ELSEVIER

Animal Reproduction Science 79 (2003) 291–317

ANIMAL REPRODUCTION SCIENCE

www.elsevier.com/locate/anireprosci

Application of transgenesis in livestock for agriculture and biomedicine

Heiner Niemann*, Wilfried A. Kues

Department of Biotechnology, Institut für Tierzucht Mariensee, FAL, 31535 Neustadt, Germany

Received 13 November 2002; received in revised form 27 January 2003; accepted 3 February 2003

Abstract

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. By targeting expression to the mammary gland, numerous heterologous recombinant human proteins have been produced in large amounts which could be purified from milk of transgenic goats, sheep, cattle and rabbit. Products such as human anti-thrombin III, α-anti-trypsin and tissue plasminogen activator are currently in advanced clinical trials and are expected to be on the market within the next few years. 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 successful employing this strategy. It is anticipated that 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 recent developments in nuclear transfer and its merger with the growing genomic data allow a targeted and regulatable transgenic production. 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. The availability of these technologies are essential to maintain "genetic security" and to ensure absence of unwanted side effects.

Keywords: Nuclear transfer; Pronuclear DNA injection; Xenotransplantation; Recombinant proteins; Gene targeting

* Corresponding author. Tel.: +49-5034-871-148; fax: +49-5034-871-101.
E-mail address: niemann@tzv.fal.de (H. Niemann).

0378-4320/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0378-4320(03)00169-6

1. Introduction: animal breeding and biotechnology

Breeding of livestock has a long standing and very successful tradition. It began with domestication by which man habituated animals to live in his proximity. Using the available methodology man has propagated useful populations of animals. The selection was predominantly done on the basis of the phenotype and specific traits. A scientifically based animal breeding has existed for approximately 50 years on the basis of the increasing knowledge in population genetics and statistics. From the early beginning, this approach incorporated biotechnological procedures for which artificial insemination (AI) is the preeminent example. In cattle AI is employed in all countries with advanced breeding programs. Recently, significant increases in AI-application are also observed in swine where currently approximately 50% of the sows are artificially inseminated. This technology allows for a more efficient exploitation of the genetic potential of valuable sires and their propagation in a given population, frequently on a truly international scale. In the 1980s, embryo transfer technology has been transferred from an experimental stage to commercial application. This technology allowed for the first time a better exploitation of the genetic potential of the female in livestock breeding. As of today, >530,000 bovine embryos are transferred annually worldwide of which half are used upon freezing and thawing. In the other livestock species only very few embryos are transferred on a yearly basis (Thibier, 2001). The efforts of animal breeders and the application of artificial insemination, embryo transfer and further biotechnological procedures have resulted in the well known and remarkable increases in performances of livestock production. However, four major limitations have to be considered:

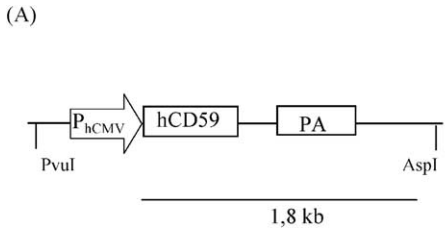
1. The genetic progress reaches only 1–3% per year and is thus relatively slow.
2. It is hardly or not possible to separate desired traits from non-desired traits.
3. A targeted transfer of genetic information between different species has not been possible.
4. Difficult adaptation to local conditions.

Biotechnological procedures which have been under development during the past 20 years opened the perspectives to overcome the limitations listed above. Today, the term “biotech-

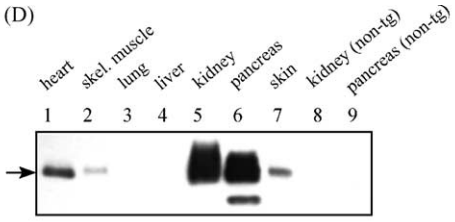
---

Fig. 1. Generation of transgenic pigs for xenotransplantation. (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 cytoplasmatic 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 h, whereas non-transgenic control kidneys failed soon after onset of perfusion due to hyperacute rejection. Mean survival times were 207.5 min for transgenic and 57.5 min for wildtype kidneys ($P < 0.005$) (Data from Niemann et al., 2001).

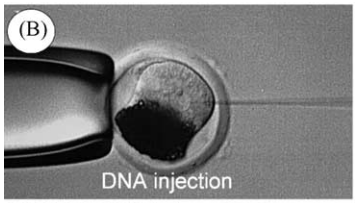
nology in livestock" comprises an arsenal of reproductive-biological and molecular-biological procedures. *Reproductive biology* includes: artificial insemination (AI), estrous synchronization, regulation of parturition, embryo transfer (ET), cryopreservation of gametes and embryos, sexing of sperm and embryos, in vitro production of embryos (IVP, including: in vitro maturation (IVM), in vitro fertilization (IVF) and in vitro culture (IVC)), embryo bisection, nuclear transfer (NT, cloning), microinjection technology (DNA, RNA,

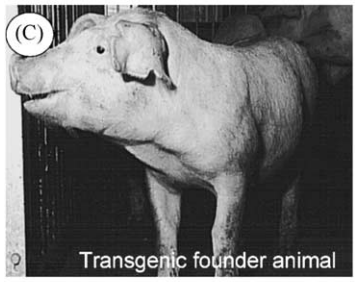


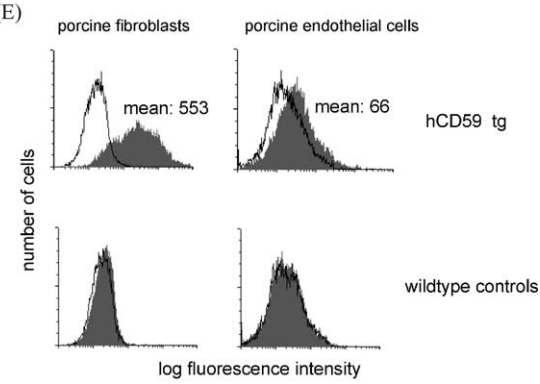
Transgene construct



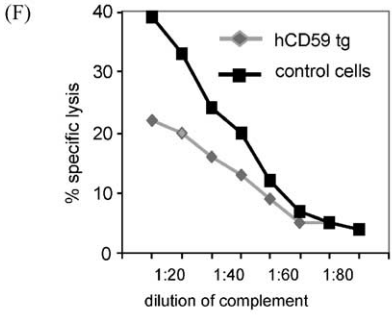
Expression of transgene (hCD59) in porcine tissues as determined by Western blot



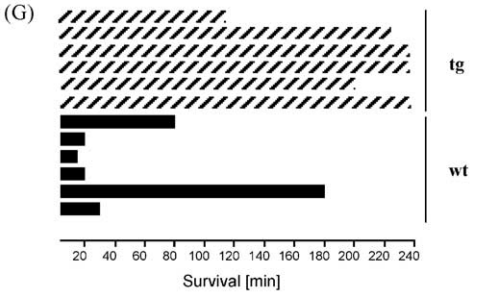




Cell surface expression of hCD59 transgenes as determined by flow cytometry



Protective effect of hCD59 against human serum



Protection of hCD59 transgenic porcine kidney in an ex vivo perfusion system

Antisense). *Molecular genetics* comprise: genome analysis (sequencing, mapping, polymorphisms), molecular diagnostics (genetic defects (MHS, malignant hyperthermia syndrome; Dumps, deficiency of uridine monophosphate synthetase; Blad, bovine leucocyte adhesion deficiency), genetic descent, genetic diversity), functional genomics (expression patterns, interactions of genes), transgenics (additive gene transfer, knockout).

Livestock biotechnology has an interdisciplinary character and comprises elements from anatomy, gynecology and obstetrics, endocrinology and physiology, andrology, ultrasound-technology, biochemistry, cell biology and molecular biology. The further development and refinement of bio- and gene technology with the goal to achieve commercial exploitation is considered to be an important tool to cope with future challenges in livestock production. A prominent example for biotechnological procedures with significant effects in animal production is the transgenic technology. Gene transfer is defined as the introduction of a protein coding DNA fragment into the host genome with the goal that the foreign DNA contributes to the protein synthesis of the host organism, e.g. the transgenic animal. Usually gene transfer involves gene constructs which are artificially combined DNA fragments consisting of regulatory and protein coding sequences. DNA microinjection and nuclear transfer are the two feasible technical approaches to generate transgenic livestock.

Microinjection involves injection of several thousands of DNA copies into pronuclei of zygotes; zygotes are transferred into recipients and offspring is screened for integration and expression of the foreign DNA (Fig. 1A–D). Although this procedure is 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 (Pursel and Rexroad, 1993; Wall, 1996). In addition, it is time-consuming and requires substantial intellectual, financial and material resources (Seidel, 1993). 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 calculated 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$ (Wall et al., 1992). Transgenic production in cattle can only be practical through in vitro production of embryos. From a total of more than 36,500 microinjected zygotes ≈2300 developed to blastocysts, upon transfer 28% resulted in pregnancy and 18 transgenic calves could be identified. To improve efficiency of the procedure the embryos were biopsied and analyzed by PCR for the presence of the transgene (Eyestone, 1999). The early detection of transgenesis in preimplantation embryos has been shown to be feasible, however, efficiency is limited due to an early incidence of mosaicism (Lemme et al., 1994). The propagation of the transgenic trait in a given cattle population can be accomplished through in vitro production techniques by using semen from a transgenic bull for in vitro fertilization and collecting oocytes by means of ultrasound-guided follicular aspiration from transgenic female founder animals and their subsequent use in IVF (Eyestone, 1999). Details of the microinjection technology and the potential applications of transgenic livestock have been extensively reviewed (Rexroad, 1992; Wall et al., 1992; Pursel and Rexroad, 1993; Niemann et al., 1994, 2002a; Wall, 1996; Murray, 1999).

Despite the inherent limitations, microinjection has allowed commercial exploitation of transgenic technology with animals for biomedical purposes and even for few agricultural traits. Substantial progress in livestock transgenesis has been 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 (Niemann and Kues, 2000).

2. Current status of animal transgenesis

2.1. Transgenic animals with agricultural traits

An Australian group has generated transgenic pigs bearing an hMt-pGH construct that can tightly be regulated by zinc feeding. The transgenic animals show significant improvements in economically important traits such as growth rate, feed conversion and bodyfat–muscle ratio (Nottle et al., 1997, 1999). Transgenic sheep carrying a keratin-IGF-I construct show expression in the skin and the clear fleece was about 6.2% greater in transgenic versus non-transgenic animals (Damak et al., 1996a,b). In both projects no adverse effects of the transgene on health or reproduction were observed. Another interesting application could be enhanced disease resistance (Müller and Brem, 1991). A mouse model in which recombinant monoclonal antibodies, which neutralize the transmissable gastroenteritis virus (TGV), are secreted into milk, provided passive protection against gastroenteric infections to the pups (Castilla et al., 1998). The verification of this model in pigs is promising. Recently, transgenic pigs expressing a bacterial phytase gene under the transcriptional control of a salivary gland-specific promoter were shown to have improved phosphate uptake (Golovan et al., 2001). The inserted phytase gene was almost exclusively expressed in the salivary gland and enabled the pigs to digest phosphorus in phytate, which could then be metabolized by the intestine. These animals require significantly fewer inorganic phosphate supplements, release substantially reduced phosphorus levels in manure and thus reduce environmental pollution. By transgenic expression of bovine alpha-lactalbumin in the mammary gland of sows, lactation performance was improved with respect to milk composition, milk yields and piglet growth (Wheeler et al., 2001). The increased survival of piglets at weaning provides significant benefits to animal welfare and the producer.

2.2. Transgenic animals in biomedicine

2.2.1. Gene pharming

By targeting expression to the mammary gland via the use of mammary gland-specific promoter elements, large amounts of numerous heterologous recombinant proteins have been produced. In the bovine mammary gland of transgenic cows at least two different pharmaceutical proteins have been produced, in the caprine mammary gland five proteins, in the ovine system four proteins, in the pig two and in transgenic rabbits seven proteins (Rudolph, 1999). These could be purified from milk of transgenic rabbits, sheep, goats and cattle. The biological activity of the recombinant proteins was assessed and the therapeutic effects have been characterized (Rudolph, 1999; Meade et al., 1999). Human lactoferrin has recently been reported to be produced in large amounts in the mammary gland of transgenic cows (van Berkel et al., 2002). Products such as anti-thrombin III (ATIII), α-anti-trypsin

Table 1
Status of selected recombinant pharmaceutical proteins produced in the mammary gland of transgenic livestock with regard to clinical testing/registration

Donor species	Product	Clinical trial	Treatment of	Expected on market	Company
Sheep	α-AT	Phase II/III	α-AT-deficiency; cystic fibrosis	2006	PPL/GTC
Goat	TPA	Phase II/III	Coronary clots	2006	GTC
Goat	AT III	Phase III	Heparin-resistance	2004	GTC
Rabbit	α-Glucosidase[a]	Phase II/III	Pompe's disease	2004	Pharming

[a] Orphan drug registration.

(α-AT) or tissue plasminogen activator (tPA) are currently in advanced clinical trials and are expected to be on the market within the next few years (Ziomek, 1998; Rudolph, 1999) (Table 1). On the basis of average expression levels, daily milk volumes and purification efficiency, 5400 cows would be needed to produce the 100,000 kg human serum albumin (HSA) that are required per year worldwide, 4500 sheep for the production of 5000 kg α-AT, 100 goats for 100 kg of monoclonal antibodies, 75 goats for the 75 kg ATIII and two pigs to produce 2 kg human clotting factor IX (Rudolph, 1999).

Although a variety of proteins has been produced in the mammary gland of transgenic animals, not every protein can obviously be expressed at the desired high amounts. Erythropoietin (EPO) could not be expressed in the mammary gland of transgenic cattle (Hyttinen et al., 1994). We have shown that human clotting factor VIII cDNA constructs can be expressed in the mammary gland of transgenic mice, rabbit and sheep. However, the recovery rates of hFVIII protein were low and dependent on the donor, storage temperature and dilution of milk samples. hFVIII was rapidly sequestered in ovine milk (Halter et al., 1993; Espanion et al., 1997; Niemann et al., 1999; Hiripi et al., 2003). These latter results show that the technology needs further improvements to achieve high level expression of extraordinary large and complex regulated genes, such as hFVIII, although higher levels of hFVIII were reported in transgenic swine (Paleyanda et al., 1997).

2.2.2. Xenotransplantation

2.2.2.1. Transplantation of solid organs. Approximately 250,000 people are currently only living because of transplantation 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 which today is the basis for a normal life of thousands of patients has led to an acute shortage of appropriate organs, while the willingness to donate organs has remained unchanged or is even slightly reduced. Estimations in the USA have revealed that approximately 45,000 people, younger than 65 years need a heart transplant whereas only 2000 human hearts are transplanted annually (Michler, 1996). In the USA, more than 74,000 people are awaiting organ transplants and a new person is added to the waiting list every 14 min. Only 21,000 patients received a transplant in the year 2000 (Petit-Zeman, 2001). In Germany approximately 2400 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) is considered as the solution of choice (Bach, 1998; Platt and Lin, 1998). 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.

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

Essential prerequisites for a successful xenotransplantation are:

1. Prevention of transmission of zoonoses from the donor animal to the human recipient. This aspect 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 (Patience et al., 1997). 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, extracorporal liver) for up to 12 years (Paradis et al., 1999). Recent intensive research revealed that PERV do not present a noticeable risk for recipients of xenotransplantation provided all necessary precautions are made (Patience et al., 1998; Dinsmore et al., 2000; Switzer et al., 2001; Martin et al., 2002). In addition, a strain of miniature pigs has been identified which does not produce infective PERU (Oldmixon et al., 2002).
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 (White, 1996a):
   (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. Well known 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-thrombic 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 administration of immunosuppressive drugs. These have to be taken by the recipients for the rest of their lives.

(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. The only remaining therapeutic option is another transplantation.

When employing a discordant donor species such as the pig, overcoming the HAR is the preeminent goal. This cannot be achieved by administering high doses of an appropriate immunosuppressive drug as these do not affect the complement regulated rejection process. The most promising strategy to overcome the HAR is the synthesis of human complement regulatory proteins in transgenic pigs (Cozzi and White, 1995; White, 1996b; Bach, 1998; Platt and Lin, 1998). 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 (Bach et al., 1997; Cozzi et al., 2000). 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 (Diamond et al., 2001). Similarly, transgenic expression of hCD59 was compatible with an extended survival of porcine hearts following transfer into primates (Fodor et al., 1994). 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 (Zaidi et al., 1998) (Table 2). These data show that HAR can be overcome in a clinically acceptable manner by successful employing this strategy (Bach, 1998).

Prior to primate experiments, extremely labor and time-consuming research is required to identify those transgenic animals with the most promising expression pattern of the transgene. Four research groups with strong links to the pharmaceutical industry have reported the generation of transgenic pigs with expression of human complement regulators. The screening of transgenic lines with suitable expression profiles is still inefficient. Previously, only one out of 30 lines showed an suitable expression pattern for xenotransplantation (Cozzi et al., 2000). A more efficient selection of transgenic pigs with an optimized expression pattern in two out of five tested lines was provided by employing the CMV promoter (Niemann et al., 2001). They provide a useful tool for the study of basic immunological

Table 2
Success rates of RCA-transgenic porcine organs upon transplantation to primate recipients

RCA	Organ/kind of transplant	Recipient	Immunosuppression	Survival
hDAF	Heart/heterotopic	Cynomologus	+++[a]	~60 days
	Heterotopic	Cynomologus	++[b]	~90 days
	Orthotopic	Cynomologus	+++	~10 days
	Heterotopic	Cynomologus	+[c]	~21 days
	Kidney/orthotopic	Cynomologus	++	~13 days (maximum 35 days)
hCD59	Heart, heterotopic	Baboon	++	~30 h
hCD46	Heart, heterotopic	Baboon	++	~23 days

[a] Heavy immunosuppression.
[b] Moderate immunosuppression.
[c] Weak immunosuppression.

questions in xenotransplantation. Transgenic pigs that show high expression of hCD59 predominantly in the heart, kidney and pancreas but also other target organs, were identified and transgenic lines established (Fig. 1D and E). Transgenic endothelial cells and fibroblasts were protected against complement mediated lysis (Fig. 1F). Perfusion studies using isolated porcine kidneys employing human blood revealed a significant protective effect against HAR (Fig. 1G). Orthotopic transplantation of a CMV-hCD59 transgenic porcine kidney into cynomologous monkey was compatible with extended survival of >20 days.

Another promising strategy towards successful xenotransplantation is the knockout of the antigenic structures on the surface of the porcine organ. These structures are known as 1,3-α-gal-epitopes and are produced from the gene for the 1,3-α-galactosyltransferase. Recently, the generation of piglets in which one allele of the α-galactosyltransferase locus had been knocked out, was reported (Lai et al., 2002; Dai et al., 2002). These animals are now in breeding programs to obtain homozygous knockout animals. The usefulness of organs from these pigs for xenotransplantation is currently being tested. The birth of piglets with disruption of both allelic loci has recently been publicly announced.

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 (Greenstein and Sachs, 1997; Auchincloss and Sachs, 1998). A promising strategy for long-term graft acceptance seems to induce a permanent chimerism via intraportal injection of embryonic stem cell like structures (Fändrich et al., 2002). Although xenotransplantation poses numerous further challenges to research, it is expected that transgenic pigs will be available as organ donors within the next 5–10 years (Jones, 1996). Guidelines for the clinical application of porcine xenotransplants are currently being developed in several countries or are already available (USA).

2.2.2.2. 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 func-

tion. Despite numerous attempts, primary human cells cannot yet be expanded well enough in culture. In the future, human embryonic stem cells may serve as a source for specific differentiated cell types that can be used in cell therapy. Xenogenic cells, in particular from the pig, hold great promise with regard to a successful cell therapy for human patients (Edge et al., 1998). These cells provide several significant advantages over other approaches, such as implantation at the optimal therapeutic location (i.e. immunoprivileged sites such as the brain), possibility for manipulation prior to transplantation to enhance cell function, banking and cryopreservation, combination with different cell types in the same graft (Edge et al., 1998).

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 (Groth et al., 1994). Porcine fetal neural cells were transplanted into the brain of patients suffering from Parkinson's disease and Huntington's disease (Deacon et al., 1997; Fink et al., 2000). 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 (Deacon et al., 1997). Further examples for the potential use of porcine neural cells are stroke and focal epilepsy (Björklund, 1991). Human, fetal neuronal cells have also been employed as transplants into Parkinson's and Huntington's disease patients. The advantages of porcine neural cells over their human counterparts are the abundant availability and the option to introduce fail safe mechanisms via suicide genes. Olfactory ensheathing cells (OECs) or Schwann cells derived from hCD59 transgenic pigs promoted axonal regeneration in rat spinal cord lesion (Imaizumi et al., 2000). Thus, cells from genetically modified pigs may serve as therapeutic measure to restore electrophysiologically functional axons across the site of a spinal cord transsection. 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 (Gunsalus et al., 1997).

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 (Thomas et al., 1997). 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 (Zawada et al., 1998). Furthermore, xenotransplantation 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 (Edge et al., 1998). In light of the emergence of more efficient protocols for genetic modification of donor pigs and new powerful immunosuppressive drugs, one can expect xenogenic cell therapy to evolve as an important therapeutic option for the treatment of human diseases.

The above description demonstrates that although the challenges to generate a transgenic animal with an optimized tailored expression pattern are enormous, within less than 20 years transgenic livestock have emerged that provide valuable contributions to human health.

With increasing knowledge about the genetic basis of agricultural traits and improvements in the technology to generate transgenic animals, numerous further useful commercial applications will be developed.

3. Improvements of transgenic technology

3.1. Inducible gene expression

A major advancement in transgenic technology would be tight control of transgene expression. Control elements that are known to regulate the activity of transgenes are the metallothionein promoter (see Nottle et al., 1999), heat shock promoter, or steroid responsive elements. However, these have been used with limited success attributed to low induction levels and physiological effects of the inducer elements (Yarranton, 1992). Significant improvements of the temporal control of gene expression could be achieved by employing the antibiotic tetracycline system (Gossen et al., 1995). This involves a transcriptional transactivator which has been created by fusion of the VP16 activation domain with a mutant Tet repressor from *Escherichia coli*. This transactivator requires the presence of tetracycline or an analogue for DNA binding and transcriptional activation (Fig. 2). It has been shown that

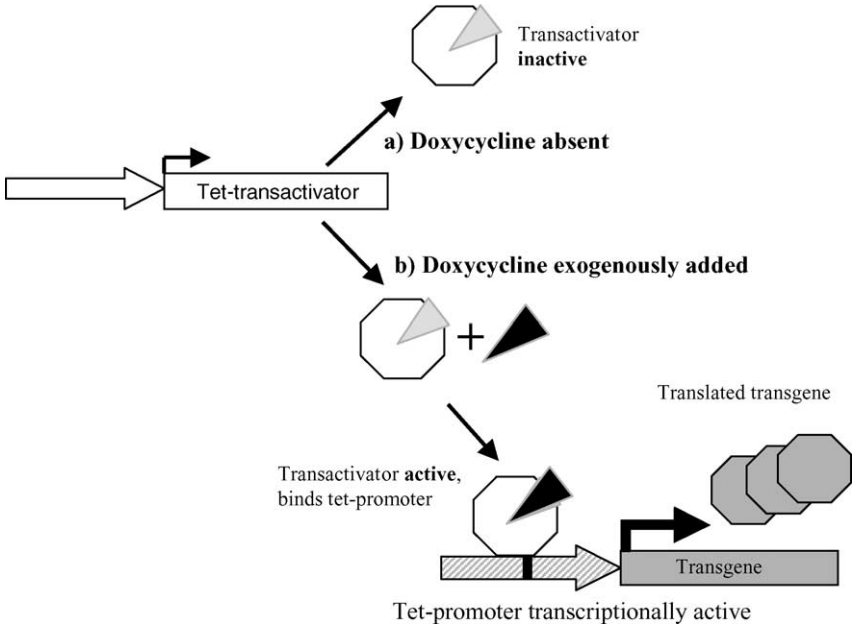


Fig. 2. Schematic drawing of Tet-on system. In the original system two independent transgene constructs were integrated in the genome. The transactivator gene is continuously transcribed and the inactive form of the transactivator is translated. By exogenous addition (feeding) of a tetracycline analogue the transactivator becomes activated, subsequently binds to the Tet-promoter and induces expression of the transgene. Removal of the compound leads to transcriptional silencing of the transgene.

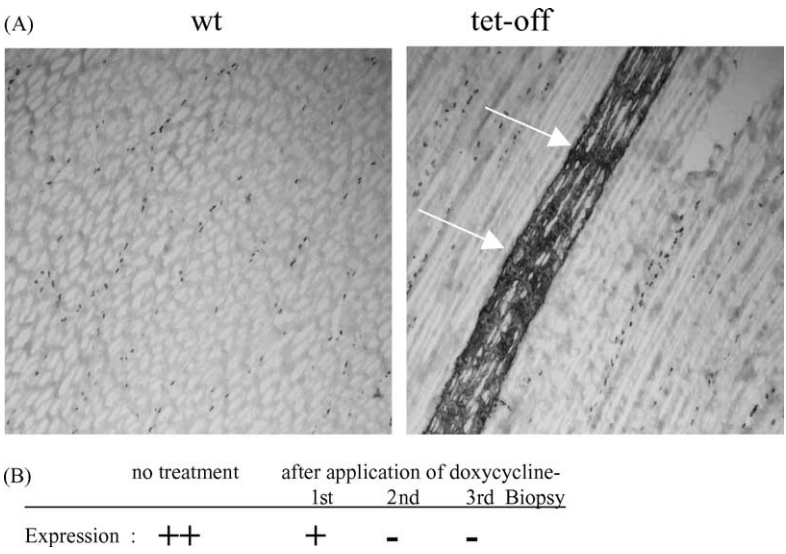


Fig. 3. Conditional transgene expression in porcine muscle. (A) Transgenic pigs carrying a Tet-off expression cassette show massive expression in skeletal muscle fibers. Muscle biopsies of a wildtype and a transgenic animal were stained with a specific antibody against the transgene. Arrows indicate a positive muscle fiber. (B) Conditional ablation of transgene expression. Expression levels of the transgene were determined in muscle biopsies before and after feeding with doxycycline. In total, four animals were treated with doxycyline and showed identical downregulation of expression.

the presence of a tetracycline analogue led to a burst of expression in cell lines and even transgenic mice (Tet-on) (Gossen et al., 1995). This system can also be modified in a way that the presence of tetracycline suppresses expression of the target gene (Tet-off). In this system, tetracycline binds to the transactivator and shuts down expression of the transgene (Furth et al., 1994). The original technology requires two constructs, which make it unfeasible for application in livestock. However, it has been shown that fusion of both control elements into one construct allows efficient and tight control of gene expression in vivo (Schultze et al., 1996). In recent own work, the transgene expression in pigs, carrying a single Tet-off gene construct, could be regulated by doxycycline feeding (Fig. 3, unpublished data). Further studies will show the usefulness of this system for the conditional expression of transgenes suitable in xenotransplantation.

3.2. Artificial chromosomes: YACs, BACs and MACs

Artificial chromosomes are DNA molecules of predictable structure which are assembled in vitro from defined constituents that are similar to natural chromosomes. The first artificial chromosomes have been constructed in yeast (*Saccharomyces cerevisiae*) (Fig. 4). They include centromeres, telomeres, and origins of replication as essential components (Brown et al., 2000). These yeast artificial chromosomes (YACs) can be introduced into cell lines (Strauss et al., 1993). They carry much larger amounts of DNA than usually can be employed in microinjection. Microinjection of a 450 kb genomic YAC harboring the

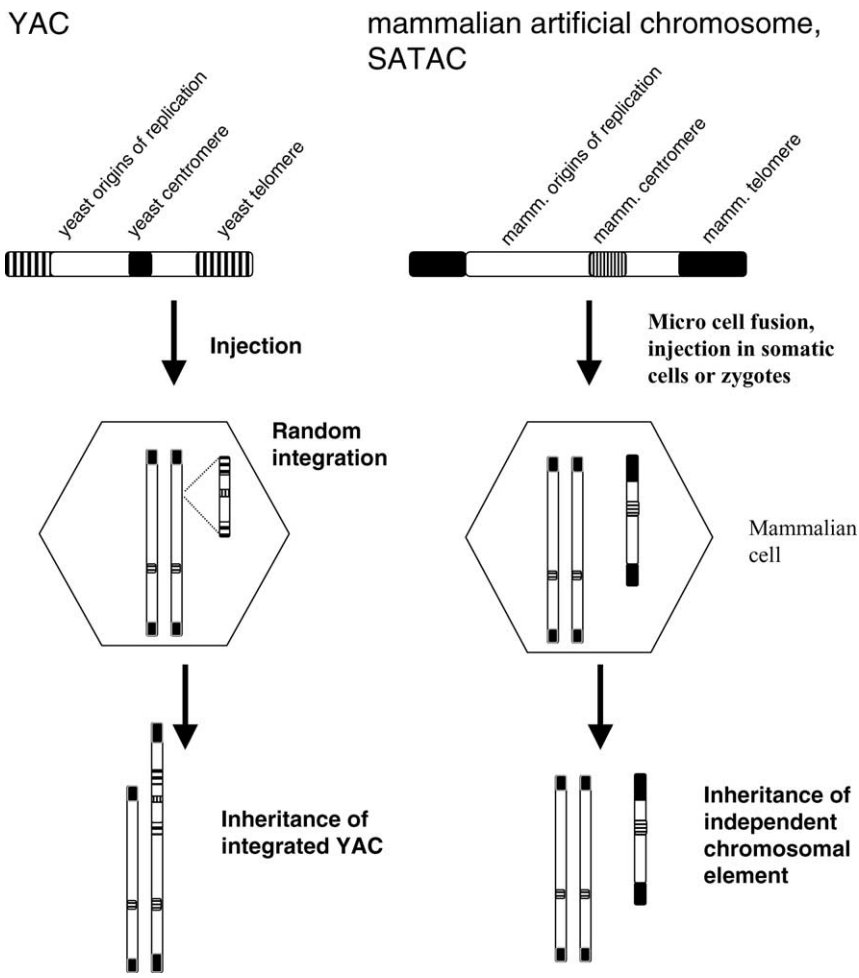


Fig. 4. Schematic comparison of yeast and mammalian artificial chromosomes used for the generation of transgenic animals. Injection of yeast or bacterial artificial chromosomes (YAC, BAC) into mammalian zygotes leads to a random integration, since their structural chromosomal elements are not functional in mammalian cells. In contrast, injection of mammalian artificial chromosomes results in the inheritance of these vectors as independent chromosomal elements.

murine tyrosinase gene resulted in transgenic mice which showed position independent and copy number dependent expression of the transgene. Albinism was rescued in transgenic mice and rabbits (Schedl et al., 1992, 1993; Brem et al., 1996). A 210 kb YAC construct has been microinjected into rat pronuclei and α-lactoglobulin and human growth factor were expressed in the mammary gland of transgenic rats (Fujiwara et al., 1997, 1999). Artificial chromosomes can also be constructed in bacteria (BACs), which can be genetically modified easier and even allow homologous recombination. Transgenic mice were generated via pronuclear injection of BACs and germline transmission and proper expression of the transgene was achieved (Yang et al., 1997). However, up to now, transgenic livestock has not been

reported upon transfer of a YAC construct. This may be attributed to the inherent problems of this technology, such as difficulties to isolate YAC DNA with sufficient purity and the inherent instability with a tendency for deleting regions from the insert (Monaco and Larin, 1994).

Mammalian artificial chromosomes (MAC) have been engineered by employing endogenous chromosomal elements from YACs or extra chromosomal elements from viruses or BACs and P1 artificial chromosomes (PACs) (Vos, 1997). MACs with a size of 1–5 Mb were formed by a de novo mechanism and segregated like normal chromosomes upon introduction into cell lines (Ikeno et al., 1998). A human artificial chromosome (HAC) containing the entire sequences of the human immunoglobulin heavy and light chain loci has been introduced into bovine fibroblasts, which were then used in nuclear transfer. Transchromosomal offspring were obtained that expressed human immunoglobulin in their blood. This system could be a significant step forward in the production of therapeutic polyclonal antibodies (Kuroiwa et al., 2002).

Satellite-DNA based artificial chromosomes (SATAC) are neochromosomes that are formed by de novo amplification of pericentric heterochromatin yielding chromosomes from 10 to 360 megabases. These can serve as chromosomal vectors for exogenous DNA (Perez et al., 2000). Transgenic mice have been generated by microinjection of SATACs into pronuclei of zygotes. The additional chromosome showed germline transmission over three generations (Co et al., 2000). Microinjection of SATACs was also compatible with the development of bovine embryos. Transgenic embryos could be identified by staining for the presence of a reporter gene and FISH detection of the extra chromosome (Co et al., 2000). Moreover, SATACs could be isolated and purified by flow cytometry due to their high AT content.

However, all efforts to assemble MACs as functional vectors for foreign DNA have met with limited success. Mini-chromosomes are considered to overcome the problems with artificial chromosomes. In contrast to artificial chromosomes, mini-chromosomes possess a defined structure, can be engineered to harbor large fragments of DNA and can go through the vertebrate germline (Brown et al., 2000). Mini-chromosomes could play an important role in the development of transchromosomal animals as models of human genetic disorders such as trisomy 21 (Down's syndrome) (Hernandez et al., 1999). Recently, mice have been engineered that contain certain fragments of human chromosomes with each of the immunoglobulin heavy chain and κ light chain gene. In these animals, the endogenous immunoglobulin genes were deleted and the mice produced humanized antibodies (Tomizuka et al., 2000). Further progress in this area will ultimately result in a variety of different vector systems which can be used for large scale transgenesis in experimental animals, agricultural livestock and crop plants.

4. Nuclear transfer and targeted genetic modification

4.1. Nuclear transfer technology: results and limitations

As microinjection has a number of significant shortcomings mentioned above, research has focused on alternate methodologies for improving the generation of transgenic livestock. These include sperm mediated DNA transfer (Gandolfi, 1998; Squires, 1999; Chang et al.,

2002; Lavitrano et al., 2002), the intracytoplasmic injection (ICSI) of sperm heads carrying foreign DNA (Perry et al., 1999, 2001), the use of retroviral vectors either by injection or infection of oocytes or embryos (Haskell and Bowen, 1995; Chan et al., 1998; Cabot et al., 2001) or the use of genetically modified donor cells from livestock in nuclear transfer (Schnieke et al., 1997; Cibelli et al., 1998a; Baguisi et al., 1999; Park et al., 2001). Further improvements may be derived from the adaptation of technologies that allow precise modifications of the murine genome. These include targeted chromosomal integration by site-specific DNA recombinases, such as Cre or FLP or homologous recombination that would enable generation of transgenic animals with a gain (knockin) or a loss of function (knockout) (Capecchi, 1989; Kilby et al., 1993). 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 base pairs have been sequenced in the year 2001. Surprisingly, the human genome only contained approximately 30–35,000 genes (Baltimore, 2001). However, RNA editing and alternative splicing significantly augments the number of proteins synthesized from a gene. Whereas only 250 out of the 6000 genes in *Saccharomyces cerevisiae* contain introns, the vast majority of the estimated human genes are thought to contain these structures (Graveley, 2001; Modrek and Lee, 2001). In contrast, in livestock species only a minority of genes have been mapped and sequenced until now (Table 3). However, the technology developed during deciphering the human genome will improve and accelerate sequencing of genomes from other species (O'Brien et al., 1999). 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 analyze even single embryos (Brambrink et al., 2002).

Reports on the generation of transgenic livestock (Schnieke et al., 1997; Cibelli et al., 1998a; Park et al., 2001) via somatic nuclear transfer had raised great expectations about this elegant approach to improve the generation of transgenic livestock. Fetal fibroblasts were transfected in vitro, screened for transgene integration and then transferred into enucleated oocytes. After fusion of both components and activation of the reconstituted nuclear transfer complexes, blastocysts were transferred to synchronized recipients and gave rise to transgenic offspring. Compared with the microinjection procedure in which screening for transgenesis and optimal expression of the transgenes takes place at the level of the offspring, cloning by nuclear transfer can accelerate the time-consuming transgenic production by rapid screening in vitro and 100% transgenic offspring. The first commercial data of the use of nuclear transfer to generate transgenic cattle show the feasibility of this approach (Forsberg et al., 2001).

Offspring from nuclear transfer have been born in all major livestock species cattle, sheep, goat and swine (Wilmut et al., 1997; Cibelli et al., 1998b; Baguisi et al., 1999; Polejaeva et al., 2000; Betthauser et al., 2000). A variety of different cell types of embryonic, fetal and somatic origin has been successfully employed as donors in nuclear transfer. However, the overall efficiency of nuclear transfer is low (Colman, 2000). Factors affecting

Table 3
Whole genome sequencing

Species	No. annotated genes	Genome size ($\times 10^6$ bp)	Sequencing completed
Milestones in genome sequencing and size of selected genomes			
Haemophilus influenca (bacteria)	~1700	1.7	1995
Saccaromyces cerevisae (fungi)	~6000	12.0	1996
Caenorhabditis elegans (nematodes)	~18000	97.0	1998
Drosophila melanogaster (insects)	~14000	137.0	2000
Arabidopsis thalia (plants)	~25000	125.0	2000
Homo sapiens[a] (mammals)	~35000	3300.0	2001
Diversity of genome sequencing approaches (10/2002)			
Archaea	Complete genomes of 16 species		
Bacteria	Complete genomes of 81 species		
Eukaryota	complete genomes of 9 species. In addition the genomes of *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Danio rerio* and some crop plants are mapped, however gene annotation and/or sequencing of untranscribed regions is still in progress		
Species	Chromosome number (haploid)	No. mapped genes	Genome size ($\times 1000$)
Human[b]	23	>30000	3.300
Mouse[c]	20	30000	2.450
Cattle[c]	30	~1000	~3.500
Sheep[c]	27	~500	~3.100
Pig[c]	19	~600	~2.400
Horse[c]	32	<200	~2.000
Dog[c]	39	~250	~2.700
Chicken[c]	39	>200	~4.000

[a] Gene annotation still in progress.
[b] February 2001 sequencing completed.
[c] Information for 2002.

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 (Wakayama et al., 1998; Wilmut et al., 2002). A better understanding of the underlying fundamental molecular and cellular processes, such as cell cycle compatibilities between recipient cytoplasm and donor nucleus (Campbell et al., 1996), cell cycle synchronization of the donor cells (Boquest et al., 1999; Kues et al., 2000), 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 presumptive optimal cell cycle stage at G_0/G_1 without compromising their viability (Kues et al., 2000, 2002; Anger et al., 2003). This contributes substantially to standardize the nuclear transfer procedures. In addition, methods have to be established that allow reliable determination

of the capacity of a given nuclear transfer embryo to develop into a normal offspring. The large offspring syndrome (LOS) including an increased peri- and postnatal mortality, is found in offspring, predominantly from ruminants, derived from nuclear transfer embryos (Wilmut et al., 1997; Young et al., 1998; Kato et al., 1998). These unwanted side effects need to be overcome prior to an eventual commercial exploitation. 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 (Young et al., 1998; Niemann and Wrenzycki, 2000; Niemann et al., 2002b).

4.2. Embryonic and adult cell lines

The fundamental tools for loss-of-function transgenics in the mouse are the availability of embryonic stem cells (ES cells), homologous recombination and the high probability with which ES cells give rise to germline contribution after injection into host blastocysts. This provides a powerful approach to introduce specific genetic changes into the murine genome (Evans and Kaufman, 1981; Martin, 1981). The essential characteristics of ES cells include derivation from the preimplantation embryo (inner cell mass cells), undifferentiated and indefinitive proliferation in vitro and the developmental potential to differentiate into all cell types under appropriate conditions.

Besides ES cells, embryonic germ (EG) and embryonic carcinoma (EC) cells have been established in the mouse model. EG cells are isolated from cultured primordial germ cells (PGC) (Matsui et al., 1992; Resnick et al., 1992) and share several characteristics with ES cells, including morphology, pluripotency, and the capacity for germline transmission. Similar to ES cells, EG cells express alkaline phosphatase and Oct-4 and can be aggregated to form embryoid bodies.

The ultimate criterion for true totipotent stem cell lines is the contribution to the germline in chimeras or starting a new development upon nuclear transfer. ES or EG-like cell lines have been isolated from sheep, goat, pig and cattle (Wheeler, 1994; Anderson, 1999). Porcine and bovine cell lines were capable to contribute to chimera formation upon injection into appropriate host blastocysts (Wheeler, 1994; Shim et al., 1997; Piedrahita et al., 1998; Cibelli et al., 1998a). However, no germline transmission has been reported so far. True totipotent stem cell lines might require specific culture conditions, growth factor supplements and probably a specific genetic background, as only few mouse strains are suitable for ES cell line isolation (Hogan et al., 1994).

Recent experiments in mice have revealed that adult cells from the hematopoetic lineage possess a greater plasticity than previously assumed (Jiang et al., 2002). Besides long-term proliferation in vitro, these mesenchymal derived cells express embryonic markers, such as Oct-4, rex and telomerase and can be differentiated into several cell types in vitro and in vivo. Upon injection into blastocysts, chimeric mice could be generated, which showed a high percentage of chimerism in nearly all organs. Similar cells were isolated from the hematopoetic system of rats and even humans, suggesting that mesenchymal stem cells could also be a useful source of research and application in livestock species.

4.3. Homologous recombination and nucleus donor cell strains

Homologous recombination in murine ES cells is the most straightforward approach to eliminate gene function and is therefore the preferred method to establish a null genotype. Several strategies for gene targeting in murine ES cells have been developed (Kühn et al., 1995; Mayford et al., 1997). More than 1000 knockout strains have been created via gene targeting in embryonic stem cells (mouse knockout and mutation database http://www.biomednet.com). The potential for a knockout technology in livestock production is highlighted by the discovery that several beef cattle breeds, like Belgian Blue and Piedmontese, are accidentally homozygous for the mutated myostatin gene, which is functionally inactive and could be referred to as a natural knockout (McPherron and Lee, 1997; Kambadur et al., 1997; Grobet et al., 1997). The similarity in phenotypes of myostatin mutated cattle and myostatin null mice (McPherron et al., 1997) is striking and suggests that myostatin could be a useful target for genetic modification in farm animals.

Nuclear transfer techniques promise to circumvent the need for true totipotent cells for the generation of loss-of-function transgenic livestock. The future challenge for the production of transgenic livestock is the isolation and handling of primary cell cultures, either from somatic or embryonic origin (Schnieke et al., 1997; Cibelli et al., 1998b; Kues et al., 1998). These could be used for sophisticated genetic modifications, clonal selection and subsequently for nuclear transfer. Gene targeting in somatic cells of livestock has important applications in combination with nuclear transfer (Fig. 5). Recent progress in the generation of gene targeted livestock clearly shows the significant potential of this field. An efficient and reproducible targeting protocol in fetal fibroblasts by which the transgenic construct β-lactoglobulin-α-anti-trypsin was placed at the ovine α1 precollagen locus was described and the production of live sheep upon nuclear transfer was reported (McCreath et al., 2000). The recombinant α-AT was highly expressed in the mammary gland. However, the proportion of embryonic and fetal losses was significantly increased in pregnancies with targeted NT derived sheep and porcine embryos. The first piglets, carrying a knockout for one allele of the α-galactosyltransferase gene did not show gross abnormalities (Lai et al., 2002; Dai et al., 2002). By performing two rounds of targeting in cell culture, both alleles can be knocked out and time-consuming breeding will be avoided in future (Fig. 5). Exploiting the targeting technology would allow to produce large amounts of human serum albumin (HSA) when inserted at the BSA locus in transgenic cattle. A knockout of the PrP gene locus could render cattle non-susceptible to BSE. Disease models in large animals would be another promising application models for targeted transgenesis. The pig is obviously a good model for human eye disease (Petters et al., 1997; Theuring et al., 1997) and sheep could be a good model for cystic fibrosis.

4.4. Conditional mutagenesis and region-specific knockouts

The above findings demonstrate the power of gene targeting, but they also point to a need for additional genetic techniques when precise genetic modifications are desired. Gene knockouts per se have no spatial or temporal restriction. As the targeted gene product

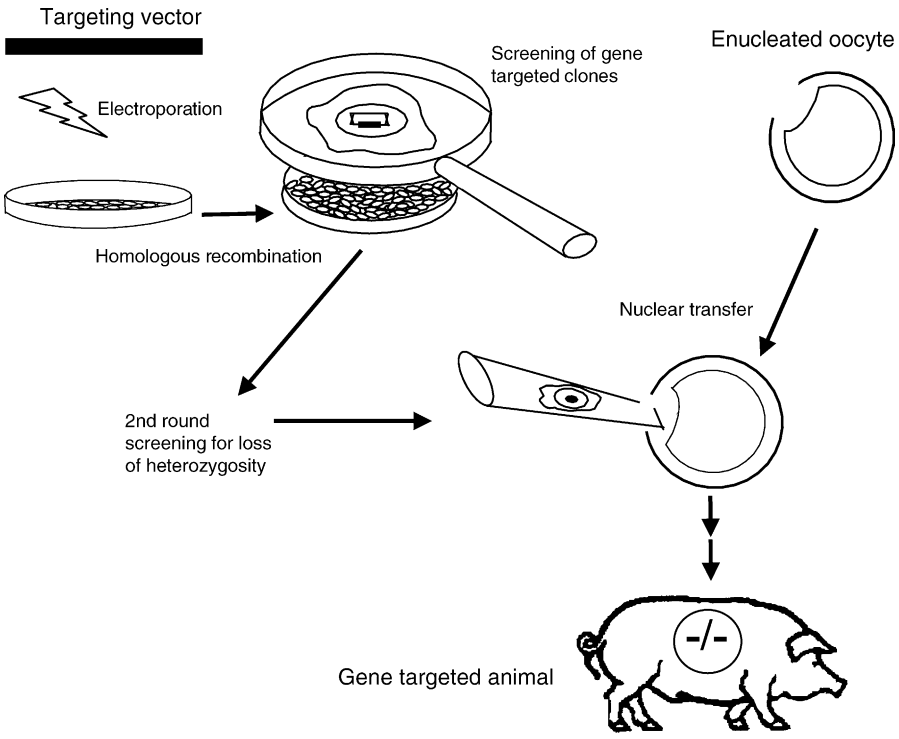


Fig. 5. Schematic drawing of gene targeting in livestock. Gene targeting of somatic primary cells by homologous recombination employing a promoterless targeting vector. Optionally, cell clones with the desired targeting event could be screened for loss of heterozygosity and subsequently be employed in nuclear transfer.

is absent for the entire life of the animal in all cells, it is difficult to assign a phenotype to a specific knockout as a mutant organism may compensate for the loss of a gene product or the knockout may have complex, secondary effects, depending on the genetic background (Gerlai, 1996), or may even result in embryonic lethality. A powerful tool for the design of genetic switches and for accelerating the creation of genetically modified animals, is the Cre site-specific DNA recombinase of bacteriophage P1 (Sauer, 1998). A single 38 kDa Cre protein is required to catalyze recombination between two loxP recognition sites, which are 34 bp DNA sequences. Recombination can occur between directly repeated loxP sites on the same molecule to excise the intervening DNA sequence, irrespective of whether the recognition sites are located on a plasmid or an mammalian chromosome (Sauer and Henderson, 1988). The combination of tissue-specific promoter elements with Cre DNA recombinase, enables a gene to be knocked out of a restricted cell type or tissue (Orban et al., 1992; Gu et al., 1994). In principle, recombinase mediated recombination should allow gene replacement, e.g. the exchange of the reading frames of milk proteins with the sequences of genes encoding pharmaceutical proteins.

5. 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 in animal production. However, major prerequisites will be the continuous refinement of reproductive biotechnologies and a rapid increase of livestock genome mapping. This would permit exploitation the great potential of bio- and gene technology with regard to a diversified animal production. One attractive example could be dairy production (Bawden et al., 1994). Apart from conventional dairy products, it could be possible to produce fat-reduced or even fat-free milk via knockout of enzymes involved in lipid metabolism, to increase curd and cheese production by enhancing expression of the casein gene family in the mammary gland, to increase efficiency of the production for creamers and liquor by increasing the proportion of β-casein in milk, to create "hypoallergic" milk by knockout of the β-lactoglobulin gene, to generate lactose-free milk via knockout of α-lactalbumin which is the key molecule in milk sugar synthesis, to produce small infant milk in which human lactoferrin is abundantly available or to produce milk with a highly improved hygienic standard via an increased level of lysozyme or other anti-microbiological substances in the udder. Lactose free-milk could render dairy products ready for consumption by a large proportion of the world's population who do not possess the active enzyme lactase in their intestine. In addition, calculations have revealed that reduction of the fat level in milk from the current level of 3.8–2% would permit an increase in the proportion of roughage in the feed from 55 to more than 80% and concomitantly to a significant reduction in the concentrated feed (Yom and Bremel, 1993). This would reduce the price and cost of animal nutrition. Such targeted production is also imaginable for other areas of animal production. However, one has to take into account that commercial application of this technology for agricultural traits will likely not be possible before the next decade. The biomedical area will see the first commercial application before the year 2010 (Table 4).

On a broader scale, bio- and gene technology as mentioned above will be important tools to cope with the challenges in animal production in the years ahead. In light of the worldwide problems in the management of a proper environment and resources, biotechnology can contribute to quantitative and qualitative increases in food production, cost reduction,

Table 4
Projections for field application of transgenic livestock[a]

	Year
Biomedical traits	
Recombinant pharmaceutical proteins	<2004
Xenogenic cells/tissue	>2005
Xenotransplantation of solid organs	>2008
Agricultural traits	
Dairy products	>2010
Meat and meat products	>2008
Wool products	>2005

[a] Application will likely be different between USA and Europe because of the controversial debate on genetic engineering in Europe.

environmental protection, maintenance of genetic resources and improvements in animal welfare as well as the above mentioned diversified production. However, it should be kept in mind that putative biomedical applications like xenotransplantation or usage of transgenic farm animals for food production will require strict standards on "genetic security" and reliable and sensitive methods for the molecular characterization of the "products". A major contribution towards this goal will come from DNA chips or arrays establishing "fingerprint" profiles at the transcriptional and/or protein level and allowing in depth insight into the proper function of a transgenic organism. Besides the technical problems, public acceptance of recombinant products, organs or food has to be taken into account. Additionally, welfare of the transgenic animals must be secured and pain or suffering due to the genetic modification could not be tolerated (Fox, 1988; Hughes et al., 1996).

Acknowledgements

The authors express their sincere gratitude to Christa Knöchelmann for the careful preparation of this manuscript.

References

Anderson, G.B., 1999. Embryonic stem cells in agricultural species. In: Murray, J.D., Anderson, G.B., Oberbauer, A.M., McGloughlin, M.M. (Eds.), Transgenic Animals in Agriculture. CABI Publishing, New York, USA, pp. 57–66.

Anger, M., Kues, W.A., Klima, J., Mielenz, M., Kubelka, M., Motlik, J., Esner, M., Dvorak, P., Carnwath, J.W., Niemann, H., 2003. Cell cycle based expression of Plk1 in synchronized porcine fetal fibroblasts, Mol. Reprod. Dev. 65, 245–253.

Auchincloss Jr., H., Sachs, D.H., 1998. Xenogenic transplantation. Annu. Rev. Immunol. 16, 433–470.

Bach, F.H., 1998. Xenotransplantation: problems and prospects. Ann. Rev. Mol. 49, 301–310.

Bach, F.H., Ferran, C., Soares, M., Wrighton, C.J., Anrather, J., Winkler, H., Robson, S.C., Hancock, W.W., 1997. Modification of vascular responses in xenotransplantation: inflammation and apoptosis. Nat. Med. 3, 944–948.

Baguisi, A., Behboodi, E., Melican, D., Pollock, J.S., Destrempes, M.M., Cammuso, C., Williams, J.L., Nims, S.D., Porter, C.A., Midura, P., Palacios, M.J., Ayres, S.L., Denniston, R.S., Hayes, M.L., Ziomek, C.A., Meade, H.M., Godke, R.A., Gavin, W.G., Overström, E.W., Echelard, Y., 1999. Production of goats by somatic cell nuclear transfer. Nat. Biotechnol. 17, 456–461.

Baltimore, D., 2001. Our genome unveiled. Nature 409, 814–816.

Bawden, W., Passey, R.J., MacKinlay, A.G., 1994. The genes encoding the major milk specific proteins and their use in transgenic studies and protein engineering. Biotechnol. Genet. Eng. Rev. 12, 89–137.

Betthauser, J., Forsberg, E., Augenstein, M., Childs, L., Eilertsen, K., Enos, J., Forsythe, T., Golueke, P., Koppang, R., Lesmeister, T., Mallon, K., Mell, G., Misica, P., Pace, M., Pfister-Genskow, M., Strelchenko, N., Voelker, G., Watt, S., Thompson, S., Bishop, M., 2000. Production of cloned pigs from in vitro systems. Nat. Biotechnol. 18, 1055–1059.

Björklund, A., 1991. Neural transplantation—an experimental tool with clinical possibilities. Trends Neurosci. 14, 319–322.

Boquest, A.C., Day, B.N., Prather, R.S., 1999. Flow cytometric cell cycle analysis of cultured porcine fetal fibroblasts cells. Biol. Reprod. 60, 1013–1019.

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

Brem, G., Besenfelder, U., Aigner, B., Müller, M., Liebl, I., Schütz, G., Montoliu, L., 1996. YAC transgenesis in farm animals: rescue of albinism in rabbits. Mol. Reprod. Dev. 44, 56–62.

Brown, W.R.A., Mee, P.J., Shen, M.H., 2000. Artificial chromosomes: ideal vectors? TIBTECH 18, 218–223.

Cabot, R.A., Kuhholzer, B., Chan, A.W., Lai, L., Park, K.W., Chong, K.Y., Schatten, G., Murphy, C.N., Abeydeera, L.R., Day, B.N., Prather, R.S., 2001. Transgenic pigs produced using in vitro matured oocytes infected with a retroviral vector. Anim. Biotechnol. 12, 205–214.

Campbell, K.H., McWhir, J., Ritchie, W.A., Wilmut, I., 1996. Sheep cloned by nuclear transfer from a cultured cell line. Nature 380, 64–66.

Capecchi, M.R., 1989. The new mouse genetics: altering the genome by gene targeting. TIG 5, 70–76.

Castilla, J., Pintado, B., Sola, I., Sánchez-Morgado, J.M., Enjuanes, L., 1998. Engineering passive immunity in transgenic mice secreting virus-neutralizing antibodies in milk. Nat. Biotechnol. 16, 349–354.

Chan, A.W.S., Homan, E.J., Ballou, L.U., Burns, J.C., Bremel, R.D., 1998. Transgenic cattle produced by reverse-transcribed gene transfer in oocytes. Proc. Natl. Acad. Sci. USA 95, 14028–14033.

Chang, K., Qian, J., Jiang, M., Liu, Y.H., Wu, M.C., Chen, C.D., Lai, C.K., Lo, H.L., Hsiao, C.T., Brown, L., Bolen Jr., J., Huang, H.I., Ho, P.Y., Shih, P.Y., Yao, C.W., Lin, W.J., Chen, C.H., Wu, F.Y., Lin, Y.J., Xu, J., Wang, K., 2002. Effective generation of transgenic pigs and mice by linker based sperm-mediated gene transfer. BMC Biotechnol. 2, 5.

Cibelli, J.B., Stice, S.L., Golueke, P.L., Kane, J.J., Jerry, J., Blackwell, C., Ponce de Leon, F.A., Robl, J.M., 1998a. Transgenic bovine chimeric offspring produced from somatic cell-derived stem like cells. Nat. Biotechnol. 16, 642–646.

Cibelli, J.B., Stice, S.L., Golueke, P.L., Kane, J.J., Jerry, J., Blackwell, C., Ponce de Leon, F.A., Robl, J.M., 1998b. Cloned calves produced from nonquiescent fetal fibroblasts. Science 280, 1256–1258.

Co, D.O., Borowski, A.H., Leung, J.D., Kaa van der, J., Hengst, S., Platenburg, G., Pieper, F.R., Perez, C.F., Jirik, F.R., Drayer, J.I., 2000. Generation of transgenic mice and germline transmission of a mammalian artificial chromosome introduced into embryos by pronuclear microinjection. Chrom. Res. 8, 183–191.

Colman, A., 2000. Somatic cell nuclear transfer in mammals: progress and applications. Cloning 1, 185–200.

Cozzi, E., White, D.J.G., 1995. The generation of transgenic pigs as potential organ donors for humans. Nat. Med. 1, 964–966.

Cozzi, E., Masroar, S., Soni, B., Vial, C., White, D.J., 2000. Progress in xenotransplantation. Clin. Neprol. 53, 13–18.

Dai, Y., Vaught, T.D., Boone, J., Chen, S.H., Phelps, C.J., Ball, S., Monahan, J.A., Jobst, P.M., McCreath, K.J., Lamborn, A.E., Cowell-Lucero, J.L., Wells, K.D., Colman, A., Polejaeva, I.A., Ayares, D.L., 2002. Targeted disruption of the alpha 1,3-galactosyltransferase gene in cloned pigs. Nat. Biotechnol. 20, 251–255.

Damak, S., Jay, N.P., Barrell, G.K., Bullock, D.W., 1996a. Targeting gene expression to the wool follicle in transgenic sheep. Biotechnology 14, 181–184.

Damak, S., Su, H., Jay, N.P., Bullock, D.W., 1996b. Improved wool production in transgenic sheep expressing insulin-like growth factor 1. Biotechnology 14, 185–188.

Deacon, T., Schumacher, J., Dinsmore, J., Thomas, C., Palmer, P., Kott, S., Edge, A., Penney, D., Kassissieh, S., Dempsey, P., Isacson, O., 1997. Histological evidence of fetal pig neutral cell survival after transplantation into a patient with Parkinson's disease. Nat. Med. 3, 350–353.

Diamond, L.E., Quinn, C.M., Martin, M.J., Lawson, J., Platt, J.L., Logan, J.S., 2001. A human CD46 transgenic pig model system for the study of discordant xenotransplantation. Transplantation 71, 132–142.

Dinsmore, L.E., Manhardt, C., Raineri, R., Jacoby, D.B., 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.

Edge, A.S.B., Gosse, M.E., Dinsmore, J., 1998. Xenogenic cell therapy: current progress and future developments in porcine cell transplantation. Cell Transplant. 7, 525–539.

Espanion, G., Halter, R., Wrenzycki, C., Carnwath, J.W., Herrmann, D., Lemme, E., Niemann, H., Paul, D., 1997. mRNA expression of human blood coagulation factor VIII (FVIII) gene constructs in transgenic mice. Transgenics 2, 175–182.

Evans, M.J., Kaufman, M.H., 1981. Establishment in culture of pluripotential cells from mouse embryos. Nature 292, 154–156.

Eyestone, W.H., 1999. Production and breeding of transgenic cattle using in vitro embryo production technology. Theriogenology 51, 509–517.

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

Fink, J.S., Schumacher, J.M., Ellias, S.L., Palmer, E.P., Saint-Hilaire, M., Shannon, K., Penn, R., Starr, P., VanHoorne, C., Kott, H.S., Dempsey, P.K., Fischman, A.J., Raineri, R., Manhart, D., Dinsmore, J., Isacson, O., 2000. Porcine xenografts in Parkinson's disease and Huntington's disease patients: preliminary results. Cell Transplant. 2, 273–278.

Fodor, W.L., Williams, B.L., Matis, L.A., Madri, J.A., Rollins, S.A., Knight, J.W., Velander, W., Squinto, S.P., 1994. Expression of a functional human complement inhibitor in a transgenic pig as a model for the prevention of xenogenic hyperacute organ rejection. Proc. Natl. Acad. Sci. USA 91, 11153–11157.

Forsberg, E.J., Betthauser, J., Strelchenko, N., Golueke, P., Childs, L., Jurgella, R., Koppang, G., Mell, G., Pace, M.M., Bishop, M., 2001. Cloning non-transgenic and transgenic cattle. Theriogenology 55, 269 (abstract).

Fox, M.W., 1988. Genetic engineering biotechnology: animal welfare and environmental concerns. Appl. Anim. Behav. Sci. 20, 83–94.

Fujiwara, Y., Miwa, M., Takahashi, R., Hirabayashi, M., Suzuki, T., Ueda, M., 1997. Position-independent and high-level expression of human alpha-lactalbumin in the milk of transgenic rats carrying a 210-kb YAC DNA. Development 124, 3621–3632.

Fujiwara, Y., Miwa, M., Takahashi, R., Hirabayashi, M., Suzuki, T., Ueda, M., 1999. High-level expressing YAC vector for transgenic animal bioreactors. Mol. Reprod. Dev. 52, 414–420.

Furth, P.A., Onge, L., Böger, H., Gruss, P., Gossen, M., Kistner, A., Bujard, H., Hennighausen, L., 1994. Temporal control of gene expression in transgenic mice by a tetracycline-responsive promoter. Proc. Natl. Acad. Sci. USA 91, 9302–9306.

Gandolfi, F., 1998. Spermatozoa, DNA binding and transgenic animals. Transgenic Res. 7, 147–155.

Gerlai, R., 1996. Gene-targeting studies of mammalian behavior: is it the mutation or the background genotype. Trends Neurosci. 19, 177–181.

Golovan, S.P., Meidinger, R.G., Ajakaiye, A., Cottrill, M., Wiederkehr, M.Z., Barney, D.J., Plante, C., Pollard, J.W., Fan, M.Z., Hayes, M.A., Laursen, J., Hjorth, J.P., Hacker, R.R., Phillips, J.P., Forsberg, C.W., 2001. Pigs expressing salivary phytase produce low-phosphorus manure. Nat. Biotechnol. 19, 741–745.

Gossen, M., Freundlieb, S., Bender, G., Müller, G., Hillen, W., Bujard, H., 1995. Transcriptional activation by tetracyclines in mammalian cells. Science 268, 1766–1769.

Graveley, B.R., 2001. Alternative splicing: increasing diversity in the proteomic world. Trends Genet. 17, 100–107.

Greenstein, J., Sachs, D.H., 1997. The use of tolerance for transplantation across xenogenic barriers. Nat. Biotechnol. 15, 235–238.

Grobet, L., Martin, L.J., Poncelet, D., Pirottin, D., Brouwers, B., Riquet, J., Schoeberlein, A., Dunner, S., Menissier, F., Massabanda, J., Fries, R., Hanset, R., Georges, M., 1997. A deletion in the bovine myostatin gene causes the double-muscled phenotype in cattle. Nat. Genet. 17, 71–74.

Groth, C.G., Korsgren, O., Tibell, A., Tollemar, J., Möller, E., Bolinder, J., Östman, J., Reinholt, F.P., Hellerström, C., Andersson, A., 1994. Transplantation of porcine fetal pancreas to diabetic patients. Lancet 344, 1402–1404.

Gu, H., Marth, J.D., Orban, P.C., Mossmann, H., Rajewsky, K., 1994. Deletion of a DNA polymerase β gene segment in T cells using cell type-specific gene targeting. Science 265, 103–106.

Gunsalus, J.R., Brady, D.A., Coulter, S.M., Gray, B.M., Edge, A.S.B., 1997. Reduction of serum cholesterol in Watanabe rabbits by xenogenic hepatocellular transplantation. Nat. Med. 3, 49–53.

Halter, R., Carnwath, J., Espanion, G., Herrmann, D., Lemme, E., Niemann, H., Paul, D., 1993. Strategies to express factor VIII gene constructs in the ovine mammary gland. Theriogenology 39, 137–149.

Haskell, R.E., Bowen, R.A., 1995. Efficient production of transgenic cattle by retroviral infection of early embryos. Mol. Reprod. Dev. 40, 386–390.

Hernandez, D., Mee, P.J., Martin, J.E., Tybulewicz, V.L., Fisher, E.M., 1999. Transchromosomal mouse embryonic stem cell lines and chimeric mice that contain freely segregating segments of human chromosome 21. Hum. Mol. Genet. 8, 923–933.

Hiripi, L., Makovics, F., Baranyi, M., Paul, D., Carnwath, J.W., Bösze, Z., Niemann, H., 2003. Expression of active human blood clotting factor VIII in the mammary gland of transgenic rabbits. DNA Cell Biol., 22, 41–45.

Hogan, B., Constantini, F., Lacy, E., 1994. Manipulating the Mouse Embryo: A Laboratory Manual. Cold Spring Harbour Laboratory, Cold Spring Harbour Press, Plainview, NY.

Hughes, B.O., Hughes, G.S., Waddington, D., Appleby, M.-C., 1996. Behavioural comparison of transgenic and control sheep: movement order, behaviour on pasture and in covered pens, behaviour on pasture and in covered pens. J. Anim. Sci. 63, 91–101.

Hyttinen, J.-M., Peura, T., Tolvanen, M., Aalto, J., Alhonen, L., Sinervirta, R., Halmekytö, M., Myöhänen, S., Jänne, J., 1994. Generation of transgenic dairy cattle from transgene-analyzed and sexed embryos produced in vitro. Biotechnology 12, 606–608.

Ikeno, M., Grimes, B., Okazaki, T., Nakano, M., Saitoh, K., Hoshino, H., McGill, I., Cooke, H., Masumoto, H., 1998. Construction of YAC-based mammalian artificial chromosomes. Nat. Biotechnol. 16, 431–439.

Imaizumi, T., Lankford, K.L., Burton, W.V., Fodor, W., Kocsis, J.D., 2000. Xenotransplantation of transgenic pig olfactory ensheathing cells promotes axonal regeneration in rat spinal cord. Nat. Biotechnol. 18, 949–953.

Jiang, Y., Jahagirdar, B.N., Reinhardt, R.L., Schwartz, R.E., Keene, C.D., Ortiz-Gonzalez, X.R., Reyes, M., Lenvik, T., Lund, T., Blackstad, M., Du, J., Aldrich, S., Lisberg, A., Low, W.C., Largaespada, D.A., Verfaillie, C.M., 2002. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418, 41–49.

Jones, I., 1996. 2010—a pig odyssey. Nat. Biotechnol. 14, 698–699.

Kambadur, R., Sharma, M., Smith, T.P., Bass, J.J., 1997. Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle. Genome Res. 7, 910–916.

Kato, Y., Tani, T., Sotomaru, Y., Kurokawa, K., Kato, J., Doguchi, H., Yasue, H., Tsunoda, Y., 1998. Eight calves cloned from somatic cells of a single adult. Science 282, 2095–2098.

Kilby, N.J., Snaith, M.R., Murray, J.A.H., 1993. Site-specific recombinases: tools for genome engineering. TIG 9, 413–421.

Kues, W.A., Carnwath, J.W., Paul, D., Niemann, H., 1998. A DNA-vector-based selection strategy for totipotent porcine cell lines. Theriogenology 49, 223 (abstract).

Kues, W.A., Anger, M., Carnwath, J.W., 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.

Kues, W.A., Carnwath, J.W., Paul, D., Niemann, H., 2002. Cell cycle synchronization of porcine fetal fibroblasts by serum deprivation initiates a nonconventional form of apoptosis. Cloning Stem Cells 4, 231–243.

Kühn, R., Schwenk, F., Aguet, M., Rajewsky, K., 1995. Inducible gene targeting in mice. Science 269, 1427–1429.

Kuroiwa, Y., Kasinathan, P., Choi, Y.J., Naeem, R., Tomizuka, K., Sullivan, E.J., Knott, J.G., Duteau, A., Goldsby, R., Osborne, B.A., Ishida, I., Robl, J., 2002. Cloned transchromosomic calves producing human immunoglobulin. Nat. Biotechnol. 20, 889–894.

Lai, L., Kolber-Simonds, D., Park, K.W., Cheong, H.T., Greenstein, J.L., Im, G.S., Samuel, M., Bonk, A., Rieke, A., Day, B.N., Murphy, C.N., Carter, D.B., Hawley, R.J., Prather, R.S., 2002. Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science 295, 1089–1092.

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

Lemme, E., Eckert, J., Carnwath, J.W., Niemann, H., 1994. Expression of 6WTK-LacZ gene construct in in vitro produced bovine embryos following microinjection into pronuclei or cytoplasm. Theriogenology 41, 236 (abstract).

Martin, G.R., 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc. Natl. Acad. Sci. USA 78, 7634–7638.

Martin, U., Tacke, S.J., Simon, A., Schröder, C., Wiebe, K., Lapin, B., Haverich, A., Denner, J., Steinhoff, G., 2002. Absence of PERV specific humoral immune response in baboons after transplantation of porcine cells of organs. Transplant. Int. 15, 361–368.

Matsui, Y., Zsebo, K., Hogan, B.L., 1992. Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell 70, 841–847.

Mayford, M., Mansuy, I.M., Muller, R.U., Kandel, E.R., 1997. Memory and behavior: a second generation of genetically modified mice. Curr. Biol. 7, 580–589.

McCreath, K., Howcroft, J., Campbell, K.H.S., Colman, A., Schnieke, A., Kind, A., 2000. Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. Nature 405, 1066–1069.

McPherron, A.C., Lee, S.J., 1997. Double muscling in cattle due to mutations in the myostatin gene. Proc. Natl. Acad. Sci. USA 94, 12457–12461.

McPherron, A.C., Lawler, A.M., Lee, S.J., 1997. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature 38, 83–90.

Meade, H.M., Echelard, Y., Ziomek, C.A., Young, M.W., Harvey, M., Cole, E.S., Groet, S., Smith, T.E., Curling, J.M., 1999. Expression of recombinant proteins in the milk of transgenic animals. In: Fernandez, J.M., Hoeffler, J.P. (Eds.), Gene Expression Systems. Academic Press, San Diego, USA, pp. 399–427.

Michler, R.E., 1996. Xenotransplantation: risks and prospects. Xeno 4, 21–26.

Modrek, B., Lee, C., 2001. A genomic view of alternative splicing. Nat. Genet. 30, 13–19.

Monaco, A.P., Larin, Z., 1994. YACs, BACs, PACs and MACs: artificial chromosomes as research tools. TIBTECH 12, 280–286.

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

Murray, J.D., 1999. Genetic modification of animals in the next century. Theriogenology 51, 149–159.

Niemann, H., Kues, A.W., 2000. Transgenic livestock: premises and promises. Anim. Reprod. Sci. 60–61, 277–293.

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 54, 21–24.

Niemann, H., Halter, R., Paul, D., 1994. Gene transfer in cattle and sheep: a summary perspective. In: Proceedings of the Fifth World Congress on Genetics Applied to Livestock Production, vol. 21, Guelph, Canada, pp. 339–346.

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

Niemann, H., Verhoeyen, E., Wonigeit, K., Lorenz, R., Hecker, J., Schwinzer, R., Hauser, H.J., Kues, W.A., Halter, R., Lemme, E., Herrmann, D., Winkler, M., Wirth, D., Paul, D., 2001. CMV early promoter induced expression of hCD59 in porcine organs provides protection against hyperacute rejection. Transplantation 72, 1898–1906.

Niemann, H., Döpke, H.H., Hadeler, K.G., 2002a. Production of transgenic ruminants by DNA microinjection. In: Pinkert, C. (Ed.), Transgenic Animal Technology: A Laboratory Handbook, 2nd ed. Academic Press, San Diego, USA, pp. 337–357.

Niemann, H., Wrenzycki, C., Lucas-Hahn, A., Brambrink, T., Kues, W.A., Carnwath, J.W., 2002b. Gene expression patterns in bovine in vitro produced and nuclear transfer derived embryos and their implication for early development. Cloning Stem Cells 4, 29–37.

Nottle, M.B., Nagashima, H., Verma, P.J., Du, Z.T., Grupen, C.G., Ashman, R.J., Macllfatrick, S., 1997. Developments in transgenic techniques in pigs. J. Reprod. Fertil. Suppl. 52, 237–244.

Nottle, M.B., Nagashima, H., Verma, P.J., Du, Z.T., Grupen, C.G., McIlfatrick, S.M., Ashman, R.J., Harding, M.P., Giannakis, C., Wigley, P.L., Lyons, I.G., Harrison, D.T., Luxford, B.G., Campbell, R.G., Crawford, R.J., Robins, A.J., 1999. Production and analysis of transgenic pigs containing a metallothionein porcine growth hormone gene construct. In: Murray, J.D., Anderson, G.B., Oberbauer, A.M., McGloughlin, M.M. (Eds.), Transgenic Animals in Agriculture. CABI Publishing, New York, USA, pp. 145–156.

O'Brien, S.J., Menotti-Raymond, M., Murphy, W.J., Nash, W.G., Wienberg, J., Stanyon, R., Copeland, N.G., Jenkins, N.A., Womack, J.E., Graves, J.A.M., 1999. The promise of comparative genomics in mammals. Science 286, 458–481.

Oldmixon, B.A., Wood, J.C., Ericcson, T.A., Wilson, C.A., White-Scharf, M.E., Andersson, G., Greenstein, J.L., Schuurman, H.J., Patience, C., 2002. Porcine endogenous retrovirus transmission characteristics of an inbred herd of miniature swine. J. Virol. 76, 3045–3048.

Orban, P.C., Chui, D., Marth, J.D., 1992. Tissue- and site-specific DNA recombination in transgenic mice. Proc. Natl. Acad. Sci. USA 89, 6861–6865.

Paleyanda, R.K., Velander, W.H., Lee, T.K., Scandella, D.H., Gwazdauskas, F.C., Knight, J.W., Hoyer, L.W., Drohan, W.H., Lubon, H., 1997. Transgenic pigs produce functional human factor VIII in milk. Nat. Biotechnol. 15, 971–975.

Paradis, K., Langford, G., Long, Z., Heneine, W., Sandstrom P., Switzer, W.M., Chapman, L.E., Lockey, C., Onions, D., The XEN 111 Study Group, Otto, E., 1999. Search for cross-species transmission of porcine endogenous retrovirus in patients treated with living pig tissue. Science 285, 1236–1241.

Park, K.W., Cheong, H.T., Lai, L., Im, G.S., Kuhholzer, B., Bonk, A., Samuel, M., Rieke, A., Day, B.N., Murphy, C.N., Carter, D.B., Prather, R.S., 2001. Production of nuclear transfer-derived swine that express the enhanced green fluorescent protein. Anim. Biotechnol. 12, 173–181.

Patience, C., Takeuchi, Y., Weiss, R.A., 1997. Infection of human cells by an endogenous retrovirus of pigs. Nat. Med. 3, 282–286.

Patience, C., Patton, G.S., Takeuchi, Y., Weiss, R.A., McClure, M.O., Rydberg, L., 1998. No evidence of pig DNA of retroviral infection in patients with short-term extracorporal connection of pig kidneys. Lancet 352, 699–701.

Perez, C., Jong de, G., Drayer, J., 2000. Satellite DNA-based artificial chromosomes—chromosomal vectors. TIBTECH 18, 402–403.

Perry, A.C.F., Wakayama, T., Kishikawa, H., Kasai, T., Okabe, M., Toyoda, Y., Yanagimachi, R., 1999. Mammalian transgenesis by intracytoplasmic sperm injection. Science 284, 1180–1183.

Perry, A.C., Rothman, A., de las Heras, J.I., Feinstein, P., Mombaerts, P., Cooke, H.J., Wakayama, T., 2001. Efficient metaphase II transgenesis with different transgene archetypes. Nat. Biotechnol. 19, 1071–1073.

Petit-Zeman, S., 2001. Regenerative medicine. Nat. Biotechnol. 19, 201–206.

Petters, R.M., Alexander, C.A., Wells, K.D., Collins, E.B., Sommer, J.R., Blanton, M.R., Rojas, G.H.Y., Flowers, W.L., Banin, E., Cideciyan, A.V., Jacobson, S.G., Wong, F., 1997. Genetically engineered large animal model for studying cone photoreceptor survival and degeneration in retinitis pigmentosa. Nat. Biotechnol. 15, 965–970.

Piedrahita, J.A., Moore, K., Oetama, B., Lee, C.K., Scales, N., Ramsoondar, J., Bazer, F.W., Ott, T., 1998. Generation of transgenic porcine chimeras using primordial germ cell-derived colonies. Biol. Reprod. 58, 1321–1329.

Platt, J.L., Lin, S.S., 1998. The future promises of xenotransplantation. In: Fishman, J., Sachs, D., Shaikh, R. (Eds.), Xenotransplantation—Scientific Frontiers and Public Policy. Ann. New York Acad. Sci. 862, 5–18.

Polejaeva, I.A., Chen, S.H., Vaught, T.D., Page, R.L., Mullins, J., Ball, S., Dal, Y., Boano, J., Walker, S., Ayares, D., Colman, A., Campbell, K.H.S., 2000. Cloned pigs produced by nuclear transfer from adult somatic cells. Nature 407, 505–509.

Pursel, V.G., Rexroad Jr., C.E., 1993. Status of research with transgenic farm animals. J. Anim. Sci. 71, 10–19.

Resnick, J.L., Bixler, L.S., Cheng, L., Donovan, P.J., 1992. Long-term proliferation of mouse primordial germ cells in culture. Nature 359, 550–551.

Rexroad Jr., C.E., 1992. Transgenic technology in animal agriculture. Anim. Biotechnol. 3, 1–13.

Rudolph, N.S., 1999. Biopharmaceutical production in transgenic livestock. TIBTECH 17, 367–374.

Sauer, B., 1998. Inducible gene targeting in mice using the Cre/lox system. Methods 14, 381–392.

Sauer, B., Henderson, N., 1988. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. Proc. Natl. Acad. Sci. USA 85, 5166–5170.

Schedl, A., Beermann, F., Thies, E., Montoliu, L., Kelsey, G., Schütz, G., 1992. Transgenic mice generated by pronuclear injection of a yeast artificial chromosome. Nucleic Acids Res. 20, 3073–3077.

Schedl, A., Montoliu, L., Kelsey, G., Schütz, G., 1993. A yeast artificial chromosome covering the tyrosinase gene confers copy number-dependent expression in transgenic mice. Nature 362, 258–261.

Schnieke, A.E., Kind, A.J., Ritchie, W.A., Mycock, K., Scott, A.R., Ritchie, M., Wilmut, I., Colman, A., Campbell, K.H.S., 1997. Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science 278, 2130–2133.

Schultze, N., Burki, Y., Lang, Y., Certa, U., Bluethmann, H., 1996. Efficient control of gene expression by single step integration of the tetracycline system in transgenic mice. Nat. Biotechnol. 14, 499–503.

Seidel Jr., G.E., 1993. Resource requirements for transgenic livestock research. J. Anim. Sci. 71, 26–33.

Shim, H., Gutierrez-Adan, A., Chen, L.R., BonDurant, R.H., Behboodi, E., Anderson, G.B., 1997. Isolation of pluripotent stem cells from cultured porcine primordial germ cells. Biol. Reprod. 57, 1089–1095.

Squires, E.J., 1999. Status of sperm-mediated delivery methods for gene transfer. In: Murray, J.D., Anderson, G.B., Oberbauer, A.M., McGloughlin, M.M. (Eds.), Transgenic Animals in Agriculture. CABI Publishing, New York, USA, pp. 87–96.

Strauss, W.M., Dausman, J., Beard, C., Johnson, C., Lawrence, J.B., Jaenisch, R., 1993. Germ line transmission of a yeast artificial chromosome spanning the murine α_1 collagen locus. Science 259, 1904–1907.

Switzer, W.M., Michler, R.E., Shanmugan, V., Matthews, A., Hussain, A.I., Wright, A., Sandstrom, P., Chapman, L., Weber, C., Safley, S., Denny, R.R., Navarro, A., Evans, V., Norein, A.J., Kwiatkowski, P., Heneine, W., 2001. Lack of cross-species transmission of porcine endogenous retrovirus infection to nonhuman primate recipients of porcine cells, tissues and organs. Transplantation 71, 959–965.

Theuring, F., Thunecke, M., Kosciessa, U., Tuern, J.D., 1997. Transgenic animals as models of neurodegenerative disease in humans. TIBTECH 15, 320–325.

Thibier, M., 2001. The animal embryo transfer industry in figures. IETS-Newsletter 19, 16–22.
Thomas, M., Northrup, R., Hornsby, P.J., 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. Nat. Med. 3, 978–983.
Tomizuka, K., Shinohara, T., Yoshida, H., Uekima, H., Ohguma, A., Tanaka, S., Sato, K., Oshimura, M., Ishida, I., 2000. Double trans-chromosomic mice: maintenance of two individual human chromosome fragments containing Ig heavy and kappa loci and expression of fully human antibodies. Proc. Natl. Acad. Sci. USA 97, 722–727.
van Berkel, P.H., Welling, M.M., Geerts, M., van Veen, H.A., Ravensbergen, B., Salaheddine, M., Pauwels, E.K., Pieper, F., Nuijens, J.H., Nibbering, P.H., 2002. Large scale production of recombinant human lactoferrin in the milk of transgenic cows. Nat. Biotechnol. 20, 484–487.
Vos, J.-M., 1997. The simplicity of complex MACs. Nat. Biotechnol. 15, 1257–1259.
Wakayama, T., Perry, A.C.F., Zuccotti, M., Johnson, K.R., Yanagimachi, R., 1998. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 394, 369–374.
Wall, R.J., 1996. Transgenic livestock: progress and prospects for the future. Theriogenology 45, 57–68.
Wall, R.J., Hawk, H.W., Nel, N., 1992. Making transgenic livestock: genetic engineering on a large scale. J. Cell. Biochem. 49, 113–120.
Wheeler, M.B., 1994. Development and validation of swine embryonic stem cells: a review. Reprod. Fertil. Dev. 6, 563–568.
Wheeler, M.B., Bleck, G.T., Donovan, S.M., 2001. Transgenic alteration of sow milk to improve piglet growth and health. Reprod. Suppl. 58, 313–324.
White, D., 1996a. Alteration of complement activity: a strategy for xenotransplantation. TIB 14, 3–5.
White, D., 1996b. hDAF transgenic pig organs: are they concordant for human transplantation. Xeno 4, 50–54.
Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J., Campbell, K.H., 1997. Viable offspring derived from fetal and adult mammalian cells. Nature 385, 803–810.
Wilmut, I., Beaujean, N., De Sousa, P.A., Dinnyes, A., King, T.J., Paterson, L.A., Wells, D.N., Young, L.E., 2002. Somatic cell nuclear transfer. Nature 419, 583–586.
Yang, Y.W., Model, P., Heintz, N., 1997. Homologous recombination based modification in *Escherichia coli* and germline transmission in transgenic mice of a bacterial artificial chromosome. Nat. Biotechnol. 15, 859–865.
Yarranton, G.T., 1992. Inducible vectors for expression in mammalian cells. Curr. Opin. Biotechnol. 3, 506–511.
Yom, H.-C., Bremel, R.D., 1993. Genetic engineering of milk composition: modification of milk components in lactating transgenic animals. Am. J. Clin. Nutr. 58 (Suppl.), 299–306.
Young, L.E., Sinclair, K.D., Wilmut, I., 1998. Large offspring syndrome in cattle and sheep. Rev. Reprod. 3, 155–163.
Zaidi, A., Schmoeckel, M., Bhatti, F., Waterworth, P., Tolan, M., Cozzi, E., Chavez, G., Langford, G., Thiru, S., Wallwork, J., White, D., Friend, P., 1998. Life-supporting pig to primated renal xeno-transplantation using genetically modified donors. Transplantation 65, 1584–1590.
Zawada, W.M., Cibelli, J.B., Chei, P.K., Clarkson, E.D., Golueke, P.J., Witta, S.E., Beil, K.P., Kane, J., Abel Ponce de Leon, F., Jerry, D.J., Robl, J.M., Freed, C.R., Stice, S.L., 1998. Somatic cell cloned transgenic bovine neurons for transplantation in Parkinson rats. Nat. Med. 4, 569–574.
Ziomek, C.A., 1998. Commercialization of proteins produced in the mammary gland. Theriogenology 49, 139–144.