 kb *pUPII* promoter fragment displayed high levels of luciferase activity in transiently transfected RT4 cells, whereas the 8.8 kb *pUPII* promoter region displayed only low levels of activity. The *pUPII*-*hEPO* expression vector was injected into the pronucleus of zygotes to produce genetically-transformed mice. To elucidate the *in vivo* molecular mechanisms controlling the tissue- and cell-specific expression of the *pUPII* promoter gene, transgenic mice containing 2.1 and 8.8 kb *pUPII* promoter fragments linked to the genomic *rhEPO* gene were generated. An erythropoietin (EPO) assay revealed that all nine transgenic mouse lines carrying the 8.8 kb construct expressed recombinant human erythropoietin (rhEPO) only in their urethra and bladder. It has also been shown that two transgenic lines carrying the 2.1 kb *pUPII* promoter displayed rhEPO expression in several organs including the bladder, kidneys, spleen, heart, and brain. These studies demonstrated that the 2.1 kb promoter contains the DNA elements necessary for high levels of expression, but lacks critical sequences necessary for tissue-specific expression. Moreover, Kwon et al. (2006) compared binding sites in the 2.1 and 8.8 kb promoter sequences and found five peroxisome proliferator responsive elements (PPREs) in the 8.8 kb promoter. Their data indicated that proliferator-activated receptor (PPAR)- γ activator treatment in RT4 cells induced the elevated expression of *rhEPO* mRNA under the control of the 8.8 kb *pUPII* promoter, but not the 2.1 kb promoter. Collectively, the results of experiments by Kwon et al. (2006) suggest that all the major trans-regulatory elements required for bladder- and urethra-specific transcription are located in the 8.8 kb upstream region. This may enhance tissue-specific protein production and be of interest to clinicians who are searching for therapeutic modalities with high efficacy and low toxicity.

Nonetheless, Lee et al. (2005) have generated male and female transgenic cloned piglets, in which the expression of fusion protein consisting of recombinant human erythropoietin (rhEPO) and eGFP-reporter protein was driven under the promoter (target-

ing vector) of porcine *UPII* gene into the urinary tract (bladder). This indicates that it is also possible to produce genetically-transformed individuals excreting biopharmaceuticals in the urine not only in laboratory animals such as mice, but also in large animals such as farm livestock species (Fig. 1).

Uromodulin, also known as Tamm-Horsfall protein (THP), is the most abundant protein in the urine of all placental mammals, with approximately 50-200 mg released per day. It is an 85- to 95-kDa glycosylphosphatidylinositol (GPI)-anchored glycoprotein secreted from the epithelial cells of the thick ascending limbs (TALs) of Henle's loop and the early distal convoluted tubule in the kidney (Zhu et al. 2002, 2003b). Physiological functions of uromodulin have remained elusive, but recent knock-out studies have suggested that it plays a role in defense against urinary tract infection. It may also have an immuno-suppressive role (Bates et al. 2004). The abundance of the uromodulin protein in urine makes the uromodulin promoter a good candidate for driving the production of recombinant human proteins in the rens and eventual excretion into the urine of transgenic animals (Zbikowska et al. 2002a). However, for such a system to be effective, expression levels should be in the range of 0.5-1 g/l to satisfy commercial applications. To date, hGH is targeted into mouse urine using mouse uroplakin II at approximately 0.5 µg/ml (Kerr et al. 1998). The same promoter has been used to target the human granulocyte macrophage-colony stimulating factor (hGM-CSF) to the kidneys of transgenic mice with a urine secretion level up to 180 ng/ml (Ryoo et al. 2001). Secretion of recombinant human α -1-antitrypsin in mouse urine at levels as high as 65 µg/ml has been achieved using the human uromodulin promoter (Zbikowska et al. 2002a,b).

In an effort to utilize the uromodulin promoter in order to target recombinant human proteins in the urine of transgenic animals Huang et al. (2005) have cloned a goat uromodulin gene promoter fragment (*GUM* promoter) and used it to drive expression of eGFP in the kidneys of genetically-engineered mice. The *GUM-eGFP* fusion gene was constructed and transgenic mice were generated in order to study the promoter's tissue specificity, the eGFP kidney specific expression and its subcellular distribution. Tissues collected from three *GUM-eGFP* transgenic mouse lines, and analysed for the presence of eGFP protein by Western blotting and fluorescence confirmed that the *GUM* promoter drove expression of eGFP specifically in the kidneys. More specifically, by using immuno-histochemistry analysis of kidney sections, it was revealed that eGFP expression was co-localized, with endogenous uromodulin protein, in the epithelial cells of the thick ascending limbs (TALs) of Henle's loop and the early distal convoluted tubules in the kidneys. In conclusion, Huang and co-workers (2005)

have shown that the goat uromodulin promoter is capable of driving recombinant biochemiluminescent protein expression in the kidneys of genetically modified mice. Furthermore, the goat uromodulin promoter fragment cloned may be a useful tool in targeting the transcriptional and translational activity of transgenes in the kidneys of other mammalian species.

Male accessory sex/genital glands of laboratory or farm animals such as the prostatic gland, vesicular glands (i.e., seminal vesicles), Cowper's bulbourethral glands and ampullae of the ductus/vasa deferentia (i.e., ampullae ductus deferentes) appear to be also sufficiently promising transgenic bioreactors (Fig. 1). Dyck et al. (1999) showed that the use of plasmid gene construct with the inserted cDNA of human growth hormone (*hGH*) under the promoter of murine *p12* gene can direct the synthesis of the human somatotropin at the vesicular glands and rens and its secretion into semen plasma. The *p12* gene encodes the 57 amino acid residue, androgen-dependent, secretory glycoprotein p12, which exhibits anti-trypsin activity. This protein is a seminal fluid or blood plasma serine protease inhibitor of molecular weight 12 kDa. Its expression is specific for the epithelial cells of the ventral prostate, seminal vesicles, coagulating gland and pancreas (Mills et al. 1987). In transgenic mice, the recombinant hGH was secreted into the ejaculated seminal fluids with the seminal vesicle lumen contents containing concentrations of up to 0.5 mg/ml (Dyck et al. 1999). As semen is a body fluid that can be collected easily on a continuous basis, the production of transgenic animals expressing pharmaceutical proteins into their seminal fluid could prove to be a viable alternative to use of the mammary gland as a bioreactor (Fig. 1).

The possibility of the excretion or secretion of xenogeneic proteins with the urine and secretory fluids of male accessory sex/genital glands may substantially reduce the costs of the production of different biopharmaceuticals. The recombinant proteins can be isolated not only from physiological excreta or secretions of genetically-transformed female specimens, but also from the urinary or reproductive tracts of males (Fig. 1). Their repeatable/reproducible extraction from these physiological fluids is already possible, before transgenic specimens have reached the stage of sexual, reproductive and somatic maturity and have undergone the first lactation. In other words, their recovery is sufficiently high even throughout the neonatal and prepubertal phase of ontogenetic development of such males and females. Additionally, the phenomenon of exogenous protein chelation, which is a physiological process of clustering the different micro- and macromolecules by casein and whey proteins in milk, does not occur in urine. For this reason, a further advantage of such production of therapeutic proteins may be the technical sim-

plification of the collection/recovery process of certain transgene expression products through both their more effective isolation/extraction and purification from urine (Lin et al. 1995, Meade and Ziomek 1998, Zbikowska et al. 2002a).

Perspectives of somatic cell cloning and transgenesis of farm ruminant species for veterinary medicine and agriculture

At present, the perspective of the propagation of transgenic mammalian species with knock-out alleles of gene encoding pathogenic (multimer) isoform of prion protein (PrP^{Sc}) is the most attractive aspect of combined technologies of targeted (*locus*-specific) mutagenesis and somatic cell cloning for animal breeding and veterinary medicine (Wrathall 2000, Denning et al. 2001a,b) (Fig. 1). The PrP^{Sc} protein is a glycoprotein anchored to the outer surface of the plasma membrane of neurons, lymphocytes and other cells. It is responsible for the induction/spread of such transmissible spongiform encephalopathies (TSEs) as *scrapie* in sheep and goats as well as bovine spongiform encephalopathy (BSE) in cattle. Initially, investigations on the application of genetic embryo engineering in the program of prion disease prophylaxis were focused on laboratory animals (mice). In this prophylactic program, misfolded prion protein diagnostic assays are based on rapid, high-throughput immunobiochemical and genetic detection techniques. These techniques utilize the epitope-specific antibody system in order to differentiate the infectious (multimer) PrP^{Sc} particle from the cellular (monomer) PrP^C form. It has been shown that homologous recombination-mediated inactivation involving both alleles of the *PrP^{Sc}* gene at *locus PRNP* does not cause any developmental disorders (lethal or sublethal) in genetically-transformed mice. Additionally, the *PrP^{Sc}* gene knock-out homozygous mice were resistant to *scrapie* following iatrogenic transmission of TSE infections (i.e., passive immunization by oral exposition to the prion agents). This resistance was also maintained following active immunization against misfolded prion protein antigens. In other words, it persisted, even after the *scrapie* pathogens had been transmitted/acquired either via intracerebral inoculation or via transfusion of blood derived from asymptomatic infected carriers (Crozet et al. 2004, Criado et al. 2005, Crecelius et al. 2008). In turn, the non-transgenic mice represented the control disease models for *scrapie* etiopathology (wild-type prion model mice). The experimental (clinical) induction of TSE distribution/tissue infectivity in these wild-type prion model mice has already led to the death of *scrapie*-afflicted specimens, after approximately 5 to 6 months. Previously, the first behavioural and immunobiochemical

symptoms of the disease had been detected by a highly-sensitive blood-based diagnostic system for PrP^{Sc} molecular screening (Weissmann and Flechsig 2003, Lobão-Soares et al. 2007, Powell et al. 2008).

Somatic cell nuclear transfer offers a new cell-based route for introducing precise genetic modifications in a range of animal species. However, significant challenges, such as establishment of somatic cell gene targeting techniques, must be overcome before the technology can be applied routinely. Denning et al. (2001a) achieved targeted deletion involving single alleles of both the α -1,3-galactosyltransferase gene and prion protein gene at the *GGTA1* and *PRNP* counterpart *loci* in primary fetal fibroblast cells from livestock (sheep and pigs). They place particular emphasis on the characteristics of the proliferative activity for the primary cell cultures of nuclear donor fibroblasts, because these are key to determining success in somatic cell cloning. Afterwards, using sheep, Denning et al. (2001b) performed reproducible and heterozygous targeted gene deficiency at two independent *loci* in fetal fibroblast cells. Vital regions were deleted from both the α -1,3-galactosyltransferase gene and the pathogenic prion protein (*PrP^{Sc}*) gene. The former gene may account for the hyperacute rejection of xenografted organs and the latter is directly associated with transmissible spongiform encephalopathies in humans and animals. SCNT-derived embryos were generated using cultures of targeted or non-targeted nuclear donor cells. Eight pregnancies were maintained to term and four *PRNP^{+/-}* gene heterozygous (i.e., PrP^{Sc}-semideficient) cloned lambs were born. Although three of these perished soon after birth, one survived for 12 days. The results of the study by Denning et al. (2001b) confirmed that lambs carrying targeted *PRNP* gene semideficiencies can be generated by somatic cell nuclear transfer (Fig. 1).

Although mice lacking PrP^{Sc} protein develop and reproduce normally and are resistant to *scrapie* infection, large animals deprived of PrP^{Sc} particles, especially those species in which TSE occurs naturally, are currently not available. Yu et al. (2006) first generated five live *PRNP^{+/-}* gene heterozygous cloned goats with the use of fetal fibroblast cells undergoing targeted mutagenesis (Fig. 1). Detailed PrP^{Sc} RNA transcription and protein expression analysis of one *PRNP^{+/-}* gene heterozygous kid revealed that one allele of the caprine *PRNP* gene had been disrupted functionally. No gross abnormal development or behaviour could be observed in these PrP^{Sc}-semideficient goats up to at least 3 months of age.

While homozygous mice devoid of the functional *PRNP* gene are resistant to *scrapie* and prion propagation, heterozygous mice for *PRNP* gene disruption still suffer from prion disease and prion deposition. As already mentioned above, Yu and co-workers

(2006) have generated transgenic heterozygous cloned goats with functional disruption of one allele within the *PRNP* locus. To obtain goats with both inactivated alleles of the gene encoding PrP^{Sc} protein, which would be completely resistant to *scrapie*, a second-round gene targeting was applied to disrupt the wild-type (WT) allele of *PRNP* locus in heterozygous *PrP^{Sc}* knock-out cloned goats (Zhu et al. 2009). By second-round gene targeting, the WT allele of the *PRNP* gene has been successfully disrupted in primary cultures of *PRNP*^{+/-} gene heterozygous (i.e., PrP^{Sc}-semideficient) caprine adult dermal fibroblast cells. As a result, a *PRNP*^{-/-} gene homozygous cell line that was deprived of PrP^{Sc} expression (i.e., with total deficiency of PrP^{Sc}) was generated. This was the first report on successful targeting modification in primary adult somatic cells of animals. These skin-derived fibroblasts were used as nuclear donor cells for somatic cell cloning to create *PRNP*^{-/-} homozygous domestic goat kids. A total of 57 morulae or blastocysts which had developed from caprine nuclear-transferred embryos were transferred to 31 recipient does. Among these, 7 surrogate females became pregnant, as was determined by ultrasonographic diagnostics at day 35 after embryo transfer. Only one cloned fetus with *PRNP*^{-/-} genotype was able to develop to day 73 of gestation, but failed to reach term. On the one hand, the research by Zhu et al. (2009) indicated that multiple genetic modifications could be accomplished by multi-round gene targeting in primary caprine dermal fibroblast cells. On the other hand, their research provided strong evidence that *PrP^{Sc}* gene knock-out in not only fetal cells, but also adult cells, could be applied to introduce precise genetic modifications in animals without destroying the conceptuses.

Given the difficulty of applying gene knock-out technology to species other than mice, Golding et al. (2006) decided to explore the utility of RNA interference (RNAi) in silencing the expression of genes in livestock. Short hairpin RNAs (shRNAs) were designed and screened for their ability to suppress the expression of caprine and bovine prion protein (PrP^{Sc}). Lentiviral vectors were used to deliver a gene construct expressing *Aequorea victoria* bioluminescent jellyfish-descended enhanced green fluorescent protein (eGFP) and a shRNA targeting PrP^{Sc} into caprine adult dermal fibroblast cells. These cells were then used as a source of donor nuclei for somatic cell cloning procedure to produce a transgenic goat fetus. This fetus was surgically recovered at day 81 of gestation and compared with an age-matched control counterpart derived by natural mating. All tissues examined in the genetically-transformed cloned fetus expressed eGFP protein, and polymerase chain reaction (PCR) analysis confirmed the presence of the transgene encoding the *PrP^{Sc}* shRNA. Most relevant, Western blot analysis performed on brain tissues com-

paring the transgenic SCNT-derived fetus with control counterparts demonstrated a significant (>90%) decrease in PrP^{Sc} expression levels. To confirm that similar methodologies could be applied to cattle, recombinant virus particles were injected into the perivitelline space of bovine metaphase II-staged oocytes. After *in vitro* fertilization and culture, 76% of the genetically-modified blastocysts exhibited eGFP expression. This indicated that the embryos expressed shRNAs targeting PrP^{Sc}. The results of the experiments carried out by Golding et al. (2006) provide strong evidence that this approach will be useful in producing transgenic livestock conferring potential prion disease (TSE) resistance. It can be also an effective strategy for suppressing *PrP^{Sc}* gene expression in a variety of large-animal models.

The production of genetically-modified SCNT-derived small and large ruminants which would be resistant to *scrapie* or bovine spongiform encephalopathy as a result of targeted insertive inactivation or RNAi-mediated silencing of *PRNP* locus has very important implications for veterinary medicine and animal reproduction biotechnology (Fig. 1). TSE infections are not transmitted by embryos (with zona pellucida, blastocel cavity or blastomeres), even if they have been transferred into the reproductive tracts of recipient females, in which prion diseases are later diagnosed. Ovine, caprine or bovine embryos with *PrP^{Sc}* gene knock-out or transcriptional repression via RNA interference can be transferred successfully to TSE-afflicted or prion early-infected recipient surrogates (at the incubation/hatching stage of the disease). These embryos have been previously recovered/flushed off from the oviducts or uteri of hormonally-stimulated transgenic founder donor females (depleted of infectious prion particles). In this case, there is no risk of transmission of pathogenic prion protein isoforms to other species originating from herds of breeding stock which are still TSE disease-free (Wrathall 2000, Denning et al. 2001b).

Perspectives of somatic cell cloning and transgenesis of pigs for xenotransplantation

Attempts at transplanting vascularized pig organs always result in their hyperacute vascular rejection (HAR) (Sharma et al. 2003). It seems that only organs of transgenic pigs, which would have the expression of genes "humanizing" their cells, would constitute desirable material for xenogeneic transplants. A major hindrance to the common use of pig organs in medicinal transplantology is immunological incompatibility, above all by way of species specific major histocompatibility complexes (MHC): human (HLA; human leukocyte antigens) and porcine (SLA; swine

leukocyte antigens). However, a deficit of organs for human allotransplantation has become a stimulus for a search for new, alternative sources of grafts. It has been postulated for a long time that genetically-modified pigs can provide (considering the high fertility and prolificacy of this species) a virtually unlimited source of xenograft donors. Pig-to-human-xenotransplantation is also an attractive option because of the compatible size, physiology and anatomy of porcine organs (Joziase and Oriol 1999). The use of targeted exogenous DNA construct introduction (*locus*-specific genetic modification) by selected transfection methods of *in vitro* cultured populations (clonal lines) of porcine somatic cells, and then fusion (or intraooplasmic microinjection) of such prepared donor nuclei with *in vitro*- or *in vivo*-matured recipient oocytes could lead to numerous pig clones. These could produce, for example, human MHC antigens (HLA), various immunosuppressive proteins, or inhibitors of cytotoxicity of the complement system (such as human C3/C5 convertase decay-accelerating factor; CD55/DAF), or “masked” epitopes by competitive inhibition of α -1,3-galactosyltransferase (α -1,3-GT) by H-transferase (α -1,2-fucosyltransferase; α -1,2-FT) (Galili 2001, Thomson et al. 2003). This could also contribute to pig clones with different genes inactivated using the “knock-out” technique (homologous recombination). The latter strategy of targeted mutagenesis involves the genes encoding enzymes, which catalyze the addition of different antigenic determinant fragments (xenogeneic oligosaccharide residues) to the plasma membrane glycoproteins and glycolipids (for example α -1,3-GT allele, *locus* *GGTA1*). The above-mentioned immunoproteins or their complexes with cell-surface plasmolemmal lipids, i.e., glycerophospholipids/phosphoglycerides, are responsible for hyperacute vascular rejection (HAR) of xenografts (xenogeneic transplants). In turn, the HAR results from their immunoreaction with human preformed xenoreactive antibodies (mainly anti-Gal antibodies, directed against galactosyl- α -1,3-galactose/ α -Gal epitopes on the surface of porcine endothelial graft cells) (Joziase and Oriol 1999, Galili 2001, Thomson et al. 2003). Transgenesis (with application of gene targeting), in conjunction with somatic cell cloning, could provide, in the near future, a basis for the generation and multiplication of a pig population with such a genetically transformed (“humanized”) immunological system and blocked expression of many epitopes. This population would be a source of xenograft donors with a substantially extended survival rate (increased resistance to HAR) in primate recipients. The birth of the first α -1,2-FT transgenic nuclear-transferred piglets with a “partially-humanized” immunological system (Bondioli et al. 2001) has indicated the approaching phase of

intensive preclinical and clinical tests on the transplantation of genetically-transformed porcine organs into non-human primates and eventually human recipients (Fig. 1).

The cloning of all livestock species (Schnieke et al. 1997, Keefer et al. 2001, Chen et al. 2002, Skrzyszowska et al. 2006) by somatic cell nuclear transfer provides an alternative means of targeted disruption or deletion of genes in mammals. The obtaining of two cloned lambs (Cupid ram and Diana ewe) with insertions of the *AATC2* gene construct inserted into the ovine α 1(I)-procollagen (*COL1A1*) *locus* showed that viable animals of both sexes in *Ovis aries* species can be produced by nuclear transfer with cultured gene-targeted fetal fibroblast cells. The *AATC2* fusion gene comprised the genomic (exon) sequence of human α -1-antitrypsin (*hAAT*) gene under the ovine β -lactoglobulin (*BLG*) promoter (McCreath et al. 2000). The single alleles of the α -1,3-GT gene and the pathological prion protein (*PrP^{Sc}*) gene have been successfully deleted at the *GGTA1* and *PRNP* counterpart *loci* in ovine nuclear donor fetal fibroblasts. *Alpha*-1,3-GT and *PrP^{Sc}* gene semideficiencies were also achieved in cloned conceptuses and lambs derived from these donor cell nuclei that had been genetically modified by homologous recombination (Denning et al. 2001b). Although all the offspring perished either immediately or up to 12 days after birth, these experiments indicated that disruption of the α -1,3-GT gene using somatic cell nuclear transplantation techniques is feasible in farm animals. Dai et al. (2002) and Lai et al. (2002) have conducted the targeted mutagenesis of one allele of the *GGTA1* *locus* in porcine fetal fibroblasts and produced a total of nine live female α -1,3-GT knock-out piglets by nuclear transfer of these cells (Fig. 1). Ramsoondar et al. (2003) have created a total of six genetically engineered male piglets that possess an α -1,3-GT knock-out allele, but four of them express additionally a randomly inserted human H-transferase (α -1,2-FT) transgene (Fig. 1). This was the first study to use Southern blot analysis to demonstrate the disruption of the α -1,3-GT gene in somatic α -1,2-FT-transgenic pig cells (fetal fibroblasts) before they were used for nuclear transfer (Ramsoondar et al. 2003). The generation of homozygous α -1,3-GT knock-out boars with the α -1,2-FT-transgenic background is underway and will be unique (Phelps et al. 2003, Ramsoondar et al. 2003). This approach intends to combine the α -1,3-GT knock-out genotype with a ubiquitously expressed α -1,2-fucosyltransferase transgene producing the universally tolerated H antigen on the cell membrane surface. Such genetic modification of cloned pigs may prove to be more effective than the α -1,3-GT null phenotype alone in overcoming hyperacute, acute or delayed xenograft rejection, since the α -Gal epitopes can be downregulated by

competitive inhibition between α -1,3-GT and α -1,2-FT for the common acceptor substrate N-acetyl lactosamine (Joziase and Oriol 1999, Ramsoondar et al. 2003, Sharma et al. 2003, Thomson et al. 2003). In the studies by Phelps et al. (2003), the second allele of the α -1,3-GT gene has also been inactivated via selection of a T-to-G single point mutation at the second base of exon 9, which resulted in knock-out of the gene. Four healthy gilts with double knock-out of the α -1,3-GT gene (one allele of *GGTA1* locus by targeted disruption and the second by selection procedure of point mutation) were obtained by nuclear transfer (Fig. 1). In contrast to α -1,3-GT-semideficient cloned pigs, complete removal of galactosyl- α -1,3-galactose epitopes in the organs of α -1,3-GT-deficient cloned piglets may be the critical step toward the success of xenotransplantation (Phelps et al. 2003).

Kolber-Simonds et al. (2004) produced healthy, totally α -1,3-GT-deficient, inbred miniature piglets with two distinct genotypes for loss of heterozygosity (LOH) in the *GGTA1* locus, using serial nuclear transfer with fibroblast cells (Fig. 1). These cells had been pre-selected for repression of transcriptional activity of the second allele of the α -1,3-GT gene from gene-targeted heterozygous cell populations. The stringent positive selection for complete disappearance of functional semideficiency of the alleles in *GGTA1* locus (i.e., disappearance/diminution of the heterozygosity) permitted efficient establishment of clonal cell lines with double α -1,3-GT knock-out from heterozygous fetal fibroblast cell cultures. This also enabled them to be sufficiently enriched from heterozygous neonatal fibroblast cell cultures for use directly in another round of SCNT procedure. Starting with *GGTA1* heterozygous fibroblasts, α -1,3-GT phenotype *null* cells with spontaneous "vanishing" of the wild-type (WT) allele were thereby isolated. The frequency of LOH-mediated gene or chromosome mutations was several orders of magnitude greater than typically expected. This phenomenon might be related to the inbred background of the *GGTA1* heterozygous specimens. The total disappearance or deficiency of heterozygosity resulted in some cases either from depletion of the WT allele or in others from chromosome structural aberrations involving somatic crossing over and gene conversion. These latter ones were compatible with chromosome recombination, chromosome loss/diminution and reduplication or interstitial deletion of chromosomal fragments. The generation of cloned gilts and boars with both hemizygous and homozygous *GGTA1*-deficient cells demonstrates that such somatic LOH mutations inducing lack/deprivation of xenogeneic α -Gal epitopes can be introduced into porcine genomes by SCNT technology (Fig. 1). Such a strategy can overcome both hyperacute rejection and subsequent acute or

chronic tissue damage of porcine grafts in preclinical tests of pig-to-primate or human xenotransplantation. The former and latter processes are associated with the binding of preformed anti-Gal antibodies to the galactosyl- α -1,3-galactose residues on the surface of endothelial cells (Phelps et al. 2003, Kolber-Simonds et al. 2004).

Somatic cell nuclear transfer in pigs seems to be the most promising technology to achieve the targeted disruption of the α -1,3-GT gene, in that donor cells can be genetically transformed before nuclear transplantation using existing technologies. The major limitation to the genetic modification of donor cell nuclei is the length of time, for which transfected cells have to be cultured *in vitro* to allow selection, clonal line growth, and genetic testing preceding nuclear transfer. Undoubtedly the ability of these cells to undergo a second cycle of gene targeting to remove a second allele could be reduced. However, genetically manipulated donor cells can be applied to produce a cloned fetus or adult animal, thus providing cells which can be used for additional rounds of targeted mutagenesis (knock-out). This gene targeting can lead either to deletion/diminution and disruption of a second allele or to insertion of additional genes encoding the immunoproteins responsible for hyperacute or acute rejection of xenogeneic grafts (Thomson et al. 2003, Wang and Zhou 2003) (Fig. 1). The prospect of transgenesis and somatic cell nuclear transfer combination also opens up new possibilities for production of pig bioreactors synthesizing and supplying, in blood and natural (physiological) body excreta and secretions (urine, semen, milk), human proteins and hormones (Fig. 1). These products include different biopharmaceuticals/therapeutic proteins such as: 1) human haemoglobin in porcine erythrocytes (Swanson et al. 1992, Sharma et al. 1994) and 2) human blood coagulation/clotting factor VIII in milk (Paleyanda et al. 1997). They also involve: 3) human blood clotting factor IX and additional recombinant isoforms of porcine lactoferrin simultaneously secreted to milk (Lee et al. 2003) as well as 4) human erythropoietin in urine (Lee et al. 2005).

Conclusions

Several transgenic cloned animal species can produce recombinant human proteins but at present three systems are in the process of implementation. The first is milk from farm transgenic mammals, which has been studied for two decades. This system allowed the ATryn[®] biopharmaceutical, i.e., recombinant human antithrombin III (rhAT), to receive the permission from both the EMEA (European Agency for the Evaluation of Medicinal Products) and the FDA (Food and Drug Administration of the United

States Department of Health and Human Services) to be marketed in the EU and USA in 2006 and 2009, respectively. The second and the third systems are urine and seminal plasma, which have recently become more attractive after essential improvement of the methods used to generate transgenic livestock (Melo et al. 2007, Niemann and Kues 2007). Nonetheless, using the new bioreactors for the expression of human genes either in the kidneys, urinary bladder or in the male accessory sex glands of transgenic mammals appears to be the most cost-effective way for the processing of valuable biopharmaceuticals. Furthermore, lentiviral vectors and small interfering ribonucleic acid (siRNA) technology are becoming important tools for genomic embryo engineering. These tools are thought to be especially suitable in biotechnological programs for efficient generating/multiplying of the genetically-transformed cloned animals which synthesize recombinant human proteins in standard or alternative bioreactors. The standard bioreactor is the mammary gland and the alternative bioreactors involve the reins, urinary bladder, prostate, seminal vesicles, Cowper's bulbourethral glands and the ampullae of the ductus/vasa deferentia (Kind and Schnieke 2008).

Transgenic cloned pigs, which are *GGTA1* locus single or double knock-out or express recombinant human enzymatic immunoproteins (among others, complement system-regulating proteins, α -1,2-FT) have been tested for their ability to serve as organ/graft donors in human transplantation medicine (i.e., swine-to-human xenotransplantation). *In vitro* and *in vivo* data convincingly indicate that the hyperacute rejection response can be overcome in a clinically acceptable manner by successfully employing this strategy. It may be anticipated that within a few years transgenic SCNT-derived pigs will be available as donors for functional xenografts including xenogeneic cells, tissues and organs (Thomson et al. 2003, Echelard et al. 2006, Kind and Schnieke 2008).

It is clear that generation of cloned transgenic farm livestock species for biomedical purposes to obtain recombinant xenogeneic proteins or organs suitable in xenotransplantation can be seen as a service to humanity. So too can the production of genetically-engineered SCNT-derived specimens in order to create cell (gene) therapy foundations for a number of animal prion diseases or serious human monogenic diseases which induce heritable (congenital) developmental anomalies.

Acknowledgments

This study was conducted as a part of research project No. N R12 0036 06, financed by the National Centre for Research and Development in Poland from 2009 to 2012.

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