Aus dem
Department für Veterinärwissenschaften
der Tierärztlichen Fakultät
der Ludwig-Maximilians-Universität München

Arbeit angefertigt unter der Leitung von
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(Re)producing transgenic pigs for xenotransplantation – selection of founder animals and establishment of breeding herds

Inaugural Dissertation
zur Erlangung der tiermedizinischen Doktorwürde
der Tierärztlichen Fakultät
der Ludwig-Maximilians-Universität München

von
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aus München

München 2011

Gedruckt mit der Genehmigung der Tierärztlichen Fakultät
der Ludwig-Maximilians-Universität München

Dekan: Univ.-Prof. Dr. Braun
Berichterstatter: Univ.-Prof. Dr. Wolf
Korreferent: Univ.-Prof. Dr. Zerbe

Tag der Promotion
30. Juli 2011

Für den Puck

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ADP	adenosine diphosphate
aPC	activated protein C
APP	amyloid precursor protein
BAC	bacterial artificial chromosome
bla	blasticidin
bp	base pair
cDNA	complementary DNA
CFTR	cystic fibrosis transmembrane conductance regulator
cm	centimetre
CTLA4-Ig	cytotoxic T-lypmphocyte antigen 4 immunoglobulin
DOP-PCR	degenerate oligonucleotide primed PCR
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
eNOS	endothelial cell nitric oxide synthase
ENU	N-ethyl-N-nitrosurea
ET	embryo transfer
ELISA	enzyme linked immunosorbent assay
F	inbreeding coefficient
FACS	fluorescence-activated cell-sorting
F1 generation	first filial generation
F2 generation	second filial generation
Gal	α1,3-galactosyl-transferase
GalKO	Gal knock-out
gDNA	genomic DNA
GIP	glucose-dependent insulinotropic polypeptide
GIPR	GIP receptor
$GIPR^{dn}$	dominant negative GIPR
GLP-1	glucagon-like peptide-1

hDAF	human decay accelerating factor
HLA-E	human leukocyte antigen E
HNF1A	hepatocyte nuclear factor 1 alpha
hPBMCs	human peripheral blood mononuclear cells
HRP	horseradish peroxidase
hTM	human thrombomodulin
INS	insulin
Ins2	mouse insulin2
kb	kilo base
l	litre
LB	lysogeny broth
M	mole
MDA	multiple displacement amplification
mg	milligramm
ml	milliliter
MODY3	maturity onset diabetes in the young
mM	millimolar
mmol	millimole
mRNA	messenger RNA
MVG	Moorversuchsgut
µg	microgram
µl	microlitre
neo	neomycin
ng	nanogram
NK cells	natural killer cells
nm	nanometer
NOD-SCID	non obese diabetic-severe combined immunodeficiency
NT	nuclear transfer
PCR	polymerase chain reaction

PEP	primer extension preamplification
PSEN1	presenilin 1
PSEN2	presenilin 2
RNA	ribonucleic acid
RNase	ribonuclease
RP	retinitis pigmentosa
RT	room temperature
RT-PCR	reverse transcription PCR
SCNT	somatic cell nuclear transfer
SLA	swine leukocyte antigen
STZ	streptozotocin
TAE	tris-acetate buffer
TFPI	tissue factor pathway inhibitor
TRAIL	tumor necrosis factor-alpha-related apoptosis-inducing ligand
UV	ultraviolet

1 INTRODUCTION

The importance of pigs in translational biomedical research has been on a constant increase, as their anatomical and physiological suitability as model animals is distinct (Aigner et al. 2010). Furthermore, pigs are considered a feasible source of replacement organs or tissues in the context of xenotransplantation (Petersen et al. 2009). But potential donor animals need to be tailored in their genetic properties as an imperative prerequisite for overcoming detrimental graft rejection processes (Sachs and Galli 2009). Somatic cell nuclear transfer has evolved into the preferential transgenic technology for achieving this (Melo et al. 2007). However, even though it is a successful method for generating novel transgenic pig lines, efficiency in large scale reproduction of already established lines has been disappointingly low (Palmieri et al. 2008). A feasible rectification of this issue can be found in the establishment of breeding herds where transgenic pigs are expanded by means of natural reproduction. By this, substantial numbers of experimental animals can be generated within a viable time frame. The conflicting matters of inbreeding and segregation of multiple transgenes, however, have to be taken into account. Rising inbreeding coefficients have been connected to lower productivity of breeding stock (Charlesworth and Charlesworth 1987; Ralls et al. 1988; Lynch 1989; 1991). While homozygosity of transgene integration sites would rectify the problem of transgene segregation and limit time requirements, it can only be achieved on the expense of inbreeding. Reproduction of already established (multiple) transgenic pigs by breeding can therefore be accomplished if the issues of time, transgene segregation and inbreeding are weighed against each other and a suitable breeding strategy that accommodates all of them is identified. When incorporating novel transgenes into already established breeding herds, selection of transgenic founder animals has to be performed on the basis of careful evaluation of genomic and expression analyses in order to be able to fully exploit cumulative effects of transgenes in multiple transgenic animals.

The aim of this work was to identify a suitable breeding strategy for already established lines of transgenic pigs for xenotransplantation research and select founders from novel transgenic lines for incorporation into the breeding herd.

2 REVIEW OF THE LITERATURE

2.1 The pig as a model species in biomedical research

Although the predominant species of animals in biomedical research are still rodents, the pig becomes an ever more important model animal for numerous applications (Swindle in Conn 2008), especially in the context of 'translational' medicine that spans the gap between basic research and clinical trials (Wehling 2008).

Rodent models may have a well defined genetic background and suit research in terms of space requirements and subsequent cost effectiveness (Rand 2010), but generally, surgery or sample taking as well as instrumentation are more easily accomplished in animals larger than a mouse (Roberts et al. 2009). This alone does not necessitate the pig as a model species for biomedical research. However, pigs share many more anatomical and physiological similarities with humans than mice, rats or other large domestic animals do. Consequently, the pig models the human situation in various ways more accurately than other species do.

Pigs are truly omnivorous animals and among all large domestic animals their gastrointestinal morphology, digestive effectiveness, as well as their energy metabolism (Aigner et al. 2010; Miller and Ullrey 1987; Spurlock and Gabler 2008) correspond to that of humans most closely, making swine the suitable candidates for research in the fields of digestion, nutrition, or metabolic syndrome. Meyer (1996) reported on the similarities in porcine and human epidermal and dermal structures, including the unpronounced body hair layer and the size, orientation and distribution of blood vessels. Wound healing in pigs has been found to resemble that in humans in many ways (Sullivan et al. 2001). Cardiovascular structures in pigs share numerous anatomical and physiological characteristics with humans, for example similar sized heart and blood vessels (Smith et al. 1990), a right side dominant conduction system as can be found in the majority of humans, and no significant collateral circulation in the coronary system (Swindle in Conn 2008). For these reasons, the pig is a popular model for research on myocardial infarction. Porcine and human lungs feature abundant common characteristics. Rogers et al. (2008) reviewed the available

information on aspects of porcine airways and lung that relate to Cystic Fibrosis, and could show that porcine and human lungs correspond with respect to volume, development of lobularity, pleural structure, and vascular supply, amongst other aspects.

In addition to these physiological and morphological analogies, Wernersson et al. could demonstrate in 2005 that on the genomic level the pig is also much more similar to humans than the mouse is. By generating ≈ 3.84 million shotgun sequences (0.66X coverage) from the pig genome, he found that almost all ultra-conserved elements in the human genome can also be detected in the pig, putting it in closer evolutionary relationship to man than rodents.

Compared to other large domestic animals, pigs are superior with respect to their reproductive performance (Aigner et al. 2010). Relatively early sexual maturity, short generation intervals, and large litters in combination with year round breeding constitute desirable traits in animal models.

Disease in pig models may be of spontaneous onset, for example artherosclerosis, obesity or gastric ulcers (Roberts et al. 2009), or it may be surgically or medicinally induced (Swindle in Conn 2008). Alternatively, pigs may be genetically modified for the development of new disease models or for applications in the field of xenotransplantation.

2.2 Established pig models

2.2.1 Neurodegenerative diseases

Alzheimer's disease is a neural disorder leading to memory loss, confusion and, ultimately, to a breakdown of bodily functions and death. While in most cases Alzheimer's is a multifactorial disease that occurs sporadically, a familial form of autosomal dominant inheritance also exists (Kragh et al. 2009). Causative genetic predispositions include mutations in the amyloid precursor protein (*APP*) gene, and in

the presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*) genes. These mutations are associated with a change in the production of the Aβ fragment of *APP* which eventually leads to neuron loss following the formation of neuritic plaques (Hardy et al. 2002).
Although mice transgenic for a mutated human *APP* gene form these neuritic plaques, extensive neuron loss does not occur in them (Takeuchi et al. 2000). Therefore, Kragh et al. (2009) postulated the generation of an Alzheimer's disease model in an animal that is evolutionarily closer to humans to obtain a more homologous model. They chose to produce a transgenic Göttingen miniature pig via so-called handmade cloning using a splice variant of human *APP* with a dominant mutation known to cause Alzheimer's disease. They gained seven healthy piglets that showed a strong expression of the transgenic protein in the brain. Neuropathological impairments are expected to develop as the pigs become older.

Huntington's disease affects specific neurons leading to impaired muscle coordination, cognitive decline and dementia. It is an autosomal dominant disorder ascribed to a mutation in the huntingtin gene (Graveland et al. 1985).
Mouse models with a targeted modification in their endogenous huntingtin gene that mimics the genetic situation in human patients fail to exhibit full Huntington phenotypes with widespread neuronal cell death (Wheeler et al. 1999). In an attempt to generate an alternative animal model, Matsuyama et al. (2000) identified and characterised the Huntington's disease gene homologue in miniature pigs and found that the disease-relevant segments of the gene and the protein expression profile were more similar to the human version than that of rodents. Five transgenic founder piglets derived from DNA microinjection into Göttingen miniature pig embryos and each with one to three integration sites of a mutated porcine huntingtin gene were generated by Uchida et al. (2001). Information about expression profiles or developing phenotypes has not been made available so far.

Retinitis pigmentosa is a group of heritable progressive retinal disorders leading to vision impairments and ultimately to blindness. A large number of different genes

have been associated with the development of retinitis pigmentosa, including mutations in the gene for rhodopsin, the visual pigment on the rod photoreceptors (Petters et al. 1997).

Various rodent models of retinal dystrophies are already available. However, differences in the number and distribution of photoreceptors, as well as in overall eye size, between the human and the rodent eye enforce limits on the usefulness of these models (Gregory-Evans and Weleber 1997). With respect to these particulars the porcine eye shares more similarities with the human eye suggesting that a pig eye model with a specific genetic flaw would react in a similar way as humans do (Gregory-Evans and Weleber 1997). Petters et al. (1997) describe the generation of transgenic pigs expressing a porcine rhodopsin with a mutation known to cause severe rod photoreceptor degeneration in man. One founder animal was established by DNA microinjection of the expression vector, and transmission and segregation of the transgenes over two generations led to two independent mutant pig lines. These pigs develop a form of retinal degeneration which closely corresponds to that of humans with the same mutation in the rhodopsin gene.

2.2.2 Cardiovascular diseases

Pig models in cardiovascular research are based largely on the many shared anatomical and physiological characteristics between pigs and humans. Most of these models make use of swine as experimental settings for invasive procedures, development of medical devices or for surgical or dietary induction of specific pathological conditions such as myocardial infarction or artherosclerosis (Swindle in Conn 2008).

However, the establishment of a transgenic pig over-expressing the endothelial cell nitric oxide synthase (eNOS) (Hao et al. 2006) provides a swine model on the basis of genetic modification for a better understanding of cardiovascular regulation. eNOS-derived nitric oxide is said to serve a wide array of important functions in the cardiovascular system. Vascular tone, vascular smooth muscle cell proliferation or thrombosis is all influenced by nitric oxide (Huang 2009). Endothelial dysfunction

involving a decrease in nitric oxide availability is a common feature of many cardiovascular risk factors, as has been confirmed by the phenotypes of numerous transgenic or knock-out rodent models (Huang et al. 1995; Moroi et al. 1998; Freedman et al. 1999). But several differences in the cardiovascular system of rodents and humans have made direct extrapolation of data to humans more feasible from a pig rather than a rodent model.

Hao et al. (2006) used additive gene transfer and subsequent somatic cell nuclear transfer to produce four Yucatan miniature pigs transgenic for a recombinant porcine eNOS protein. Expression of the transgenic eNOS on the vascular endothelium and distinction from the endogenous protein could be demonstrated. These pigs are to be used as models in long term studies further clarifying the role of eNOS in the cardiorespiratory system.

2.2.3 Diabetes mellitus

The term diabetes mellitus stands for a number of metabolic disorders with multifactorial genetic, immunological and lifestyle aetiology. They all share the common characteristic of elevated blood glucose levels due to insufficient availability of insulin in the sufferer. A distinction has to be made between type 1 diabetes, where the main causative matter is an inability of the pancreatic beta-cells to produce sufficient amounts of insulin (Tuomi 2005), and type 2 diabetes which results from a peripheral insulin resistance (Martin et al. 1992) leading to progressive pancreatic beta-cell dysfunction (Prentki et al. 2006). Other types of diabetes with various causes are less common.

In the past, numerous swine models with a diabetic pathogenesis that show symptoms of altered glucose tolerance and insulin resistance, or elevated blood glucose and insulin levels, had been established by selective breeding of certain strains of Yucatan (Phillips et al. 1982), Chinese Guizhou (Xi et al. 2004) and Göttingen (Johansen et al. 2001; Larsen et al. 2004) miniature pigs. Other diabetic swine models utilising Sinclair (Dixon et al. 1999) and Göttingen (Larsen et al. 2003) miniature pigs, developed the disease following induction by Streptozotocin or Alloxan

administration (Yamamoto et al. 1981).

Umeyama et al. (2009) established the first genetically modified pigs exhibiting the pathophysiological characteristics of diabetes. These pigs carry a dominant-negative mutation of the hepatocyte nuclear factor 1 alpha (HNF1A), causing the so-called type 3 of maturity-onset diabetes in the young (MODY3). Previously, this correlation had already been demonstrated in a comparable mouse model (Watanabe et al. 2007). A combined method of intracytoplasmic sperm injection-mediated gene transfer and somatic cell nuclear transfer was used to generate four viable transgenic animals that showed persistently elevated non-fasting blood glucose levels and abnormal oral glucose tolerance tests.

Recently, a transgenic pig model expressing a dominant-negative glucose-dependent insulinotropic polypeptide receptor ($GIPR^{dn}$) (Renner et al. 2010) has been generated and is expected to shed light on a feature in the clinical picture of type 2 diabetes that can be universally found in human patients: an impaired incretin function (Meier et al. 2001). The two incretin hormones glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) enhance glucose-induced insulin secretion in response to the presence of nutrients (Baggio and Drucker 2007). Earlier experiments of Herbach et al. (2005) indicated that over-expression of a dominant-negative GIP-receptor in mice leads to a severe diabetic phenotype with early-onset diabetes and a pronounced reduction and structural alteration in pancreatic islets. However, these findings contrast the effects of a lack of functional GIP receptor expression described for other mouse models (Miyawaki et al. 1999). Renner et al. (2010) generated $GIPR^{dn}$ pigs in order to clarify the role of GIP receptor signalling in the pathogenesis of impaired pancreatic islet function. Lentiviral vectors were used to generate transgenic pigs that mimic important aspects of human type 2 diabetes mellitus. These pigs initially display a reduced GIP action with impaired oral and, subsequently, also impaired intravenous glucose tolerance tests, and progress to a reduction in pancreatic beta-cell proliferation and overall beta-cell mass.

Additionally, Aigner et al. (2010) report on the ongoing establishment of a pig model with disturbed intravenous glucose tolerance and reduced insulin secretion due to a point mutation in the insulin (*INS*) gene. The corresponding mutation in the mouse

insulin2 (*Ins2*) causes a progressive diabetes mellitus with a pronounced reduction in total pancreatic islet and beta-cell volume (Herbach et al. 2007). Comprehensive data on the characterisation of this novel pig has not been made available yet.

2.2.4 Cystic Fibrosis

The pathology of cystic fibrosis involves multiple organs, including the pancreas, intestine, liver, vas deferens and, most commonly, the lung. Persistent airway inflammation and chronic bacterial infections leading to progressive lung destruction and pancreatic disease cause most of the morbidity and mortality in cystic fibrosis patients (Elston et al. 2007; Rogers et al. 2008a).

Cystic fibrosis is a disease of autosomal recessive inheritance of mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (*Cftr*) (Rogers et al. 2008; Meyerholz et al. 2010). More than 1000 different mutations in the *Cftr* gene are associated with the manifestation of cystic fibrosis (Welsh et al. 2009). The deletion of phenylalanine at position 508 (ΔF508) in the *Cftr* gene is the most common one (Davis et al. 1996), accounting for approximately 70% of cystic fibrosis alleles (Zielenski et al. 1995). The ΔF508 mutation causes different defects on human *Cftr*. In *Cftr*/ΔF508 patients, most of the mutant protein is retained within the endoplasmic reticulum and its maturation to the plasma membrane is prevented (Dorwart et al. 2004). In addition, chloride channel activity of the remaining processed *Cftr* is reduced, and the protein's stability on the cell surface is impaired (Swiatecka-Urban et al. 2005). The combination of these deficiencies results in the pathological manifestation of cystic fibrosis. However, the exact mechanisms underlying these processes and the extent to which each of the defects is responsible for the development of cystic fibrosis pathology, is as of now still largely unclear (Meyerholz et al. 2010a).

A number of genetically engineered mouse models with the ΔF508 mutation in their *Cftr* gene, displaying varying phenotypes from almost absent to near 100% mortality rate before maturity, have been characterised since the early 1990s (Guilbault et al. 2007). None of these mouse models develop the chronic lung inflammations that are

characteristic for the human cystic fibrosis pathology. Neither do any of them exhibit evidence of pancreatic disease (Colledge et al. 1995; Zeiher et al. 1995). Ostedgaard et al. (2007) postulate that the severity of the *Cftr*/ΔF508 processing defect is much more pronounced in humans than in mice, accounting for the obvious compensation mechanisms in some mutant mouse lines that prevent marked phenotypes. Their data also suggests that in pigs the effect of a ΔF508 mutation on the posttranslational *Cftr* processing is less than in humans but still a lot more profound than in mice.

The pig lung has already served as an excellent model for the normal and diseased human lung, or the effect of therapeutics, in many ways (Rogers et al. 2008). Structural and size similarities between porcine and human lungs (Jones et al. 1975) facilitate extrapolation of porcine data to the human situation. In the context of cystic fibrosis, particulars of electrolyte transport by airway epithelia are a critical point. In vitro experiments demonstrated quantitative similarities in epithelial ion transport between pig and man (Liu et al. 2007). However, even though defects in electrolyte transport due to a limited availability and reduced activity of the *Cftr* channels are seen as a hallmark of cystic fibrosis (Quinton 2007), knowledge about electrolyte transport in the human cystic fibrosis lung, especially during the neonatal period before onset of associated lung symptoms, is extremely limited (Rogers et al. 2008). Other parameters, such as mucociliary clearance defects, also call for detection before the development of severe lung disease. This, too, has been difficult to achieve in humans. On that account, the pig might become a useful tool in clarifying the (patho)mechanisms of the abnormal processes in cystic fibrosis affected lungs, and align the onset of symptoms to biochemical or morphological changes in the airways.

Rogers et al. (2008b) chose this species to establish two new animal models of cystic fibrosis because the pig has become increasingly popular in biomedical research, and its similarities with man in terms of anatomical, histological, biochemical, and physiological features are distinct.

Pigs with *Cftr*-null alleles were generated to lack any *Cftr* function, so a full porcine cystic fibrosis phenotype could be observed. Adeno-associated virus-mediated gene targeting and subsequent somatic cell nuclear transfer were employed to insert the targeting vector into the *Cftr* gene via homologous recombination. Nine male

heterozygous *Cftr*-null piglets were gained, which sired numerous heterozygous male and female offspring. By mating male and female heterozygous animals to each other, Rogers et al. (2008a) established homozygous *Cftr*-null pigs. Loss of CFTR chloride channel activity could be detected in newborn piglets, as well as the development of meconium ileus, exocrine pancreatic insufficiency, focal biliary cirrhosis and gall bladder abnormalities. This phenotype mimics that of human newborns with cystic fibrosis, however the symptoms appear to be accelerated and more severe in these particular pigs (Meyerholz et al. 2010a). Lung affection could also be demonstrated in homozygous *Cftr*-null piglets. Evidence for defects in eradicating bacteria from the lung was found shortly after birth. Over time, the pigs developed spontaneous lung disease that was largely similar to that observed in human patients, such as spontaneous inflammation, airway remodelling, mucus accumulation and chronic bacterial infection (Stoltz et al. 2010).

Additionally, Rogers et al. (2008b) attempted the establishment of a pig carrying the ΔF508 mutation in its *Cftr* gene. The techniques applied in the generation of these pigs were the same as were used for the *Cftr*-null model, and produced four transgenic animals. This model might offer a progression towards understanding the mechanisms of *Cftr* metabolism. However, so far a phenotypic evaluation of these pigs has not been made available.

2.3 Pigs as donor animals in the context of xenotransplantation

In xenotransplantation lies a great potential for providing life-saving treatment for patients with many end-stage diseases leading to organ failure. The gap between demand and availability of appropriate allogeneic organs for transplantation to treat these patients is ever increasing as life expectancy rises (Petersen et al. 2009). As a result, non-human sources of replacement organs and tissues have to be explored and exploited (Ekser et al. 2009).

Pigs are considered a feasible source because of their physiological and organ size

similarities to humans, in addition to their growth capacity and favourable breeding characteristics (Petersen et al. 2009; Sachs and Galli 2009). To date, the biggest hurdle in utilising pigs as donor animals for organs or tissue remains in the immunological barriers between the porcine and human organism (d'Apice and Cowan 2009; Sprangers et al. 2008; Yang and Sykes 2007). Immunological rejection of the xenograft that cannot be controlled by immunosuppressant regimens is the result.

Various strategies for genetically altering pigs in order to overcome these immunological incompatibilities (Sachs and Galli 2009) have already been, or are currently being, pursued. Building on the availability of a number of different techniques for modification of the porcine genome that can be applied without posing major ethical problems (Sprangers et al. 2008), significant progress in graft survival in pig to non-human primate transplantation settings has already been achieved (Petersen et al. 2009). The mechanisms leading to xenograft rejection include several different immunological processes that have to be addressed by tailored modification of the donor pig in order to be successful in prolonging graft survival.

2.3.1 Hyperacute xenograft rejection

Hyperacute rejection occurs within seconds to minutes or hours of transplantation (Rand 2010) and is characterised by an almost immediate loss of graft function and distinct changes in the physical appearance of the graft (Petersen et al. 2009). It is mediated primarily by natural antibodies that are directed against carbohydrate epitopes synthesised by the enzyme α1,3-galactosyl-transferase (Gal) (Klymiuk et al. 2010). Most species, including pigs, express this enzyme. However, humans, apes and Old World Monkeys do not. Moreover, they possess natural preformed antibodies that are able to bind these Gal epitopes on porcine vascular endothelium, leading to an activation of the complement system and coagulation cascade, and the subsequent destruction of the xenograft (Yang and Sykes 2007).

By generating pigs lacking a functional Gal expression, the antigens become absent from the donor organs and cannot trigger a reaction by the recipient. Various approaches in targeting the gene for Gal in pigs have been described (Dai et al. 2002;

Lai et al. 2002; Harrison et al. 2004; Ramsoondar 2003; Sharma et al. 2003) . Phelps et al. (2003) reported on the establishment of the first Gal knock out (GalKO) pigs completely deficient of a functional version of the enzyme. These pigs were generated by two rounds of homologous recombination with a knockout targeting vector and subsequent somatic cell nuclear transfer. Expression analysis including an in-vivo immunogenicity test in GalKO mice demonstrated the absence of Gal epitopes on the porcine cells. Other transgenic pigs lacking functional Gal expression through loss of heterozygosity mutations have been established since (Kolber-Simonds et al. 2004).

A range of different organs derived from GalKO pigs, from kidneys (Yamada et al. 2005) and lungs (Schroeder et al. 2005) to hearts (Kuwaki et al. 2005; Hisashi et al. 2008; Shimizu et al. 2008), have already been put to test in various xenogenic transplantation and perfusion settings, demonstrating the prolongation of graft survival in the absence of Gal epitopes, and the lowered requirements for immunosuppression (Tseng et al. 2005).

Lowering the incidence of Gal epitopes in porcine tissues by expressing enzymes that utilise the same substrate as Gal and therefore compete with it (Koike et al. 1997; Sharma et al. 1996; Costa et al. 1999; Miyagawa et al. 2001), constitute an alternative approach to combating the effect preformed xenoreactive antibodies have on the donor organ or tissue. However, since even smallest amounts of Gal epitopes trigger immunological reactions in the graft recipients, this method seems insufficient in preventing hyperacute rejection (Yang and Sykes 2007).

The expression of one or more human complement regulators on porcine tissue is a critical component in creating the optimal donor pig (d'Apice and Cowan 2009). The cross-species incompatibilities in controlling complement activation mean that porcine complement regulators are not efficient in directing human complement activation and vice versa (Miyagawa et al 1988). The expression of human decay accelerating factor (hDAF), CD59 and/or CD46 on porcine cells have been the most favoured strategies for overcoming this problem.

Expressing hDAF on porcine tissues has been shown to suppress endothelial activation and reduce thrombin generation in transplants (Miwa et al. 2010). Different attempts at generating pig lines transgenic for hDAF have been made by a number of

groups (Cozzi et al. 1994; Langford et al. 1994; McCurry et al. 1995; Lavitrano et al. 2002), each being successful at expressing the protein endothelial cell specifically.
Transgenesis for the complement membrane attack complex inhibitor CD59 has been used by others in order to protect pig organs from recipient complement (Fodor et al. 1994; McCurry et al. 1995; Diamond et al. 1996).
CD46 is an inhibitory complement receptor which, in the physiological situation, prevents cells from damage through autologous complement (Liszewski et al. 1996). Diamond et al. (2001) describe the establishment of a transgenic pig line expressing high levels of human CD46 in a pattern similar to the endogenous situation in man. In-vivo experiments indicated the effectiveness of this approach in overcoming hyperacute rejection. Comparable results were achieved by Zhou et al. (2002), who subsequently used these pigs to generate a double transgenic line with hDAF. Kidney transplantation experiments into baboons using organs of CD46 transgenic pigs established by Loveland et al. (2004) provided further evidence for the effective protection of transgenic tissues against antibody and complement-mediated lysis or damage.
In addition, a variety of multiple transgenic pigs expressing two or three genes of the above have been generated by Byrne et al. (1997), Chen et al. (1999) or Cowan et al. (2000), among others.

2.3.2 Acute humoral xenograft rejection

If hyperacute rejection is prevented, acute humoral rejection develops (Schuurman et al. 2003), likely induced by low levels of natural and elicited xenoreactive antibodies (Yang and Sykes 2007). The exact mechanisms that trigger the ensuing complement activation and thrombotic microangiopathy are not known yet, however, results of Shimizu et al. (2006) indicate that incomplete cross-species regulation of complement activation might contribute to the process. Cowan and d'Apice (2008) point out that the ultimate killer of most xenografts has proven to be thrombosis. Immune mediated endothelial injury converts the normally anticoagulant endothelial surface to a procoagulant state (Bach et al. 1994). This is regarded as the most important aspect in

the development of microvascular thrombosis occurring during xenograft rejection. A number of factors modulating platelet activation and the coagulation cascade have been under discussion for being able to prevent the destruction of the xenograft by coagulopathies.

CD39 is responsible for the inhibition of ADP-induced platelet aggregation. Studies have shown that transgenic expression of CD39 is able to protect transplants from thrombosis (Dwyer et al. 2004).

Tissue factor pathway inhibitor (TFPI) targets tissue factor, which is the initiator of the extrinsic activation of the coagulation cascade. Contrary to earlier findings of Kopp et al. (1997), Lee et al. (2008) could show that porcine TFPI exercises similar anticoagulant activity in human coagulation as does human TFPI. Since studies were performed in-vitro, however, the in-vivo effect still remains to be elucidated.

Human thrombomodulin (hTM) is a membrane protein on endothelial cells. It is an important factor in the generation of the anticoagulant activated protein C (aPC). By forming a thrombin/thrombomodulin complex, it enhances the activation level of protein C 20-fold (Taylor et al. 2001). As had been suggested in in-vitro studies, porcine thrombomodulin is unable to efficiently bind human thrombin (Siegel et al. 1997; Kopp et al. 1998), resulting in an inadequate activation of human protein C and subsequently in increased coagulation. Roussel et al. (2008) could show that even though porcine thrombomodulin binds to human thrombin and reduces the procoagulant characteristics of thrombin significantly, its cofactor activity in the thrombin/thrombomodulin complex for protein C activation is only ~10% that of human thrombomodulin. Incorporating hTM in xenogeneic transplant tissues by expressing it on endothelia might therefore offer benefits in terms of coagulation cascade inhibition. As Petersen et al. (2009) report, fibroblasts isolated from hTM transgenic pigs showed an elevated production of activated protein C in an in-vitro coactivity assay. So far, possible in-vivo effects have not been published yet.

2.3.3 Cell-mediated xenograft rejection

In allotransplantation, cellular rejection mechanisms are the major issues that have to be dealt with. This chronic rejection process occurs between days and weeks after transplantation and is mainly mediated by T- lymphocytes and macrophages (Petersen et al. 2008). It can be combated by detailed immunosuppressive protocols. However, incompatibilities between human and porcine cell interactions may limit the effectiveness of these protocols in cross-species transplantation (Petersen et al. 2009). Transgenic strategies to overcome this cell-mediated rejection of xenografts build on the expression of T-cell modulating genes in donor pigs.

Different approaches including the selective inhibition of $CD4^+$-T-cell activation by proteins such as CTLA4-Ig or LEA29Y (Mirenda et al. 2005; Huurman et al. 2007; Phelps et al. 2009) have been employed in achieving this. Martin et al. (2005) established transgenic pigs that express CTLA4-Ig brain-specifically to be used as neuron donors for potential treatment of neurodegenerative disorders.

A different attempt used human tumor necrosis factor-alpha-related apoptosis-inducing ligand (TRAIL), which acts as an apoptosis inducing agent (Ursini-Siegel et al. 2002) and cell cycle inhibitor on human lymphocytes (Song et al. 2000). As Klose et al. (2005) demonstrated, lymphocytes derived from human TRAIL transgenic pigs are able to induce apoptosis in a line of immortalised T-lymphocytes in-vitro. Neutralising TRAIL with antibodies indicated the TRAIL-specificity of the effect.

Natural killer (NK) cells play a diverse role in cellular rejection processes in xenotransplantation settings. Apart from activating the porcine endothelium upon direct contact (Goodman et al. 1996), they have also been shown to infiltrate graft tissue (Khalfoun et al. 2000) and exercise direct and antibody-mediated cytotoxicity upon porcine cells (Rieben and Seebach 2005). The xenogeneic cytotoxic effect of human NK cells can be diminished by expression of the human major histocompatibility complex class I molecule human leukocyte antigen (HLA)-E on endothelial cells of pig organs, as demonstrated in-vitro by a number of authors (Matsunami et al. 2002; Rieben and Seebach 2005; Lilienfeld et al. 2007). HLA-E binds specifically to the inhibitory receptor CD94/NKG2A on activated human NK

cells and thereby prevents reactivity (Forte et al. 2005). To make use of this effect, Weiss et al. (2009) generated transgenic pigs by pronuclear microinjection of genomic fragments of HLA-E and human β2-microglobulin into zygotes. These animals showed a strong, consistent expression of HLA-E in endothelia of multiple organs. In-vitro NK cell cytotoxicity assays revealed that the capacity of NK cells to lyse HLA-E expressing target cells was markedly reduced, making a contribution of this transgene towards ameliorating xenograft survival likely.

Organs or tissue derived from transgenic pigs appear to be the most imminent option in the pursuit of utilising xenotransplantation in order to ameliorate transplant supply for our aging population. As many research groups and authors have demonstrated in the past years, this designated target seems to be drawing closer.

2.4 (Re)producing transgenic pigs

Genetic modification of pigs has been utilised for more than 25 years to refine and tailor large animal models for applications in translational biomedicine and xenotransplantation. A number of different techniques for additive gene transfer or targeted alterations of the porcine genome are available.

First successful attempts in producing transgenic large animals were made with pronuclear DNA microinjection (Brem et al. 1985; Hammer et al. 1985) whereby the gene of interest is directly injected into one of the pronuclei of a zygote. This technique, however, harbours some problems. Apart from the general inefficiency in producing viable embryos with this method (Nottle et al. 2001; Hofmann et al. 2003), the unpredictability of the integration site of the transgene leads to great variations in expression levels (Clark et al. 1994) or even mosaicism in the resulting embryos (Keefer 2004).

Gene transfer employing the infection capacity of lentiviruses allows integration of the transgene into very early embryos (Pfeifer 2004). In lentiviral transgenesis the viral genome is integrated into the host chromosome, providing a prerequisite for

stable transgene expression (Pfeifer and Hofmann 2009; Pfeifer et al. 2010). Efficiency of this method has proven higher than with pronuclear DNA microinjection (Hofmann et al. 2003; Whitelaw et al. 2004), however, stable expression of the transgene may be compromised by epigenetic silencing of promoter regions or coding sequences through DNA methylation (Hofmann et al. 2006).

Sperm-mediated gene transfer makes use of the ability of spermatozoa to incorporate exogenous DNA and pass it on to the oocyte during fertilisation (Lavitrano et al. 2006). In pigs this method has shown a comparably high efficiency while at the same time being very cost-effective (Lavitrano et al. 2003). Incorporation of multiple transgenes at the same time (Webster et al. 2005) is feasible but success of the procedure depends greatly on the selection of sperm donors and incubation parameters (Lavitrano et al. 2003).

Presently, the leading technique in generating transgenic livestock is somatic cell nuclear transfer, also known as cloning (Melo et al. 2007). Genetically altered cells originating from foetal, neonatal or adult donors are transferred into enucleated oocytes and the resulting embryos are subsequently transferred to recipient animals. Campbell et al. (1996) reported the first live offspring derived from this method in sheep. Since then, numerous attempts have been successful in applying this technique of generating transgenic animals to a variety of species, including pigs (Betthauser et al. 2000; Onishi et al. 2000; Polejaeva et al. 2000; Lagutina et al. 2007; Kurome et al. 2008). The great advantage of somatic cell nuclear transfer compared to other ways of generating transgenic animals is the option of site specific introduction of transgenes via homologous recombination (Lai et al. 2002; Rogers et al. 2008). So far, this method has been the only one that offers such a possibility.

Campbell et al. (2005) point out that judging the overall efficiency of cloning in producing transgenic offspring is difficult because of the large differences in experimental protocols, embryo selection and data presentation applied in the various reports. However, the percentage of live offspring derived from transferred embryos is generally quoted as below 5% (Palmieri et al. 2008; Aigner et al. 2010) across all examined species. A number of reasons have been suggested for this limited efficiency in the production of cloned piglets. Cloning procedure and in-vitro

manipulations might affect the embryos so embryonic signalling to the recipient becomes too weak (King et al. 2002; Petersen et al. 2008). Reduced intrauterine transport of the embryos (Schmidt et al. 2010) or breed differences between embryos and recipients have also been discussed as contributing factors (Estrada et al. 2008; Kurome et al. 2008; Koo et al. 2009). The major focus, however, has been on a perceived reduction in epigenetic reprogramming of the embryos attributed to the cloning procedure. This has been investigated and discussed extensively by numerous groups and authors (Dean et al. 2001; Humpherys et al. 2001; Khosla et al. 2001; Young et al. 2001; Jiang et al. 2007; Bonk et al. 2008). They have also linked a variety of developmental abnormalities to insufficient epigenetic reprogramming of the somatic donor cell (reviewed in Tian et al. 2009). However, cloned pigs that are actually born alive and grow to maturity appear to show no difference in reproductive characteristics compared to wild-type controls. Gestation length, litter size, birth and weaning weights are similar, as reported in a variety of studies (Martin et al. 2004; Mir et al. 2005; Williams et al. 2006; Shibata et al. 2006).

The low numbers of transgenic animals that can be generated through somatic cell nuclear transfer or any of the other methods make these procedures valuable tools for introducing new transgenes into the porcine genome. However, for routinely reproducing such animals, these methods are as of now too inefficient and cost intensive. Reproduction of already established transgenic lines by breeding seems a feasible alternative.

Cross-generational stability of transgene expression levels has been shown in transgenic cattle and pigs derived from somatic cell nuclear transfer (Bordignon et al. 2003; Brunetti et al. 2008), leading to the conclusion that continuous generation of transgenic animals by breeding might be an affordable and reproducible alternative to in-vitro techniques.

2.4.1 Pig breeds and pig breeding

In contrast to mouse models where one century of standardised breeding experience already exists and numerous suitable strains have been established and characterised,

systematic breeding of experimental herds for large animal models is only in the early stages of development. A large proportion of current knowledge about pig breeding derives from reproduction of conventionally utilised pig breeds in agriculture. The term breed refers to a group of animals of a defined species that has been selected by people for their heritable similarity in terms of appearance or productive and reproductive characteristics (Porter 1993).

In Europe, directed breeding of conventional pig lines for pork or lard production developed in England during the 18th century, when the increasing industrialisation led to urbanisation and an enhanced demand of food supplies that could not be derived from self-subsistence. Pigs from a number of different countries were imported to England and a crossbreed between Asian and Italian pigs with the existing landraces turned out the first modern pig breed, the Leicester pig. Small, Middle and Large (=Yorkshire) White followed during the first half of the 19th century. These breeds were spread throughout Europe and were used to improve the prevalent landraces (Nickels 1997). In Germany, a wide variety of new breeds developed until the mid-20th century. The late 1950s brought a change in the demographics of pig breeds. Pigs that had been bred for lard production were successively eliminated and breeding began to centralise on meat production to satisfy the increasing demand during the economic miracle (v. Lengerken and Wicke in v. Lengerken et al. 2006). German Landrace became the predominant breed by far. Other breeds such as Hampshire, Duroc, Angler Saddle Pig or Swabian-Hall were at some point during the 1970s on the verge of extinction. However, over the course of the 1980s, a few of these breeds were rediscovered (Nickels 1997). Today, the most common commercial pig breeds in Germany are German Landrace, German Large White and Pietrain or, comprising the majority of animals in conventional pig production, hybrids of these breeds (Horst and Gregor in Kräußlich and Brem 1997). Some of the rediscovered breeds such as Swabian-Hall, Duroc or Hampshire pigs constitute niche populations in pork production on the one hand, and an important component in the conservation of old farm animal breeds on the other hand.

In contrast to pig livestock, miniature pigs have been bred specifically for experimental use and, later and to a lesser extent, as pets. Miniature pig is the generic

term for growth-restricted pig breeds or lines with varying heights. Generally, pigs with an adult weight of less than 100 kg are called miniature pigs, even though the majority are much smaller, most weighing around 15-45 kg when they reach sexual maturity (Fisher 1993). The goal in miniature pig breeding was to create a conveniently sized animal for research that would be able to compete with other experimental animal species and make use of the anatomical and physiological similarities between pigs and humans but without the disadvantages of commercial pig breeds. Miniature pigs require less housing space, are easier to handle and are more cost-effective in terms of feed and experiments due to lower material requirements, compared to larger pigs (Swindle 2007). In 1949 the Hormel Institute of the University of Minnesota initiated a project to develop a breed of miniature swine specifically for use in biomedical research (Bustad 1966). One of the first miniature pigs, the Minnesota miniature pig, arose from this effort. Today, there are over 50 other breeds of miniature pigs worldwide, but only a few of them are regularly used for research. The most commonly utilised breeds include the Göttingen, Sinclair, Yucatan, and Hanford miniature pigs (Swindle in Conn 2008).

The Hanford miniature pig, for example, has served as a model for a number of neonatal and paediatric diseases (Glauser 1966; Cohen et al. 1990; 1991). Yucatan and Sinclair pigs have been extensively used in diabetes research (Phillips et al 1982; Dixon et al. 1999; 2002) and as models of cardiovascular disease (Wissler and Vesselinovitch 1968; Gal et al. 1990). Sachs et al. (1976) reported on the establishment of a partially inbred herd of miniature swine homozygous for a specific swine leukocyte antigen allele. This model has been put to use in various transplantation settings, such as skin grafting (Leight et al. 1978), bone marrow transmissions (Pennington et al. 1988) and liver transplantations (Flye et al. 1999). By sequential brother-sister matings, the co-ancestry, also known as inbreeding, of this herd has reached levels greater than 90% (Mezrich et al. 2003; Cho et al. 2007).

This so-called inbreeding is defined by the probability that the two alleles at an autosomal locus of an individual are identical by descent, meaning that the parents of an individual must have one or more common ancestor for that individual to be termed inbred. Inbreeding is measured by the inbreeding coefficient (Wright 1922).

Dickerson et al. (1954) showed that in pigs inbreeding in the mother leads to a reduction in litter size of approximately 0.2 piglets per 10% rise in the inbreeding coefficient. As Dettmers and Rempel reported in 1968, this correlation became apparent during the recurrent mass selection for small size in the establishment of the Minnesota miniature pig breed. Although litter size underlies great variation depending on the exact breed but also on environmental factors, large, commercial pig breeds generally farrow more than 10 live piglets per litter (v. Lengerken and Wicke in v. Lengerken et al. 2006). Contrary to this, miniature pigs produce average litters of no more than 5-6 piglets (Swindle 2007). Due to the limited population size of miniature pig breeds, inbreeding becomes inevitable if specific characteristics of the breed are to be conserved. Other reproductive parameters such as the number of stillborn piglets per litter, the piglets raised in a litter or the average weight gain until weaning have been equally shown to negatively correlate with an increase in the inbreeding coefficient (Bradford et al. 1958). These unwanted effects, in addition to others, are summarised under the term inbreeding depression.

2.4.2 Inbreeding depression

Various studies have indicated that inbreeding causes a shift in mean phenotypes towards a reduction in fitness related characters, either taking shape in the form of distinct abnormalities or, in a less overt manner, in lower fertility, survival and growth rates of individuals with high inbreeding coefficients (Charlesworth and Charlesworth 1987; Ralls et al. 1988; Lynch 1989; 1991). This is called inbreeding depression. This phenomenon has been studied extensively in a variety of species, both theoretically (Charlesworth et al. 1990; Bataillon and Kirkpatrick 2000) and experimentally (Bradford et al. 1958; Bereskin et al. 1968; Casellas et al. 2009) because it appears to play an important role in the evolution of mating systems and challenge the viability of restricted populations (Glemin et al. 2003).

Inbreeding depression is triggered by increased homozygosity of individuals (Charlesworth and Willis 2009). The two rivalling hypotheses of partial dominance and overdominance are discussed as explanation for the lower overall fitness of inbred

individuals. Deleterious but normally recessive and rare traits phenotypically manifest themselves in individuals that are homozygous for these particular alleles. Because the likelihood for homozygosity at any given gene locus increases with inbreeding, recessive traits are more prevalent in inbred populations (Charlesworth and Charlesworth 1999). The inbred line becomes fixed for recessive detrimental alleles, leading to an overall reduced fitness of the population (Charlesworth and Charlesworth 1987). This explanation of partial dominance as the origin of inbreeding depression was first outlined by the maize scientist Davenport (1908).

In the hypothesis of overdominance, inbreeding depression is ascribed to the superiority of heterozygotes over homozygotes at specific gene loci (Charlesworth and Charlesworth 1987). Recessive or partially recessive alleles that in homozygosity result in a phenotype that decreases fitness can survive in a population because their heterozygous occurrence produces an advantage for the affected individual. For example, the overdominant sickle-cell allele in humans protects against malaria in heterozygotes, providing a distinct advantage in malaria-endemic regions. On the other hand, homozygosity for this partially recessive allele causes sickle-cell anaemia (Currat et al. 2002), greatly shortening the life expectancy of patients. In an inbred population, homozygosity for overdominant alleles increases and disadvantageous phenotypes become prevalent.

The level to which each of these phenomena contribute to inbreeding depression has been discussed extensively since these theories were formulated in the early 20th century, with shifting emphasis over the decades. In recent years, molecular evolutionary studies and fine mapping of genes involved in fitness variation support the notion that the main cause for inbreeding depression is to be found in an accumulation of recessive deleterious mutations at many gene loci, attributing a major part of inbreeding depression to the theory of partial dominance. Charlesworth and Willis (2009) argue that the higher fitness of heterozygotes rarely derives from single overdominant loci but only appears to do so because mapping of so-called quantitative trait loci does as yet not offer a resolution high enough to exactly define the responsible gene loci. Thus, the overall extent to which overdominant genes contribute to the phenomenon of inbreeding depression remains unclear, as it has not

been possible so far to identify single responsible genes and draw conclusions upon the extent of their effects.

It has been proposed that inbreeding can purge deleterious alleles when it is combined with selection pressure (Boakes et al. 2007; McParland et al. 2009), thereby avoiding the phenomenon of inbreeding depression. A notable example supporting this hypothesis is a study of the Chillingham cattle in Northern England. This herd of 93 (www.chillinghamwildcattle.com 2010) animals is genetically almost uniform as it has experienced no additions to its genetic pool in at least 300 years. Studies of blood groups and biochemical polymorphisms as well as microsatellite markers known for their polymorphisms in cattle (Visscher et al. 2001), demonstrated that the homozygosity of this herd far exceeds that of other cattle breeds or wild species. The continuing viability of this herd (animal numbers rose from 49 in 2000 to 93 in 2010) is seen as evidence for purging of deleterious alleles.

Heterosis is often described as the opposite of inbreeding depression (Glodek in Kräußlich 1994). This effect occurs as a result of outbreeding. Crossing individuals of two inbred lines or separate populations (for example two breeds) will lead to offspring that, on average, possesses greater fitness than the parent generation did (Brem in Kräußlich and Brem 1997). Analogously to inbreeding depression theory, this effect is attributed to a shift in allele distribution, in the case of heterosis in an increase in heterozygosity in the offspring generation. Again, as with inbreeding depression, the two concepts of partial dominance and overdominance have been employed in elucidating the genetic basis for heterosis (Charlesworth and Willis 2009).

2.4.3 Inbreeding in pig livestock and experimental animals

Animal genetic resources have contracted dramatically over the past decades, especially in areas of great economic importance and vast commercial impact such as the pig livestock industry (Welsh et al. 2010). As the number of core swine breeds employed in meat production has decreased, selection for highly specific productivity traits within these breeds has intensified at the same time. Meuwissen and Woolliams

(1994) suggest that fitness of offspring may decrease either due to inbreeding depression or as a negatively correlated response to artificial selection. According to Bereskin et al. (1968) the so-called margin-of-safety effect appears to provide a certain amount of resistance against the detrimental effects of homozygosity in the lower levels of inbreeding in pigs. This, however, does not apply to inbreeding levels beyond approximately 10%. When Donald (1955) gave an account on data drawn in Britain on the effect of inbreeding pig lines of the Large White breed, several productive and reproductive characteristics were shown to be negatively influenced by inbreeding. Abnormalities in new born piglets, for example, were about twice as prevalent in the inbred lines as they were in the outbred controls. Similarly, litter parameters deteriorated with increased inbreeding. Elaborating on this, Bereskin et al. (1968) differentiated between the effect inbreeding in the sire, dam or litter has on reproductive performance. While an inbred sire was shown to be of little consequence for litter size at farrowing, inbreeding in the dam significantly depressed not only litter size, but also average piglet weight and total litter weight. On the other hand, the inbreeding in the litters themselves effected an increased influence on weaning traits rather than on farrowing.

Closed swine breeding company nucleus herds supplying animal breeding stock for commercial swine producers undergo intense selection for specific traits (Rathje 2000), leading to greater likelihoods of elevated inbreeding levels that hamper productivity and are subsequently transferred to commercial pork production herds. For the European swine population, Laval et al. (2000) examined 11 European pig breeds with respect to their genetic diversity using microsatellite markers. They observed a strong clustering into individual breeds, each with a common genetic background, and significantly reduced heterozygosity in the case of the German Landrace breed as well as in the Swabian-Hall breed. Consanguineous mating, heightening inbreeding levels, is proposed as the likely cause for these findings. In a follow-up project, San Cristobal et al. (2006) presented data on a total of 58 European pig breeds and lines confirming and elaborating on the previously published material. Similarly, a study of Welsh et al. (2010), who analysed the pedigree of five swine breeds in the United States, reported that, presently, more than 99% of the surveyed

pigs are inbred. The majority display inbreeding coefficients of less than 10% but a notable exception was found in Landrace pigs, which feature a mean inbreeding coefficient of almost 18%. An earlier work of Hubbard et al. (1990) determined the rates of inbreeding in a number of performance tested breeds in Canada in the 1970s and 80s. They found increasing numbers of inbred animals in each of the tested herds as well as rising average inbreeding coefficients over the years.

Today, the intense selection pressure and competition within the swine industry makes strategies for controlling the accumulation of inbreeding ever more important (Rathje 2000). Blackburn and Welsh (2010) conclude that the actions of breeders are the key in managing the extent of inbreeding because it is within their scope to determine which animals to mate to one another in order to reach the best possible equilibrium between productivity and selection for other traits. Even though the avoidance of matings between close relatives does not lower the eventual rate of inbreeding within a herd, it nevertheless delays it (Meuwissen 2009). The frequently occurring shifts in breeding objectives may also contribute to delaying inbreeding in commercially exploited pig breeds even further.

Experimental pig herds, on the other hand, are used as resource populations to supply animals for biomedical research. Initiation of those herds and selection for required traits usually resulted from comparably small animal numbers. For example, a Sinclair miniature swine herd susceptible to the development of cutaneous malignant melanoma (Gomez-Raya et al. 2008) originated from only 10 animals. Similarly, the founding animals of Göttingen miniature pigs were a group of 16 Minnesota miniature and Vietnamese potbellied pigs (Simianer and Kohn 2010). The main breeding objective usually includes a very clearly defined physiological parameter such as body weight (Dettmers et al. 1965; Panepinto and Phillips 1981; Kohn et al. 2008) that is being selected for, or a certain genetic property for which populations need to be kept fairly closed in order to maintain the desired trait. In small populations this often results in a continuous increase in inbreeding and loss of alleles (Simianer and Kohn 2010) similar to that of closed seed stock herds in commercial pig production.

Reproductive capacity may be further impaired by a genetic and physiological antagonism between litter size and body weight. In a study analysing the

developmental capacity of cloned embryos, Koo et al. (2009) found that genes related to implantation and maintenance of pregnancy were significantly down-regulated in miniature pig fetuses compared to domestic pigs. In earlier experiments Diehl et al. (1986) had already concluded that the comparably small litters of miniature pigs are due to lower ovulation rates and an unfavourable uterine environment not wholly attributable to a depression originating in the 40% inbreeding coefficient of the examined miniature pig herd.

From an experimental viewpoint, variations in the genetic background of animals constitute factors that contribute to heterogeneity of experimental results (Sachs in Swindle 1992). Inbred strains of rodents are defined by at least 20 consecutive brother-to-sister matings resulting in homozygosity close to 100%. In mice, the resulting inbreeding depression has been shown to be most evident in intermediate generations (Issa and Seeland 2001) with inbreeding coefficients of around 40%. Over time, selection for fitness and reproductive capacity may offset the negative effect an increasing inbreeding coefficient has. Certain miniature swine herds have been established as highly inbred lines (Mezrich et al. 2003; Wang et al. 2006). However, fertility problems make reproduction in these herds a trying venture. A SLA homozygous miniature pig line first reported on by Sachs et al. (1976) has reached inbreeding levels greater than 90% (Mezrich et al. 2003; Cho et al. 2007). Data on their reproductive performance in 1992 already showed average litter sizes that were reduced to 4.5 piglets per sow (Koketsu et al. 1994). Similarly, an inbred herd of Westran pigs with an estimated inbreeding coefficient of 98% (O'Connell et al. 2005) has average litters of only 4 piglets. For domestic pig breeds Soe et al. (2008) also report on deteriorating reproductive performance in the establishment of a highly inbred SLA homozygous Duroc pig line. In a review on pigs utilised in xenotransplantation settings, d'Apice and Cowan (2009) pointed out that most groups that had generated GalKO pigs found that breeding these animals to homozygosity for this gene and establishing a GalKO herd proved difficult because the inbreeding necessary to achieve this resulted in very low fertility.

Consequently, breeding management of experimental pig herds must pursue the conservation of the desired physiological or genetic characteristics but it must also

include the goal of keeping enough genetic variation within the population in order to maintain a satisfactory reproduction level (Prather et al. 1997; Wang et al. 2006; Gomez-Raya et al. 2008; Simianer and Kohn 2010).

2.5 Selection of breeding material – expression analysis

Producing transgenic pigs by any of the earlier mentioned techniques, resulting in germline stability of the transgene in the genome, offer the opportunity to expand transgenic pig herds by means of natural reproduction. Additive gene transfer entails random integration of the transgene, thus leading to the possibility of an integration site influence on the expression potential of the transgene. In a first step, breeding schedules for transgenic pigs therefore require the identification of founder animals with appropriate transgene expression. Taking into account the potential of multiple integration loci and the resulting segregation during breeding, founder animals ideally carry only one integration site, necessitating genomic analysis in addition to expression profiles. In contrast to site-directed mutagenesis, the unpredictability of integration sites in additive gene transfer effects unknown endogenous genomic flanking regions of the transgene. Several possibilities exist for clarifying the adjacent genomic sequence.

Chromosome-walking based polymerase chain reactions (PCR) for DNA fragment walking adjacent to known sequences are available as different approaches. Inverse PCR (Ochman et al. 1988; 1990) or ligation-mediated PCR (Dai et al. 2000) are both based on genomic DNA fragmented by restriction digest. The first uses two specific primers that bind on the transgene sequence and face apart on the genomic DNA but subsequently face towards each other after circularisation by ligation. The latter uses only one transgene-specific primer as well as a primer binding on a synthetic adapter which is ligated to the genomic DNA fragments after digestion. Both methods have their limitations, mainly due to the unpredictable length of the genomic fragments and, especially in the case of the adapter-based method, amplification of unspecific

fragments. The use of nested PCR overcomes these limitations at least partially. Further methodological improvements have been presented (Ren et al. 2005; Bryda et al. 2006; Liang et al. 2008), but in all cases the increase in efficiency is paid for by a significant rise in effort. An alternative approach avoiding the unpredictable length of genomic DNA fragments has been described by Liu and Whittier (1995). The method is based on significantly different annealing temperatures of the primers used. In sequential steps, the high melting and transgene-specific primer is used for linear and single strand amplification of the transgene plus adjacent region, whereas the complementary strand is synthesised in a later step using the annealing of degenerate primers at lower temperatures. Several rounds of this procedure using semi-nested PCRs shift the proportion of unspecific amplicons to that of defined length towards the latter, again making the attainment of specificity a laborious process.

Libraries of bacterial artificial chromosomes (BACs) covering the genome of the founder animals constitute a different approach in clarifying transgene integration sites. This method relies on the cloning of large genomic DNA fragments into plasmid vectors and their multiplication in bacteria. By generating probes that recognise the transgene sequence, it then becomes possible to identify the BACs that carry the transgenic DNA, sequence them and thereby identify the surrounding genomic DNA sequence. Construction of porcine BAC libraries is being conducted regularly (Liu et al. 2010; Suzuki et al. 2000), however, the intense effort this technique involves makes it feasible only on the small scale. Analysis of large numbers of different transgenic lines would be extremely time consuming, as the whole procedure has to be repeated for each individual founder animal.

Whole genome sequencing involves whole genome amplification of limited DNA quantities and the subsequent sequencing using high-throughput techniques (Coskun and Alsmadi 2007). Currently, this method is mainly used in preimplantation diagnostics (Zheng et al. 2011), but has also found application in population genetics (Pool et al. 2010). Alignment of amplified and sequenced whole genomic DNA with genome databases can determine transgene integration sites. Different techniques for amplifying the genomic DNA are being routinely utilised. Primer extension preamplification (PEP) (Zhang et al. 1992; Dietmaier et al. 1999) and degenerate

oligonucleotide primed PCR (DOP-PCR) (Telenius et al. 1992) both rely on thermal cycling using *Taq* polymerase. Multiple displacement amplification (MDA), on the other hand, is an isothermal method dependent on bacteriophage *phi29* DNA polymerase (Dean et al. 2002). In contrast to the former methods, this polymerase offers better proofreading activity, making preferential amplification of one allele or random amplification failure of alleles (allele drop out) less likely. Further improvements in the accuracy of whole genome amplification are currently being discussed (reviewed in Zheng et al. 2011). High-throughput sequencing technology is then necessary to master the huge amounts of data derived from whole genome amplification. A variety of systems such as pyrosequencing or reversible terminator technology are already in use (reviewed in Kircher and Kelso 2010), with new developments constantly increasing efficiency. Initial studies demonstrated the proof of principle for whole genome sequencing in the determination of ENU-induced mutations in mice as well as in *C. elegans* (Flibotte et al. 2010; Arnold et al. 2011). Applications in integration site determination of transgenes, however, currently find their limit in the high costs involved in this technique as well as in the availability of comprehensive sequencing data for the genome of the species in question, or lack thereof. For the pig, the Swine Genome Sequencing Consortium initiated a whole genome sequencing project in 2006 (Chen et al. 2007) which has been underway since. Archibald et al. (2010) report on the preliminary release of an annotated draft sequence but so far no whole porcine genome sequence has been made available. Whole genome amplification is therefore currently not an option in clarifying transgene integration sites in the pig.

Expression analysis focuses either on the RNA or the protein level. While the latter is more significant for the performance of the transgene, the RNA level is easier to determine and to quantify. Although RNA is a much less stable poly-ribonucleic acid compared to DNA, isolation of transcripts have been described decades ago (Chirgwin et al. 1979, Han et al. 1987). First quantification experiments were performed by Northern blotting (Alwine et al. 1977; 1979) which was developed analogously to the DNA analysis described by Southern (Southern 1975). However, a more systematic and precise quantification has become available by the combination of reverse

transcription and PCR (RT-PCR) (Han et al. 1987, Simpson et al. 1988). By using viral reverse transcriptase enzymes, complementary DNA (cDNA) is synthesised that constitutes a suitable template for PCR amplification. Several steps have improved this method from a semi-quantitative approach that evaluated one single sample per reaction to a technique that quantifies the sample more precisely, and eventually to an application addressing the expression levels of the whole transcriptome on one single chip (Schena et al. 1995). Transcriptome analysis has thus evolved to an automatised high-throughput technology for expression analysis. However, in contrast to quantification of expression levels, the localisation of expression is more difficult to achieve in transcriptome analysis. Even though hybridisation of tissue sections with labelled probes has long been employed, the technology is still laborious, time-consuming and error prone (Coghlan et al. 1985). Additionally, biological limitations have to be taken into account. As the action of a given gene conventionally takes place at the protein level, the transcription status gives only limited information about the protein activity. Numerous post-transcriptional regulatory mechanisms, such as mRNA stability, translational control, intra-cellular protein trafficking or protein activation/de-activation are essential, too.

From the technological point of view, the protein level is much more complicated to address than the RNA level. In contrast to RNA, which represents a homogenous pool of poly-ribonucleic acids with almost identical biochemical features, proteins are more diverse regarding their solubility, their sensitivity for degradation and their accessibility for detection. Even more, whereas RT-PCR represents a powerful tool for detection of even low amounts of particular molecules, such an amplification technology does not exist for proteins, limiting the sensitivity of protein analysis. Antibody based detection methods such as Western blotting, immunohistochemistry, enzyme-linked immunosorbent assay (ELISA) or fluorescence-activated cell-sorting (FACS) are the most widely employed applications. While Western blotting has evolved as a routine technology in many laboratories for detection or semi-quantification of differently sized proteins, or the study of their glyocosylation patterns (Renart et al. 1979), immunohistochemistry addresses the cellular, or even sub-cellular localization of specific proteins (reviewed in Ramos-Vara 2005). ELISA

has been established as the most reliable quantification method for particular proteins from tissue samples (Engvall and Perlmann 1971) whereas FACS was developed for protein analysis on the surface of cells in liquids (Sugarbaker et al. 1979).
Alternative methods for protein analysis have emerged over the last decade, aiming for increased sensitivity and a higher throughput. While microarrays address the analysis of whole proteome samples by an antibody based means (reviewed in Lopez and Pluskal 2003), sensitivity of protein analysis has been increased by implementing the principle of mass-spectrometry detection (Sickmann et al. 2002). However, both approaches, similar to advanced RNA-technologies, require cost-intensive equipment and profound know-how. Genomic and transcriptome or proteome analyses thus provide diverse possibilities for examining potential founder animals with respect to their suitability for breeding transgenic pig lines.

Since continuous reproduction of transgenic pigs by cloning had proven itself as too inefficient in terms of animal numbers that can reasonably be generated by it, the aim of this thesis was to identify novel transgenic founder animals by genomic and expression analysis to subsequently re-establish them by cloning for utilisation in breeding.
Concurrently, already existing transgenic founder pigs for xenotransplantation were to be expanded into multitransgenic breeding herds while accounting for the two conflicting issues of transgene segregation and rising inbreeding coefficients.

3 ANIMALS, MATERIALS AND METHODS

3.1 Animals

In this work genetically engineered pigs displaying the following modifications to their genome were investigated and used for the establishment of transgenic breeding herds. All animal experiments were carried out in compliance with the German Animal Protection Law (AZ: 211-2531-58/97 and AZ: 55.2-1-54-2531-54/08, Regierung von Oberbayern).

3.1.1 GalKO

Original founder animals of the Large White breed were established at *Revivicor Inc.*, Blacksburg, VA. As described in detail in Dai et al. (2002) and Phelps et al. (2003), targeting of the catalytic domain in exon IX of the α1,3-galactosyl-transferase gene by homologous recombination initiated a loss of gene function. This was achieved by using a targeting vector consisting of two homologous arms of 4.9 kb and 1.9 kb, respectively. Targeting efficiency was increased by promoter trapping with an *IRES-neo*-polyadenylation cassette. Somatic cell nuclear transfer (SCNT) and embryo transfer (ET) yielded founder animals.

3.1.2 CD46

CD46 transgenic pigs were established by Loveland et al. (2004). The transgene vector was assembled from a genomic human CD46 fragment containing exons I and II as well as a cDNA fragment of exons III-XII and a SV40 polyadenylation site. This CD46 minigene was used for pronuclear microinjection into fertilised oocytes which were subsequently transferred to oestrus-synchronised recipients. These CD46 transgenic pigs were mated to homozygous GalKO pigs at *Revivicor Inc.*, Blacksburg, VA, generating double transgenic founder animals (Hara et al. 2008). Primary cells of a double transgenic boar were transferred to the Chair of Molecular Animal Breeding and Biotechnology, LMU Munich. These cells were subsequently used for SCNT according to the protocol of Kurome et al. (2006) and, following that, ET into oestrus-

synchronised German Landrace gilts as described in Besenfelder et al (1997). First cloned GalKO/CD46 founders were born at the Moorversuchsgut (MVG), Badersfeld in 2006. Nuclear transfers as well as embryo transfer experiments at the MVG were performed by Dr. Mayuko Kurome and Dr. Barbara Kessler. Cloned offspring has since been used for xenotransplantation experiments and for the establishment of the breeding herd.

3.1.3 hTM

The transgene vectors for human thrombomodulin (hTM) were established at the Chair for Molecular Animal Breeding and Biotechnology, LMU Munich and have as of now not been published yet. The DNA constructs consist of a 6 kb porcine thrombomodulin promoter, the coding sequence of human thrombomodulin, a poly-adenylation cassette from the bovine growth hormone gene and a neomycin or blasticidin resistance cassette for positive selection. The hTM-neo construct (Figure 3.1 (A)) was transfected into primary kidney cells from a wild-type German Landrace boar, whereas the hTM-bla construct (Figure 3.1 (B)) was transfected into primary kidney cells of a neomycin resistant GalKO/CD46 transgenic boar cloned from the above described *Revivicor* cells. Both types of hTM-transgenic cells were then used for SCNT and subsequent ET into oestrus-synchronised German Landrace gilts as described above in order to generate hTM and GalKO/CD46/hTM transgenic boars. Vectors were constructed by Dr. Nikolai Klymiuk and cell culture experiments were performed by Dr. Annegret Wünsch, both at the MVG.

3.1.4 HLA-E

Human leukocyte antigen (HLA)-E transgenic pigs were established by Weiss et al (2009) using a 7.7 kb genomic fragment of HLA-E with a HLA-B7 signal sequence and a 15 kb genomic human β2 microglobulin fragment. Both vectors were microinjected simultaneously into the pronucleus of zygotes with which ET to oestrus-synchronised recipient gilts was performed. Transgenic founder animals were generated at the MVG, Badersfeld in 2002 according to the methods described in this publication. These pigs have been reproduced at the MVG by breeding since.

3.1.5 LEA29Y

Pigs expressing the CTLA4-Ig derivate LEA29Y were generated at the Chair for Molecular Animal Breeding and Biotechnology, LMU Munich. The coding region of LEA29Y was commercially synthesised (Bio&Sell, Nürnberg). For ubiquitous expression LEA29Y was inserted into an expression cassette between the CAG-promoter and the rabbit haemoglobin beta polyadenylation box according to Matsuda and Cepko (2004) (Figure 3.1 (C)). For beta-cell-specific expression LEA29Y was placed under a 1.3 kb insulin promoter and linked to a poly-adenylation cassette derived from the bovine growth hormone (Grzech et al. 2010) (Figure 3.1 (D)). A neomycin resistance cassette facilitates positive selection of transgenic cells in both cases. Vectors were designed and constructed by Dr. Nikolai Klymiuk at the MVG, Badersfeld. Transgenesis of primary cells, nuclear transfer as well as embryo transfer were performed analogously to the procedures described for the hTM constructs.

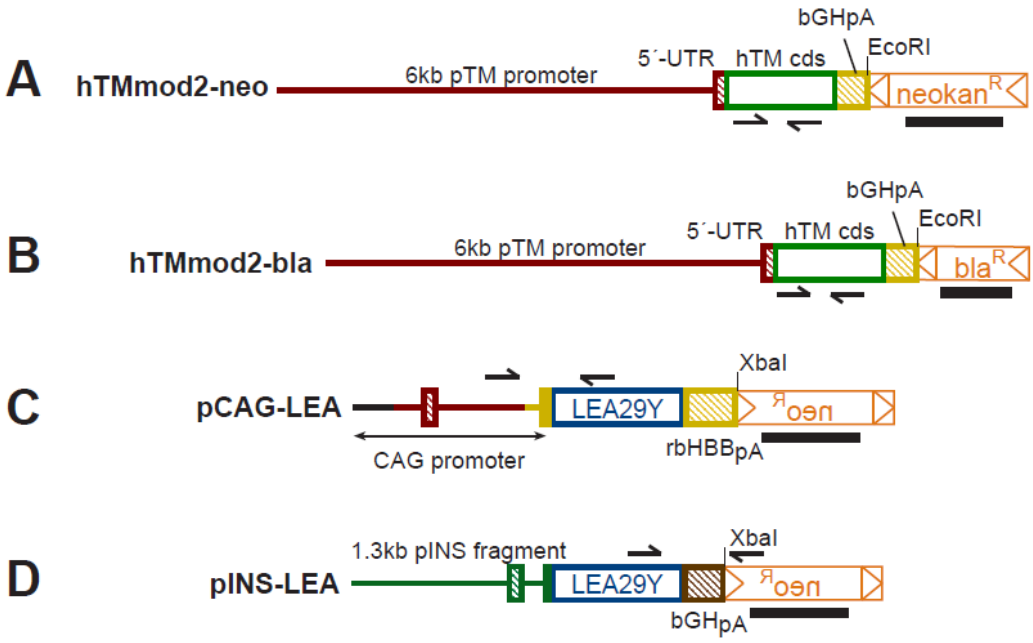


Figure 3.1: DNA constructs for establishment of novel transgenic pig lines. hTM constructs contain either a neomycin resistance for transfection into wild-type cells (A) or a blasticidin resistance for transfection into GalKO/CD46 background (B). Both LEA29Y constructs are neomycin resistant; a CAG promoter is used for ubiquitous expression (C) and an insulin promoter for β-cell specific expression (D). Primer binding sites for genotyping are illustrated by arrows; localisation of probes for Southern blotting is indicated by bars and the relevant restriction sites are shown.

3.2 Materials

3.2.1 Chemicals

All chemicals were used in p.a. quality unless noted otherwise.

Acetic acid (glacial)	Merck, Darmstadt
Agar-agar	Roth, Karlsruhe
Agarose Universal	Bio&SELL, Nürnberg
Agarose UltraPure™	Invitrogen, Karlsruhe
Ampicillin	Roth, Karlsruhe
Bromophenolblue	Roth, Karlsruhe
Chloroform (Trichlormethan)	Merck, Darmstadt
D-Mannitol	Sigma, Steinheim
DTT (Dithiothreitol)	Biomol, Hamburg
EDTA (Ethylenediaminetetraacetic acid)	Roth, Karlsruhe
Ethanol	Roth, Karlsruhe
Ethidiumbromide (1mg/ml)	Merck, Darmstadt
Formaldehyde solution, 37%	Roth, Karlsruhe
Formamide	Roth, Karlsruhe
Glucose	Roth, Karlsruhe
Glycerol	Roth, Karlsruhe
Hydrochloric acid, 37%	Roth, Karlsruhe
Isoamylalcohol	Roth, Karlsruhe
Magnesium chloride	Merck, Darmstadt
Paraformaldehyde	Merck, Darmstadt
PEG 8000	Roth, Karlsruhe
Peptone/Tryptone	Roth, Karlsruhe
Phenol	Roth, Karlsruhe
Phosphoric acid	Roth, Karlsruhe
PIPES (Piperazin-1,4-bis-(2-ethansulfonic-acid))	Sigma, Steinheim
Potassium acetate	Roth, Karlsruhe

Potassium chloride	Sigma, Steinheim
2-Propanol	Roth, Karlsruhe
SDS (Sodiumdodecylsulfate), ultrapure	Roth, Karlsruhe
Sodium acetate-trihydrate	Merck, Darmstadt
Sodium chloride	Roth, Karlsruhe
tri-Sodium citrate-2-hydrtae	Roth, Karlsruhe
Sodiumdihydrogenphosphate-1-hydrate	Merck, Darmstadt
di-Sodiumhydrogenphosphate-2-hydrate	Roth, Karlsruhe
Sodium hydroxide (2N)	Roth, Karlsruhe
D(+)-Sucrose	Roth, Karlsruhe
Sulfuric acid	Sigma, Steinheim
Tris (Tris-(hydroxymethyl)-aminomethan)	Roth, Karlsruhe
Tween® 20	Sigma, Steinheim
Yeast extract	Roth, Karlsruhe

3.2.2 Consumables

ABgene® PCR plates, 96-well	Thermo Scientific, UK
Centrifuge tubes (15ml, 50ml)	Falcon®, Becton Dickinson, Heidelberg
Coated filter paper	Roth, Karlsruhe
Culture dishes	Roth, Karlsruhe
Developing and fixing solutions for x-rays	AGFA-Gevaert N.V., Belgium
Hybond-N+ nylon membrane	GE Healthcare, Munich
Maxisorp 96-well immuno plates	Nunc™, Denmark
Parafilm® M	American Can Company, USA
PCR reaction tubes (0.2ml)	Braun, Wertheim
Pipet tips	Eppendorf, Hamburg
Pipet tips with filter	Axygen Inc., USA
SafeGrip® Latex gloves	SLG, Munich

Safe-Lock reaction tubes (1.5ml, 2ml)	Eppendorf, Hamburg
Saran Barrier food wrap	Dow, USA
Self-adhesive plastic covers	Nunc™, Denmark
Sephadex G-50 columns	Amersham, UK
Super RX Fuji medical x-ray film	FujiFilm Corp., Japan
Whatman paper	Roth, Karlsruhe

3.2.3 Devices

AccuJet® pro Pipetman	Brand, Wertheim
Agarose gel electrophoresis chamber	OWL Inc., USA
Centrifuge 5415 D	Eppendorf, Hamburg
Centrifuge 5417 R	Eppendorf, Hamburg
Chyo scales	YMC Co., Japan
Glass tubes 80mm	Zefa, Harthausen
Eppendorf HH Mastercycler Gradient	Eppendorf, Hamburg
GeneQuant Pro spectrophotometer	Amersham, UK
Gel documentation system	BioRad, Munich
GFL 3031 shaker	Hilab, Düsseldorf
Glass pipets	Hirschmann, Eberstadt
Hybrid mini 38 hybridisation oven	H. Saur, Reutlingen
37°C incubator	Memmert, Schwabach
60°C incubator	Memmert, Schwabach
Labofuge M centrifuge	Heraeus, Osterode
Microprocessor pH meter	WTW, Weilheim
MS1 minishaker	IKA Labortechnik, Staufen
Multitip pipet (300µl)	Eppendorf, Hamburg
Pipets (1000µl, 200µl, 20µl)	Gilson Inc., USA
Polytron homogeniser	Kinematica, Switzerland
Power Pac 300 gel electrophoresis unit	BioRad, Munich
REAX2 Automatic swivel unit	Hilab, Düsseldorf
RH Basic heating plate with magnetic stirrer	IKA Labortechnik, Staufen

Severin 900 microwave	Severin, Sundern
Tecan Sunrise ELISA reader with Magellan software	Tecan, Austria
Thermomixer 5436	Eppendorf, Hamburg
UV cross-linker	Vilber-Lourmat, France
Varioklav 400 autoclave	H+P Labortechnik, Oberschleißheim
WB6 water bath	Firmengruppe Preiss-Daimler, Medingen
X-ray cassette	Rego, Augsburg

3.2.4 Antibodies, drugs, enzymes, oligonucleotides, standards

3.2.4.1 Antibodies

Primary antibodies

Polyclonal rabbit anti-human IgG	Dako, Denmark

Secondary antibodies

Polyclonal rabbit anti-human IgG/HRP	Dako, Denmark

3.2.4.2 Drugs

Azaperon (Stresnil®)	Janssen Pharmaceutica, Belgium
Ketamine hydrochloride (Ursotamin®)	Serumwerk Bernburg, Bernburg
T61	Intervet, Unterschleissheim

3.2.4.3 Enzymes

BigDye® Terminator v3.1	Applied Biosystems,

	Weiterstadt
Herculase® II and buffer (5x)	Agilent, Böblingen
Klenow fragment exo$^-$ (5U/µl) and buffer (10x)	Fermentas, St. Leon Roth
Taq Polymerase (5U/µl) and buffer (10x)	Agrobiogen, Hilgertshausen
Proteinase K (20mg/ml)	Roth, Karlsruhe
Restriction enzymes and recommended buffers:	Fermentas, St. Leon Roth
Alw44I	
BamHI	
EcorI	
HindIII	
NcoI	
PstI	
PvuII	
TaqI	
XbaI	
Ribonuclease A (RNase A) (0.2U/µl)	Roche, Mannheim
T4 DNA Ligase (2000U/µl) and buffer (10x)	Fermentas, St. Leon Roth

3.2.4.4 Oligonucleotides

Thermo Scientific, UK

CD46ex2F	5-GGC TAC CTG TCT CAG ATG AC-3
CD46ex3R	5-CCA TTT GCA GGG ACT GCT TG-3
GGTAr21	5-GCC ACC TCT TCT CGG ACT TGA TCT C-3
GGTAf22	5-CAT CCA GGC ACA TAC AGC ACA AG-3
hTMf1a	5-CTT CAT GGC ATT TC-3
hTMf1b	5-TGC TAG TCA ATC TT-3

hTMf2a	5-GTG ACC ATG GCT TT-3
hTMf2b	5-AGC TCT GAT TCA AC-3
hTMs3f	5-TTG GAA GTT CCT GGG ACA GAG-3
hTMs6f	5-GGT TTC AGG TTC GAT TCC TG-3
hTMf21	5- GGC CAT AAC TGA CAT CCC GTT C-3
hTMf22	5-ACA GTC ACA GAG TTG CCT CTG C-3
hTMf23	5-GAA TCC CAG CCA CAG CTT TGA C-3
hTMf24	5-TGG ATG AAC TCA GAC CAA ACA G-3
hTMf25	5-GAG ATG GAC TGG CCC TCT GCA AG-3
hTMf26	5-GAC AGT GAT GGA CAG CCG ACG GCA G-3
hTMf27	5-CGA CAG GTG CTT CTC GAT CTG-3
hTM43f1	5-CAG TGT TTG TCT TCT TAA CTA TC-§
hTM1101f	5-CGA CGC AGT CCT GCA ACG A-3
hTMr1	5-CAG CTA TCC TGA AC-3
hTMr2	5-AGC GCC TTT ATC CT-3
hTM44r1	5-ATC ACT ATG TCA TTC AAG AGC TC-3
hTM44r4	5-TCG TTT CTG TCC AAA ACC AGA AG-3
hTM1608r	5-CCG GAG TCA CAG TCG GTG CCA A-3
hTM-pA2050r	5-GGG CAA ACA ACA GAT GGC TG-3
hTM-pA2100r	5-AAA GGA CAG TGG GAG TGG CA-3
pTMSr	5-AAG CGC ACC AGC TGA AAG-3
neokanf	5-GAC AAT AGC AGG CAT GCT G-3
neokanR	5-GTG GAT GTG GAA TGT GTG C-3

bla241r	5-TTC CGA TCG CGA CGA TAC AAG TCA G-3
bla343r	5-GCT GTC CAT CAC TGT CCT TCA CT-3
PGKr	5-GCT GCT AAA GCG CAT GCT CCA GAC-3
bGHpAf1	5-AGG TGC CAC TCC CAC TGT CTT TTC-3
bGHpAr3	5-GAT GGC TGG CAA CTA GAA GGC AC-3
bGHpAr4	5-GTC GAG GCT GAT CAG CGA GCT C-3
CAGf	5-CTC TGC TAA CCA TGT TCA TG-3
Neof	5-TGA TTC CCA CTT TGT GGT TC-3
NeoPf	5-CAG CTG TGC TCG ACG TTG TC-3
HLAE-f	5-CCC AAG TGA AAT ACC CTG GCA-3
HLAE-r	5-CGA AGA TTC CCT GAC AAT CCC-3
LEAf	5-CCA GCA CCT GAA CTC CTG-3
LEAr	5-GGC TTT GTC TTG GCA TTA TG-3
pACTB954f	5-CGC TCG TGG TCG ACA ACG-3
pACTB1919r	5-CTG GAT GGC CAC GTA CAT G-3

3.2.4.5 Protein standards

Bovine serum albumin (BSA) Fraction V	Roth, Karlsruhe
Human serum protein calibrator	Dako, Denmark

3.2.5 Buffers, media, solutions

Water, deionised in a Millipore machine (EASYpure® II, pure Aqua, Schnaitsee) and termed aqua bidest., was used as solvent, unless indicated otherwise.

Chloroform-isoamylalcohol (CiA)

96 ml chloroform

4 ml isoamylalcohol

Stored at 4°C protected from light

DNA loading buffer (10x)

10% glycerol in aqua bidest.

1 spatula tip of bromophenolblue

Add 0.5 M NaOH until colours turns blue

Stored aliquoted at 4°C

dNTP-mix

2 mM dATP, dCTP, dGTP, dTTP

in aqua bidest.

Stored aliquoted at -20°C

ELISA coating buffer

0.35 g NaH_2PO_4, H_2O

1.34 g Na_2HPO_4, $2H_2O$

8.47 g NaCl

Ad 1000 ml aqua bidest.

Adjust pH to 7.2 with 5M NaOH

Stored at 4°C

ELISA dilution and washing buffer

0.35 g NaH_2PO_4, H_2O

1.34 g Na_2HPO_4, $2H_2O$

29.22 g NaCl

Ad 1000 ml aqua bidest.

1 ml Tween 20

Adjust pH to 7.2 with 5M NaOH

Stored at 4°C

ELISA stop solution

0.5 M Sulphuric acid

Stored at room temperature

LB medium

5 g yeast extract

10 g peptone/tryptone

5 g NaCl

Ad 1000 ml aqua bidest.

pH 7.0 (adjust with 5 M NaOH)

autoclave

Medium was stored at room temperature.

LB-agar plates

1000 ml LB pH 7.0

15 g agar-agar

Autoclave

Cool to 50°C

Add 1 ml ampicillin (500 mg/ml)

Pour into sterile 10 cm culture dishes

After setting, plates were stored at 4°C.

4% Paraformaldehyde in 0.1 M Phosphate Buffer

1. Prepare 0.2 M Phosphate Buffer (PB), pH 7.4:

21.8 g Na_2HPO_4

6.4 g NaH_2PO_4

Ad 1000 ml aqua bidest.

Adjust pH with 5 M NaOH

2. Prepare 0.1 M PB pH 7.4:
500 ml 0.2M PB
Ad 1000 ml aqua bidest.
40 g paraformaldehyde was added to 1000 ml 0.1 M PB, pH 7.4 and heated to 60-65ºC while stirring under the hood. A few drops of 1 N NaOH cleared the solution. After cooling the solution was filtered and stored at 4°C.

PEG-$MgCl_2$
40% (w/v) PEG 8000
30 mM $MgCl_2$
Stored at room temperature

Phenol-chloroform-isoamylalcohol (PCiA)
25 ml phenol
25 ml CiA
Stored at 4°C protected from light

Plasmid A
50 mM glucose
25 mM Tris/HCl pH 8.0
10 mM EDTA/NaOH pH 8.0
Stored at room temperature

Plasmid B
0.1 M NaOH
0.5% (w/v) SDS
Prepare freshly

Plasmid C
3 M KOAc
pH 4.8 with 9 M HOAc

autoclave

Stored at room temperature

Sequencing buffer (5x)

17.5 ml 1 M Tris/HCl (pH 9.0)

125 µl 1 M $MgCl_2$

Ad 50 ml aqua bidest.

Stored aliquoted at -20°C

Sodium-phosphate Buffer 1M

4 ml 85% H_3PO_4

89 g Na_2HPO_4, $2H_2O$

Ad 1000 ml aqua bidest.

Southern Blot Church buffer

1% BSA

1 mM EDTA, pH 8.0

500 mM Sodium-phosphate Buffer pH 7.2

7% SDS

Southern Blot depurination solution

0.3M HCl

Southern Blot high stringency buffer

1 mM EDTA, pH 8.0

40 mM Sodium-phosphate Buffer pH 7.2

1% SDS

Southern Blot low stringency buffer

0.5% BSA

1 mM EDTA

40 mM Sodium-phosphate Buffer pH 7.2
5% SDS

Southern Blot neutralisation solution
0.5 M Tris
1.5 M NaCl
pH 7.5

Southern Blot 20x SSC
0.3 M Na-Cit
3 M NaCl

Southern Blot strand break solution
0.5 M NaOH
1.5 M NaCl

STE
10 mM Tris/HCl pH 8.0
0.1 M NaCl
1 mM EDTA/NaOH pH 8.0
Stored at room temperature

TAE buffer (50x)
242 g Tris
100 ml 0.5M EDTA (pH8.0)
57 ml AcOH
Ad 1000 ml aqua bidest
Buffer was stored at room temperature and diluted to single concentration with aqua bidest. for use.

10 mM Tris/HCl pH 8.0

10 mM Tris

Adjust pH to 8.0 with HCl

Stored at room temperature

3.2.6 Kits

CloneJET™ PCR Cloning Kit	Fermentas, St. Leon Rot
Nexttec™ Genomic DNA Isolation Kit	Nextecc, Leverkusen
Qiaex® II Gel Extraction Kit	Qiagen, Hilden
Roti®-Quant	Roth, Karlsruhe

3.2.7 Others

α^{32}P-dCTP	Perkin-Elmer, Netherlands
dNTPs (dATP, dCTP, dGTP, dTTP)	Fermentas, St. Leon Roth
Gene Ruler™ (1 kb DNA ladder)	Fermentas, St. Leon Roth
DNA molecular weight standard	
6 x DNA loading dye	Fermentas, St. Leon Roth
Random primers (3µg/µl)	Invitrogen, Karlsruhe
TMB Solution (HRP substrate)	Uptima, France
TOP 10 chemically competent E.coli	Invitrogen, Karlsruhe

3.2.8 Software

BioEdit Sequence Alignment Editor

FinchTV Version 1.3.1, Geospiza Inc.

Macromedia Freehand MX

3.3 Methods

3.3.1 Genomic analysis

3.3.1.1 Genotyping of founder animals and F1 generation

In order to identify transgenic pigs, genotyping PCR was performed on tissue samples of piglets. For this, ear punches were obtained from three-day-old piglets and stored at -20°C until further processing.

For genotyping of founder animals, genomic DNA was isolated in 1.5 ml reaction tubes from approximately 100 mg of shredded tissue using the following protocol. All steps were carried out at room temperature unless indicated otherwise.

Composition of lysis buffer
160 mM Sucrose
80 mM EDTA pH 8.0
100 mM Tris/HCl pH 8.0
0.5% SDS

Add 400 µl lysis buffer and 30 µl Proteinase K (10 mg/ml) to tissue, mix
Incubate at 60°C overnight
Centrifuge 5 min, 16,100 g
Transfer supernatant to fresh tubes
Add 400 µl sodium chloride 4.5 M, mix
Add 300 µl phenol-chloroform-isoamylalcohol (PCiA)
Pivot in automatic swivel unit 15 min
Centrifuge 10 min, 16,100 g
Transfer aqueous phase to fresh tubes
Repeat PCiA extraction twice: add 300 µl PCiA, pivot 10 min, centrifuge 2.5 min
Add 550 µl 2-propanol to precipitate DNA
Transfer DNA successively into 2 tubes with 1 ml EtOH 70% each
Incubate in EtOH 70% overnight

Discard EtOH 70%
Air dry DNA pellet 10 min
Resolve DNA in 55 µl 10 mM Tris/HCl
Mix repeatedly for 1 hour

DNA concentration was measured at 260 nm using a GeneQuant Pro spectrophotometer. For genotyping PCR analysis, isolated gDNA was diluted with 10 mM Tris/HCl to a concentration of 100 ng/µl.
Genomic DNA of F1 generation pigs was isolated using Nexttec™ Genomic DNA Isolation Kit (Nextecc Biotechnologie GmbH, Leverkusen) according to the manufacturer's protocol.

In both cases genotyping PCR was performed in 0.2 ml reaction tubes in a total volume of 25 µl as follows:

Composition of reaction mix
2.5 µl 10x PCR-Buffer
2.5 µl $MgCl_2$ (25mM)
2.5 µl dNTPs (2mM)
0.4 µl forward + 0.4 µl reverse primer (10 µM)
0.2 µl Taq polymerase (5 U/µl)
1 µl DNA-template
15.5 µl aqua bidest.

Cycler protocol

2 min 95°C	denaturation
30 sec 95°C	denaturation
30 sec xx°C	annealing at PCR specific temperature
1 min 72°C	elongation
GO TO Step 2 34x	
10 min 72°C	final elongation

15 min 4°C termination of reaction

Transgene specific primers were used to identify the respective transgenes. In addition, a house-keeping gene, the ubiquitously expressed β-actin, was amplified in parallel to verify integrity of isolated genomic DNA. For details of genotyping primers used and their respective annealing temperatures, see Table 3.1.

3.3.1.2 Duplex PCR

For discrimination of homozygous from heterozygous transgenic offspring, duplex PCRs were established for the GalKO and the hTM locus. One transgene specific and two wild-type sequence specific primers were employed in each duplex PCR. Details of the primers used are given in Table 3.1.
PCRs were performed in 0.2 ml reaction tubes in a total volume of 25 µl as follows:

Composition of reaction mix
2.5 µl 10x PCR-Buffer
2.5 µl $MgCl_2$ (25 mM)
2.5 µl dNTPs (2 mM)
0.4 µl first + 0.4 µl second + 0.4 µl third primer (10 µM)
0.2 µl Taq polymerase (5 U/µl)
1 µl DNA-template
15.5 µl aqua bidest.

Thermocycling for duplex PCR was performed according to the cycler protocol detailed above for initial genotyping PCR.

Table 3.1: Details of genotyping primer pairs used in the identification and characterisation of transgenic founders and offspring.

Transgene	Primer pairs	Annealing temperature	Amplicon size
CAG-LEA	CAGf/LEAr	56°C	790 bp
CD46	CD46ex2F/CD46ex3R	62°C	425 bp
HLA-E	HLAEf/HLAEr	62°C	500 bp
hTM	hTM1101f/hTM1608r	58°C	508 bp
hTM$^{+/-}$	hTM43f1/hTM44r1/hTM44r4	56°C	570 bp/1200 bp
INS-LEA	LEAf/neof	61°C	993 bp
GalKO$^{+/-}$	neoPf/GGTAf22/GGTAr21	60°C	940 bp/570 bp
Actin	pACTB954f/pACTB1919r	58°C	971 bp

$^{+/-}$ indicates zygosity discriminating PCR

3.3.1.3 Agarose Gel Electrophoresis

After completion of PCR amplification, a 0.7% agarose gel was prepared from a 0.7% Universal Agarose/1x TAE buffer solution in a microwave. After cooling the mixture to approximately 55°C, ethidiumbromide (0.5 µg/ml) was added and the gel was left to set in a gel electrophoresis chamber (OWL Inc., USA). 2.5 µl of DNA loading buffer (10x) were added to each 25 µl PCR sample. The gel electrophoresis chamber was filled with 1x TAE buffer and samples and a DNA molecular weight standard were loaded into individual gel slots. By applying an electric current (Power Pac 300 Gel Electrophoresis Unit; BioRad, Munich) to the gel electrophoresis chamber, DNA fragments were separated according to their size and could be visualised afterwards under UV-light. This allowed analysis of PCR products in relation to the DNA molecular weight standard.

3.3.1.4 Southern Blot

For determination of transgene copy numbers and numbers of integration sites, southern blotting was carried out on genomic DNA of founder animals, involving the visualisation of genomic DNA fragments containing a defined sequence.

DNA separation:

For this, genomic DNA was digested by restriction enzymes in 1.5ml reaction tubes in a total volume of 60 µl per sample as follows:

15 µg gDNA
4 µl restriction enzyme
Ad 60 µl aqua bidest.
Incubate overnight at 37°C

The next day, 7 µl of DNA loading buffer (10x) were added to each sample, which were then loaded onto a 1% Universal Agarose/TAE gel prepared as described above. DNA fragments were separated according to their size and analysed in relation to a DNA molecular weight standard.
For details of restriction enzymes used see Table 3.2

Table 3.2: Conditions for genomic fragmentation and probe establishment for Southern blotting.

Transgene	Enzyme	Probe	Annealing temperature	Length	Hybridisation
hTM-neo	EcoRI	neoSENf/neoSr	56°C	707 bp	58°C
hTM-bla	HindIII	PGK41f/bla343r	56°C	893 bp	56°C
CAG-LEA	XbaI	neoSENf/neoSr	56°C	707 bp	58°C
INS-LEA	XbaI	neoSENf/neoSr	56°C	707 bp	58°C

Blotting:

For further processing the DNA was transferred to a Hybond-N+ Nylon Membrane (GE Healthcare, Munich). To achieve this, the DNA in the agarose gel was first treated as follows:

Incubate 45 min in depurination solution
Wash 2x with aqua bidest.
Incubate 45 min in strand break solution
Wash 2x with aqua bidest.

Incubate 20 min in neutralisation solution
Wash 2x with aqua bidest.
Incubate 20 min in 5x SSC

Subsequently, the DNA was blotted onto the membrane in a semi-dry manner. For this, the gel was placed upside down onto cling film (Saran Barrier Food Wrap; Dow, USA). Two layers of Whatman paper (Roth, Karlsruhe) and multiple layers of absorbent tissue were placed on top. To facilitate capillary transfer of the DNA onto the membrane, additional weight was applied on top and transfer was carried out over 24 hours.
Then DNA was crosslinked to the membrane under 0.120 J/cm^2 UV light (Vilber-Lourmat, France) and stored at room temperature.

Hybridisation:
DNA fragments containing the transgene were detected by probes specific for the neomycin or blasticidin resistance cassette in the transgene construct. For this, the probes were amplified by PCR from plasmids containing the vectors.
PCR was carried out according to a standard protocol as described for genotyping PCRs above. For details of primers and annealing temperatures used in the establishment of probes for the different transgenes, see Table 3.2.

The PCR products were separated on 1% Universal Agarose/TAE gels prepared as described above, eluated from the gel using QiaexII Gel Extraction Kit (Qiagen, Hilden), and DNA concentration of eluates was determined in relation to a DNA molecular weight standard on 1% Universal Agarose/TAE gels.

Prior to hybridisation the membranes were placed in 80 mm glass tubes (Zefa, Harthausen), pre-wetted with 5x SSC and incubated in 30 ml prehybridisation buffer (Church Buffer) under permanent rotation at 58°C in an incubator (H. Saur, Reutlingen) for 1 hour.
The probes were then radio-labelled with α^{32}P-dCTP (Perkin-Elmer, Netherlands)

according to the following protocol:

7 µl Klenow 10x Bu
50-100 ng DNA probe
10 µl Random primer (3 µg/µl)
Ad 50 µl aqua bidest.

Probe DNA was denaturated at 97°C for 10 minutes. After incubation on ice for 2 minutes, 20 µl of hybridisation mix was added to each sample.

Hybridisation mix:
3 µl C-Mix (0.33 mM of each dATP, dGTP, dTTP)
5 µl α^{32}P-dCTP (3000 Ci/mmol)
1 µl Klenow exo$^-$
Ad 20 µl aqua bidest.

Probe labelling was performed at 37°C for one hour. Unincorporated nucleotides were separated from the labelled probes by centrifugation through Sephadex G-50 columns (Amersham, UK). The purified and labelled probes were denaturated at 97°C for 5 minutes and cooled on ice. Meanwhile, the prehybridisation buffer was exchanged for fresh Church Buffer in which the labelled probes were diluted.
Hybridisation of the probes to the membrane was carried out overnight at 58°C under permanent rotation.
The next day, the hybridisation solution was discarded and the membrane was washed twice with low-stringency buffer at room temperature and afterwards twice with high-stringency buffer at 58°C. Then the membrane was placed on a coated filter paper (Roth, Karlsruhe), wrapped in cling film, and exposed to x-ray film (FujiFilm Corp., Japan) in an x-ray cassette (Rego, Augsburg) for at least one day at -80°C.
The exposed x-ray films were developed using developing and fixing solution (AGFA-Gevaert, Belgium) according to the manufacturer's instructions and subsequently analysed.

3.3.1.5 Inverse Polymerase Chain Reaction (inverse PCR)

Inverse PCR was performed in order to clarify the unknown genomic DNA flanking regions of the transgene at the insertion site. Figure 3.2 gives an overview of inverse PCR procedure.

All steps were carried out at room temperature unless indicated otherwise.

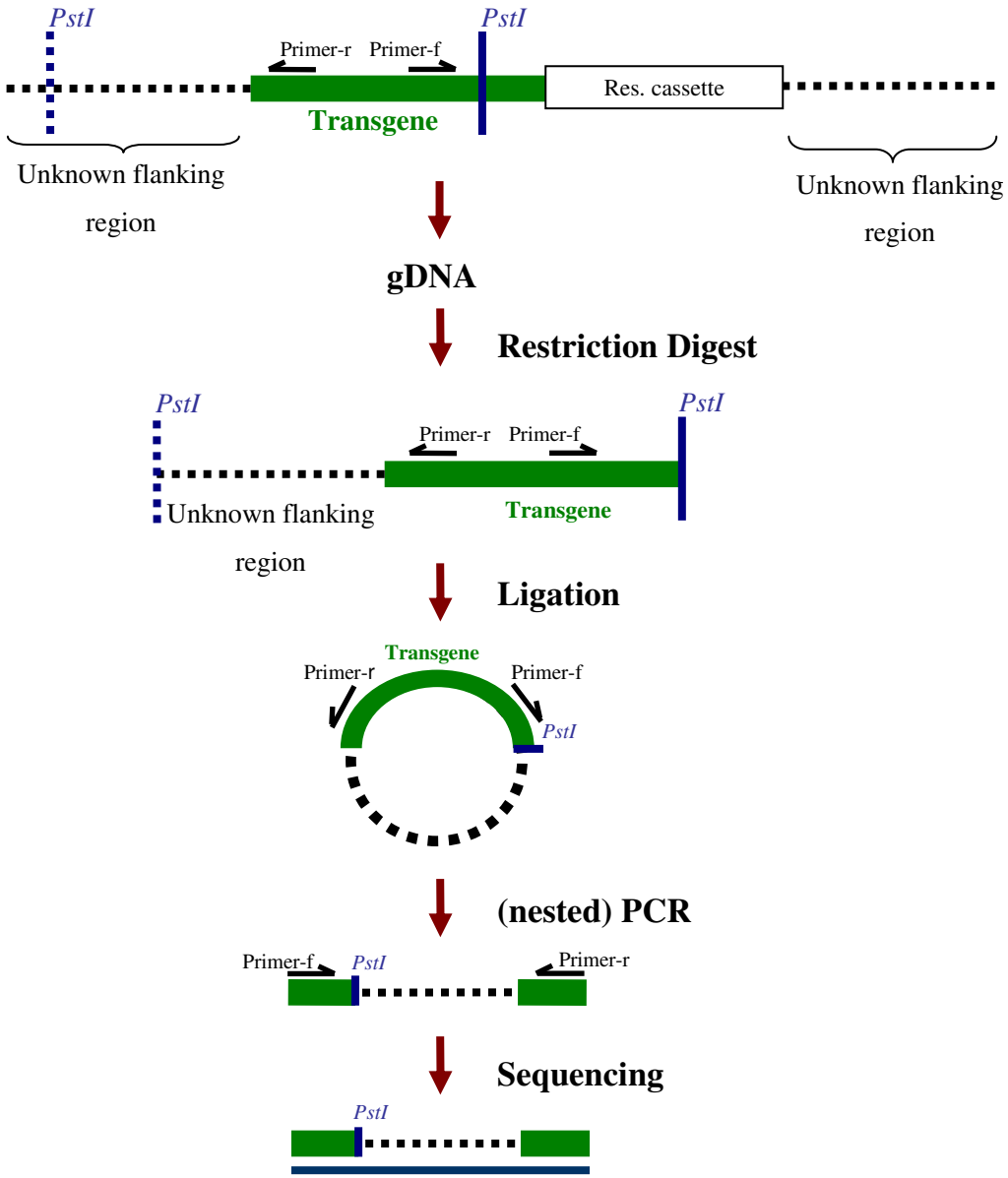


Figure 3.2: Inverse PCR overview. Genomic DNA was isolated from cells of transgenic animals. Isolated gDNA was then digested with restriction enzymes (here: PstI) and, following that, ligated to form circular DNA fragments. Primer pairs complementary to the transgene sequence were designed to face apart on linear DNA fragments but face towards each other once DNA has been circularised. Two rounds of PCR amplification using nested primer pairs were then performed on the circularised DNA. PCR amplicons were sequenced to clarify the previously unknown gDNA flanking regions of the transgene. Primer binding sites are indicated by black arrows.

3.3.1.5.1 Genomic DNA isolation

Genomic DNA was isolated in 1.5 ml reaction tubes from cultured cells of transgenic founders grown to confluence on a 10 cm cell culture dish analogously to the protocol used for gDNA isolation from tissue samples of founder animals.
After completion of the isolation process, DNA concentration was measured at 260 nm using a GeneQuant Pro spectrophotometer and adjusted to 500 ng/µl by diluting with 10 mM Tris/HCl.

3.3.1.5.2 DNA fragmentation

Restriction digest of genomic DNA was performed to generate linear DNA fragments with defined ends. For details of restriction enzymes used see Table 3.3. The total volume of 30 µl per digestion sample was composed as follows in 1.5 ml reaction tubes:

3 µl Restriction enzyme buffer
1 µl Restriction enzyme
24 µl aqua bidest.
2 µl gDNA (equals 1 µg gDNA)

After mixing carefully, samples were incubated at 37°C overnight. The next day PCiA extraction according to the following protocol was carried out:

Adjust volume to 150 µl with aqua bidest.

Add 100 µl PCiA
Extract by pivoting for 1 min
Centrifuge 2.5 min, 16,100g
Transfer aqueous phase to new tube
Add 15 µl 3M NaOAc
Add 400 µl EtOH 100%
Store at -80°C 30 – 60 min
Centrifuge 30 min, 16,100g, 4°C
Wash in EtOH 70% overnight
Centrifuge 2.5 min, 16,100g
Discard supernatant
Air dry DNA pellet 6 min
Resolve in 30 µl 10 mM Tris/HCl

10 µl of resolved, digested gDNA was mixed with 12.5 µl aqua bidest. and 2.5 µl DNA loading buffer (10x) and loaded onto a 1% agarose gel to verify digestion success. The gel was prepared from a 1% Universal Agarose/TAE buffer solution as described above.
The remaining 20 µl of digested gDNA were diluted with 100 µl 10 mM Tris/HCl and used for further processing.

3.3.1.5.3 Circularisation of DNA fragments

Digested and diluted gDNA was ligated overnight at room temperature in a total volume of 40 µl in 1.5 ml reaction tubes to circularise DNA. Each reaction mix was composed as follows:

4 µl 10x ligation buffer
4 µl T4 DNA ligase
12 µl aqua bidest.
20 µl digested gDNA
Termination of the reaction by 15 min 65°C

3.3.1.5.4 Nested PCR

For amplification of DNA sequences on circularised fragments, (nested) PCR amplification was performed in a total volume of 25 µl in 0.2 ml reaction tubes. For general principle of nested PCR see Figure 3.3.

Composition of reaction mix

5.0 µl 5x Herculase-Buffer
2.5 µl dNTPs
0.35 µl forward + 0.35 µl reverse Primer
0.2 µl Herculase II
1 µl DNA-template
15.6 µl aqua bidest.

Cycler-protocol

2 min 95°C	denaturation
30 sec 95°C	denaturation
30 sec 58°C	annealing
3 min 72°C	elongation
GO TO Step 2 39x	
10 min 72°C	final elongation
15 min 4°C	termination of reaction

After completion of PCR amplification, samples were used as DNA templates for nested PCR amplification. For this, each sample was used undiluted and in a 1:100 dilution with aqua bidest. Reaction mix and cycler protocol were the same as in the first amplification. Primers used were nested.
For an overview of restriction enzymes and primer pairs used for the different transgenes in the inverse PCR process, see Table 3.3

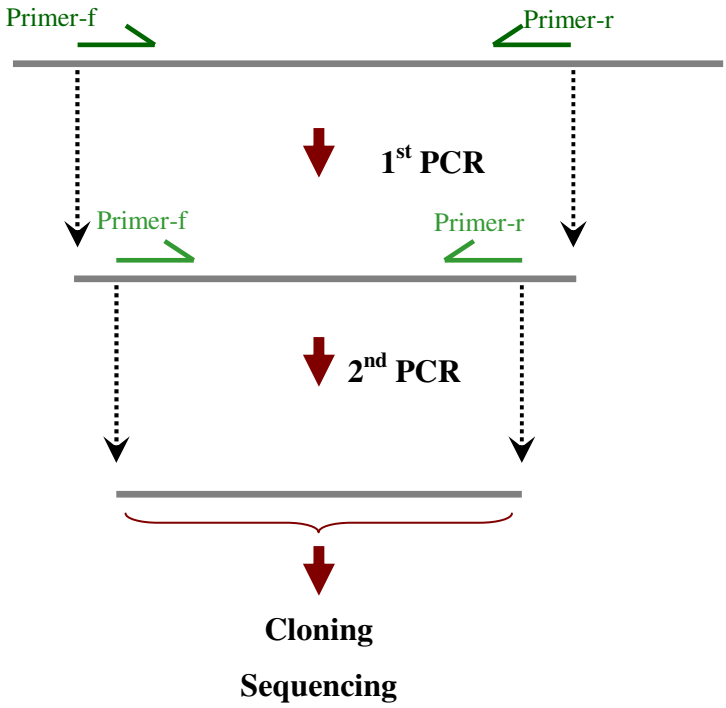


Figure 3.3: Nested PCR.

Sense and antisense primers are designed complementary to known sequence. Amplification of DNA sequence between primers is carried out by PCR. A second primer pair complementary to sequence of DNA amplicons is designed (= nested primers). DNA fragments are amplified a second time. PCR products can be further processed for cloning and sequencing. Primers are indicated by green arrows.

Table 3.3: Restriction enzymes and primers used for nested inverse PCR

Transgene	Founder	Restriction enzyme	Primers 5' end		Primers 3' end	
			1. PCR	**2.PCR**	**1.PCR**	**2.PCR**
hTM	#9943	EcorI	hTMr1 hTMf25	hTMr2 hTMf24	neokanr bGHpAf1	PGKr neokanf
		HindIII	hTMr1 hTMf22	hTMr2 hTMf21	hTM2100r bGHpAf1	hTM2050r neokanf
		NcoI	hTMr1 hTMfs3f	hTMr2 hTMf23	bla241r bGHpAf1 bGHpAr3	bla343r neokanf bGHpAr4
		PstI	nd	nd	bGHpAf1	neokanf
		XbaI	hTMr1 hTMf27	hTMr2 hTMf26	bGHpAr3 bGHpAf1	bGHpAr4 neokanf
	#9948	BamHI	hTMr1 hTMf1b	pTMSr hTMf2a	bGHpAr3 bGHpAf1	bGHpAr4 neokanf
		EcorI	hTMr1 hTMf25	hTMr2 hTMf24	bGHpAr3 bGHpAf1	bGHpAr4 neokanf
		HindIII	hTMr1 hTMf22	hTMr2 hTMf21	hTM2100r bGHpAf1	hTM2050r neokanf
		NcoI	hTMr1 hTMfs3f	hTMr2 hTMf23	bGHpAr3 bGHpAf1	bGHpAr4 neokanf
		PstI	hTMr1 hTMs6f	pTMSr hTMf2b	bGHpAr3 bGHpAf1	bGHpAr4 neokanf
		TaqI	hTMr1 hTMf1b	pTMSr hTMf1a	nd	nd
		XbaI	hTMr1 hTMf27	hTMr2 hTMf26	bGHpAr3 bGHpAf1	bGHpAr4 neokanf
	#9949	BamHI	hTMr1 hTMf1b	pTMSr hTMf2a	bGHpAr3 bGHpAf1	bGHpAr4 neokanf
		EcorI	hTMr1 hTMf25	hTMr2 hTMf24	bGHpAr3 bGHpAf1	bGHpAr4 neokanf
		HindIII	hTMr1 hTMf22	hTMr2 hTMf21	hTM2100r bGHpAf1	hTM2050r neokanf
		NcoI	hTMr1 hTMfs3f	hTMr2 hTMf23	bGHpAr3 bGHpAf1	bGHpAr4 neokanf
		PstI	hTMr1 hTMs6f	pTMSr hTMf2b	bGHpAr3 bGHpAf1	bGHpAr4 neokanf
		TaqI	hTMr1 hTMf1b	pTMSr hTMf1a	nd	nd
		XbaI	hTMr1 hTMf27	hTMr2 hTMf26	bGHpAr3 bGHpAf1	bGHpAr4 neokanf

3.3.1.5.5 DNA Eluation

After preparation of a 1% UltraPure™Agarose/TAE buffer gel analogously to the method described above, 2.5 µl DNA loading dye (10x) was added to each of the samples from the nested PCR, which were then loaded onto the gel. DNA separation was performed as detailed above. After visualisation, appropriate DNA strands were excised from the gel and extracted using a QiaexII Gel Extraction Kit (Qiagen, Hilden) following the manufacturer's protocol. Then

2 µl DNA eluate
2 µl DNA loading buffer (10x)
15 µl aqua bidest.

were loaded onto a 1% Universal Agarose/TAE buffer gel prepared as described above. A DNA molecular weight standard allowed determination of DNA concentration in eluate.

3.3.1.5.6 Ligation

Eluated DNA fragments were inserted into pJet plasmid cloning vector (Fermentas, St. Leon Roth) in a ligation reaction as follows:

2 µl 10x ligation buffer
1 µl T4 DNA ligase
1 µl pJet (5ng/µl)
15-30 ng DNA eluate
Ad 20 µl aqua bidest.

Ligation was carried out overnight at room temperature. Ligase was deactivated by 15 min 65°C.

3.3.1.5.7 Heat Shock Transformation

Plasmid vectors containing the DNA inserts were transformed into *E.coli* TOP10 competent cells according to the following protocol:

Thaw up competent cells carefully on ice
Add 5 µl of ligation
Mix carefully, avoid re-pipetting
20 min on ice
45 sec at 42°C
2 min on ice
Add 1 ml LB-Medium
45 min 37°C
Centrifuge 5 min, 2,300g
Resuspend pellet in 100 – 200 µl of the supernatant
Plate on LB-Amp (50 µg/ml) agar dishes
Cultivate overnight at 37°C

The next day, individual colonies were picked from the agar dishes and inoculated individually into 2.5 ml LB-Amp (50 µg/ml) medium each. Cultivation was carried out at 37°C overnight whilst shaking.

3.3.1.5.8 Plasmid preparation

Preparation of plasmids from the overnight liquid cultures was carried out as follows:

Centrifuge overnight cultures 10 min, 1,300g, discard supernatant
Resuspend pellet in 750 µl STE and transfer to 1.5 ml tubes
Centrifuge 5 min, 4,500g, discard supernatant
Resuspend pellet in 200 µl Plasmid A
Add 400 µl Plasmid B, 5 – 7 x mix (don´t vortex), 5 min on ice
Add 300 µl Plasmid C, 5 – 7 x mix (don´t vortex), 3 min on ice
Centrifuge 10 min, 16,100g

Transfer supernatant to new tube
Add 4 µl RNase A (10 mg/ml)
Incubate 45 min 37°C
Add 300 µl PCiA, 1 min shake
Centrifuge 2.5 min, 16,100g
Transfer aqueous phase into new tube
Add 650 µl 2-propanol, invert
Centrifuge 10 min, 16,100g, discard supernatant
Wash pellet in 700 µl EtOH 70%
Centrifuge 2.5 min, 16,100g, discard supernatant
Air dry pellet 6 min
Resolve pellet in 55 µl 10 mM Tris/HCl

DNA concentration was measured in a GeneQuant Pro spectrophotometer at 260 nm, and approximately 2 µg were used for qualitative analysis by restriction digestion.

3.3.1.5.9 Restriction digest

2 µl 10x restriction enzyme buffer
0.2 µl restriction enzyme
2 µg Plasmid
Ad 20 µl aqua bidest.

After overnight incubation at 37°C samples were loaded onto a 1% Universal Agarose/TAE buffer gel prepared as described above to determine ligation and transformation success. Samples displaying appropriate restriction digestion patterns were chosen for further processing.

3.3.1.5.10 PEG precipitation

Precipitation of DNA for sequencing was carried out as follows:

20 µl plasmid preparation
20 µl PEG-$MgCl_2$
20 µl aqua bidest.

Equilibrate 10 min
Centrifuge 20 min 15,700g
Wash in 100 µl EtOH 70% overnight
Centrifuge 2.5 min, 16,100g
Air dry 6 min
Resolve in 20 µl 10 mM Tris/HCl

DNA concentration was measured at 260 nm in a GeneQuant Pro spectrophotometer. Subsequently, samples were diluted with 10 mM Tris/HCl to a DNA concentration of 30 ng/µl to serve as template for sequencing.

3.3.1.5.11 Sequencing

Sequencing of samples was performed in 0.2 ml reaction tubes in a total reaction volume of 10 µl. Each sample was sequenced twice, once with a pJet forward primer and another time with a pJet reverse primer.

Composition of each reaction mix
4 µl 5x sequencing buffer
1 µl Big Dye
1 µl primer (10 µM)
2 µl template
2 µl aqua bidest.

Cycler-protocol

1 min 95°C	denaturation
5 sec 95°C	denaturation
10 sec 50°C	annealing

4 min	60°C	elongation
GO TO Step 2 39x		
15 min	4°	termination of reaction

3.3.1.5.12 EtOH precipitation

After sequencing amplification, DNA was precipitated from the reaction mix as follows:

Add 2.5 µl 125 mM EDTA into PCR-lid, spin down
Add 30 µl EtOH 100%, transfer to 1.5 ml tube
Incubate on ice 5 min
Centrifuge 30 min, 15,700g, 4°C
Wash pellet in 50 µl EtOH 70%
Centrifuge 2.5 min, 15,700
Air dry pellet 6 min
Resolve DNA in 30 µl aqua bidest. and transfer to sequencing plate (Abgene® PCR Plates; Thermo Scientific, UK)

Capillary electrophoretic separation of DNA samples was carried out at the German Mouse Clinic, Helmholtz Centre, Neuherberg.

Nucleotide sequences were bioinformatically analysed using FinchTV Version 1.3.1, Geospiza Inc. and BioEdit Sequence Alignment Editor.

3.3.2 Expression analysis

For transgene expression analysis tissue was either obtained in the form of ear punches or as an organ spectrum.
Ear punches of approximately 0.5 cm^2 were taken from three-day-old manually

fixated piglets. Tissue was either shock frozen in liquid nitrogen and stored at -80°C or transferred to 15 ml reaction tubes filled with 4% PFA.
Pigs sampled for organ spectrum analysis were anaesthetised with Ketamine (2 ml/10 kg) and Azaperon (0.5 ml/10 kg) i.m. Following that the animals received a vein catheter and were then euthanized with T61 (1 ml/10 kg) i.v. Organs were explanted postmortally without delay and samples of approximately 1 cm^3 were either shock frozen in liquid nitrogen and stored at -80°C or transferred to 4% PFA.

3.3.2.1 Protein isolation

For protein isolation frozen tissue was pulverised using a mortar and pestle and approximately 100 mg were homogenised in a sterile 2 ml reaction tube with 750 µl homogenising buffer of the following composition:

1.25 ml 1 M Mannitol
0.2 ml 2 M Sucrose
0.1 ml 0.5 M EDTA (pH 8.0)
0.5 ml 0.1M PIPES/Tris (pH 6.7)
Ad 10 ml aqua bidest.

After 30 min centrifugation at 4,100g, 4°C, supernatant was transferred to fresh reaction tubes and protein concentration was measured using bovine serum albumin (BSA) as standard protein. For this, 500 mg BSA was dissolved in 5 ml homogenising buffer of the above composition and dilutions of 25 µg/ml, 20 µg/ml, 15 µg/ml, 10 µg/ml, 5 µg/ml and 1 µg/ml were prepared.
Standards and samples were measured in a GeneQuant Pro spectrophotometer at 595 nm using Roti®-Quant according to the manufacturer's instructions.

3.3.2.2 Enzyme-Linked Immunosorbent Assay (ELISA)

In order to perform qualitative and quantitative evaluation of transgenic protein, ELISAs were carried out in 96 well immuno plates (Maxisorp; Nunc™, Denmark).

Experiments were performed for CAG-LEA transgene expression analysis.
Antibodies used were directed against the human IgG fragment of the transgene construct and reaction was measured against a human serum protein calibrator (Dako, Denmark) in dilutions of 1:11,900, 1:59,500, 1:297,500, 1:1,487,500 and 1:7,437,500. For reaction development chromogenic substrate for the horseradish peroxidase system was used. The following protocol was employed in carrying out the experiments:

Dilute primary antibody (polyclonal rabbit anti-human IgG; Dako, Denmark) 1:570 in coating buffer
Add 100 µl to each well
Cover with self-adhesive plastic covers and incubate overnight at 4°C
Discard well contents
Wash 3x with 200 µl washing buffer per well
Dilute standard and samples to appropriate concentrations in washing/dilution buffer
Add 100 µl to each well
Cover and incubate 2 hours at room temperature
Discard well contents
Wash 3x with 200 µl washing buffer per well
Dilute secondary antibody (polyclonal rabbit anti-human IgG/HRP; Dako, Denmark) 1:4800 in washing/dilution buffer
Add 100 µl to each well
Cover and incubate 1 hour at room temperature
Discard well contents
Wash 3x with 200 µl washing buffer per well
Add 100 µl 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Uptima, France) to each well
Cover and incubate 7 min at room temperature
Add 100 µl Stop solution

Colour development was read immediately by measuring extinction at 490 nm and

620 nm reference wave length through the bottom of the microwell plate in an ELISA reader (Tecan, Austria). Protein concentrations in samples were calculated by Magellan software (Tecan, Austria) using the standard as a reference.

3.3.2.3 Immunohistochemistry

For immunohistochemistry, samples were initially preserved in 4% PFA as described above.

Immunohistochemical staining and evaluation of tissue samples was carried out by Dr. Nadja Herbach (Department of Veterinary Pathology, Ludwig-Maximilians-Universität, München) for CAG-LEA and INS-LEA transgenes, and Dr. Claudius Faber (Institute of Pathology, Ludwig-Maximilians-Universität, München) for GALKO, CD46 and hTM according to their specific protocols.

Briefly, samples were taken from 4% PFA, placed in embedding cassettes and embedded in paraffin. A series of tissue sections was cut from paraffin blocks using a microtome and mounted onto glass slides. These sections were then stained immunohistochemically using specific antibodies. After visualisation of immunoreactivity and counterstaining, samples were analysed for putative transgene expression on protein level.

Particulars of antibodies, chromogens and counterstains employed in the different stainings are given in Table 3.4

Table 3.4: Immunohistochemical procedures

Antigen	Primary antibody	Detection system	Chromogen	Counterstain
Gal	mouse anti-α-Gal-epitope, monoclonal, 1:5	goat anti-mouse IgM, biotinylated 1:100	DAB	Hematoxylin
CAG-LEA	rabbit anti-human IgG, polyclonal, 1:50	swine anti-rabbit IgG, HRP-conjugated 1:100	DAB	Hemalaun
CD46	mouse anti-human CD46, monoclonal, 1:10	Histofine Simple Stain Rat MAX PO	AEC	Hematoxylin
hTM	mouse anti-human TM, monoclonal, 1:300	Histofine Simple Stain Rat MAX PO	AEC	Hematoxylin
INS-LEA	rabbit anti-human IgG, polyclonal, 1:50	swine anti-rabbit IgG, HRP-conjugated 1:100	DAB	Hemalaun

DAB: 3,3'diaminobenzidine tetrahydrochloride; AEC = aminoethylcarbazole

3.3.3 Calculation of inbreeding coefficient

For calculation of the inbreeding coefficient, an ancestral table of the individual in question was drawn (Figure 3.4 (A)) according to the so called point system described in LeRoy (1966). This system leads to a calculation of the unadjusted inbreeding coefficient F'_x according to the following formula:

$$F'_x = \sum (½)^{n+n'+1}$$

n = number of generations between parental generation and common ancestor on the paternal side

n' = number of generations between parental generation and common ancestor on the maternal side

This system yields the correct inbreeding coefficient if none of the common ancestors are inbred themselves. Otherwise the resulting value has to be adjusted accordingly.

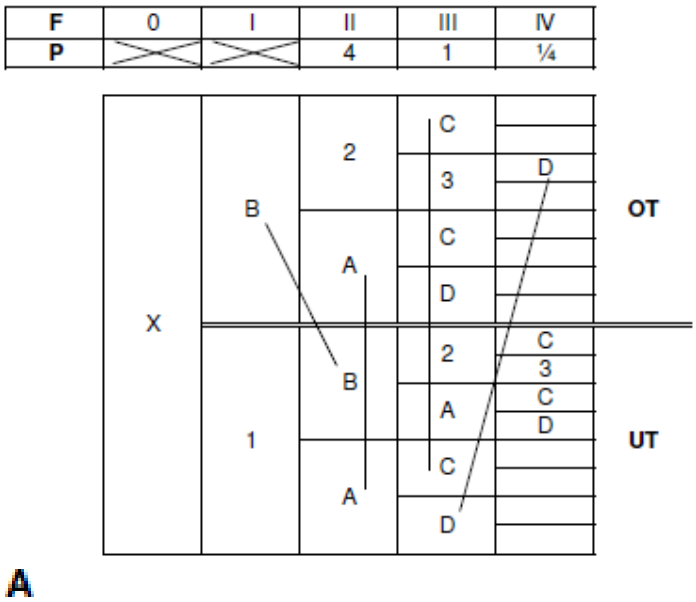
Ancestry of the founder animals investigated in this thesis and employed in establishing transgenic breeding herds is not known. We defined this inbreeding coefficient as basal level *0*.

The table drawn for calculation of the inbreeding coefficient of transgenic offspring was divided in an upper (OT) and a lower half (UT) for the maternal and the paternal ancestry. An individual is only then inbred if a common ancestor is to be found in both halves of the table. In order to calculate the inbreeding coefficient each ancestral generation (F0 –Fxx) is given a point weight (P) and the total point value of a common ancestor is calculated according to the following rules:

- common ancestors in OT and UT are connected across the central dividing line
- if the offspring of a specific ancestor is the same in OT and UT, the connection between the ancestor in OT and UT is cancelled
- if parts of the ancestry repeat themselves in OT and UT, all connections between common ancestors are cancelled except the ones that are furthest to the left of the table
- connecting lines within the same generation are given the point value indicated for that generation at the top of the table
- connecting lines between two adjacent generations are allocated the value of

the generation on the right times two

- if a connecting line skips a generation the value of the generation to the right is timed by four (or six if two generations are skipped)
- all point values are added up and the inbreeding coefficient is taken by inserting the total calculated point value into Figure 3.4 (B)



P	F'_x
0	0
1	3.1
2	6.2
3	9.4
4	12.5
5	15.6
6	18.8
7	21.9
8	25.0
9	28.1
10	31.2
11	34.4
12	37.5
13	40.6
14	43.8
15	46.9
16	50.0

B

Figure 3.4: Example of tables for calculation of inbreeding coefficient of individual 'x'. (A) Ancestral table for individual 'x'; connecting lines of common ancestors on maternal and paternal side have been drawn; (B) Table for reading of unadjusted inbreeding coefficient F'_x
Tables adapted from LeRoy (1966)

3.3.4 Design of breeding schedules

Breeding schedules were designed using Macromedia Freehand MX software while taking into account the calculated inbreeding coefficients of potential offspring and segregation patterns of transgenes according to Mendelian rules of inheritance.

4 RESULTS

4.1 Identification of suitable breeding herds

In order to satisfy demand for a regular supply of donor animals for xenotransplantation experiments, breeding herds of pigs displaying xenotransplantation-relevant transgene expression were to be established. Planned experiments included donor animals with GalKO/CD46/HLA-E expression for blood perfusion studies utilising the fore limbs, hearts and kidneys of these animals and GalKO/CD46/hTM transgenic pigs for pig-to-baboon heart transplantation and pig-to-cynomolgus monkey kidney transplantation. Up until now donor animals for these studies had been derived from recloning experiments of the original founders with proven transgene expression. This strategy, however, had been too inefficient and cost-intensive to ensure regular supply of donor animals in sufficient quantities. Creating a feasible breeding strategy that satisfies the demand for transgenic offspring provided a suitable alternative. All currently performed experiments are covered by the DFG Transregio Research Unit "Xenotransplantation" (FOR 535) which provided the basis for calculation of required numbers of donor animals per year. An outline of the number of experiments with each of the different transgene combinations is given in Table 4.1. This compilation served as a ground for estimating the size of the breeding herds according to aspired numbers of litters per year.

In these experiments the eventual goal was to utilise donor animals transgenic for GalKO/CD46 and either HLA-E or hTM. However, double instead of triple transgenic animals were to be used during a transition period where breeding had not produced the full set of transgenics yet.

Table 4.1: Planned number of xenotransplantation experiments

Transgene	Pig-to-baboon heart	Pig-to-cyn. monkey kidney	Perfusion heart	Perfusion kidney	Perfusion limb
GalKO/CD46	8*				
GalKO/CD46/hTM	8	12			
hTM			3	5	
CD46/HLA-E			3		5*
GalKO/CD46/HLA-E			3		5

*Double transgenic animals will be used until triple transgenic variants are available.

Additionally, animals expressing only one or two of the transgenes in varying combinations were to be used in the organ perfusion experiments in order to provide an opportunity for comparison of the singular and cumulative effects of the different transgenes.

Taking into account that average litter sizes similar to those in wild-type breeding herds are to be expected in the breeding of transgenic pigs (Martin et al. 2004; Mir et al. 2005; Shibata et al. 2006; Williams et al. 2006) and that transgenes adhere to Mendelian laws of inheritance (Aigner et al. 1999), a mean litter size of 10 piglets with 25% desired transgene combinations was assumed. Thus four litters of GalKO/CD46/HLA-E and 10 litters of GalKO/CD46/hTM transgenic pigs per year were aimed for in order to fulfil the requirements for transgenic donors.

Since it can be expected that one sow will generate two litters per year, a breeding strategy involving two GalKO/CD46 boars, two GalKO/HLA-E sows and five GalKO/hTM sows was designed. For ease of handling and decline of fertility with age, boars in the breeding herd are to be replaced every two years and sows every three.

4.2 Selection of founder animals

For establishment of transgenic breeding herds, suitable founder animals were chosen from the available material on the basis of genomic and expression analysis with regard to transgene status.

GalKO/CD46 boars with well-characterised transgenic properties (Ekser et al. 2010;

Lin et al. 2010) that had been obtained from *Revivicor Inc.* were re-established at the MVG, Badersfeld. From these efforts, two GalKO/CD46 transgenic boars were gained in June (#9872) and August (#9896) 2009 which were raised until sexual maturity and are now being utilised in breeding.

HLA-E transgenic pigs had been established at the MVG, Badersfeld and were characterised for their transgene expression as reported by Weiss et al (2009). Breeding of these initial founders generated three HLA-E transgenic sows in June 2008 (#9713) and June 2009 (#9864, #9869). These three animals are now being employed in establishing a GalKO/CD46/HLA-E breeding herd.

4.3 Expression and functional analysis of novel transgenic lines

Additionally, potential founder pigs for new transgenic lines from litters derived from nuclear and embryo transfer have been characterised for their transgenic properties on the basis of genomic and expression analysis. The conclusions drawn from these analyses provided the foundation for the choice of founder animals which were to be re-cloned and utilised in the breeding of pigs carrying the novel transgenes.

4.3.1 hTM

hTM transgenic founder animals were derived from two different strategies. In one case the hTM-neo vector was used to establish single transgenic pigs. Three litters generated by NT and ET and born in August and December 2008 provided a total of eight piglets. Genotyping PCR on genomic DNA isolated from ear punches of three days old piglets determined seven of them as hTM transgenic (Figure 4.1 (A)). Immunohistochemical staining of tissue sections demonstrated strong transgene expression in all examined organs of animal #9781, while the litter mates #9780 and #9782 did not show consistent expression across all organs (Table 4.2).

In order to receive information about the transgene copy number and the number of integration sites in the genome, Southern blotting on isolated genomic DNA was conducted. Taking into account the restriction sites within the transgene vector and the

feasible array of transgene copies in the genome this revealed the integration of only a few copies of the transgene at one single site in founder #9781, while it can be assumed that the examined litter mates display a more complicated transgene integration pattern (Figure 4.2). Boar #9781 was therefore chosen for re-cloning. Table 4.3 outlines the NTs and ETs performed in re-establishing boar #9781, which led to the birth of boar #9943 in December 2009. This animal has been utilised as a founder in the establishment of the GalKO/CD46/hTM breeding herd.

At the same time, animal #9744 has undergone re-cloning attempts (Table 4.3). This boar had also been genotyped positive for the hTM-neo vector (Figure 4.1 (A)) and southern blotting indicated a single integration site of one copy of the transgene (Figure 4.2). Transgene expression capacity was verified by immunohistochemical staining of tissue sections, where this particular animal showed strong hTM expression in all examined organs (Table 4.2).

Additionally, the hTM-bla vector was established on the GalKO/CD46 background obtained from *Revivicor Inc.* From this effort, four potential founder pigs were gained in January 2010 of which three were analysed positive for the hTM-bla transgene in a genotyping PCR (Figure 4.1 (B)). Immunohistochemical staining of tissue sections demonstrated strong hTM expression across all examined organs in animals #9947 and #9948. Animal #9949 showed equivalent expression in ear and heart tissue, however, staining of other organs was markedly weaker than in the two litter mates (Table 4.2). Boar #9948 was therefore chosen as suitable founder animal for GalKO/CD46/hTM breeding. Re-cloning efforts for this animal are summarised in Table 4.4. Boar #1103, a clone of #9948 born in December 2010, is currently being raised for breeding.

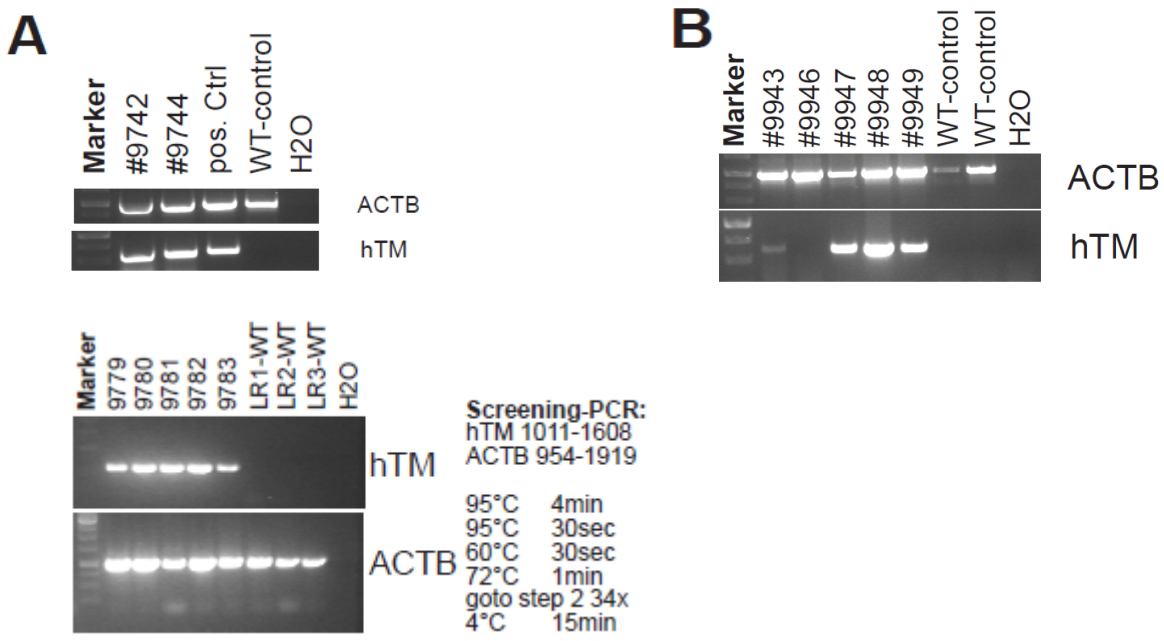


Figure 4.1: Genotyping of genomic DNA isolated from hTM transgenic founder animals and littermates. (A) Genotyping PCR for hTM-neo founders; (B) Genotyping PCR for hTM-bla founders; A 508 bp band is amplified if the transgene has been incorporated into the genome; wild-type DNA produces no signal; β-actin served as a control for DNA integrity. PCR conditions were as shown.

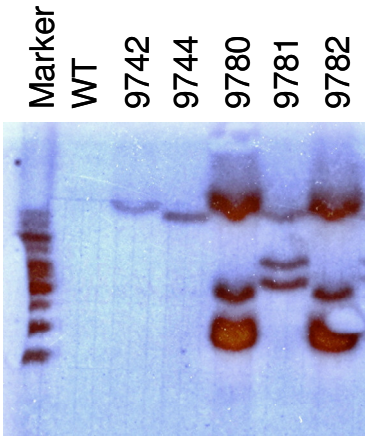


Figure 4.2: Southern Blot of genomic DNA derived from hTM transgenic founder animals. Southern blotting of hTM-neo founders and wild-type control. Whereas #9780 and #9782 show an identical transgene integration pattern, the other animals constitute independent founders.

Table 4.2: Transgene expression levels of hTM founder animals determined by immunohistochemical staining of organ sections.

Founder	Transgene	Ear	Heart	Lung	Muscle	Kidney	Liver	Spleen
#9742	hTM	3	3	3	3	3	3	3
#9743	hTM	0	0	0	0	0	0	0
#9744	hTM	3	3	3	3	3	3	3
#9780	hTM	3	2-3	1	nd	3	0	nd
#9781	hTM	3	3	3	nd	3	3	nd
#9782	hTM	3	2-3	1	nd	3	1	nd
#9947	Gal	0	0	0	0	0	0	0
	CD46	3	3	2	2	1	3	2
	hTM	3	3	3	3	3	3	3
#9948	Gal	0	0	0	0	0	0	0
	CD46	3	1	1	1	1	1	1
	hTM	3	3	3	nd	3	3	3
#9949	Gal	0	0	0	0	0	0	0
	CD46	3	1	1	1	1	3	1
	hTM	3	3	1	nd	2	1	0

Expression grading: 0 = absent; 3 = very strong

Data provided by Dr. Julius Faber, Institute of Pathology, LMU Munich

Table 4.3: Re-cloning of hTM-neo founders

Founder	NT/ET	Pregnancy	Delivery	Offspring	Weaned
#9744	Dec. 2008	-	-	-	-
	Dec. 2008	-	-	-	-
	Jan. 2009	+	+	2	0
	Jun. 2009	-	-	-	-
	Jun. 2009	-	-	-	-
#9781	Jun. 2009	+	-	-	-
	Aug. 2009	+	+	1	**#9943**
	Aug. 2009	-	-	-	-
	Sep. 2009	-	-	-	-
	Sep. 2009	+	+	1	0

Data provided by Dr. Mayuko Kurome and Dr. Barbara Keßler

Table 4.4: Re-cloning of hTM-bla founder

Founder	NT/ET	Pregnancy	Delivery	Offspring	Weaned
#9948	Mar. 2010	-	-	-	-
	April 2010	-	-	-	-
	April 2010	+	+	1	0
	May 2010	+	+	6	3*
	June 2010	+	+	4	3*
	June 2010	+	+	2	1*
	Aug. 2010	+	+	4	**#1103**
	Aug. 2010	+	+	4	2*
	Aug. 2010	+	+	3	0
	Sep. 2010	-	-	-	-
	Sep. 2010	-	-	-	-
	Sep. 2010	+	+	4	0
	Oct. 2010	+	+	4	1*
	Oct. 2010	+	-	-	-
	Nov. 2010	+	+	1	nd
	Nov. 2010	+	+	1	nd

* Offspring was utilised as donor in xenotransplantation experiments

Data provided by Dr. Mayuko Kurome and Dr. Barbara Keßler

4.3.1.1 Clarification of hTM transgene integration site

In order to aid selection of breeding material, clarification of the hTM transgene integrations sites in the genome was attempted with the aim of establishing zygosity specific duplex PCRs that allow for the discrimination of hemi- from homozygous transgenic animals. For this, inverse PCR was performed on genomic DNA isolated from hTM founder animals.

In pig #9781 two hTM-neo transgene-transgene links could be identified. Both were in a head to tail orientation, however, while in one case transgene ends on both sides of the connection were complete, the other link suggested that one transgene copy was missing 9.2 kb of its head part (Figure 4.3 (A)). Additionally, DNA sequences of 915 bp and 467 bp were generated that did, in part, not match the transgene sequence, making amplification into the adjacent regions of the 5' and 3' ends of the transgene likely. DNA data base searches of the porcine genome with the unknown flanking sequence did not provide an indication of the region concerned. However, the 915 bp fragment amplified from across the 5'end of the transgene sequence into the genomic

region contained a pig specific repetitive element, thereby confirming the genomic nature of the unknown sequence.

In a subsequent step, primers were designed complementary to the identified unknown DNA sequences beyond the 5' and 3' end of the transgene. From these a DNA fragment was amplified by conventional PCR from wild-type alleles that overlapped the sequence generated by inverse PCR on each end. Using this 1075 bp wild-type fragment in conjunction with the flanking regions identified beyond the primer binding sites, a new data base search was conducted with a DNA fragment of 2278 bp length. In this case the porcine genome again yielded no matching sequence. However, searches in the closely related bovine and the well characterised human genome identified a region within the *CCDC132* gene around exon 21 that matched the unknown sequence to between 76% and 78%. More detailed analysis of the region concerned revealed an hTM-neo transgene integration site 182 bp upstream of exon 21 of the *CCDC132* gene. Additionally, it became obvious that in transgenic alleles 790 bp of the wild-type sequence had been replaced by the transgene (Figure 4.3 (B)). The absence of any other amplicons from inverse PCR that could not be correlated with the integration site in the *CCDC132* gene in conjunction with the previously analysed Southern blot strongly supports the notion that this integration site is the only one in animal #9781. Combining the knowledge gained from inverse PCR and Southern blotting, the integration of three transgene copies, one of them truncated, the other two complete, at a single integration site is the most likely explanation. Within this site, two different arrays would fit the restriction pattern of both the Southern blot and the transgene connections found by inverse PCR (Figure 4.4 (A)(B)).

For the hTM-bla vector integration site, genomic DNA of two animals was examined by inverse PCR. Integration locus clarification has not been possible in either of them so far. However, numerous transgene-transgene links have been found in animal #9948, several of them indicating a row of complete transgene copies whereas some appeared to be truncated at the 5´ end. Most of the transgene-transgene connections were found to be assembled in a head to tail orientation, but one link was of a tail to tail nature (Figure 4.5 (A)). #9949 displayed a presumably less complicated integration pattern compared to #9948. Only one head to tail transgene link could be

identified in which case both transgenes appeared to be complete (Figure 4.5 (B)).

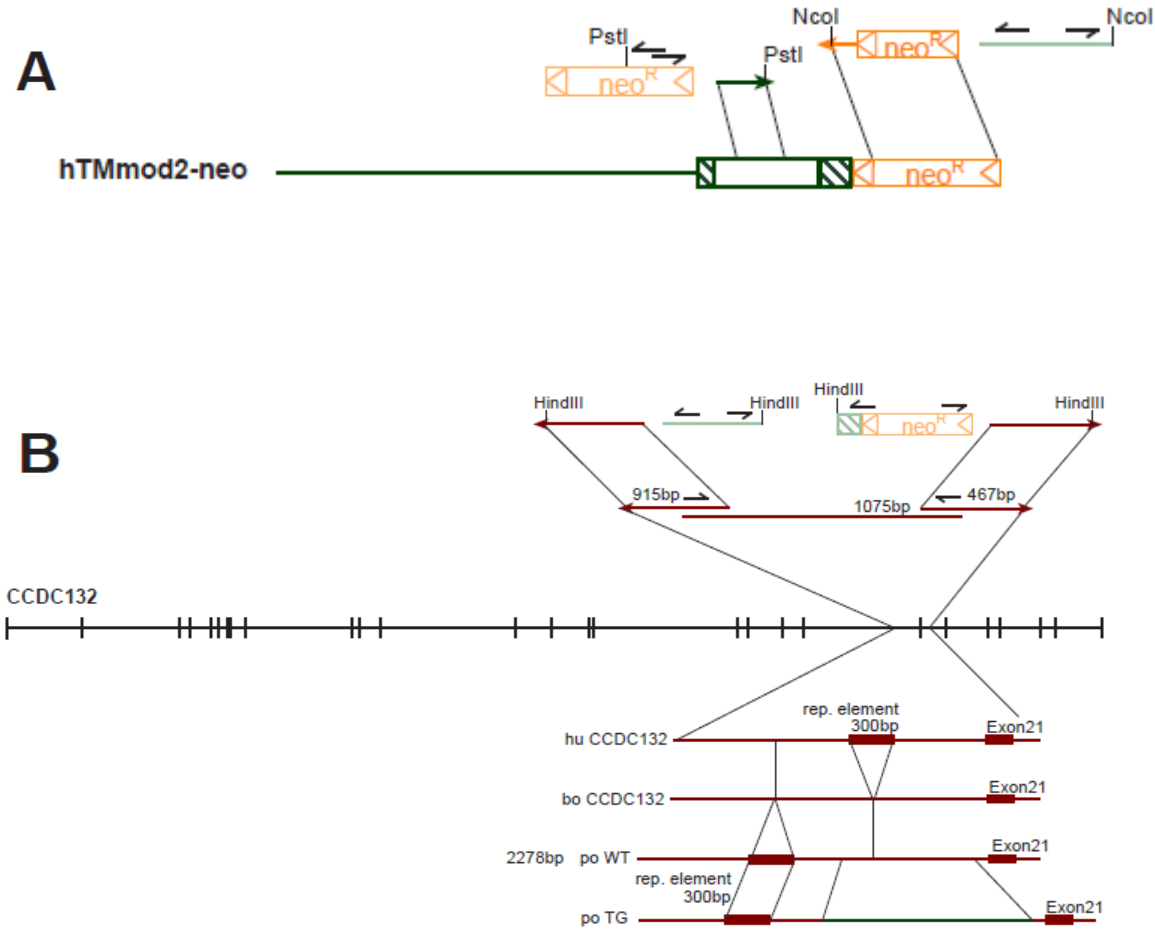


Figure 4.3: Integration site determination of hTM-neo. Either the 5´ end or the 3´ end of the transgene served as an anchor in inverse PCR using primers indicated as black arrows. The restriction sites used for fragmentation of genomic DNA are shown. The constructs are in accordance with Figure 3.1. (A) Two different transgene-transgene links in head-to-tail orientation could be identified; the homologies of the flanking sequences to the transgene construct are represented by thin lines. (B) Two different transitions of transgene to genomic sequence were identified. Conventional PCR revealed that these sequences are in close proximity to each other on wild-type alleles. Homology of the unknown porcine sequence to the human and bovine genome suggested integration of the transgene within the porcine *CCDC132* gene near exon 21.

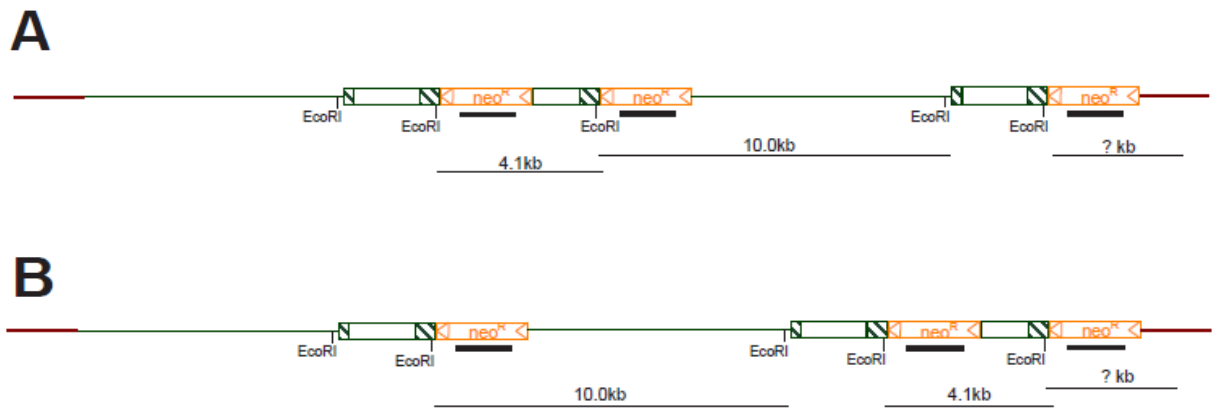


Figure 4.4: Feasible transgene integration patterns of hTM-neo. Combining the findings in inverse PCR and Southern blotting where three distinct bands were identified (see Figure 4.2), three transgene copies have presumably been integrated in founder #9781, two complete, one truncated. The truncated transgene is either placed in the middle (A) or at the 3' end of the integration site (B). The constructs are in accordance with Figure 3.1 with the positions of the EcoRI sites used for genomic fragmentation in Southern blotting indicated. The localisation of the neomycin-resistance-specific probe is displayed as a black bar and the lengths of the hybridised fragments are indicated.

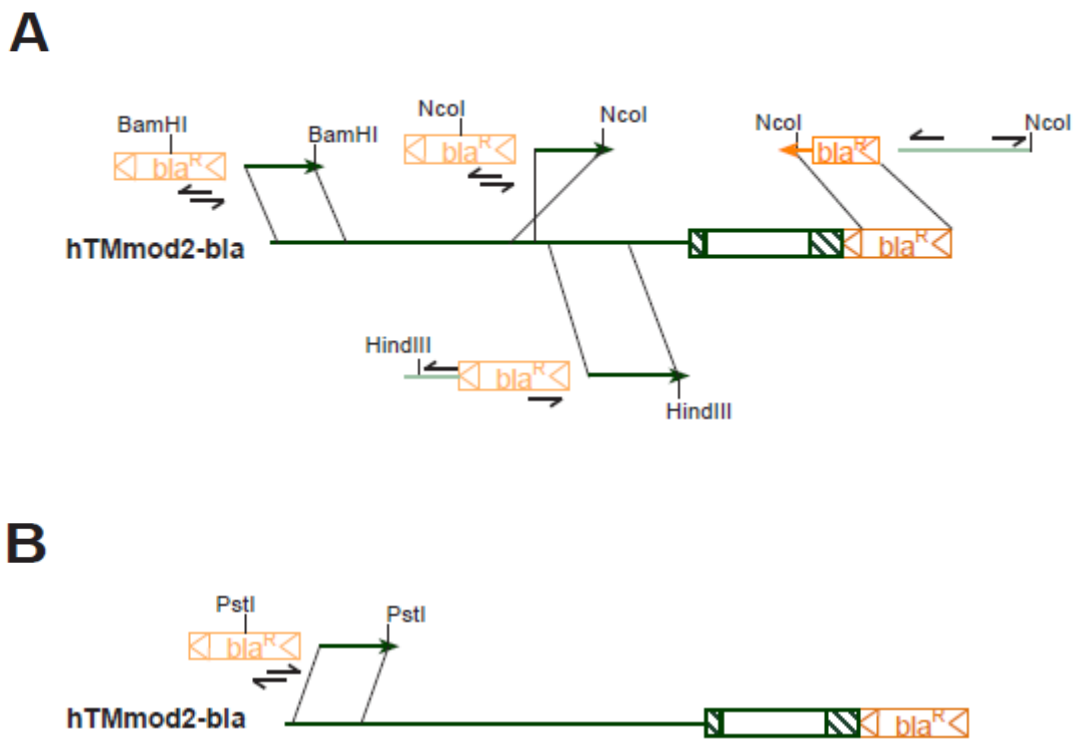


Figure 4.5: hTM-bla transgene-transgene links in founder animals. Either the 5´ end or the 3´ end of the transgene served as an anchor in inverse PCR using the primers indicated as black arrows. The restriction sites used for fragmentation of genomic DNA are shown. The constructs are in accordance with Figure 3.1. While #9948 displays a complex integration pattern with either head-to-tail or tail-to-tail links (A), inverse PCR revealed only one transgene-transgene transition in #9949 in head-to-tail orientation (B).

4.3.2 INS-LEA

Two founder litters for INS-LEA yielded nine piglets in February 2009. These animals were initially examined for their transgene status. For this, a genotyping PCR was performed on genomic DNA isolated from ear punches taken from three day old piglets (Figure 4.6 (A)). Seven piglets could be shown to carry the INS-LEA transgene in their genome. Southern blotting on genomic DNA was conducted and showed a different restriction pattern for each of the examined individuals. Thus they all constituted independent founders with unique integration sites (Figure 4.6 (B)). Copy numbers of integrated transgenes were estimated by analysis of the specific

restriction pattern and comparison of signal intensity to wild-type DNA mixed with stoichiometric equivalents of the transgene vector construct. From this, #9816 and #9818 were found to contain only one copy of the transgene, while the others contained more.

Animals #9813, #9814, #9816 and #9818 survived to an age of three months and were then sacrificed for organ spectrum expression analysis. Figure 4.7 shows immunohistochemical stainings of pancreas and kidney sections of these animals. Because the INS-LEA construct contains a porcine insulin promoter which controls the transgene, expression should be confined to pancreas islets, where this particular promoter is active. Founders #9813 and #9814 showed strong transgene expression islet specifically (Figure 4.7 (A), (B)), while pancreas sections of #9816 and #9818 stained only weakly for the transgene product (Figure 4.7 (D)). As expected, expression was absent from all kidney sections (Figure 4.7 (C)). In integration site analysis, Southern blot patterns led to the assumption that both well expressing pigs #9813 and #9814 contained four copies of the transgene at only one integration site, making them equivalent in their suitability as founders for an INS-LEA transgenic line. Both animals were therefore chosen to be re-cloned. Table 4.5 provides an overview of NTs and ETs conducted in re-cloning efforts for these two founder pigs. So far, boars #1044 and #1050, both re-cloned #9814, have been born in September 2010 and will be raised to be utilised for breeding.

In-vivo studies determined the functional effect of pancreas islet specific LEA29Y expression. For this, islet like clusters were isolated from pancreata of neonatal INS-LEA transgenic piglets, cultured for several days and subsequently transplanted under the kidney capsule of NOD-SCID mice that suffered from a streptozotocin induced diabetes mellitus. After regaining normogylcaemia, hPBMCs were administered to these mice in order to restore their immune system in a humanised way. While mice transplanted with wild-type islets showed steadily increasing blood glucose levels, mice with LEA29Y transgenic islets stayed within normal ranges (Figure 4.8).

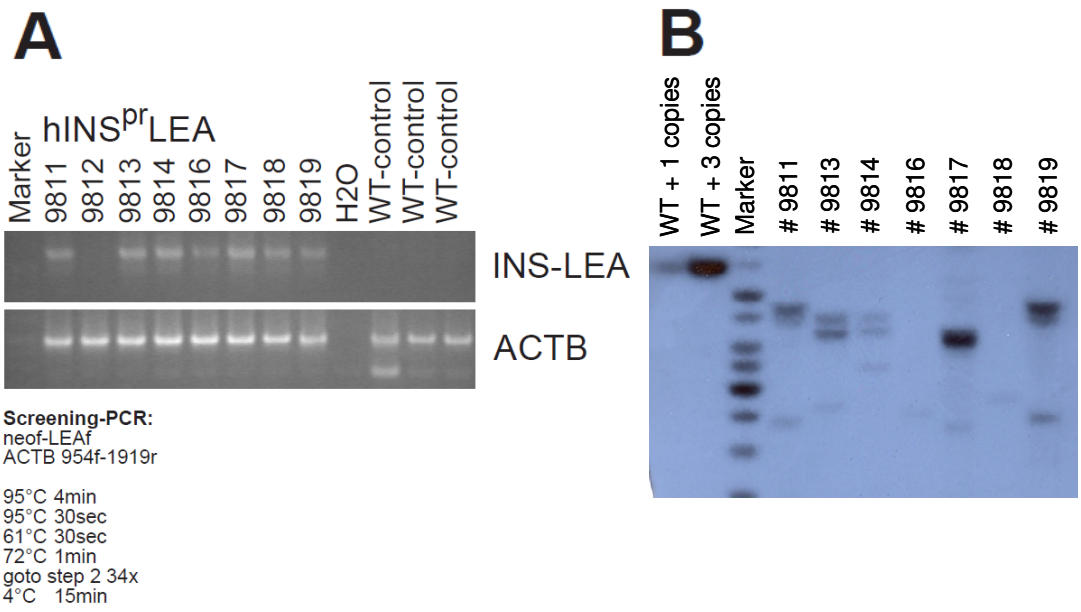


Figure 4.6: Genotyping and Southern Blotting of genomic DNA isolated from INS-LEA transgenic founder animals and littermates. (A) Genotyping PCR: A 993 bp band is amplified if the transgene has been incorporated into the genome; wild-type DNA produces no signal; β-actin served as a control for DNA integrity; PCR was performed according to the conditions shown. (B) Southern blotting of INS-LEA founders and wild-type DNA mixed with stoichiometric equivalents of the transgenic construct. All founders display independent transgene integration patterns and all except #9816 and #9818 contain more than one copy of the transgene.

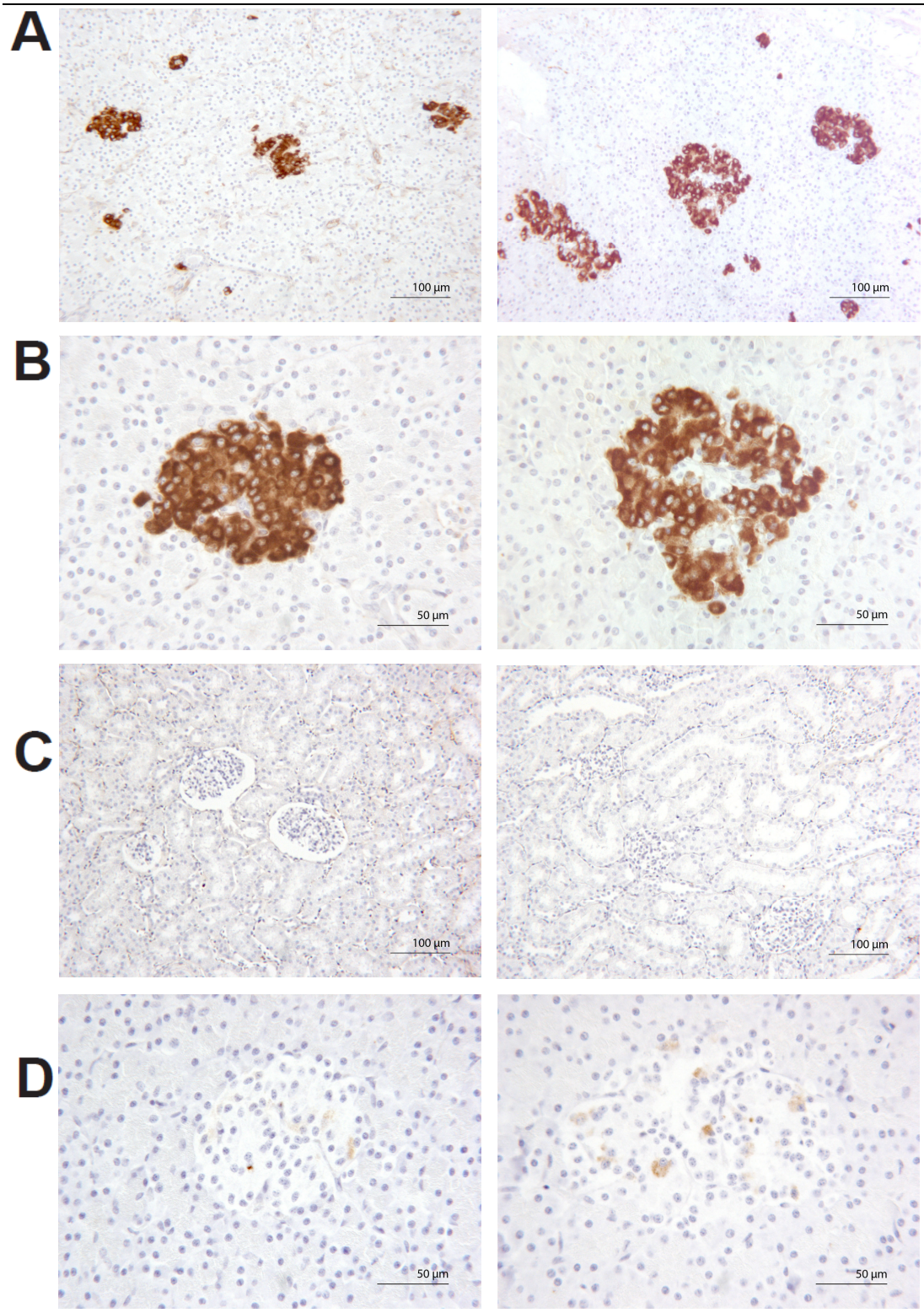


Figure 4.7: INS-LEA expression. Immunohistochemical staining with human IgG antibody reveals pancreas islet specific expression of LEA29Y in founder animals; (A) & (B) Pancreas sections of founder animals #9813 (left) and #9814 (right); (C) Kidney sections of founder animals #9813 (left) and #9814 (right); (D) Pancreas sections of weakly expressing littermates #9816 (left) and #9818 (right). Data provided by Dr. Nadja Herbach, Institute of Veterinary Pathology, LMU Munich.

Table 4.5: Re-cloning of INS-LEA founders

Founder	NT/ET	Pregnancy	Delivery	Offspring	Weaned
#9813	Mar. 2010	-	-	-	-
	Mar. 2010	-	-	-	-
	Mar. 2010	-	-	-	-
	Apr. 2010	-	-	-	-
	July 2010	-	-	-	-
#9814	Jan. 2010	+	-	-	-
	Jan. 2010	-	-	-	-
	Mar. 2010	-	-	-	-
	May 2010	+	+	2	0
	May 2010	+	+	3	#1050
	May 2010	+	+	4	#1044
	Sep. 2010	-	-	-	-
	Sep. 2010	-	-	-	-
	Oct. 2010	+	+	1	0

Data provided by Dr. Mayuko Kurome and Dr. Barbara Keßler

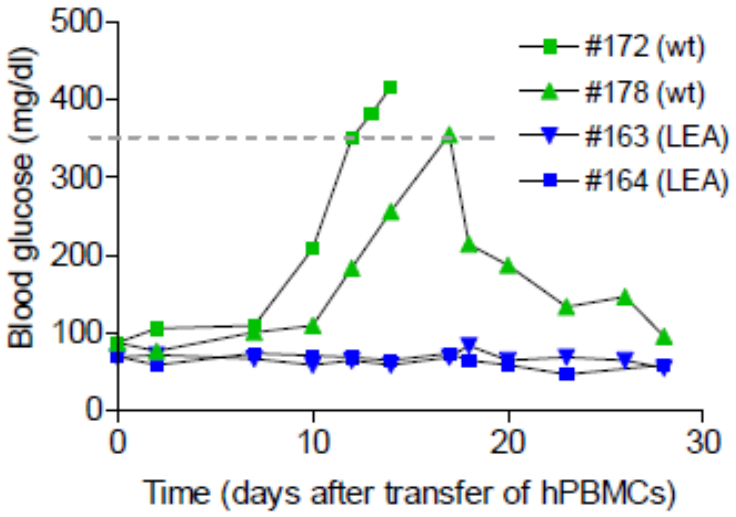


Figure 4.8: Pig-to-humanised mouse islet transplantation. INS-LEA transgenic islets retain their functionality after hPBMCs administration while wild-type controls fail to uphold normoglycaemia; hPBMCs = human peripheral blood mononuclear cells. Data provided by Lelia v. Bürck, LMU Diabetes Centre, Klinikum Innenstadt

4.3.3 CAG-LEA

In September 2009 one litter of three CAG-LEA transgenic pigs was generated by NT and ET. Genotyping PCR demonstrated that all three had the transgene incorporated into their genome (Figure 4.9). Southern blot analysis indicated the integration of single transgene copy in founders #9808 and #9810, whereas founder #9809 contained presumably two transgene copies. Preliminary expression analysis was performed by immunohistochemical staining of ear tissue sections taken from three day old piglets. This revealed strong LEA29Y expression in animal #9908, whereas staining of the littermates showed no difference to the wild-type control (Figure 4.10). Because the controlling promoter on the CAG-LEA construct is ubiquitously active, transgene expression was to be expected in all organs. The piglets were therefore sacrificed at an age of three months and a whole organ sample spectrum was preserved and examined for LEA29Y expression. Immunohistochemistry again indicated well detectable transgene expression in animal #9908, but staining was absent from samples of littermates across all examined organs (Figure 4.11). The superior expression capacity of pig #9908 was further verified by measuring transgenic protein in serum samples and total protein extracts taken from CAG-LEA transgenic pigs and wild-type control. For this, an ELISA detecting the human IgG fragment of the LEA29Y protein was employed. Here it became obvious that pigs #9909 and #9910 did after all display transgene expression, albeit a weak one (Table 4.6). The difference in expression level ranged between three to nine times as much protein measured in samples of the strongest expressing animal #9908 compared to the weakest expressing animal #9909. An in-vitro assay utilising cell cultures grown from animal #9908 additionally demonstrated the functionality of CAG-LEA transgene expression by examining the binding capacity of LEA29Y expressed from transgenic cells to a porcine B-cell line (Figure 4.12).

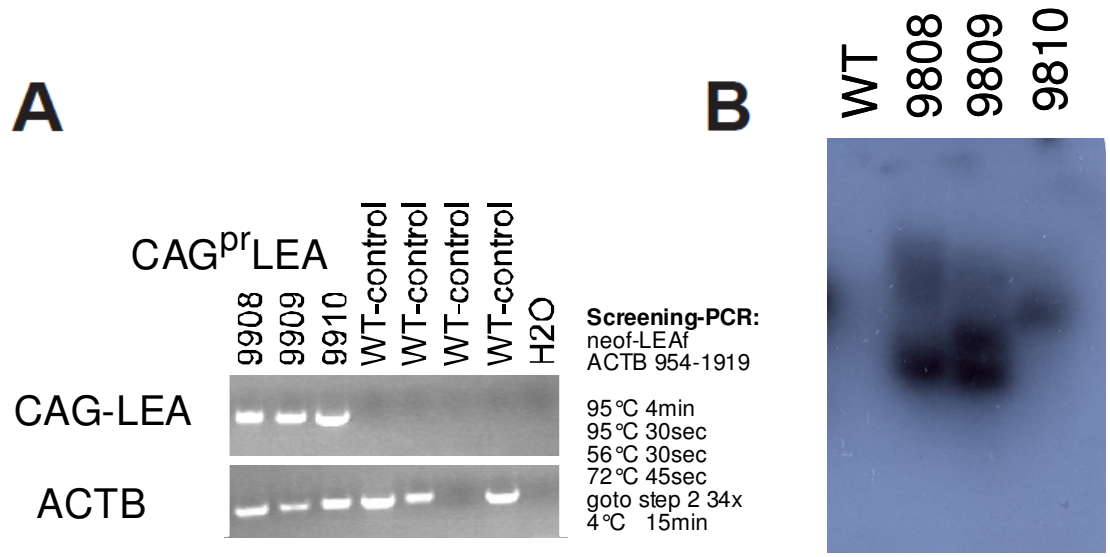


Figure 4.9: Genotyping PCR of CAG-LEA founder animals. (A) A 790 bp fragment is amplified from genomic DNA of transgenic animals; wild-type controls generate no signal; β-actin served as a control for DNA integrity. PCR was performed according to the conditions shown. (B) Southern blotting of the three transgenic and a wild-type animal was performed. While #9808 and #9810 indicated a single transgene copy, #9809 appeared to contain two transgenes.

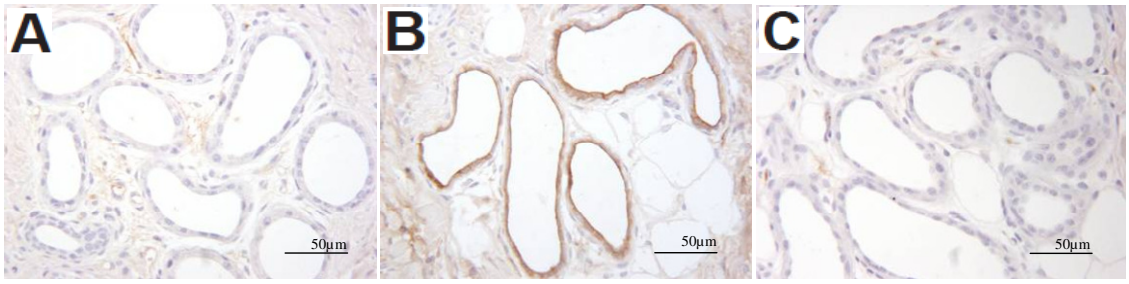


Figure 4.10: LEA29Y expression in CAG-LEA transgenic founder pigs and control. Immunohistochemical staining of ear tissue sections with human IgG antibody reveals expression of LEA29Y in founder animals; (A) Wild-type control; (B) Well-expressing transgenic founder #9908; strong staining for human IgG can be detected around sweat glands; (C) Transgenic founder #9909; no specific staining for human IgG detectable. Data provided by Dr. Nadja Herbach, Institute of Veterinary Pathology, LMU Munich

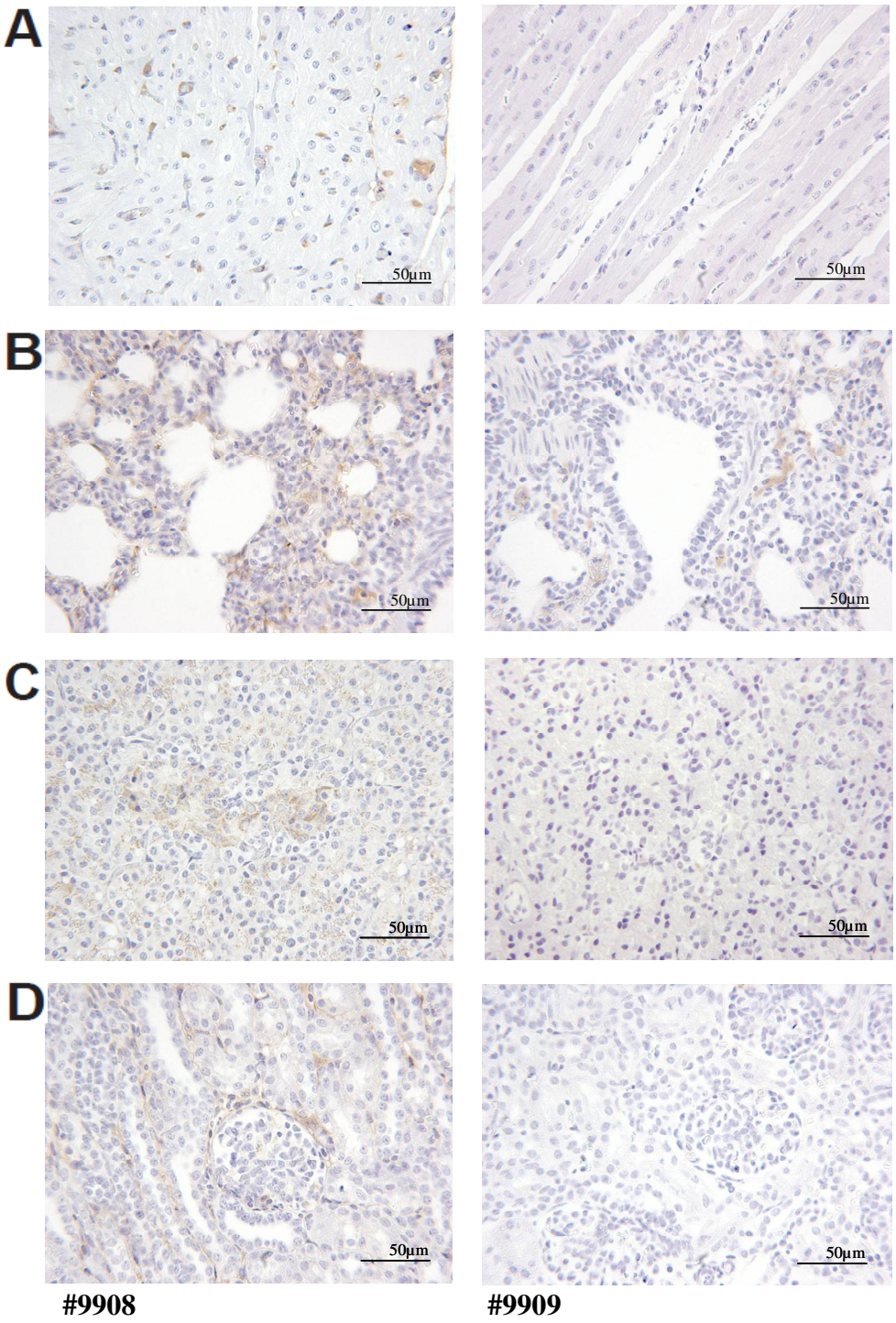


Figure 4.11: LEA29Y expression in organ spectrum of CAG-LEA transgenic founder pigs. Immunohistochemical staining with human IgG antibody reveals different levels of LEA29Y expression in founder animals #9908 (left column) and #9909 (right column); (A) Heart; (B) Lung; (C) Pancreas; (D) Kidney. Data provided by Dr. Nadja Herbach, Institute of Veterinary Pathology, LMU Munich.

Table 4.6: Human IgG concentration in serum and total protein extracts of organs of CAG-LEA transgenic founder pigs.

Sample	Serum	Heart	Kidney	Pancreas	Liver	Lung	Muscle	Spleen	Intestine
#9908	2.15	2.83	2.99	4.22	2.14	8.05	2.20	2.47	5.05
#9909	0.32	0.87	0.32	0.65	0.72	1.39	0.26	0.69	0.91
#9910	1.22	1.27	0.88	0.63	0.84	1.42	0.64	1.38	1.32
WT control	0.04	0.01	0.01	0.01	0.00	0.06	0.02	0.01	nd
Positive control	15.45	nd	nd	nd	nd	nd	nd	nd	nd

Concentration in µg/ml was determined by ELISA using human IgG antibodies. Human serum from a healthy donor was used as positive control and protein samples from a wild-type pig as negative control.

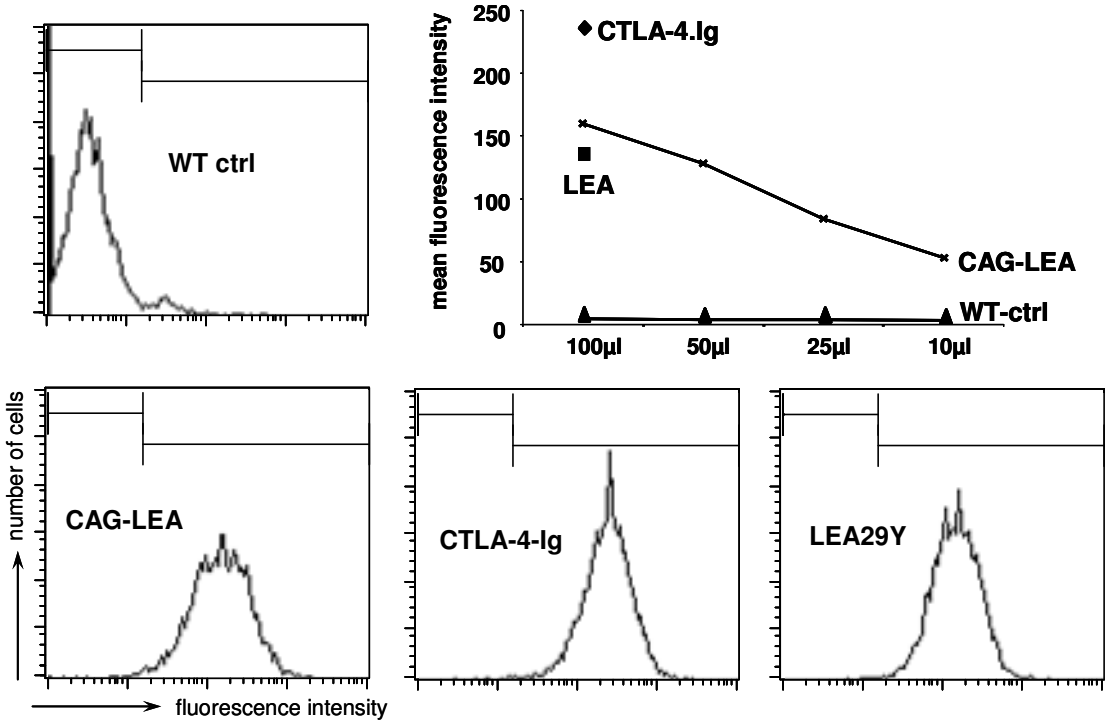


Figure 4.12: Binding of transgene expressed from CAG-LEA construct to antigen presenting cells. Binding capacity of transgenic protein from cell culture supernatant to the porcine B-cell line L23 was compared to capacity of cell culture supernatant from porcine wild-type cells and to the synthetic drugs CTLA4-Ig and LEA29Y. Titration of cell culture supernatants revealed a linear regression of CAG-LEA binding capacity. Data provided by Dr. Reinhard Schwinzer, MHH Hannover

4.4 Breeding schedules

Breeding schedules based on the initial availability of GalKO/CD46 boars obtained from *Revivicor Inc*. and HLA-E sows were designed with the aim of generating a GalKO/CD46/HLA-E breeding herd in a first instance. Because hTM transgenic animals were not available for breeding yet when breeding of transgenic herds started, the aim was to integrate this transgene lateron. Since the common background of all breeding herds is GalKO/CD46, it was decided to preserve these characteristics paternally and add HLA-E and hTM transgenes from the maternal side. Additionally, sows employed in breeding must also display a GalKO, because the effectiveness of this trait requires a full knock-out of the αGal gene, thereby necessitating homozygosity for this characteristic, whereas HLA-E, CD46 and hTM expression is detectable irrespective of their zygosity status. Figure 4.13 shows different breeding schedules aiming at the establishment of intial GalKO/CD46 and GalKO/HLA-E breeding animals derived from the available GalKO/CD46 boars and HLA-E sows.

Generation of animals displaying the full set of required transgenes can only be achieved by breeding over two generations. The calculated probabilities for specific transgene combinations depending on transgene segregation and the level of inbreeding in the breeding herd both have to be taken into account when deciding on a suitable breeding method. Three different breeding approaches are feasible in establishing the breeding herds. Full-sibling mating of the F1 generation (Figure 4.13 (A)) results in calculated 3.125% for each desired transgene combination and sex distribution in the F2 generation that represents the breeding herd. Similar probabilities can be achieved with half-sibling matings in F1 (Figure 4.13 (B)). However, inbreeding coefficients in the breeding herds would be twice as high with full-sibling matings (0.25) compared to half-sibling matings (0.125). In order to increase the proportions of transgenic animals in F2, father-daughter matings can be employed in the F1 generation (Figure 4.13 (C)). This would produce 6.25 % animals of each suitable sex and transgene composition in F2. Resulting inbreeding, however, would be comparable to F1 full-sibling matings.

Because a rise in inbreeding coefficients of the breeding material has been shown to negatively correlate with productivity traits (Charlesworth and Charlesworth 1987;

Ralls et al. 1988; Lynch 1989; 1991), thereby decreasing numbers of available animals derived from the breeding herd, it was decided to favour breeding schedules that keep inbreeding levels as low as possible. Generation of initial breeding by half-sibling matings of F1 generations was thus given preference over the other options.

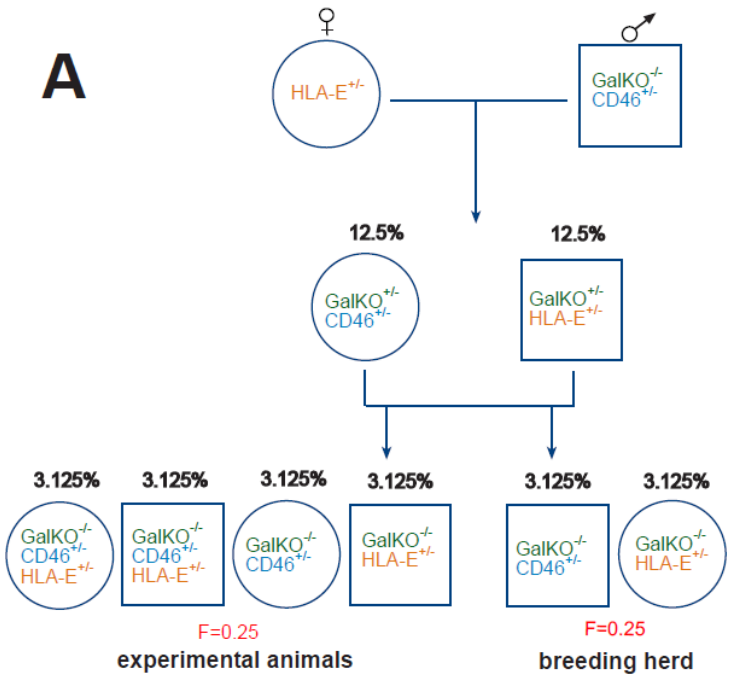


Figure 4.13: Breeding schedules for establishment of GalKO/CD46/HLA-E breeding herd. (A): Full-sibling matings of F1 generation; Circles indicate sows, squares indicate boars; percentages given are the probability for specific sex and transgene conformation in offspring; F = Inbreeding coefficient; $^{+/-}$ = hetero- or hemizygous knock out or transgene; $^{-/-}$ = homozygous knock out.

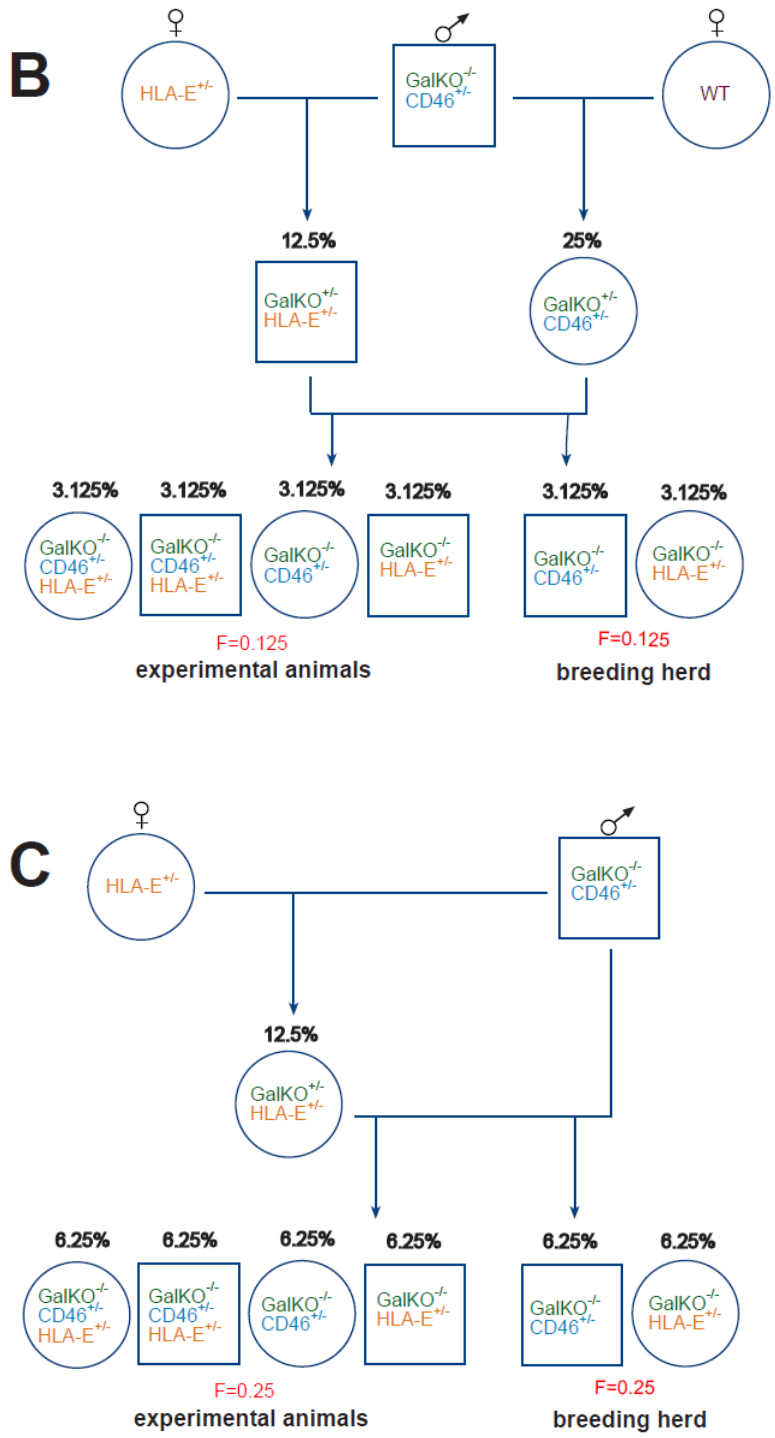


Figure 4.13: Breeding schedules for establishment of GalKO/CD46/HLA-E breeding herd. (B): half-sibling matings of F1 generation; (C): father-daughter matings of F1 generation. Circles indicate sows, squares indicate boars; percentages given are the probability for specific sex and transgene conformation in offspring; F = Inbreeding coefficient; $^{+/-}$ = hetero- or hemizygous knock out or transgene; $^{-/-}$ = homozygous knock out.

Integration of the hTM transgene into the breeding herd can be achieved by two different strategies. In one case, hTM is derived from boars generated by transfection of the hTM vector construct into GalKO/CD46 cells from *Revivicor Inc*. In this case the genetic background of the resulting GalKO/CD46/hTM boars is identical to the GalKO/CD46 boars employed in the establishment of the initial breeding herds. Figure 4.14 (A) shows a breeding schedule built on this option. Inbreeding coefficients in breeding material derived from mating a GalKO/CD46/hTM boar to an F1 sow of the half-sibling breeding schedule in Figure 4.13 will result in an inbreeding coefficient of the offspring of 0.25. Additionally, probabilities for a GalKO/hTM sow that could be utilised within the breeding herd calculate to only 3.125%.

However, since the hTM vector construct had also been transfected into a wild-type cell line, a boar transgenic only for hTM was also available for furthering the breeding herd. In this schedule, shown in Figure 4.14 (B), the establishment of GalKO/hTM sows takes one generation longer than in the other case. But probabilities for the correct transgene combination are doubled and, even more importantly, the inbreeding coefficient of the resulting individuals had been calculated at only 0.062. Therefore, preference was given to introducing the hTM transgene into the breeding herd via the more time-consuming route.

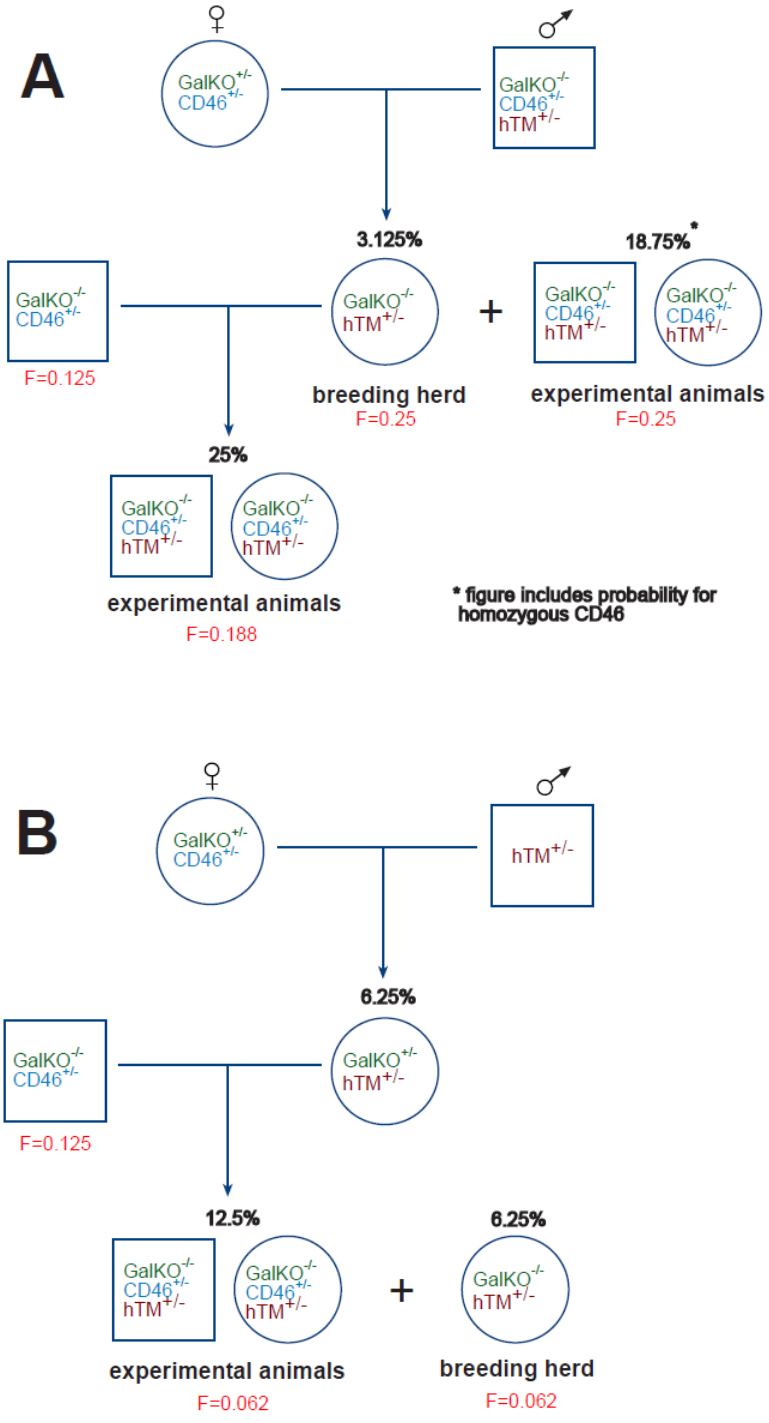


Figure 4.14: Breeding schedules for incorporation of hTM into GalKO/CD46 breeding herd. (A): hTM derived from boar #9948, triple transgenic founder with hTM on GalKO/CD46 background; (B) hTM derived from boar #9943, single transgenic hTM founder. Circles indicate sows, squares indicate boars; percentages given are the probability for specific sex and transgene conformation in offspring; F = Inbreeding coefficient; $^{+/-}$ = hetero- or hemizygous knock out or transgene; $^{-/-}$ = homozygous knock out.

In addition, simultaneous outbreeding of the breeding herd with wild-type animals is supposed to keep inbreeding at levels as low as feasible. As can be seen in Figure 4.15, outbreeding of GalKO/CD46 boars over two or three generations with wild-type sows prior to backcrossing offspring into breeding herd will lower inbreeding levels within the breeding herd itself significantly to 0.062 or 0.031 for GalKO/CD46/HLA-E combinations and 0.047 or 0.023 for GalKO/hTM, respectively if half-sibling matings are chosen for the establishment of breeding herds and hTM is derived from the single transgenic hTM boar.

Since GalKO/CD46 had been chosen as the common background for all transgene combinations, outbreeding is being performed with respect to these transgenes, building on the original founder boars available and introducing wild-type blood on the maternal side of the breeding schedule.

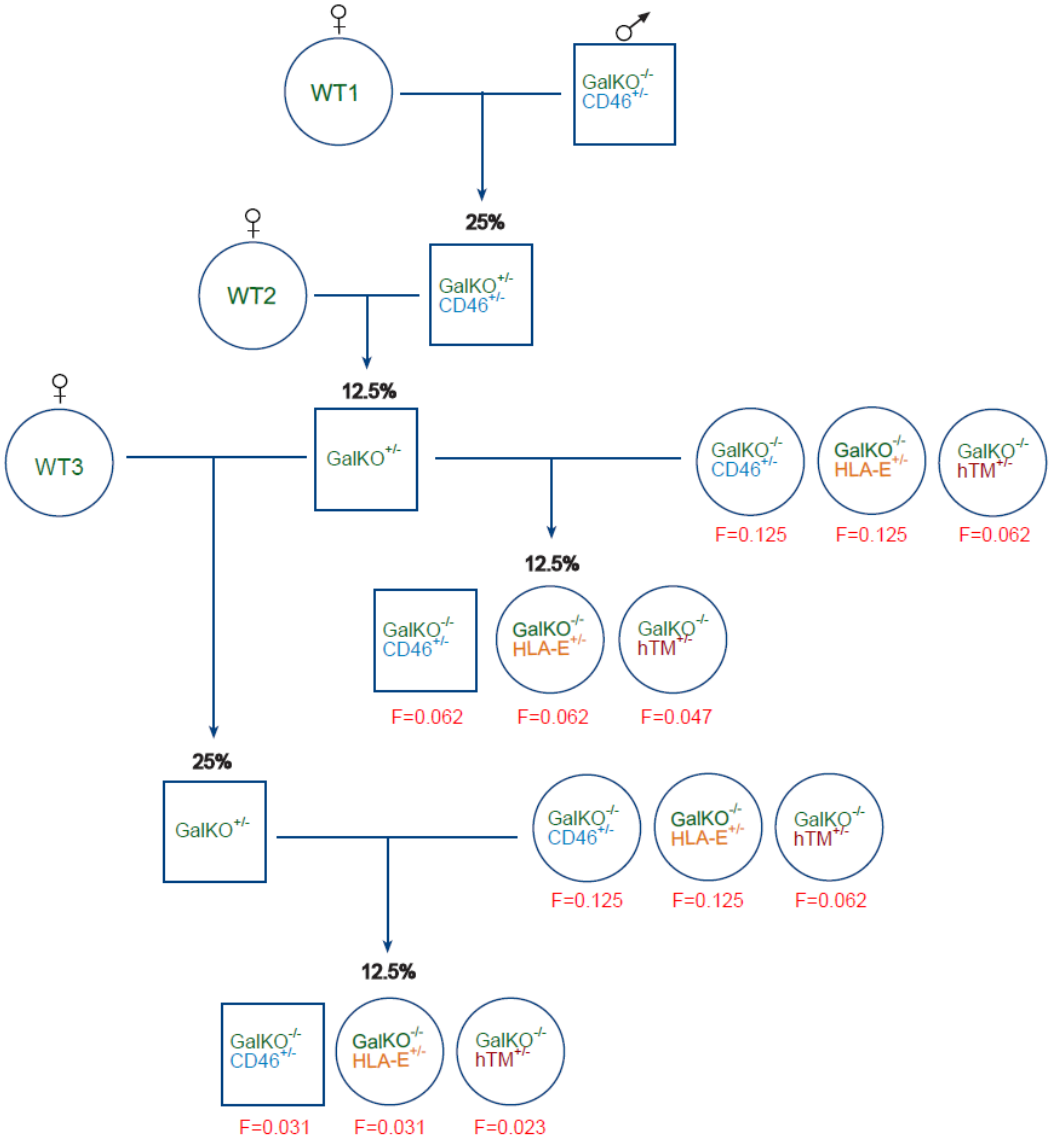


Figure 4.15: Outbreeding schedule for lowering inbreeding coefficients in transgenic breeding herds. Effects of outcrossing GalKO/CD46 boars with wild-type sows over two or three generations on inbreeding coefficients within breeding herds; circles indicate sows, squares indicate boars; percentages given are the probability for specific sex and transgene conformation in offspring; F = Inbreeding coefficient; $^{+/-}$ = hetero- or hemizygous knock out or transgene; $^{-/-}$ = homozygous knock out.

4.5 Breeding & analysis of intermediate (F1) generations

Table 4.7 provides an overview of matings conducted between the founder animals and their F1 generation offspring. These offspring are being employed in the establishment of triple transgenic breeding herds. Over the course of eight months ten matings took place which yielded a total number of 100 piglets.

Table 4.7: Matings

Mating	Boar	Sow	Date	Delivery	Offspring	Weaned
1	#9896	WT #NT53	16.03.2010	09.07.2010	13	9
2	#9872	#9864	16.03.2010	08.07.2010	7	7
3	#9896	WT #475	13.04.2010	05.08.2010	12	10
4	#9872	#9869	08.06.2010	29.09.2010	10	10
5	#9896	#9713	08.06.2010	01.10.2010	13	12
6	#9896	#9864	17.08.2010	09.12.2010	6	6
7	#9943	WT #NT122	01.10.2010	28.01.2011	7	5
8	#9943	WT #SH2	01.11.2010	24.02.2011	9	9
9	#9896	#9869	18.11.2010	11.03.2011	11	nd
10	#9896	#9713	18.11.2010	12.03.2011	12	nd

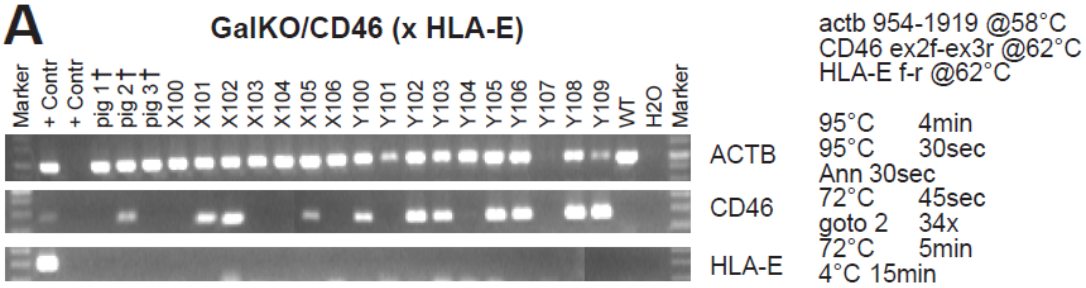
All piglets were genotyped to determine their transgene status. For this, genomic DNA isolated from ear punches taken from three day old piglets was used as template in genotyping PCRs employing primer pairs specific for the respective transgenes. Transgenic DNA between the primer binding sites was amplified and could be made visible as bands of a defined size on agarose gels after PCR if the individual had the transgene in question incorporated into its genome. In each PCR, genomic DNA of an animal previously genotyped positive for the respective transgene served as a positive control in order to determine a successful amplification process. Furthermore, negative controls consisting of wild-type genomic DNA and aqua bidest. were utilised to demonstrate absence of unspecific amplifications or contamination of reaction mixtures. In addition, each DNA sample was amplified with a primer pair specific for the β-actin gene in order to verify DNA integrity. A 971 bp fragment could be generated if the genomic DNA template was intact.

Two matings between one of the GalKO/CD46 boars and a wild-type sow were conducted to facilitate outbreeding of the GalKO/CD46 transgene. These matings

produced a total of 25 offspring, of which 13 piglets could be shown to have inherited the CD46 transgene from the father. All offspring were heterozygous for the GalKO since the boar was homozygous for this trait (Figure 4.16 and 4.17).

The initial matings for generating the core breeding herd for GalKO/CD46/HLA-E constituted six matings of GalKO/CD46 boars to HLA-E sows which yielded 59 piglets. Of these piglets, 26 carried the CD46 and 22 the HLA-E transgene. Nine of the CD46 and the HLA-E transgenic piglets had inherited both transgenes. Again, as the boars were homozygous GalKO, all piglets were heterozygous for it (Figure 4.16, 4.18 and 4.19).

Immunohistochemical stainings of ear tissue sections of representatively selected animals demonstrated the absence of Gal epitopes in founder boar #9896 but heterozygous GalKO offspring displayed Gal epitope staining (Figure 4.20).



B

9896 × WT NT53

#	Gal	CD46	HLA-E	hTM
pig 1 †	+/-	-	-	-
pig 2 †	+/-	+	-	-
pig 3 †	+/-	-	-	-
Y100	+/-	+	-	-
Y101	+/-	-	-	-
Y102	+/-	+	-	-
Y103	+/-	+	-	-
Y104	+/-	-	-	-
Y105	+/-	+	-	-
Y106	+/-	+	-	-
Y107	+/-	-	-	-
Y108	+/-	+	-	-
Y109	+/-	+	-	-

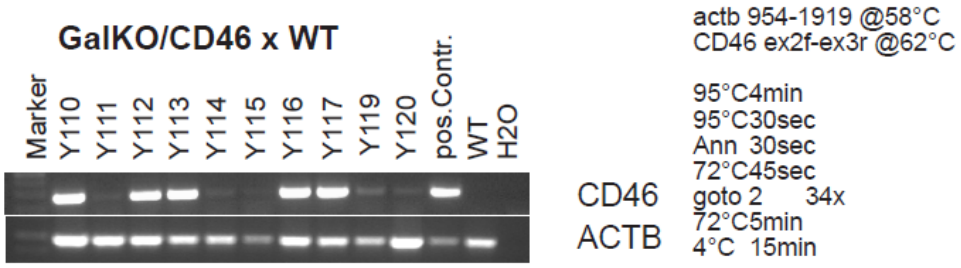
C

9872 × 9864

#	Gal	CD46	HLA-E	hTM
X100	+/-	-	-	-
X101	+/-	+	-	-
X102	+/-	+	-	-
X103	+/-	-	-	-
X104	+/-	-	-	-
X105	+/-	+	-	-
X106	+/-	-	-	-

Figure 4.16: Genotyping of F1 generation for establishment of breeding herd. (A) Genotyping PCR amplified a 425 bp DNA fragment from CD46 transgenic pigs and a 500 bp DNA fragment from HLA-E transgenic pigs; wild-type animals yielded no signal; β-actin served as control for DNA integrity; (B) One litter from a GalKO/CD46 mating with wild-type for outbreeding provided 13 piglets; eight were CD46 transgenic; (C) One litter of a GalKO/CD46 mating with HLA-E had seven piglets; three were CD46 transgenic, but none HLA-E. PCR conditions were as shown.

A



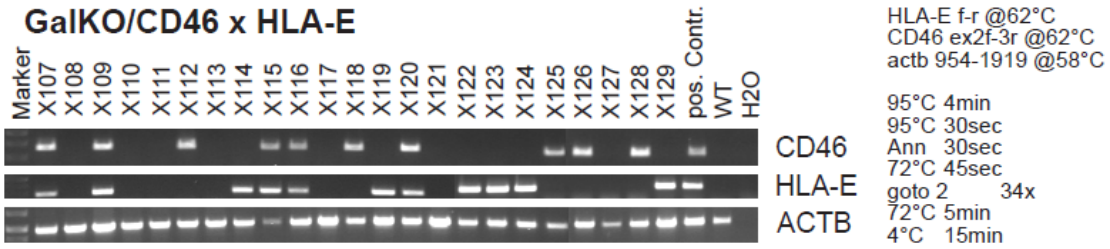
B

9896 × WT 475

#	Gal	CD46	HLA-E	hTM
pig 1 †	+/-	nd	nd	-
Y110	+/-	+	-	-
Y111	+/-	-	-	-
Y112	+/-	+	-	-
Y113	+/-	+	-	-
Y114	+/-	-	-	-
Y115	+/-	-	-	-
Y116	+/-	+	-	-
Y117	+/-	+	-	-
Y118	+/-	-	-	-
Y119	+/-	-	-	-
Y120	+/-	-	-	-

Figure 4.17: Genotyping of F1 generation for outbreeding. (A) Genotyping PCR amplified a 425 bp DNA fragment from CD46 transgenic animals; wild-type animals yielded no signal; β-actin served as a control for DNA integrity; (B) One litter from a GalKO/CD46 mating with wild-type for outbreeding purposes consisted of 12 piglets; five were transgenic for CD46. PCR conditions were as shown.

A



B

9872 × 9869

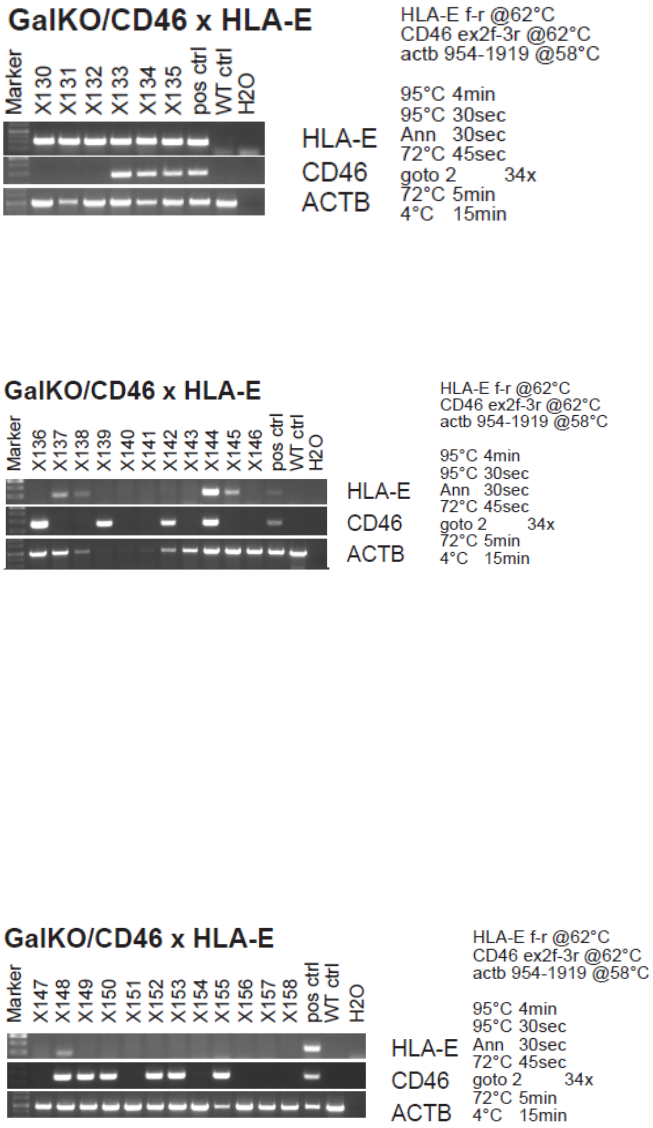
#	Gal	CD46	HLA-E	hTM
X107	+/-	+	+	-
X108	+/-	nd	nd	-
X109	+/-	+	+	-
X110	+/-	-	-	-
X111	+/-	-	-	-
X112	+/-	+	-	-
X113	+/-	-	-	-
X114	+/-	-	+	-
X115	+/-	+	+	-
X116	+/-	+	+	-

9896 × 9713

#	Gal	CD46	HLA-E	hTM
X117	+/-	-	-	-
X118	+/-	+	-	-
X119	+/-	-	+	-
X120	+/-	+	+	-
X121	+/-	-	-	-
X122	+/-	-	+	-
X123	+/-	-	+	-
X124	+/-	-	+	-
X125	+/-	+	-	-
X126	+/-	+	-	-
X127	+/-	-	-	-
X128	+/-	+	-	-
X129	+/-	-	+	-

Figure 4.18: Genotyping of F1 generation for establishment of breeding herd. (A) Genotyping PCR amplified a 425 bp DNA fragment from CD46 transgenic animals and a 500 bp DNA fragment from HLA-E transgenic animals; wild-type animals yielded no signal; β-actin served as a control for DNA integrity; (B) Two litters from GalKO/CD46 matings with HLA-E yielded a total of 23 piglets; ten were transgenic for CD46, 11 for HLA-E. PCR was performed according to the conditions shown.

A



B

9896 × 9864

#	Gal	CD46	HLA-E	hTM
X130	+/-	-	+	-
X131	+/-	-	+	-
X132	+/-	-	+	-
X133	+/-	+	+	-
X134	+/-	+	+	-
X135	+/-	+	+	-

9896 × 9869

#	Gal	CD46	HLA-E	hTM
X136	+/-	+	-	-
X137	+/-	-	+	-
X138	+/-	-	+	-
X139	+/-	+	-	-
X140	+/-	-	-	-
X141	+/-	-	-	-
X142	+/-	+	-	-
X143	+/-	-	-	-
X144	+/-	+	+	-
X145	+/-	-	+	-
X146	+/-	-	-	-

9896 × 9713

#	Gal	CD46	HLA-E	hTM
X147	+/-	-	-	-
X148	+/-	+	+	-
X149	+/-	+	-	-
X150	+/-	+	-	-
X151	+/-	-	-	-
X152	+/-	+	-	-
X153	+/-	+	-	-
X154	+/-	-	-	-
X155	+/-	+	-	-
X156	+/-	-	-	-
X157	+/-	-	-	-
X158	+/-	-	-	-

Figure 4.19: Genotyping of F1 generation for establishment of breeding herd. (A) Genotyping PCR amplified a 425 bp DNA fragment from CD46 transgenic animals and a 500 bp DNA fragment from HLA-E transgenic animals; wild-type animals yielded no signal; β-actin served as a control for DNA integrity; (B) Three litters from GalKO/CD46 matings with HLA-E yielded a total of 29 piglets; 13 were CD46 transgenic, 10 HLA-E. PCR conditions were as shown.

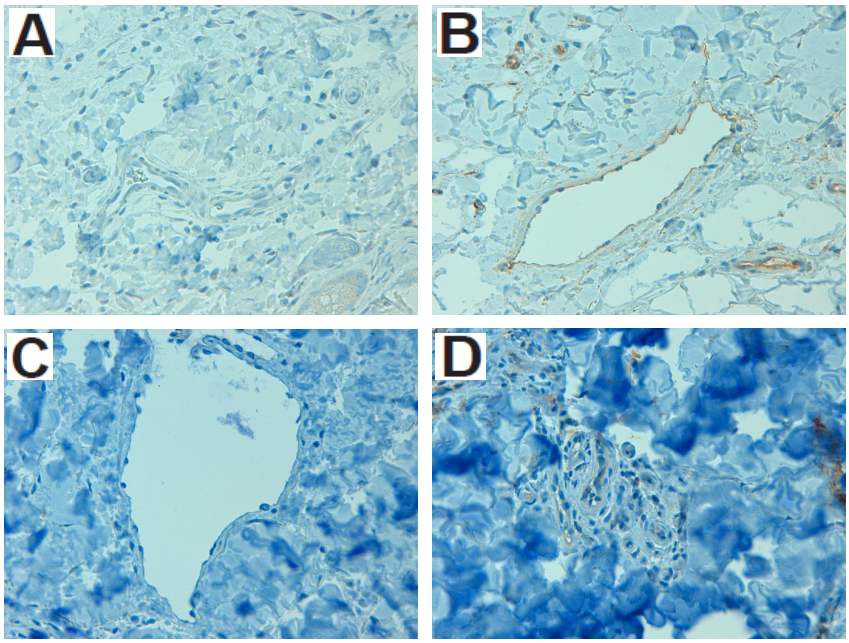
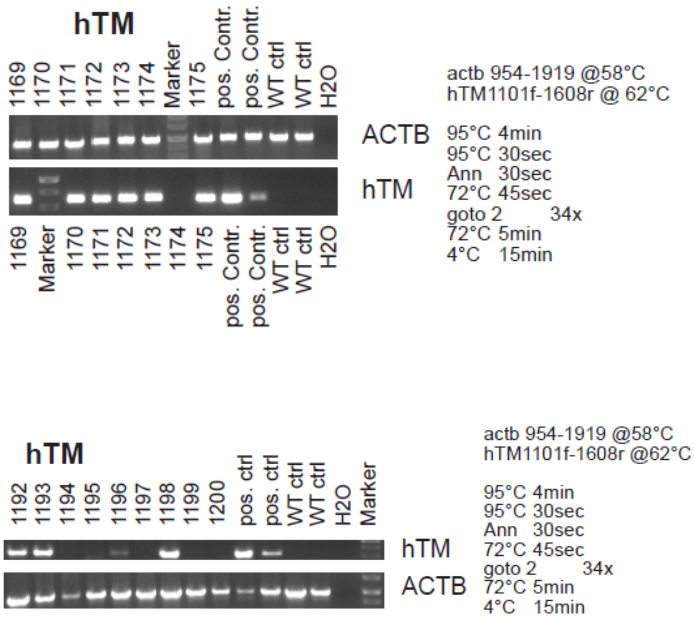


Figure 4.20: Inheritance of GalKO. Representative display of GalKO inheritance on immunohistochemically stained ear sections of founder and F1 offspring. (A) negative control; (B) positive control; (C) homozygous GalKO #9896; (D) heterozygous GalKO F1 generation offspring. Data provided by Dr. Julius Faber, Institute of Pathology, LMU Munich.

Additionally, production of hTM single transgenic offspring was accomplished by mating the available hTM transgenic boar to two wild-type sows. This was done in order to provide hTM offspring for experimental purposes and generate a replacement boar for the current hTM boar. A total of 16 piglets were gained from these two matings, of which nine had inherited the hTM transgene from their father (Figure 4.21). In order to determine inheritance of the integration pattern, all hTM-neo transgenic offspring were analysed by Southern blotting on genomic DNA (Figure 4.22). Identical restriction patterns could be observed in founder #9781, its re-established clone #9943, and F1 generation offspring of #9943.

A representative selection of immunohistochemical stainings of ear tissue samples demonstrated inheritance of hTM expression capacity in transgenic offspring (Figure 4.23).

A



B

9943 × WT NT122

#	Gal	CD46	HLA-E	hTM
1069	+/+	-	-	+
1070	+/+	-	-	+
1071	+/+	-	-	+
1072	+/+	-	-	+
1073	+/+	-	-	+
1074	+/+	-	-	-
1075	+/+	-	-	+

9943 × WT SH2

#	Gal	CD46	HLA-E	hTM
1192	+/+	-	-	+
1193	+/+	-	-	+
1194	+/+	-	-	-
1195	+/+	-	-	-
1196	+/+	-	-	-
1197	+/+	-	-	-
1198	+/+	-	-	+
1199	+/+	-	-	-
1200	+/+	-	-	-

Figure 4.21: Genotyping of hTM single transgenic offspring. (A) Genotyping PCR amplified a 508 bp DNA fragment from hTM transgenic animals; wild-type pigs produced no signal; β-actin served as a control for DNA integrity; (B) Two matings of hTM transgenic boar #9943 with wild-type sows yielded 16 piglets; nine were hTM transgenic. PCR conditions were as shown.

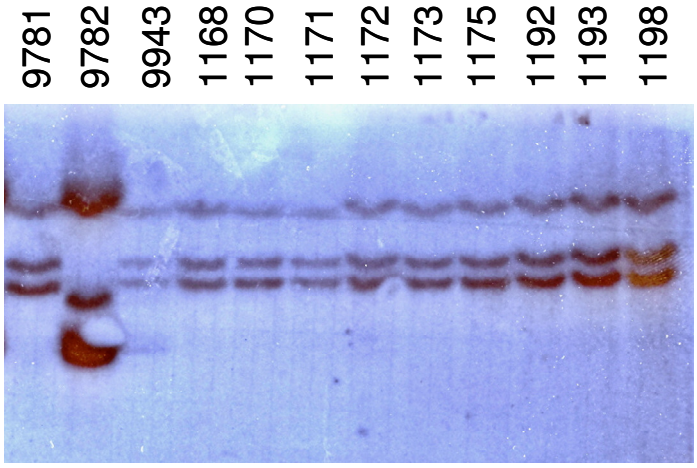


Figure 4.22: Inheritance of transgene integration pattern. #9943 was re-cloned from #9781 and mated to two unrelated wild-type sows. All transgenic offspring (#1168-#1198) showed the same restriction pattern in Southern blotting, indicating a single transgene integration locus.

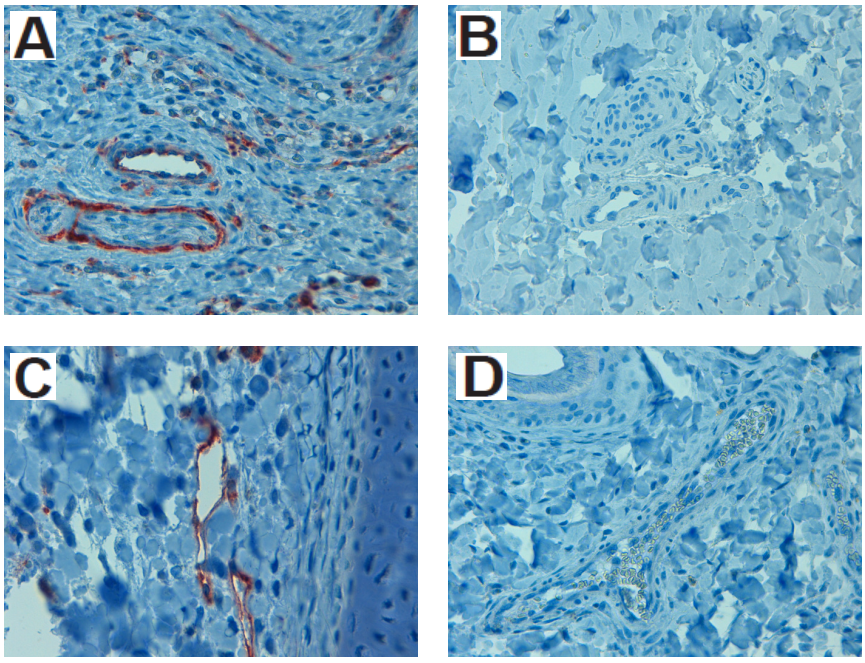


Figure 4.23: Inheritance of hTM expression capacity. Representative example of immunohistochemically stained ear tissue sections of hTM transgenic offspring and wild-type littermates. (A) positive control; (B) negative control; (C) hTM transgenic offspring, (D) wild-type littermate. Data provided by Dr. Julius Faber, Institute of Pathology, LMU Munich.

4.6 F2 generations and hTM incorporation into breeding herd

For breeding of the F2 generation and incorporation of the hTM transgene into the breeding herds, potential breeding material was chosen from F1 generation litters after genotyping and expression analysis by immunohistochemistry on tissue derived from ear punches of the animals (Figures 4.16 – 4.23). In order to generate the aspired GalKO/CD46/HLA-E breeding herd, half-siblings of the F1 generation with a heterozygous GalKO, and either transgenic for CD46 or for HLA-E, were to be mated to one another.

Taking into account the only 3.125% probability of offspring with the correct sex and transgene composition, seven litters were calculated to be necessary for obtaining the required two homozygous GalKO/HLA-E transgenic sows. Additionally, these litters will produce homozygous GalKO/CD46 boars as replacements for the currently utilised GalKO/CD46 boars (#9872/#9896). Outbreeding will be accomplished by generating heterozygous GalKO boars that have been outbred over two generations and can be mated to sows from the breeding herd. For this, one mating of an outbred F1 generation heterozygous GalKO/CD46 sow with a wild-type boar has so far been scheduled.

Incorporating the hTM transgene into the breeding herd will be achieved over two generations. Seven litters of F1 generation heterozygous GalKO/CD46 sows gained from mating with the available hTM boar (#9943), or one of his already generated offspring, will be necessary to produce four heterozygous GalKO/hTM sows. These sows can then be mated to F2 generation homozygous GalKO/CD46 boars and will have to produce a total of eight litters in order to generate the five homozygous GalKO/hTM sows essential in the breeding herd.

Table 4.8 depicts the currently scheduled matings for establishment of the breeding herds.

Table 4.8: Mating schedule for establishment of transgenic breeding herds

Mating	Boar #	Sow #	Date	Purpose
1	X101	X129	April 2011	Gal/CD46/HLA-E
2	WT	Y100	April 2011	outbreeding
3	9943	X105	April 2011	Gal/CD46/hTM
4	9943	X128	April 2011	Gal/CD46/hTM
5	X130	Y102	June 2011	Gal/CD46/HLA-E
6	X131	Y103	June 2011	Gal/CD46/HLA-E
7	X132	Y106	June 2011	Gal/CD46/HLA-E
8	X130	Y108	June 2011	Gal/CD46/HLA-E
9	X101	X129	October 2011	Gal/CD46/HLA-E
10	X131	X145	October 2011	Gal/CD46/HLA-E
11	9943	X142	October 2011	Gal/CD46/hTM
12	9943	X149	October 2011	Gal/CD46/hTM
13	9943	X150	October 2011	Gal/CD46/hTM
14	9943	X152	October 2011	Gal/CD46/hTM
15	9943	X153	October 2011	Gal/CD46/hTM

4.7 Identification of transgene zygosity

In order to define the zygosity status of transgenic offspring with respect to the transgenes carried, duplex PCRs were established for the GalKO and hTM. This allowed discrimination of hetero- or hemizygous GalKO and hTM transgenic animals from their homozygous or wild-type littermates.

4.7.1 GalKO duplex PCR

As is demonstrated in Figure 4.24 (A), a duplex PCR on an exemplary selection of homozygous GalKO animals, their heterozygous offspring and unrelated wild-type animals illustrates homozygosity for GalKO in pigs #1061, #1066, #9948, #9949, #1103 and #1104, heterozygosity in pigs #X108, #X110, #X126, #X127 and #X128 and wild-type in pigs #1197, #1198, #1199 and #9943. In each case, a 940 bp DNA fragment was amplified from the GalKO allele and a 570 bp band from the wild-type counterpart. Homozygous animals for either GalKO or wild-type would therefore generate only one band of the defined size while heterozygous animals would generate both fragment sizes. Primer locations on the transgenic and wild-type sequences are

depicted in Figure 4.24 (B). It can be observed that the primers located on the wild-type allele should theoretically also generate a DNA fragment on the GalKO locus, covering the whole length of the transgene. This, however, is not the case because the given PCR conditions do not allow the amplification of a DNA fragment that large. Consequently, only the DNA fragment between primers neoPf and GGTAr21 will be amplified from the GalKO allele.

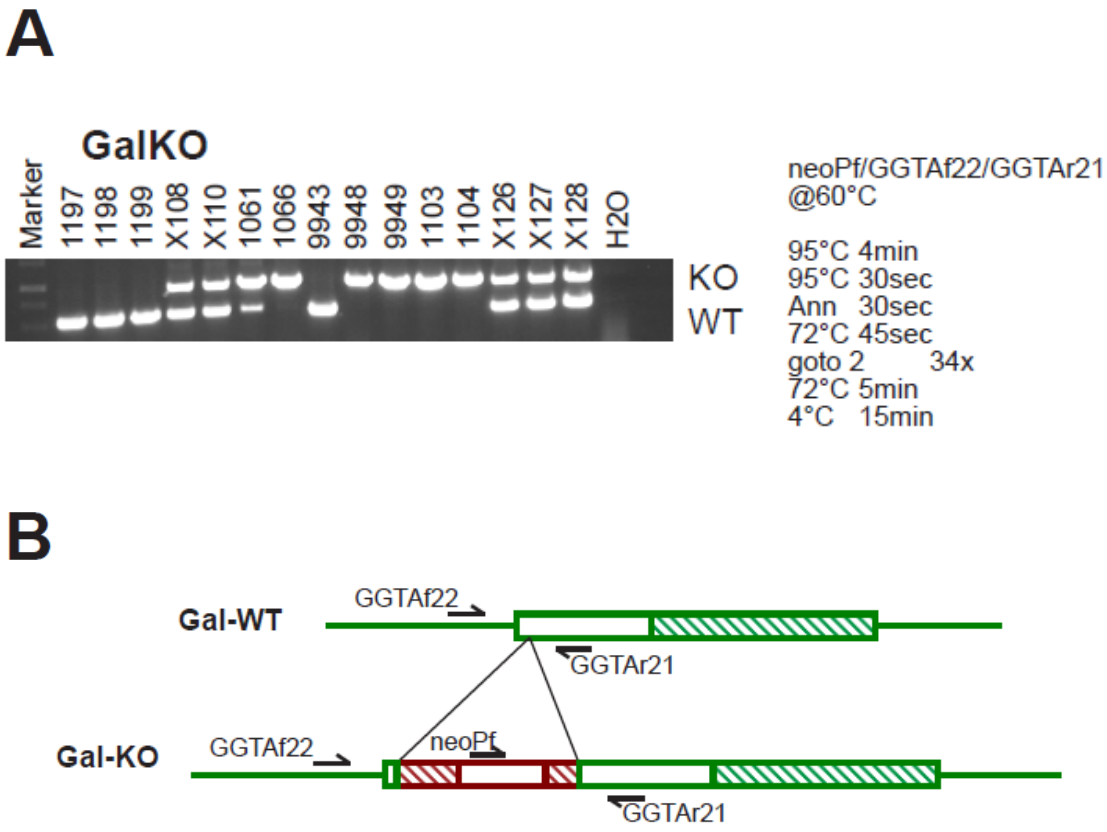


Figure 4.24: Zygosity of GalKO alleles. (A) A duplex PCR was established to discriminate a 570 bp fragment amplified from the Gal-wild-type allele from a 940 bp fragment generated from the GalKO allele. #1197, #1198, #1199 and #9943 are transgenic animals without GalKO. #X108, #X110, #X126, #X127 and #X128 are offspring of a homozygous GalKO boar with WT sows regarding the Gal locus. #1061, #1066, #9948, #9949, #1103 and #1104 are animals re-cloned from homozygous GalKO pigs. The faint WT band in animal #1061 is presumably due to DNA contamination. Additionally, band intensity appears to provide semi-quantitative information about the zygosity of wild-type and knock-out alleles. (B) The localisation of the primers is shown for the wild-type as well as for the knock-out allele. In the knock-out allele the primers GGTAf22/GGTAr21 should theoretically generate an additional 2.3 kb fragment but this is not amplified under the given PCR conditions detailed in (A).

4.7.2 hTM duplex PCR

Analogously, a duplex PCR for the hTM-neo locus was established. Here, the information gained from the integration locus clarification by inverse PCR was utilised in order to design primer pairs that amplify a 570 bp DNA fragment from the wild-type allele and a 1200 bp fragment from the transgenic allele within one PCR reaction. Figure 4.25 shows a duplex PCR on an exemplary selection of hTM-neo heterozygous offspring of founder boar #9943 and their wild-type littermates. Heterozygosity is demonstrated for animals #1192, #1198, 1171, #1172 and #1173 while animals #1195, #1197, #1199, #1200 and #1174 are homozygous wild-type. Genomic DNA of founder #9943 was used as positive control for heterozygosity, and genomic wild-type DNA as control for amplification of the wild-type sequence. No control for a homozygous hTM-neo locus could be applied because no such animal was available at the given time point.

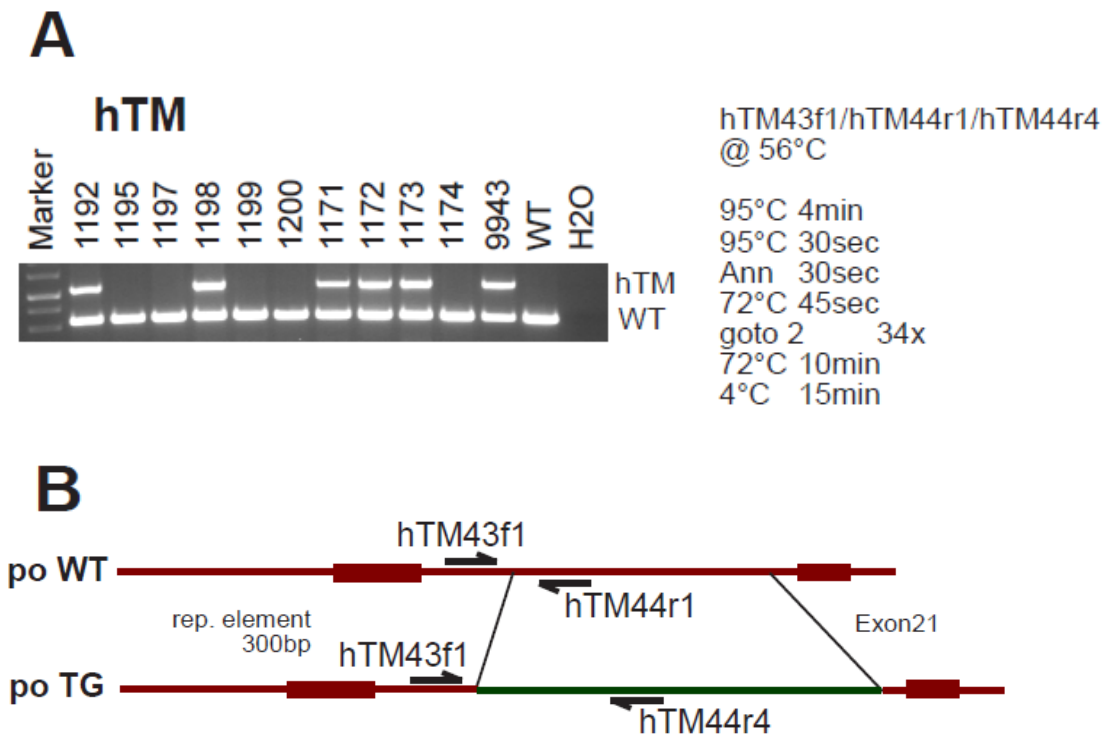


Figure 4.25: Zygosity of hTM-neo transgene. (A) A duplex PCR was established to discriminate a 570 bp fragment amplified from the wild-type allele from a 1200 bp fragment generated from the hTM-neo transgenic allele and thus identify wild-type as well as hemi- and homozygous transgenic animals. #1192, #1198, #1171, #1172, #1173 and #9943 are hemizygous transgenic animals. #1195, #1197, #1199, #1200 and #1174 are wild-type. (B) The localisation of the primers is shown for the wild-type as well as for the knock-out allele.

5 DISCUSSION

The notion that pigs are among the most suitable species for biomedical translational research and as potential donors in the context of xenotransplantation has been prevalent for some time and has been discussed extensively by numerous authors (reviewed in Aigner et al. 2010). This is owed to the fact that the organ systems of pigs share many anatomical and physiological characteristics with humans (Smith et al. 1990; Rogers et al. 2008; Spurlock and Gabler 2008). Additionally, their size corresponds to human dimensions more closely than that of rodents does, which also makes sampling and handling a less materially limited and demanding task (Roberts et al. 2009). In their guidelines, national and international regulating authorities such as the European Medicines Agency require the use of a non-rodent mammal as experimental animal in clinical development trials (www.ema.europa.eu). Genetically modified pigs therefore occupy an ever increasing place in biomedical translational research and xenotransplantation. However, in order to draw meaningful conclusions from experiments, sizeable numbers of animals are required. Lead times in the preparation or specific time frames in the conduction of experiments demand predictable groups of experimental animals of specific size and at specific time points. This has proven to be a problematic issue in providing transgenic pigs for experimental purposes.

Novel transgenic lines have been and are being established by a variety of technologies based on additive gene transfer with unpredictable transgene integration sites or site directed knock-out or knock-in methods. Somatic cell nuclear transfer followed by embryo transfer into oestrus synchronised recipient sows has become the leading technique in generating transgenic pigs (Melo et al. 2007). This method features beyond 90% transgenesis rates and pooling of transgenic cells derived from independent integration events leads to more than 90% independent founders, as long term experience at the MVG has shown (unpublished data). However, it has become apparent that the process is also very inefficient in terms of total animals numbers produced. Rates of less than 5% live offspring compared to the total number of transferred embryos have been reported across the literature (Polejaeva et al. 2000; Oback 2008; Palmieri et al. 2008) which makes the cloning procedure appropriate for

establishing novel transgenic pigs but raises the question if this technique is suitable for reproducing already established lines by nuclear and embryo transfer.

As Tables 4.3, 4.4 and 4.5 demonstrate, re-cloning of pigs after expression analysis of founder litters and selection of the most suitable founders at the MVG, Badersfeld has proven to be a tedious issue. For example, six attempts at re-cloning hTM-neo founder pig #9781 delivered only one single animal which, fortunately, survived the neonatal period and could be raised until sexual maturity (Table 4.3). Re-cloning INS-LEA founders #9813 and #9814 has been equally difficult. 14 nuclear and embryo transfers resulted in only four pregnancies that actually delivered. From these, only two animals (#1044, #1050) were weaned (Table 4.5). Re-cloning of hTM-bla founder boar #9848 appears to have generated slightly more live offspring. However, taking into account that 16 nuclear and embryo transfers dates produced only 11 weaned piglets that could be utilised in experiments or raised for breeding, this calculates to an average of 0.7 piglets derived from one nuclear and embryo transfer . Or, if only nuclear and embryo transfers from which a pregnancy was established are taken into account, this number still averages at less than one piglet per NT/ET (Table 4.4). Therefore, this method had been discarded as a means for routinely re-producing transgenic pigs for experimental purposes, as outcome is too unpredictable and, consequently, too time, space and human and material resource intensive.

Breeding transgenic pigs on a demand-based level rectifies these limitations. As has been reported before, transgenic animals generated by cloning and their offspring behave similarly in terms of reproductive capacity as their wild-type counterparts (Martin et al. 2004; Mir et al. 2005; Shibata et al. 2006; Williams et al. 2006). Additionally, transgenes integrated into the genome appear to be passed on to the next generation according to Mendelian rules of inheritance (Aigner et al. 1999). Breeding of transgenic pigs that has been conducted during the preparation of this thesis has confirmed both of these notions. Litter sizes of transgenic pigs mated to either another transgenic or to wild-type individuals averaged 10 piglets per litter which is similar to what has been reported for wild-type litters (McGlone and Pond 2003) and also reflects the wild-type breeding experience gained at the MVG.

Transgene inheritance appeared to adhere to Mendelian rules. As has been shown in

Figures 4.16, 4.18 and 4.19, eight matings of GalKO/CD46 boars to either wild-type or HLA-E transgenic sows generated a total of 84 piglets. Calculated numbers of offspring transgenic for CD46 and/or HLA-E should have constituted 27.25 CD46, 14.75 HLA-E and 14.75 CD46/HLA-E transgenic piglets. Effectively, gained transgenic offspring represented 29, 11 and 10, respectively. Even though total numbers for HLA-E and CD46/HLA-E transgenic offspring are slightly less than expected, individual single litters have brought more transgenic offspring than calculated, while others have produced less. Matings involving the hTM-neo boar #9943 have produced nine transgenic piglets when, mathematically, only eight should have been generated (Figure 4.21).

Since transgenes are passed on to the next generation adhering to Mendelian rules of inheritance, they also segregate accordingly. This lowers probabilities of specific transgene combinations in multiple transgenic animals as long as each transgene is incorporated as a single vector construct into the genome. However, it also effects segregation of multiple integration sites of a single transgene. This can become a problem in offspring generations, as transgene expression capacity of the individuals cannot be foreseen. Detailed expression analysis would therefore have to be conducted on each pig, which is in many cases not practicable as animals would have to be sacrificed for this and could therefore not be utilised in breeding or experiments. Consequently, segregation of transgenes has to be avoided. This is only possible if animals are selected for breeding that only possess one single transgene integration site in the first instance. Segregation of multiple copies of the transgene at one integration site would only be possible if these transgenes were relocated to multiple integration sites by recombination first, which would then be able to segregate. This, however, is a theoretical possibility but extremely unlikely. As Aigner et al. (1999) have demonstrated, transgene integration sites apparently remain stable in over 20 generations of breeding without reassortment of individual copies. Southern blotting of the hTM-neo founder and his offspring demonstrated that indeed all transgenic piglets derived from this boar displayed the same restriction pattern as their father (Figure 4.22), suggesting cross-generational stability in integration conformation of the transgenes. Selection of founder animals displaying a single integration site, even

with more than one transgene copy, is therefore a viable means of preventing segregation.

If avoidance of transgene segregation is accomplished, expression patterns of founder animals can be assumed to be passed on to offspring (Bordignon et al. 2003; Brunetti et al. 2008). Even though epigenetic silencing of promoters or coding sequences has been observed in lentiviral transgenesis (Kearns et al. 2000, Hofmann et al. 2006), this effect seems to occur mainly with viral sequences. F1 generation offspring of GalKO/CD46 and hTM-neo boars could therefore have been expected to display transgene expression on a level comparable to their fathers'. Indeed, analysis of immunohistochemically stained ear sections (Figure 4.23) of F1 hTM-neo offspring reinforced the notion that expression patterns of transgenes are inherited to the next generation. Thus, expression analysis of founder animals with a single transgene integration site presumably constitutes a sufficient method of ensuring reliable expression levels also in later generations.

Furthermore, with the knowledge of transgene vector construction, namely the promoter part of it, expression of the transgene can be anticipated in cell types or tissues in which the controlling promoter is active. Therefore, it might appear to be an option to confine expression analysis of potential founder animals to tissues that can be harvested without sacrificing the animals in question. For example, CAG-LEA founders were expressing the immunomodulatory LEA29Y transgene ubiquitously. Initial expression analysis on ear tissue sections (Figure 4.10) and in blood serum samples (Figure 4.6) gave a good indication of the expression capacity of each of the three founders and could be gained without killing the animals. This can be seen as an important finding, as the extremely low efficiency in re-cloning primary cells from animals killed for expression analysis hinders generation of breeding stock. However, specific projects often require the site-specific expression of a particular transgene. Potential founder animals for INS-LEA transgenic pigs, for example, had to be sacrificed for analysis because expression of the transgene was expected to be limited to pancreatic ß-cells which could not feasibly be obtained from living pigs. Moreover, during the analysis of hTM-bla founders it became apparent that even though initial expression analysis in the form of immunohistochemically stained ear sections (Figure

4.2) would lead to the assumption that all three potential founders were equal in their transgene expression capacity, further examination proved this conjecture wrong. In depth expression analysis on the basis of immunohistochemical staining of organ spectra brought to light that expression was indeed on a comparable level in some, but not in all investigated tissues. For example, animal #9849 expressed hTM well in heart tissue on a level similar to that of the two litter mates but staining of lung, liver or kidney tissue was markedly weaker. The same phenomenon could be observed in the selection of hTM-neo founders. Ear tissue sections of litter mates #9780, #9781 and #9782 all stained equally well in immunohistochemical evaluation. However, staining of a range of organs showed the differing expression capacity of the litter in, for example, lung and liver tissue. In both cases, the animal that expressed best in all examined organs was chosen as founder boar, for hTM-bla pig #9848 and for hTM-neo pig #9781. This choice, however, would not have been possible, had expression analysis been confined to a more superficial level.

In-depth expression analysis thus appears to be a prerequisite in the selection of founder animals that are to be utilised in breeding transgenic pig lines. Choosing suitable founders with respect to their transgene expression capacity and limiting the likelihood of transgene segregation in future generations by only employing animals with a single integration site in breeding ensures viable transgene inheritance. This is the case for single transgenic animals; however, if multiple transgenic vectors have been incorporated into the genome, segregation of these transgenes becomes inevitable. As a result, probabilities for specific transgene combinations in offspring can decrease to very low numbers, necessitating many litters in order to generate a certain number of particular pigs.

The low probability of multi-transgene inheritance might be overcome by choosing only animals for breeding that carry the transgenes on both alleles. In this case, all offspring would be hemizygous for each transgene. This, however, requires knowledge of transgene zygosity, which in turn can only be determined if integration sites are known. Additionally, even though employing homozygous transgenic animals in breeding or mating animals with identical transgenic properties to each other increases the numbers of transgenic offspring it also heightens inbreeding levels.

This is due to the fact that only a very limited amount of breeding stock is available in the case of transgenic pigs and founder animals are usually confined to only one genetic background for each transgene. Consequently, homozygosity for a trait can only be achieved by mating related animals and pigs with identical transgenic properties will necessarily have been derived from the same founder. But rising inbreeding coefficients lower productivity of breeding stock and the viability of offspring (Charlesworth and Charlesworth 1987; Ralls et al. 1988; Lynch 1989; 1991). This collides with requirements for large numbers of offspring if probabilities for transgene combinations are low.

Inbreeding should therefore be kept as low as possible within a breeding herd. Thus matings between animals that are related to each other should be avoided as far as possible and unrelated wild-type blood should be introduced from the outside on a regular basis. However, both of these measures again lower probabilities for specific transgene combinations and make them even impossible, as is the case with traits that have to be present in homozygosity in order to take effect, such as the GalKO. Hence it can be concluded that transgene inheritance and inbreeding are two conflicting issues that cannot be accounted for fully both at the same time. Rather a common ground has to be identified on which either of these issues presents the least possible problem.

In order to examine the differing effect a distinct emphasis on either increasing transgene combination probabilities or on keeping inbreeding levels low can have on breeding outcome, breeding schedules for establishment of GalKO/CD46/HLA-E and GalKO/CD46/hTM herds were designed that account for both issues to a different extent (Figure 4.13). Because GalKO/CD46 was only available in the form of founder boars generated by re-cloning from cells of one single founder established at *Revivicor, Inc.* these transgenes had to be derived from these two boars. On the other hand, HLA-E was only available as sows derived from breeding the original founders established in 2006. And, finally, hTM had been established as a new transgene on two different backgrounds, once by transfecting the hTM-bla vector onto the *Revivicor* GalKO/CD46 background and once as hTM-neo on a wild-type cell line, both again resulting in male animals. As the GalKO needs to be bred to homozygosity

for an applicable effect, mating of related animals in F1 generation was inevitable because the GalKO could only be derived from the paternal side. Since the other transgenes are already functioning in a hemizygous conformation, they could be obtained from either the paternal or the maternal side. Figure 4.13 (A) and (B) demonstrate that the choice between half-sibling and full-sibling matings in F1 was inconsequential for the proportion of suitable transgene combinations in F2. However, it did have a substantial effect on the development of the inbreeding coefficient in F2 offspring. While half-sibling matings resulted in an inbreeding coefficient of 0.125, it doubled to 0.25 for full-sibling matings. This can be seen as a considerable difference if it is kept in mind that several authors have reported average inbreeding coefficients in commercial pig breeds of below 0.1 (Welsh et al. 2010) and that every ten percent rise in inbreeding of the sow has been correlated with a 0.2 piglet reduction in litter size (Dickerson et al. 1954). Father-daughter matings, on the other hand, would have been able to double probabilities for specific transgene combinations in offspring (Figure 4.13 (C)). But they, too, would have resulted in inbreeding coefficients of 0.25 in offspring. The decision was thus made to employ half-sibling matings to generate homozygous GalKO offspring with CD46, HLA-E or both transgenes.

An even greater effect could be observed in deciding on where to derive the hTM transgene for integration into the breeding herd from. Utilising the GalKO/CD46/hTM founder boar, suitable animals for breeding would already have been generated within one generation. However, the genetic background of this founder is identical to the GalKO/CD46 founders, as all of them are clones of the initial *Revivicor* cell population. Thus, mating of F1 generation sows from half-sibling matings to the GalKO/CD46/hTM founder boar would have constituted father-daughter matings, resulting in 0.25 inbreeding (Figure 4.14 (A)). On the other hand, employing the single transgenic hTM founder and mating him to the exactly the same sows will not generate breeding animals in the following generation as it will be impossible to establish a homozygous GalKO. But offspring one generation later will not only display the required transgenes with twice as high a probability than in (A) but will also feature an inbreeding coefficient of only 0.062 (Figure 4.14 (B)). This constitutes a value comparable to two generations GalKO/CD46 outbreeding with wild--type

sows as depicted in Figure 4.15 which can thus be achieved simultaneously with integrating the hTM transgene into the breeding herd.

Zygosity of transgenes becomes an important issue if transgenes are only effective in a homozygous conformation or if, on the contrary, homozygosity of a trait implicates severe disadvantages for the affected individual. For calculation of expectable proportions of transgenic piglets in offspring, zygosity of the parents is also the determining factor. In rodents, it is feasible to determine zygosity by out crossing the individuals in question. This, however, does not appear to be an option in pigs. Pregnancy lengths of approximately 115 days in pigs would make this a time-consuming undertaking. Depending on overall body weight, European regulations dictate minimum floor areas for sows of up to 2.5 m^2 per sow (Directive 2010/63/EU) for experimental animals, thus implying substantial space requirements. Furthermore, it is doubtful if animal welfare concurs with producing numerous litters of piglets solely for determining transgene zygosity of the parents. A reliable method for genomic analysis of the zygosity status of individuals with respect to transgenes must therefore be identified.

In the establishment of the transgenic breeding herds that are being covered by this thesis, zygosity was not relevant for the F1 offspring generation. Zygosity of founder animals was known in each case and since no identical transgenes on the maternal and the paternal side were present, offspring was necessarily hemi- or heterozygous for the respective transgene. However, in F2 generation offspring the question of GalKO zygosity will arise and later on the other transgenes will also become relevant. Zygosity specific duplex PCRs that are able to amplify two different DNA fragments from transgenic and wild-type alleles in one PCR reaction and thus discriminate between homo- and heterozygous transgenic individuals were therefore to be established.

In the case of the GalKO the location of the relevant DNA sequence within the genome was already clear, as site directed knock out of a specific gene had been performed. Thus, primers could be designed that cover the region in question and from which DNA fragments of two different sizes were amplified from the transgenic and the wild-type allele. As has been shown in Figure 4.23, this method is able to

reliably discriminate between hetero- and homozygous GalKO pigs and generates a 570 bp DNA fragment from the wild-type locus and a 940 bp fragment from the knock out sequence. Because the reverse primer was placed downstream of the known integration site of the gene disruption vector, it could be utilised for both alleles. Two forward primers were placed either upstream of the Gal gene or on the knock out vector sequence. Consequently, DNA fragments from the wild-type allele were amplified from the reverse and the forward primer that had been placed upstream of the gene, and DNA fragments from the knock out allele were generated from the reverse and the second forward primer on the vector sequence. The forward primer upstream of the Gal gene was also able to bind on the knock out allele; however, since the vector sequence was larger than 4 kb, the distance between this primer and the reverse primer downstream of the integration site would have been too long for *Taq* polymerase to cover under the used PCR conditions. Thus, no amplicons were generated from this primer pair.

In order to establish a zygosity specific duplex PCR for the hTM transgene the integration loci in the founder boars had to be identified first so the comparable wild-type sequence would be known. Thus, inverse PCR was chosen as a means of determining integration sites of transgenes in the founders employed in breeding. From a technological point of view, this method is rather simple and straightforward (Ochman et al. 1988; 1990). Genomic DNA is fragmented, fragments are circularised and DNA is amplified from primers binding to the transgene sequence. Flanking regions of the transgene copy thereby become visible. Using this technique, it has been possible to successfully identify the hTM-neo integration site in founder #9781 (Figure 4.3) and several transgene-transgene transitions in hTM-bla founders #9948 and #9949 (Figure 4.5), including a number of truncated transgene copies. Additionally, inverse PCR amplified porcine genomic regions including the porcine thrombomodulin and the Gal gene. This can be ascribed to the porcine thrombomodulin promoter and the bGH-polyadenylation site of the resistance cassettes present in the transgene vectors on which the primer binding sites could be found.

Identification of the genomic flanking region of transgenes by inverse PCR is based

on the assumption of complete individual transgene copies. Only then are primer binding sites, which need to be placed close to the 5' or the 3' end of the transgene, reliably available. Furthermore, increasing numbers of transgene copies at one integration site lower the probability of ascertaining the inverse PCR amplicons that contain parts of the genomic flanking region. Thus, failure to identify the hTM-bla integration locus in founder pigs #9948 and #9949 might be due to (i) the possibility that only truncated transgene copies are present on either transition site to genomic DNA or (ii) the fact that such a large number of transgene copies has been inserted at one site that probabilities of identifying the genomic flanking regions are extremely low anyway, or (iii) the circularised genomic DNA fragments are too large to be amplified by the PCR reaction. Several restriction enzymes for genomic fragmentation and a number of different primer pairs were used in each case but only a minority delivered amplicons after inverse PCR, which suggests that circularised DNA fragments might indeed have been too large in many cases for successful amplification. On the other hand, up to eight different restriction enzymes were tested in each approach with all of them resulting in calculatory mean fragment sizes of below 3 kb. Therefore it seems unlikely that so many of the fragments should have been too large for amplification. In the case of founder animal #9949, only one single transgene-transgene link could be identified (Figure 4.5 (B)) at all, indicating that failure to identify genomic flanking regions might have been due to severely truncated transgene copies on either transition site to the genomic sequence. In founder #9948, a combination of truncated transgene copies and the sheer number of copies in the integration site appear to be a probable explanation that the hTM-bla integration locus could not be clarified. The same can be said for examination of the CD46 integration locus in Gal/CD46 founder boars, of which results have not been included in this thesis. Here, too, a number of transgene-transgene transitions could be revealed but no genomic flanking regions.

In order to rectify problems with too many inserted transgene copies, it might be a possibility to choose restriction enzymes that result in comparably large fragments within the transgenic sequence but possess a relatively high restriction frequency within genomic DNA. Thus fragments resulting from transgene-transgene links might

become too large for amplification by inverse PCR while fragments from genomic DNA might be of a suitable size. The majority of amplicons generated would therefore possibly contain genomic flanking regions. However, this approach would not account for the presence of truncated transgene copies, limiting the prospects of success for inverse PCR in clarification of transgene integration sites. Alternative measures could perhaps be found in the generation of BAC libraries for the founder animals in question, where probes that recognise parts of the transgenic sequence would be used to seperate the BACs that contain the transgene from a pool of large genomic DNA fragments. Sequencing of these particular BACs would then reveal the surrounding genomic region. Several authors have reported on the construction of porcine BAC libraries (Liu et al. 2010; Suzuki et al. 2000) however, high throughput methods for sequencing would have to be employed in order to process the masses of data this approach would deliver. A third option for clarifying the integration site of a transgene might be deep sequencing methods which enable the sequencing of whole genomes or at least large portions of it without requiring extraordinary amounts of time or money. These methods, however, are still under development and require in depth knowledge of the genome analysed. Since the presently available sequence of the porcine genome still contains large gaps, these methods are currently not feasible but might find their application in the future.

In conclusion, the first systematic approach to establishing breeding herds for production of donor animals with multiple functional transgenes for xenotransplantation has been described. The selected breeding schedules represent a compromise between the conflicting parameters of rising inbreeding coefficients and efficiency of transgene inheritance. Additionally, novel transgenic pig lines have been identified that are to be integrated into the breeding herds and duplex PCR screening methods have been established to facilitate future breeding by discrimination of transgene zygosity.

6 SUMMARY

(Re)producing transgenic pigs for xenotransplantation – selection of founder animals and establishment of breeding herds

Xenotransplantation is discussed as an alternative treatment for end-stage organ failure. The pig has been widely accepted as a feasible donor species. However, it has to be genetically modified in order to overcome incompatibilities of the human and the porcine immune systems. Although profound experience in long-term breeding of multi-transgenic animals exists for mouse models in biomedical research, the situation in breeding multi-transgenic pigs is far more complicated. In contrast to small animal models, several limitations have to be taken into account for the pig: (i) inbreeding has a detrimental effect on fertility and litter size in pigs while congenic mouse strains are being bred with small limitations; (ii) generation times of approximately 12 months and pregnancies of 115 days in pigs necessitate careful breeding management; (iii) costs and space requirements in pig maintenance far exeed those for mice. In addition, insight into xenograft rejection mechanisms is still limited and further transgenes or novel transgene combinations might be required in the future. Thus, effective breeding schedules have to be designed that accomodate these limitations and allow for adaptation to changing transgene requirements.

This thesis describes the design and establishment of multi-transgenic GalKO/CD46/HLA-E and GalKO/CD46/hTM donor herds and the characterisation of novel hTM and LEA29Y transgenic lines for future incorporation into the breeding herd. In a first step, two fertile GalKO/CD46 boars and three fertile HLA-E sows were selected as founders for an initial GalKO/CD46/HLA-E breeding herd into which further xeno-relevant transgenes were to be incorporated later on. Breeding schedules that accommodate the conflicting issues of inbreeding, transgene segregation and time requirements to different extents were designed and the most feasible strategy was identified. Furthermore, options for incorporation of already fertile hTM transgenic founder pigs into the core breeding herd were analysed.

Chosen GalKO/CD46 and HLA-E founder animals were mated to each other and half-sibling matings of the F1 generation offspring were selected as a method for

establishment of a homozygous GalKO with CD46 or HLA-E in F2 generation offspring. GalKO/CD46 boars were also mated to wild-type sows to facilitate outbreeding and thus restrict inbreeding levels within the herd. Additionally, the selected method for integration of the hTM transgene into the breeding herd necessitated matings of the hTM founder boar to wild-type sows. All F1 generation offspring was analysed on a genomic level to determine inheritance of transgene integration patterns. Additionally, inheritance of expression capacity was examined immunohistochemically. This facilitated selection of breeding animals for generation of F2 offspring.

In parallel, analyses of potential founder animals for the novel transgenic pig lines hTM, INS-LEA and CAG-LEA were performed to select the most suitable individuals for incorporating these transgenes into the breeding herd. In each case, animals were chosen where Southern blotting indicated the presence of only one single transgene integration site to avoid segregation of integration loci in offspring. Expression of the transgene was examined by immunohistochemistry and ELISA in a broad range of organs to determine the individuals that exhibited the best expression capacity. By combining results of genomic with expression analysis, founders for the novel lines were chosen which were subsequently re-cloned to be raised for breeding. While the generation of a fertile founder had already been accomplished for the hTM transgene, INS-LEA founders have not reached sexual maturity yet.

Comprehensive genomic analysis included the determination of transgene integration loci in hTM founder animals. For this, inverse PCR was chosen as a method for determining unknown genomic flanking regions of transgenes. The transgene integration site could be identified in the hTM founder that is being employed in breeding. Additionally, two further potential hTM founders were analysed and complex transgene integration patterns were clarified. However, the integration locus itself could not be found in these two cases.

The information gained from hTM integration locus determination in the founder boar employed in breeding was utilised for establishing a duplex PCR that discriminates between an hTM transgenic allele and its wild-type counterpart in one single PCR reaction. This was done in order to facilitate discrimination of hemizygous from

homozygous transgenic pigs, which will be necessary from F2 generation onwards. A zygosity specific duplex PCR was also established for the genomically assigned GalKO, discriminating the wild-type from the hetero- or homozygous knockout.

In conclusion, this thesis decribes the systematic reproduction of multi-transgenic donor pigs for xenotransplantation experiments. While breeding for the well-characterised transgenes has already commenced, the established schedule also allows for integration of the additionally analysed transgenes once fertile founder animals become available.

7 ZUSAMMENFASSUNG

Transgene Spenderschweine für die Xenotransplantation – Auswahl von Foundertieren und Etablierung von Zuchtherden

Xenotransplantation gilt angesichts des notorischen Mangels an Spenderorganen als mögliche Behandlungsmethode für Organversagen im Endstadium. Das Schwein wird als best-mögliches Spendertier gesehen, allerdings geht man davon aus, dass mehrere genetische Modifikationen vorgenommen und kombiniert werden müssen, um die zahlreichen Inkompatibilitäten zwischen humanem und porzinem Immun- und Gerinnungssystem zu überwinden. Solche multi-transgenen Tiere durch somatischen Kerntransfer herzustellen bedeutet einen immensen Aufwand. Zucht von multi-transgenen Tieren wird seit langem für Mausmodelle durchgeführt, aber die Übertragung dieser Erfahrungswerte auf Schweine unterliegt mehreren Beschränkungen : (i) während kongene Mausstämme mit wenigen Einschränkungen gezüchtet werden können, beeinträchtigt schon eine vergleichsweise geringe Erhöhung des Inzuchtkoeffizienten im Schwein Fertilität und Wurfgrößen; (ii) eine Generationszeit von 12 Monaten und Trächtigkeitsdauern von 115 Tagen erfordern eine genaue Zuchtplanung; (iii) Kosten und Raumbedarf in der Schweinehaltung übersteigen die der Mäusehaltung bei weitem. Zusätzlich zu diesen zucht-spezifischen Einschränkungen kommt, dass die Abstossungsmechanismen in der Xenotransplantation bisher nur rudimentär verstanden sind. Man kann davon ausgehen, dass in Zukunft weitere Transgene und/oder neue Transgenkombinationen erprobt werden müssen. Zuchtplanungen müssen deshalb diese Limitationen berücksichtigen und ausserdem an neue Anforderungen angepasst werden können.

Diese Dissertation behandelt Planung und Etablierung von multi-transgenen Schweineherden mit den Transgenkombinationen GalKO/CD46/HLA-E und GalKO/CD46/hTM sowie die Charakterisierung neuer hTM- und LEA29Y-transgener Linien für die zukünftige Implementierung in die Zuchtherden. Aufgebaut wurden die Bemühungen auf fünf zuchtfähigen Tieren, zwei GalKO/CD46 Ebern und drei HLA-E Sauen. Mögliche Zuchtpläne wurden auf die gegensätzlichen Aspekte von Inzucht, Transgensegregation und Zeitaufwand hin untersucht und Anpaarungen nach der

günstigsten Variante durchgeführt. Verschiedene Varianten des Einzüchtens von hTM in die Zuchtherden wurden ebenfalls erwogen.

Die Strategie basierte auf einer männlichen GalKO/CD46 und einer weiblichen GalKO/HLA-E Linie. Für deren Erstellung wurden GalKO/CD46 und HLA-E Foundertiere verpaart und Halbgeschwisterverpaarungen aus den resultierende Würfen geplant. Um den Zuwachs an Inzucht zu reduzieren, wurden Auszuchten von GalKO/CD46 Ebern mit Wildtypsauen durchgeführt. Alle F1 Tiere wurden genotypisiert und das Expressionsverhalten wurde immunohistologisch analysiert, um die Auswahl der Zuchttiere für F2 zu treffen.

Parallel zur Erstellung der ersten Zuchtherde wurden neue Foundertiere mit den Transgenen hTM, INS-LEA und CAG-LEA untersucht, um die besten Tiere für die Einbringung dieser Transgene in die Zuchtherden auszuwählen. Dafür wurde die Transgenintegration durch Southern Blotting analysiert und das Expressionsverhalten in einer Reihe von Organen immunhistologisch und mit ELISA bestimmt. Nach der Auswahl der Founderlinien wurden Tiere für die Zucht durch Reklonierung erzeugt. Während für hTM ein zeugungsfähiger Eber schon vorhanden ist, stehen zwei INS-LEA Eber kurz vor der Fertilität.

Für die Aufklärung von Transgen-Integrationsorten wurden inverse PCR durchgeführt. Für den hTM Eber, der in die Zuchtherden integriert werden soll, konnten die Anzahl der integrierten Kopien und die flankierenden genomischen Sequenzen bestimmt werden. Für zwei weitere hTM Founder konnten ebenfalls Transgen-Transgen Verknüpfungen bestimmt werden, allerdings bleibt der Integrationsort unklar.

Mit der F2 Generation können sowohl hemi- als auch homozygote Varianten von additiven Transgenen auftreten. Um diese unterscheiden zu können, wurde für die hTM-Linie mit dem bekannten Integrationsort eine Duplex-PCR etabliert, die das Wildtyp- vom transgenen Allel unterscheidet. Analog dazu wurde für den GalKO ebenfalls eine Duplex-PCR entwickelt, die in der Zucht auftretende Wildtyptiere von hetero- und homozygoten Knockouttieren unterscheidet.

Diese Doktorarbeit beschreibt die systematische Erzeugung von multi-transgenen Spenderschweinen für die Xenotransplantation. Bereits beschriebene Transgene wurde

nach einem Zuchtplan verpaart, der auch die Einbringung neuer Transgene erlaubt.

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10 REFERENCES

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11 ACKNOWLEDGEMENTS

I would like to thank Prof. Dr. Eckhard Wolf and all the people at the Moorversuchsgut for giving me the opportunity to complete this thesis and all their support and help.