

H. Niemann, W. A. Kues

Progress in Xenotransplantation Research Employing Transgenic Pigs

Microinjection of foreign DNA into pronuclei of a fertilized oocyte has predominantly been used for the generation of transgenic livestock. This technology works reliably, but is inefficient and results in random integration and variable expression patterns in the transgenic offspring. Nevertheless, remarkable achievements have been made with this technology with regard to xenotransplantation. Transgenic pigs that express human complement regulating proteins have been tested in their ability to serve as donors in human organ transplantation (i.e. xenotransplantation). In vitro and in vivo data convincingly show that the hyperacute rejection response can be overcome in a clinically acceptable manner by successfully employing this strategy. The recent developments in nuclear transfer and its merger with the growing genomic data allow targeted and regulatable transgenesis. Systems for efficient homologous recombination in somatic cells are being developed and the first knock-out pigs, carrying a deletion in the α -galactosyltransferase gene, were recently generated. It is anticipated that poly-transgenic pigs will be available as donors for functional xenografts within a few years. Similarly, pigs may serve as donors for a variety of xenogenic cells and tissues. The availability of these technologies is essential to maintain "genetic security" and to ensure absence of unwanted side effects.

Key words:

transgenic pigs, xenotransplantation, microinjection, nuclear transfer

Wissenschaftliche Fortschritte auf dem Weg zur Organspende aus transgenen Schweinen

Die Mikroinjektion von DNA-Konstrukten in die Vorkerne von Zygoten war bisher die bevorzugte Methode, um transgene Großtiere zu erzeugen. Diese Technologie liefert zwar zuverlässig transgene Nachkommen, ist jedoch mit erheblichen Nachteilen behaftet, wie große Ineffizienz (< 5% transgene Nachkommen), zufällige Integration des Fremdgens und variable Expressionsmuster in den transgenen Nachkommen. Trotzdem sind mit dieser Technologie beachtliche Fortschritte erzielt worden, insbesondere im Hinblick auf die Xenotransplantation. Humane komplementregulatorische Proteine sind erfolgreich in transgenen Schweinen exprimiert und diese Tiere im Hinblick auf ihre Eignung in der humanen Organtransplantation (Xenotransplantation) geprüft worden. Umfangreiche In vitro- und In vivo-Daten haben gezeigt, dass die hyperakute Abstoßungsreaktion mit dieser Strategie zuverlässig und in klinisch wirksamer Form überwunden werden kann. Damit ist ein

Department of Biotechnology, Institut für Tierzucht (FAL) Mariensee, Neustadt, Germany

wichtiger Schritt im Hinblick auf ein längerfristiges Überleben porciner Xenotransplantate gemacht worden. Die jüngsten Entwicklungen im Bereich des somatischen Kerntransfers in Verbindung mit den wachsenden genomischen Daten werden zukünftig eine zielgenaue und regulierbare transgene Expression auch in Großtieren erlauben. Systeme für eine effiziente homologe Rekombination in somatischen Zellen werden z.Zt. entwickelt und die ersten „Knock-out“ Schweine mit einer Deletion des α -Galactosyltransferase-Gens sind kürzlich generiert worden. Damit sollte eine weitere Verbesserung im Hinblick auf eine Kontrolle der hyperakuten Abstoßungsreaktion erreicht werden können. Es wird erwartet, dass polytransgene Schweine in einigen Jahren als Spender für funktionsfähige Xenotransplantate zur Verfügung stehen werden. Solche transgenen Schweine werden nicht nur als Spender für solide Organe, sondern auch für xenogene Zellen und Gewebeanteile dienen können. Die Verfügbarkeit dieser neuen Technologien wird wesentlich für eine Standardisierung der transgenen Effekte sein und sicherstellen, dass unerwünschte Nebeneffekte vermieden werden.

Schlüsselwörter:

transgene Schweine, Xenotransplantation, Mikroinjektion, Kerntransfer

1. Introduction

Microinjection of foreign DNA into pronuclei of a fertilized oocyte has predominantly been used for the generation of transgenic livestock. Microinjection involves injection of several thousands of copies of a given gene construct into pronuclei of zygotes; zygotes are transferred into recipients and resulting offspring are screened for integration of the foreign DNA. Although this procedure works reliable, it is inefficient (1-4% transgenic offspring/transferred microinjected zygotes), results in random integration into the host genome and variable expression due to position effects [1,2]. In addition, it is time consuming and requires substantial intellectual, financial and material resources [3]. Attributed to the enormous amounts of resources needed for transgenic livestock production, the costs for one expressing transgenic animal are extraordinary high. It has been estimated that one expressing transgenic mouse requires average expenses of 121 US\$ whereas one expressing transgenic pig would amount to 25,000 US\$, one transgenic sheep 60,000 US\$ and

one transgenic cow 546,000 US\$ [4]. Details of the microinjection technology and the potential applications of transgenic livestock have been extensively reviewed [1,2,4,5,6,7,8]. Despite its inherent limitations, microinjection has allowed commercial exploitation of transgenic technology primarily with animals for biomedical purposes. Substantial progress in livestock transgenesis can be made through application of somatic nuclear transfer. It is anticipated that the merger of nuclear transfer with molecular tools, such as targeted genetic modification and conditional gene expression already explored in mice, will provide another boost to livestock transgenesis [9,10]. Here, we review the present state of research aiming at developing transgenic pigs for clinical xenotransplantation and outline future perspectives.

2. Xenotransplantation

2.1 Transplantation of Solid Organs

Approximately 250,000 people are currently only living because of transplan-

tation of an appropriate human organ (e.g. allotransplantation). In most cases no alternative therapeutic treatment was available and the recipients would have died without the organ transplantation. However, the enormous progress in organ transplantation technology has led to an acute shortage of appropriate organs. Estimations in the USA have revealed that approximately 45,000 people, younger than 65 years need a heart transplant whereas only 2,000 human hearts are transplanted annually [11]. In the U.S.A., more than 74,000 people are awaiting organ transplants and a new patient is added to the waiting list every 14 minutes. Only 21,000 patients received a transplant in the year 2000 [12]. In Germany approximately 2,400 kidneys, 730 livers, 540 hearts and 180 pancreas are transplanted annually. However, the demand is twice as high as these figures. This has led to the sad and ethically challenging situation that several thousand patients die every year who could have survived if appropriate organs would have been available.

To close this growing gap between demand and availability of appropriate organs, xenotransplantation (= the transplantation of organs between discordant species e.g. from animals to human) employing porcine xenografts is considered as the solution of choice [13,14]. The pig seems to be the optimal donor animal because

- the organs have a similar size as human organs,
- porcine anatomy and physiology are not too different from those in humans,
- pigs have short reproduction cycles and large litters,
- pigs grow rapidly,
- maintenance is possible at high hygienic standards at relatively low costs,
- pigs are a domesticated species
- and transgenic techniques are established to modify the immunogenicity of porcine cells and organs.

The process of evaluating transgenic pigs as potential donors for xenotransplants involves a variety of complex steps and is extremely time-, labour- and resource-intensive (Table 1).

Essential prerequisites for a successful xenotransplantation are:

1. Prevention of transmission of zoonoses from the donor animal to the human recipient. This aspect

Tab. 1: Steps involved in testing transgenic swine as donors for xenotransplantation

1. Generation and propagation of transgenic pigs
2. Determination of the transgenic expression pattern (mRNA, protein, organ specificity, etc.)
3. Selective breeding to expand homozygous transgenic lines
4. In vitro tests to assess the protective function of the transgene against HAR
5. Tests to assess the "safety" of the donor lines (exogenous pathogens, endogenous viruses, PERV, etc.)
6. Perfusion studies with isolated porcine organs using human blood
7. In-vivo studies using non-human primates
- physiological compatibility
- potential transmission of pathogens
8. Registration as a therapeutic treatment
9. Clinical application

Tab. 2: Success rates of RCA-transgenic porcine organs upon transplantation to primate recipients

RCA	Organ/kind of transplant	Recipient	Immuno-suppression	Survival (days)
hDAF	heart/heterot.	Cynomologus	+++	~ 60 d
	heterotopic	"	++	~ 90 d
	orthopic	"	+++	~ 10 d
	heterotopic	"	+	~ 21 d
	kidney/orthotopic	"	++	~ 13 d (max. 35 d)
hCD59	heart, heterotopic	Baboon	++	~ 30 h
hCD46	heart, heterotopic	Baboon	++	~ 23 d

+ = weak immunosuppression; ++ = moderate immunosuppression; +++ = heavy immunosuppression

gained particular significance since a few years ago it was shown that porcine endogenous retroviruses (PERV) can be produced by porcine cell lines and can even infect human cell lines [15]. However, until today no infection has been found in patients that had received various forms of living porcine tissues (e.g. islet cells, insulin, skin, extracorporeal liver) for up to 12 years [16]. Recent intensive research revealed that PERV do not present a noticeable risk for recipients of xenotransplantation provided all necessary precautions are made [17,18,19, 20]. In addition, a strain of miniature pigs has been identified which does not produce infective PERV [21].

2. Compatibility of the donor organs in anatomy and physiology with

the human organ system, e.g. lifespan differences, growth rate, expected body weight.

3. Overcoming of the immunological rejection of the transplanted organ. The immunological hurdles are as follows [22]:

a) *Hyperacute rejection response* (HAR) occurs within seconds or minutes. In the case of a discordant organ, e.g. from pig to human, naturally occurring antibodies react with antigenic structures on the surface of the porcine organ and induce HAR by activating the complement cascade which is achieved via the antigen-antibody-complex. Ultimately, this results in the formation of the membrane attack complex (MAC). However, the complement cascade

can be shut down at various points by expression of regulatory genes which prevent the formation of MAC. Regulators of the complement cascade are CD55 (= Decay Accelerating Factor DAF), CD46 (= Membrane Cofactor Protein, MCP) or CD59. MAC disrupts the endothelial cell layer of the blood vessels which leads to lysis, thrombosis, loss of vascular integrity and ultimately to rejection of the transplanted organ.

- b) *Acute vascular rejection* (AVR) occurs within days. Induced xenoreactive antibodies are thought to be responsible for AVR. The endothelial cells of the graft's microvasculature lose their anti-thrombotic properties, attract leucocytes, monocytes and platelets leading to anemia and organ failure.
- c) *Cellular rejection* occurs within weeks after transplantation. In this process the blood vessels of the transplanted organ are damaged by T-cells which invade the intercellular spaces and destroy the organ. This rejection is observed after allotransplantation and normally is suppressed by life-long administration of immunosuppressive drugs.
- d) Chronic rejection is a complex immunological process resulting in the rejection of the transplanted organ after several years. This process is slow and progressive and its etiology is largely unknown.

When employing a discordant donor species such as the pig, overcoming the HAR and AVR are the preeminent goals. The most promising strategy to overcome the HAR is the synthesis of human complement regulatory proteins in transgenic pigs [13,14,23,24]. Following transplantation, the porcine organ would produce the complement regulatory protein and can thus prevent the complement attack of the recipient. Pigs transgenic for DAF have been generated and their hearts have been transplanted either heterotopically, e.g. in addition to the recipient's own organ or orthotopically (= life supportive) into non-human primates. Upon heterotopic

transplantation, the average survival of the recipients reached a maximum of 40-90 days whereas the non-transgenic control organs were destroyed within a few minutes. The primates had to be treated with high doses of immunosuppressive drugs to maintain survival of the xenotransplant. Following moderate doses of immunosuppression survival rates of 20-25 days could be obtained [25,26]. Employing the genomic clone of hCD46 (MCP), transgenic pigs showed a similar expression pattern for the transgene as found for the endogenous gene of the patients. Survival of a hCD46 porcine heart upon transplantation to baboons exceeded 23 days [27]. Similarly, transgenic expression of hCD59 was compatible with an extended survival of porcine hearts following transfer into primates [28]. Transplantation of hDAF-transgenic porcine kidneys was compatible with an extended survival of the recipients. The physiological function of the kidneys was maintained for up to 3-4 weeks [29] (Table 2). These data show that HAR can be overcome in a clinically acceptable manner by successful employing this strategy [13].

Four research groups with strong links to the pharmaceutical industry have reported the generation of transgenic pigs with expression of human complement regulators. In our experiments such transgenic pigs were produced without restrictions imposed from the pharmaceutical industry [30]. Our transgenic pigs show high expression of hCD59 predominantly in the heart, kidney and pancreas but also other target organs; several transgenic lines established (Figure 1). Transgenic endothelial cells and fibroblasts were protected against complement mediated lysis. Perfusion studies using isolated porcine kidneys employing human blood revealed a significant protective effect against HAR. Orthotopic transplantation of a CMV-hCD59 transgenic porcine kidney into cynomolgous monkey was compatible with extended survival of >20 days. The use of the CMV promoter provided a more efficient selection of transgenic pigs with an optimized expression pattern in 2 out of 5 tested lines [30]. Previously, only one out of 30 lines showed an expression pattern that was considered to be compatible with a successful xenotransplantation [26]. A novel promising strategy towards successful xenotransplantation is the

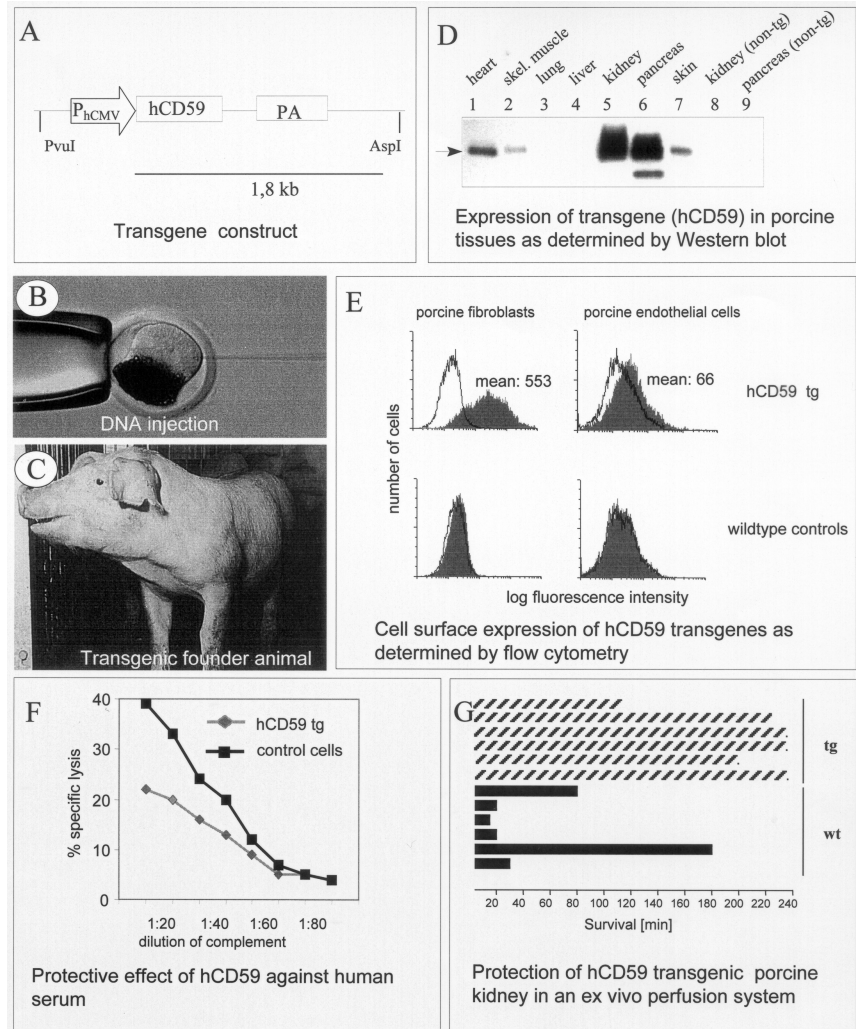


Fig. 1: Generation of transgenic pigs for xenotransplantation (data from Niemann et al., 2001).

A) Minigene construct for microinjection, P_{CMV}: cytomegalovirus immediate early promoter, hCD59: human CD59 (regulator of complement) cDNA, PA: polyadenylation site, PvuI and AspI: flanking restriction enzyme sites.

B) DNA microinjection into one pronucleus of a porcine zygote. Prior to microinjection the zygote was centrifuged to separate the dark lipids from the cytoplasmic fraction to allow optic identification of the pronuclei.

C) Transgenic founder animal identified by Southern blotting of an ear sample.

D) Organ-specific expression of hCD59 protein in an F1-offspring animal determined by Western blotting with a specific monoclonal antibody.

E) Cell surface expression of hCD59 in porcine primary cells as determined by flow cytometry. Grey shadowed curves demonstrated presence of hCD59 by usage of a monoclonal antibody, white curves indicate background values by usage of an isotype matched control antibody.

F) Functional expression of human CD59 on transgenic porcine cells as demonstrated by cytotoxicity assay. Porcine cells were incubated with heat treated human serum and a dilution series of human complement. Specific lysis of porcine cells were measured by chromium release.

G) Ex vivo perfusion of porcine kidneys from F1-animals with human blood. Nearly all transgenic porcine kidneys could be perfused for 4 hours, whereas non-transgenic control kidneys failed soon after onset of perfusion due to hyperacute rejection. Mean survival times were 207.5 minutes for transgenic and 57.5 minutes for wildtype kidneys (p<0.005).

knockout of the antigenic structures on the surface of the porcine organ that cause HAR (Figure 2). These structures are known as 1,3- α -gal-epitopes produced by activity of the 1,3- α -galactosyltransferase. The generation of piglets in which one allele of the α -galactosyltransferase locus had been knocked out by homologous recombination in primary donor cells employed in nuclear transfer, was recently reported [31,32]. The birth of four healthy piglets with disruption of both allelic loci for α -galactosyltransferase has also been published. In this study, the high cytotoxicity of toxin A from *Clostridium difficile* was used to establish a functional screen for cells without gal-epitopes. Toxin A binds to gal-epitopes and effectively kills all wildtype cells. Applying toxin A on cells which already carried one deleted α -galactosyltransferase allele, selected a cell clone, which carried an inactivating point mutation on the second allele [33]. The strength of the cellular response to xenografts can be so great that it is unlikely to be fully controlled by immunosuppressive treatment and transgene expression. Further improvements of the success in xenotransplantation might arise from the possibility of inducing a permanent tolerance across xenogenic barriers [34,35]. A promising strategy for long-term graft acceptance seems to induce a permanent chimerism via intraportal injection of embryonic stem cell like structures [36]. Although xenotransplantation poses numerous further challenges to research, it is expected that transgenic pigs will be available as organ donors within the next five to ten years [37]. Guidelines for the clinical application of porcine xenotransplants are currently being developed in several countries or are already available (USA).

2.2 Use of Xenogenic Cells and Tissue

Another promising area of application for transgenic animals will be the supply of xenogenic cells and tissue. Several intractable diseases, disorders and injuries are associated with irreversible cell death and/or aberrant cellular function. Despite numerous attempts, differentiated human cells cannot yet be expanded well enough in culture. In the future, human embryonic stem-like cells may serve as a source for specific

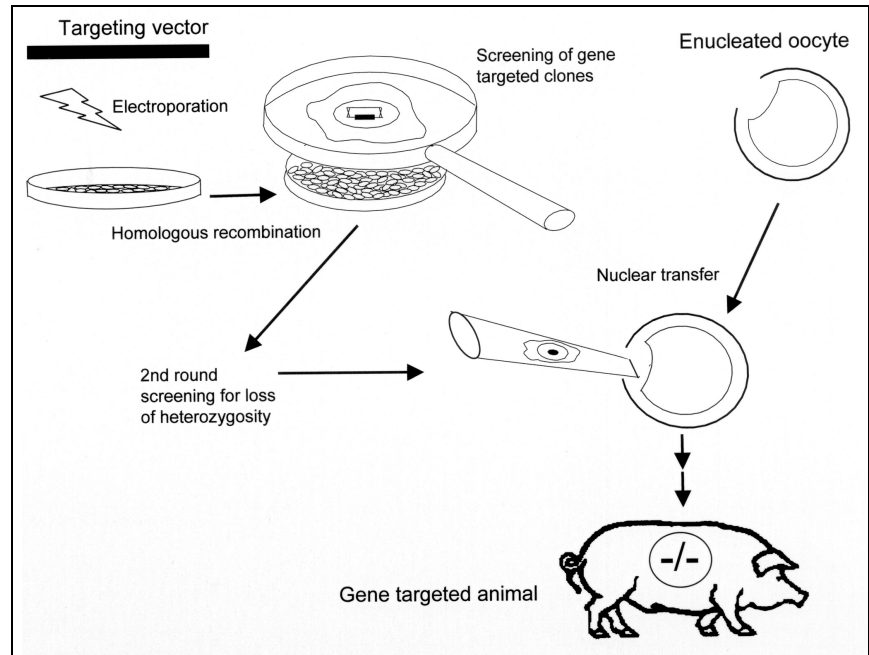


Fig. 2: Schematic drawing of gene targeting in livestock

Gene targeting of somatic primary cells by homologous recombination employing a promoterless targeting vector. Optionally, cell clones with the desired targeting event could be screened for loss of heterozygosity and subsequently be employed in nuclear transfer.

differentiated therapeutic cell types. Xenogenic cells, in particular from the pig, hold great promise with regard to a successful cell therapy for human patients [38]. These cells provide several significant advantages, such as the possibility for manipulation prior to transplantation to enhance cell function, banking and cryopreservation, the combination with different cell types in the same graft and the option to introduce fail safe mechanisms via suicide genes [38].

There are already numerous examples for successful application of xenogenic cell therapy. Porcine islet cells have been transplanted to diabetic patients and were shown to be at least partially functional over a limited period of time [39]. Porcine fetal neural cells were transplanted into the brain of patients suffering from Parkinson's disease and Huntington's disease [40,41]. In a single autopsied patient the graft survived for more than 7 months and the transplanted cells formed dopaminergic neurons and glial cells. Pig neurons extended axons from the graft site into the host brain [40]. Further examples for the potential use of porcine neural cells are stroke and focal epilepsy [42]. Human, fetal neuronal cells have also been employed as transplants into Parkin-

son's and Huntington's disease patients. Olfactory ensheathing cells (OECs) or Schwann cells derived from hCD59 transgenic pigs promoted axonal regeneration in rat spinal cord lesion [43]. Thus, cells from genetically modified pigs may serve as therapeutic measure to restore functional axons across the site of a spinal cord transection. Xenogenic porcine cells may also be useful as novel therapy for liver diseases. Upon transplantation of porcine hepatocytes to Watanabe heritable hyperlipidemic (WHHL) rabbits (a model for familial hypercholesterolemia), the xenogenic cells migrated out of the vessels and integrated into the hepatic parenchyma. The integrated porcine hepatocytes provided functional LDL receptors and thus reduced cholesterol levels by 30-60% for at least 100 days [44].

A clone of bovine adrenocortical cells restored adrenal function upon transplantation to adrenalectomized SCID mice. This finding shows that functional endocrine tissue can be derived from a single somatic cell [45]. Bovine neuronal cells were collected from transgenic fetuses, transplanted into the brain of rats and resulted in significant improvements of symptoms of Parkinson's disease [46]. Furthermore, xeno-

transplantation of retinal pigment epithelial cells holds promise to treat retinal diseases such as macular degeneration which is associated with photoreceptor losses. Porcine or bovine fetal cardiomyocytes or myoblasts may provide a therapeutic approach for the treatment of ischemic heart disease. Similarly, xenogenic porcine cells may be valuable for the repair of skin or cartilage damage [38].

3. Improvements of Transgenic Technology by Nuclear Transfer

In light of the recent advances, somatic nuclear transfer holds the greatest promise for significant improvements in the generation of transgenic livestock. A major prerequisite is the availability of suitable primary cells or cell lines compatible with techniques for precise genetic modifications either for gain or loss of function. Another prerequisite is a significantly improved knowledge of gene sequences and organization of the livestock genome, which currently is lagging much behind that of mouse and human. In the latter, the putative 3 billion bp have been sequenced in the year 2001. Surprisingly, the human genome only contained approximately 30-35.000 genes [47]. However, RNA editing and alternative splicing significantly augments the number of proteins synthesized from a gene [48,49]. In contrast, in livestock species only a minority of genes has been mapped and sequenced until now. However, the technology developed during deciphering the human genome will improve and accelerate sequencing of genomes from other species [50]. Even the currently limited genetic information in livestock species allows the application of cDNA array technologies and or high density DNA chips to obtain gene expression profiles of nearly any tissue of interest. Improvements of RNA isolation and unbiased amplification of tiny amounts of mRNA (picogram) enable to analyse even single preimplantation embryos [51].

Offspring from nuclear transfer have been born in all major livestock species cattle, sheep, goat and swine [52-56]. Our laboratory has cloned cattle already several years ago, and recently obtained the first cloned piglets from in vitro matured oocytes (Hoelker et al., in preparation). This forms the basis for future studies on novel approaches towards genetic modification for xenotransplantation. A variety of different cell types of embryonic, fetal and somatic origin has been successfully employed as donors in nuclear transfer. Factors affecting the success of nuclear transfer are poorly defined and the average percentage of live offspring does not exceed 1-3% of the transferred reconstituted embryos [57,58]. A better understanding of the underlying fundamental molecular and cellular processes, such as cell cycle compatibilities between recipient cytoplasm and donor nucleus [59], cell cycle synchronization of the donor cells [60,61], reprogramming and the relevance of differentiation versus totipotency is urgently needed. Upon serum deprivation or treatment with chemical cell cycle inhibitors, the majority of porcine donor cells was synchronized at the pre-

sumptive optimal cell cycle stage at G₀/G₁ without compromising their viability [61,62]. Recently, we have identified expression of Polo-like kinase-1 (Plk-1) to be a good marker to indicate the appropriate cell cycle stage of porcine donor cells [63]. This contributes substantially to standardize the nuclear transfer procedures.

In cloned offspring predominantly from ruminants approximately 30% are afflicted by the large offspring syndrome (LOS) which includes an increased peri- and postnatal mortality and various other pathologies [52,64,65]. These unwanted side effects need to be overcome prior to an eventual commercial exploitation of somatic nuclear transfer. Aberrations of the well orchestrated pattern of gene expression are thought to be involved in the high incidence of LOS. A primary mechanism may be alterations in the methylation of genes, including those that are subject to imprinting [64,66,67]. The first piglets, carrying a knock-out for one allele of the α -galactosyltransferase gene did not show gross abnormalities [31-33].

4. Perspectives and Outlook

The merger of the recent advancements in the reproductive technologies with the tools of molecular biology opens the horizon for a completely new era for genetic modification of pigs significant for clinical application of xenotransplantation. The recent developments in nuclear transfer and the growing genomic data will allow the generation of loss-of function animals, precise targeting of transgenes to defined chromosomal positions, and the establishment of multi-transgenic pigs for xenotransplantation. We are currently testing the usefulness of the tetracycline system for the conditional expression of transplantation related human genes in transgenic pigs [68]. Systems for efficient homologous recombination in somatic cells are being developed and the adaptation of sophisticated molecular tools, already explored in mice, for transgenic livestock production is underway in the international research community. The availability of these technologies is essential to maintain "genetic security" and to ensure absence of unwanted side effects.

5. Acknowledgements

Research reported in this article has been funded by grants from the BMBF and the DFG. The authors like to acknowledge the important contribution from many members of the laboratory and on the experimental farm.

References

- Pursel VG, Rexroad CE Jr (1993) Status of research with transgenic farm animals. *J Anim Sci* 71: 10-19
- Wall RJ (1996) Transgenic livestock: Progress and prospects for the future. *Theriogenology* 45: 57-68
- Seidel GE Jr (1993) Resource requirements for transgenic livestock research. *J Anim Sci* 71: 26-33
- Wall RJ, Hawk HW, Nel N (1992) Making transgenic livestock: Genetic engineering on a large scale. *J Cell Biochem* 49: 113-120
- Rexroad CE Jr (1992) Transgenic technology in animal agriculture. *Anim Biotech* 3: 1-13
- Niemann H, Halter R, Paul D (1994) Gene transfer in cattle and sheep: A summary perspective. *Proc. 5th World Congress 'Genetics Applied to Livestock Production', Guelph (Canada), Vol 21: pp 339-346*
- Murray JD (1999) Genetic modification of animals in the next century. *Theriogenology* 51: 149-159
- Niemann H, Döpke HH, Haderl KG (2002) Production of transgenic ruminants by DNA microinjection. In: Pinkert C (Ed.) *Transgenic Animal Technology: A Laboratory Handbook (2nd Edition, pp. 337-357)*. San Diego, USA: Academic Press
- Niemann H, Kues WA (2000) Transgenic livestock: premises and promises. *Anim Reprod Sci* 60-61: 277-293
- Niemann H, Kues WA (2003) Application of transgenesis in livestock for agriculture and biomedicine. *Anim.Reprod Sci (in press)*
- Michler RE (1996) Xenotransplantation: Risks and prospects. *Xeno* 4: 21-26
- Petit-Zeman S (2001) Regenerative medicine. *Nature Biotech* 19: 201-206
- Bach FH (1998) Xenotransplantation: Problems and prospects. *Ann Rev Mol* 49: 301-310
- Platt JL, Lin SS (1998) The future promises of xenotransplantation. In: Fishman J, Sachs D, Shaikh R (Eds.) *Xenotransplantation – Scientific frontiers and public policy*. *Annals of the New York Academy of Sciences* 862: 5-18
- Patienc C, Takeuchi Y, Weiss RA (1997) Infection of human cells by an endogenous retrovirus of pigs. *Nature Med* 3: 282-286
- Paradis K, Langford G, Long Z, Heneine W, Sandstrom P, Switzer WM et al. (1999) Search for cross-species transmission of porcine endogenous retrovirus in patients treated with living pig tissue. *Science* 285: 1236-1241
- Patienc C, Patton GS, Takeuchi Y, Weiss RA, McClure MO, Rydberg L (1998) No evidence of pig DNA of retroviral infection in patients with short-term extracorporeal connection of pig kidneys. *The Lancet* 352: 699-701
- Dinsmore LE, Manhardt C, Raineri R, Jacoby DB, Moore A (2000) No evidence for infection of human cells with porcine endogenous retrovirus (PERV) after exposure to porcine fetal neuronal cells. *Transplantation* 71: 132-142

- Switzer WM, Michler RE, Shanmugan V, Matthews A, Hussain AI, Wright A, et al. (2001) Lack of cross-species transmission of porcine endogenous retrovirus infection to nonhuman primate recipients of porcine cells, tissues and organs. *Transplantation* 71: 959-965
- Martin U, Tacke SJ, Simon A, Schröder C, Wiebe K, Lapin B et al. (2002) Absence of PERV specific humoral immune response in baboons after transplantation of porcine cells of organs. *Transplantation Int* 15: 361-368
- Oldmixon BA, Wood JC, Ericsson TA, Wilson CA, White-Scharf ME, Andersson G et al. (2002) Porcine endogenous retrovirus transmission characteristics of an inbred herd of miniature swine. *J Virology* 76: 3045-3048
- White D (1996) Alteration of complement activity: a strategy for xenotransplantation. *TIB* 14: 3-5
- Cozzi E, White DJ (1995) The generation of transgenic pigs as potential organ donors for humans. *Nature Med* 1: 964-966
- White D (1996) hDAF transgenic pig organs: are they concordant for human transplantation? *Xeno* 4: 50-54
- Bach FH, Ferran C, Soares M, Wrighton CJ, Anrather J, Winkler H et al. (1997) Modification of vascular responses in xenotransplantation: Inflammation and apoptosis. *Nature Med* 3: 944-948
- Cozzi E, Masroar S, Soni B, Vial C, White DJ (2000) Progress in xenotransplantation. *Clin Nephrol* 53: 13-18
- Diamond LE, Quinn CM, Martin MJ, Lawson J, Platt JL, Logan JS (2001) A human CD46 transgenic pig model system for the study of discordant xenotransplantation. *Transplantation* 71: 132-142
- Fodor WL, Williams BL, Matis LA, Madri JA, Rollins SA, Knight JW et al. (1994) Expression of a functional human complement inhibitor in a transgenic pig as a model for the prevention of xenogeneic hyperacute organ rejection. *Proc Natl Acad Sci USA* 91: 11153-11157
- Zaidi A, Schmoeckel M, Bhatti F, Waterworth P, Tolan M, Cozzi E et al. (1998) Life-supporting pig to primated renal xenotransplantation using genetically modified donors. *Transplantation* 65: 1584-1590
- Niemann H, Verhoeven E, Wonigeit K, Lorenz R, Hecker J, Schwitzer R et al. (2001) CMV early promoter induced expression of hCD59 in porcine organs provides protection against hyperacute rejection. *Transplantation* 72: 1898-1906
- Lai L, Kolber-Simonds D, Park KW, Cheong HT, Greenstein JL, Im GS et al. (2002) Production of alpha-1,3-galactosyltransferase knock-out pigs by nuclear transfer cloning. *Science* 295: 1089-1092
- Dai Y, Vaught TD, Boone J, Chen SH, Phelps CJ, Ball S et al. (2002) Targeted disruption of the alpha1,3-galactosyltransferase gene in cloned pigs. *Nature Biotechnol* 20: 251-255
- Phelps CJ, Koike C, Vaught TD, Boone J, Wells KD, Chen SH et al. (2003) Production of α 1,3-Galactosyltransferase-deficient pigs. *Science* 299: 411-414
- Greenstein J, Sachs DH (1997) The use of tolerance for transplantation across xenogeneic barriers. *Nature Biotechnol* 15: 235-238
- Auchincloss H Jr, Sachs DH (1998) Xenogeneic transplantation. *Ann Rev Immunol* 16: 433-470
- Fändrich F, Lin X, Chai G, Schulze M, Ganten D, Bader M et al. (2002) Preimplantation-stage stem cells induce long-term allogeneic graft acceptance without supplementary host conditioning. *Nature Med* 8: 171-178

37. Jones I (1996) 2010 – a pig odyssey. *Nature Biotechnol* 14: 698-699
38. Edge ASB, Gosse ME, Dinsmore J (1998) Xenogeneic cell therapy: Current progress and future developments in porcine cell transplantation. *Cell Transplantation* 7: 525-539
39. Groth CG, Korsgren O, Tibell A, Tollemar J, Möller E, Bolinder J et al. (1994) Transplantation of porcine fetal pancreas to diabetic patients. *The Lancet* 344: 1402-1404
40. Deacon T, Schumacher J, Dinsmore J, Thomas C, Palmer P, Kott S et al. (1997) Histological evidence of fetal pig neural cell survival after transplantation into a patient with Parkinson's disease. *Nature Med* 3: 350-353
41. Fink JS, Schumacher JM, Ellias SL, Palmer EP, Saint-Hilaire M, Shannon K et al. (2000) Porcine xenografts in Parkinson's disease and Huntington's disease patients: preliminary results. *Cell Transplant* 2: 273-278
42. Björklund A (1991) Neural transplantation – An experimental tool with clinical possibilities. *Trends Neurosci* 14: 319-322
43. Imaizumi T, Lankford KL, Burton WV, Fodor W, Kocsis JD (2000) Xenotransplantation of transgenic pig olfactory ensheathing cells promotes axonal regeneration in rat spinal cord. *Nature Biotechnol* 18: 949-953
44. Gunsalus JR, Brady DA, Coulter SM, Gray BM, Edge ASB (1997) Reduction of serum cholesterol in Watanabe rabbits by xenogeneic hepatocellular transplantation. *Nature Med* 3: 49-53
45. Thomas M, Northrup R, Hornsby PJ (1997) Adrenocortical tissue formed by transplantation of normal clones of bovine adrenocortical cells in scid mice replaces the essential functions of the animals' adrenal glands. *Nature Med* 3: 978-983
46. Zawada WM, Cibelli JB, Chei PK, Clarkson ED, Golueke PJ, Witta SE et al. (1998) Somatic cell cloned transgenic bovine neurons for transplantation in parkinson rats. *Nature Med* 4: 569-574
47. Baltimore D (2001) Our genome unveiled. *Nature* 409: 814-816
48. Graveley BR (2001) Alternative splicing: increasing diversity in the proteomic world. *Trends in Genetics* 17: 100-107
49. Modrek B, Lee C (2001) A genomic view of alternative splicing. *Nature Genet* 30: 13-19
50. O'Brien SJ, Menotti-Raymond M, Murphy WJ, Nash WG, Wienberg J, Stanyon R et al. (1999) The promise of comparative genomics in mammals. *Science* 286: 458-481
51. Brambrink T, Wabnitz P, Halter R, Klocke R, Carnwath JW, Kues WA et al. (2002) Application of cDNA arrays to monitor mRNA profiles in single preimplantation mouse embryos. *Biotechniques* 33: 376-378
52. Wilmut I, Schmieke AE, McWhir J, Kind AJ, Campbell KH (1997) Viable offspring derived from fetal and adult mammalian cells. *Nature* 385: 810-803
53. Cibelli JB, Stice SL, Golueke PL, Kane JJ, Jerry J, Blackwell C et al. (1998) Cloned calves produced from nonquiescent fetal fibroblasts. *Science* 280: 1256-1258
54. Baguisi A, Behboodi E, Melican D, Pollock JS, Destrempes MM, Cammuso C et al. (1999) Production of goats by somatic cell nuclear transfer. *Nature Biotech* 17: 456-461
55. Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S et al. (2000) Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature* 407: 505-509
56. Bethhauser J, Forsberg E, Augenstein M, Childs L, Eilertsen K, Enos J et al. (2000) Production of cloned pigs from in vitro systems. *Nature Biotech* 18: 1055-1059
57. Wakayama T, Perry ACF, Zuccotti M, Johnson KR, Yanagimachi R (1998) Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* 394: 369-374
58. Wilmut I, Beaujean N, De Sousa PA, Dinnyes A, King TJ, Paterson LA et al. (2002) Somatic cell nuclear transfer. *Nature* 419: 583-586
59. Campbell KH, McWhir J, Ritchie WA, Wilmut I (1996) Sheep cloned by nuclear transfer from a cultured cell line. *Nature* 380: 64-66
60. Boquest AC, Day BN, Prather RS (1999) Flow cytometric cell cycle analysis of cultured porcine fetal fibroblasts cells. *Biol Reprod* 60: 1013-1019
61. Kues WA, Anger M, Carnwath JW, Paul D, Motlik J, Niemann H (2000) Cell cycle synchronization of porcine fetal fibroblasts: Effects of serum deprivation and reversible cell cycle inhibitors. *Biol Reprod* 62: 412-419
62. Kues WA, Carnwath JW, Paul D, Niemann H (2002) Cell cycle synchronization of porcine fetal fibroblasts by serum deprivation initiates a nonconventional form of apoptosis. *Cloning and Stem Cells* 4: 231-243.
63. Anger M, Kues WA, Klima J, Mielenz M, Kubelka M, Motlik J et al. (2003) Cell cycle dependent expression of Plk1 in synchronized porcine fetal fibroblasts. *Mol Reprod Dev* 65: 245-253
64. Young LE, Sinclair KD, Wilmut I (1998) Large offspring syndrome in cattle and sheep. *Rev Reprod* 3: 155-163
65. Kato Y, Tani T, Sotomaru Y, Kurokawa K, Kato J, Doguchi H et al. (1998) Eight calves cloned from somatic cells of a single adult. *Science* 282: 2095-2098
66. Niemann H, Wrenzycki C (2000) Alterations of expression of developmentally important genes in preimplantation bovine embryos by in vitro culture conditions: Implications for subsequent development. *Theriogenology* 53: 21-34
67. Niemann H, Wrenzycki C, Lucas-Hahn A, Brambrink T, Kues WA, Carnwath JW (2002) Gene expression patterns in bovine in vitro produced and nuclear transfer derived embryos and their implication for early development. *Cloning and Stem Cells* 4: 29-37
68. Niemann H, Hauser HJ, Wirth D, Wonigeit K, Schwitzer R, Kues WA (2002) Expression von TET-hCD59 und TET-hCD55 Konstrukten in transgene Schweine. *Transplantationsmedizin (Suppl. DTG Jahrestagung Hannover, 24.-26.10.2002)*: 94

Prof. Dr. Heiner Niemann
Abteilung für Biotechnologie
Institut für Tierzucht
Mariensee
D-31535 Neustadt
E-mail: niemann@tzv.fal.de