Pigs as model systems for biomedical research

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Pigs have a long standing and very successful history as biomedical model for studying human diseases and developing novel therapies mainly attributed to the many genetic, anatomical and physiological similarities with humans. Non-transgenic pig models have long been used for a wide range of human organ systems and diseases, and even complex metabolic disorders and have served as model for developing novel surgical techniques and endoscopic approaches, such as NOTES (natural orifice transluminal endoscopic surgery). The availability of the porcine genome and novel tools to add or delete specific genes significantly expands the potential for transgenic pig production. Somatic cell nuclear transfer has emerged as the preferred method for transgenesis. Well characterized transgenic pig models have been reported for Cystic fibrosis, the eye disease Retinitis Pigmentosa, atherosclerosis and diabetes. Transgenic pigs have been produced for modeling neurological diseases, including Alzheimer and Huntington disease, specific forms of cancer, and skin diseases. Transgenic pigs play an important role in developing functional porcine xenografts to combat the growing shortage of appropriate human organs for transplantation. Other important transgenic pig models include immunodeficient pigs and Oct4/GFP transgenic pigs for studies of reprogramming. Pig models will not replace the already existing mouse models but can provide significant novel insight into a variety of diseases, as mouse models frequently do not mimic the human situation. Transgenic pigs will also soon play an increasing role in the development of novel therapies based on stem cell technology. The biomedical use of pigs will also facilitate transgenic pig production for agricultural production.

Introduction

Pigs have a long standing and very successful history as biomedical model for studying human diseases and developing novel therapies. Domestic pigs and minipigs are the main categories that have been used as biomedical models. Usually minipigs are in shorter supply than domestic pigs and thus more expensive compared with domestic pigs, which cost more due to housing, feed and medication (Litten-Brown *et al.*, 2010). The preferred use of pigs as model in biomedical research is attributed to the many anatomical and physiological similarities with humans. As humans, the pig is a monogastric omnivore. As result of a long domestication process a great variety of pig phenotypes exists worldwide that could be relevant for current human health research priorities, including obesity, diabetes and cardiovascular diseases. Given the high degree of similarity, many diagnostic, surgical or other medical techniques can be directly

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transferred from the pig into the clinic to help human patients. Another great advantage is the ability to maintain pigs under strict hygienic conditions, such as specific pathogen free (SPF) or gnotobiotic (completely sterile) conditions.

In addition, the high fertility of the pig makes it an attractive species for use in biomedical model application. Moreover, effective protocols are established for artificial insemination and embryo transfer for a long time. More recently, somatic cell nuclear transfer (SCNT) methodology has been improved and refined protocols for genetic modification of pigs have been established (Petersen *et al.*, 2008; Hauschild *et al.*, 2011; Garrels *et al.* 2012).

Worldwide, thousands of pigs are being used in biomedical research every year. In Germany, an average of 12 -13.000 pigs is being used per year in biomedical research. A Google search, using the three words "pig, model, research" yielded 140.000 hits in 2007, 6.7 million hits in 2008 and 29.9 million hits in 2013. This clearly shows the rapidly growing interest in the use of the pig as a biomedical model for the benefit of humans. One can discriminate between non-transgenic and transgenic pig models. The following gives a brief overview on the wide range of non-transgenic pig models followed by an update of the recent development of transgenic pig models.

Non-transgenic pig models

Pigs have been used as appropriate biomedical models due to genetic, anatomical and physiological similarities to humans (Litten-Brown *et al.*, 2010). Tests have frequently been undertaken to investigate pharmacokinetics and pharmacodynamics of specific drugs. However, swine also have unique characteristics and husbandry requirements which must be taken into account when using the species as a biomedical model. Non-transgenic pig models have been employed for a wide range of human organ systems and diseases.

Head and brain injuries

Pig models have been developed for traumatic brain injury, including brain death to define critical parameters of ischemia and to study systemic reperfusion as a model for human brain death (Purins *et al.*, 2011, 2012). Even four weeks old piglets have been used as model for studying traumatic brain injury for pediatric purposes (Friess *et al.*, 2011). The optimal resuscitation strategy was developed in a domestic pig model of traumatic brain injury and hemorrhagic shock (Jin *et al.*, 2012). Two days old piglets were also used to study the effectiveness of anti-inflammatory drugs for treatment of head injury (Friess *et al.*, 2012).

Eye diseases

The porcine eye is very similar to the human eye showing an area of increased cone density arranged in a central horizontal band considered analogous to the human macula. Porcine eyes have been used to study age-related macular degeneration and to develop ophthalmological surgical treatments for human patients (Pennesi *et al.*, 2012). An inducible photoreceptor damage porcine model was developed using chemical toxins. Sodium iodate (NaIO3) was an effective toxin for the pig eye and could thus serve as model to develop treatments to replace damaged photoreceptors (Noel *et al.*, 2012).

Cardiovasculatory diseases

The pig has been widely used in preclinical studies to develop novel treatments for cardiovascular diseases that are a common reason for death of human patients. Most prominent are models for

myocardial infarction and reperfusion, the hibernating myocardium, and for vulnerable plaques (Suzuki *et al*., 2011). Specific aspects of the treatment of coronary injuries were also investigated in the pig model, with emphasis on endothelial denudation and stent placement (von Bary *et al*., 2011). Novel treatments involving the application of stem cells and growth factors have been tested in the pig to study survival and regeneration of the infarcted pig heart. The combination of IGF-1 and HGF seems to be beneficial in this respect (Ellison *et al*., 2011). Multipotent stromal cells were successfully applied in a pig model to improve the situation after chronic myocardial infarction (Sato *et al*., 2011). Immediate implantation of bone marrow-derived cells into minipig myocardium after coronary artery ligation promoted neovascularization and improved myocardial viability (Ko *et al*., 2011). The intracoronary delivery of mesenchymal stem cells (MSCs) into the ischemic heart reduced malignant ventricular arrhythmias and improved cardiac performance (Wang *et al*., 2011). A novel coronary guidewire was successfully tested in a porcine model and emerged as an effective tool to improve transcoronary pacing (Heinroth *et al*., 2011). The pig has also served as model to study cardiac arrest and to develop strategies to overcome this pathology by applying therapeutic hypothermia and selective heart cooling (Wang *et al*., 2012; Li *et al*., 2012).

Vasculatory diseases

The pig has successfully been used as model to study the effects of implantation of MSCs into an aortic aneurysma injury. The orthologous implantation was successful, but long term effects remain to be investigated (Turnbull *et al*., 2011). Type B aortic dissection, which is the most common acute disease of the aorta and a life-threatening condition, has been studied in the pig model (Okuno *et al*., 2012). The pig was also successfully used as a model for ultrasound enhanced recombinant tissue plasminogen activator mediated thrombolysis in a carotid artery model (Hitchcock *et al*., 2011).

Drug-coated balloons have been tested as therapeutic approach for treating vasculatory diseases in familial hypercholesterolemic swine. These balloons were effective in reducing proliferation of the neointima cells (Granada *et al*., 2011). Intramural injection of complex lipids into the coronary arteries of pigs induced symptoms similar to human atherosclerosis (Tellez *et al*., 2011).

Pulmonary diseases

Transesophageal upper pulmonary lobectomy was successfully established in the domestic pig and is considered as a novel strategy towards scar free pulmonary lobectomy (Moreira-Pinto *et al*., 2012). A specific extracorporeal cardiopulmonary support system was tested in its effectiveness to rescue patients after massive pulmonary embolism. However, the optimal treatment is still unknown (Kjærgaard *et al*., 2012). New lung ventilator strategies, including an "open lung" system, were developed in the pig and turned out to be superior to standard strategies treating lung injuries (Albert *et al*., 2011). The pig has also served as model for developing new treatments of intra-abdominal hypertension, which is an important factor leading to increased morbidity and mortality in human patients. Application of positive end-expiratory pressure did not yield beneficial results (Regli *et al*., 2012). The pig also served as a model to test adenosine A_{2A} receptor agonists for treating reperfusion injury in a preclinical lung transplantation model (LaPar *et al*., 2011). In a pig model for human pulmonary diseases, surfactant administration improved important parameters of pulmonary function and specific ventilators were needed for improving lung function (Bhatia *et al*., 2011; Dickson *et al*., 2011). Pigs have also been used as model for studying etiology and to develop treatments for

various pulmonary diseases, including acute respiratory distress syndrome and specific forms of pneumonia (Ballard-Croft *et al.*, 2012, Martinez-Olondris *et al.*, 2012).

Kidney diseases

The pig is an excellent model for kidney transplantation studies, specifically using an ischemia reperfusion model. Porcine and human kidneys are anatomically very similar due to their multi-lobular structure, which is in contrast to rodent and dog kidneys (Giraud *et al.*, 2011). The pig has also extensively been tested in a kidney autotransplantation model with all its facets from medical anesthesia to surgical intervention and postoperative management and analgesia (Post *et al.*, 2012). The technical feasibility and safety of trajectory image-guided percutaneous renal cryoablation were demonstrated in a porcine model (Rebuck *et al.*, 2012). Specific aspects of renal artery stenosis and treatment via specific magnetic resonance technologies were investigated in a pig model (Morelli *et al.*, 2012). By feeding hydroxyproline or a gelatin diet, a model was established for studying oxalate urolithiasis in human patients (Patel *et al.*, 2012).

Liver diseases

New hemostatic dressing has been successfully tested in a porcine model of liver injury (De Castro *et al.*, 2010). The porcine model of short bowel disease syndrome revealed severe liver pathology, similar to symptoms in human patients (Hua *et al.*, 2012). Small bowel grafts were used in a pig allotransplantation model and graft viability was shown (Yandza *et al.*, 2012). Steatotic porcine livers could be successfully preserved for prolonged period under normothermic conditions (Jamieson *et al.*, 2011).

Pancreatic diseases

High intensity ultrasound was successfully used to ablate the pancreas and emerged as a safe and effective approach for use in human patients with pancreatic disease (Xie *et al.*, 2011). Hypertonic saline resuscitation was successfully used in a porcine model to improve symptoms of acute pancreatitis, but organ damage could not be prevented (Ni *et al.*, 2012). The insulin injection site was shown to cause minor perturbation of local glycemia in a minipig model without diabetes (Rodriguez *et al.*, 2011).

Hemorrhagic shock

Hemorrhage accounts for ∼ 40% of trauma death and is the most common cause of preventable death after injury. The optimal fluid strategy for early treatment of trauma patients was tested by applying hypertonic saline with dextran in a pig model of uncontrolled hemorrhagic shock (Riha *et al.*, 2011). The porcine femoral artery injury is well suited for evaluation of new hemostatic agents (Kheirabadi *et al.*, 2011).

Wound repair

To avoid significant contracture formation after skin injuries, keratinocytes alone or in combination with fibroblasts were successfully applied in a vivo porcine model (Eldardiri *et al.*, 2012). Negative pressure therapy improved characteristics of primarily closed porcine wounds (Meeker *et al.*, 2011).

Cartilage and bones repair

Cartilage repair was attempted by applying mesenchymal stem cells either in an undifferentiated stage or in a more differentiated stage. Undifferentiated MSCs were superior to other treatments

(Chang *et al.*, 2011). The pig also served as a model for developing a novel flexor tendon repair treatment (Zetlitz *et al.*, 2012). Tap water washouts turned out to be effective for treatment of contaminated open bone fractures in a porcine model (Gaines *et al.*, 2011). Adipose-derived mesenchymal stem cells enhanced healing of defects in the mandibles in a pig model (Wilson *et al.*, 2012).

Endoscopy and NOTES-techniques

Endoscopical techniques have been successfully used in the pig for gastrojejunostomy and liver resection (von Renteln *et al.*, 2011; Zijlmans *et al.*, 2012). We have explored the possibilities and limitations of NOTES-techniques (Natural orifice transluminal endoscopic surgery) which would have major advantages over standard laparoscopic techniques, because of significantly less postsurgical pain, fewer wounds and abdominal wall infections, avoiding hernias, fewer adhesions, shorter recover periods and improved cosmetic results. This technology is intensively explored in the domestic pig in the own laboratory and has been used to improve esophagus repair treatments (Fritscher-Ravens *et al.*, 2008, 2011).

Obesity

Hypothalamic deep brain stimulation was shown to reduce weight gain in a porcine obesity model. The low frequency ventromedial hypothalamus electrical stimulation could emerge as a potential strategy for modulation of body weight (Melega *et al.*, 2012). Laser technology was successfully applied to induce subdermal lipolysis and collagen deposition in an in vivo pig model (Levi *et al.*, 2011). The drug meloxicam was successfully tested in a kaolin inflammation model (Fosse *et al.*, 2010).

Infectious diseases

The pig is considered a successful model for studying a variety of human infectious diseases. The pig has numerous advantages for studies of infectious diseases and vaccines for a wide range of organ systems (Meurens *et al.*, 2011). The pig is also a useful model studying parasitic diseases such as human amebiasis, and could help to increase understanding the intestinal and extraintestinal symptoms of this specific disease (Girard-Misguich *et al.*, 2011).The pig has served as a model to study the effects of specific plasma separation filtration techniques in a sepsis model (Sauer *et al.*, 2012). The pig is even useful as a model for studying the effects of specific diet components in a post-operative infection situation (Langerhuus *et al.*, 2012).

This brief and non-exhaustive survey of the recent literature clearly demonstrates the extensive use of pigs in medical research. Pig models are obviously extremely useful for a better understanding of specific human diseases or morphology and to develop and validate novel therapies. This will further increase with the advent of novel cell-based therapies derived from stem cell technologies.

Transgenic pig models

The assembly and annotation of the porcine genome has recently been published, including a comparison of genomes from wild and domestic pigs from Europe and Asia (Groenen *et al.* 2012). As other large animals, pigs have approx. 22.000 protein coding genes. The porcine genome shows a high degree of homology with that of human, dogs, horses and other large mammals. Naturally occurring mutations further expand the potential to use pigs as biomedical models (see above). At least 112 positions in the porcine genome were identified in which

the porcine protein had the same amino acid as human diseases (Groenen *et al*. 2012). These genetic changes were associated with an increased risk of multifactorial traits, including obesity, diabetes or late-onset diseases such as Parkinson disease and Alzheimer disease in humans (Groenen *et al*. 2012). The availability of the porcine genome significantly expands the potential for transgenic pig production. This is facilitated by effective new tools to add or delete specific genes to the porcine genome, including specific nucleases such as zinc finger nucleases (Figure 1), TALEN and transposons (Hauschild *et al*. 2011; Flisikowska *et al*. 2011; Garrels *et al*. 2012; Pennisi 2012).

- Nucleases must dimerize to cut DNA, 2 ZFN molecules necessary , specific recognition of 18 bp

5'-CAG TTG GCA TAC **CATGGT** TCA TAC TAG ACC- 3'
3'-GTC AAC CGT ATG **GTACCA** AGT ATG ATC TGG- 5'

5'-CAG TTG GCA TAC **CAT**   **GGT** TCA TAC TAG ACC- 3'
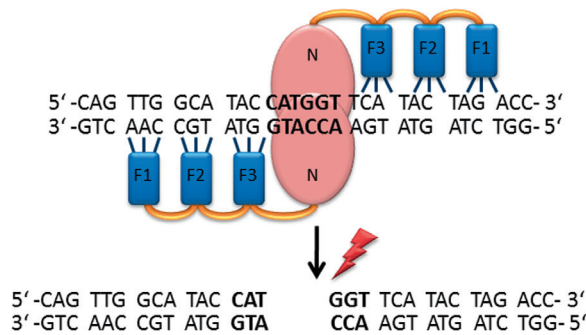3'-GTC AAC CGT ATG **GTA**   **CCA** AGT ATG ATC TGG- 5'

Fig. 1 Mechanism of Zinc-finger-nucleases (ZFN) in the editing of DNA. Here the bi-allelic knock-out of a specific DNA sequence is shown.

The in-depth knowledge of the structure and organization of the porcine genome and further refinements of molecular tools will soon allow the production of a great variety of transgenic pig phenotypes similar to the laboratory mouse. Pigs are more expensive to keep than rodents and reproduce more slowly than rodents. However, similarities between human and pig genetics, anatomy and physiology much outweigh these limitations. Porcine eyes are similar in size with photoreceptors that are similarly distributed in the retina as in the human eye. Thus the pig became the first transgenic model for Retinitis Pigmentosa (RP) which is an important cause of human blindness. More than 4 years ago the first transgenic pig model of cystic fibrosis was reported which stimulated the interest of researchers in porcine disease models (Abbott 2012). Additional stimulation of transgenic pig production will arise from the availability of pluripotent stem cells that are superior to the currently available somatic cells for any genetic modification procedure. Germ line contribution of pluripotent cells has not yet been reported for the pig. However, significant progress has been reported towards this goal (Nowak-Imialek *et al*. 2011; Nowak-Imialek and Niemann 2013). The following is an update of the available literature on the production of transgenic pigs for biomedical models. The topic has also been reviewed recently (Wolf 2012; Prather *et al*. 2013).

Porcine models for the genetic diseases cystic fibrosis and hereditary tyrosinemia type 1

Transgenic pigs carrying a mutated CFTR (cystic fibrosis transmembrane conductance regulator) gene are present the best characterized model for a human genetic disease. Cystic fibrosis

is caused by a genetic defect in the CFTR which encodes the cyclic AMP activator chloride channel in epithelial cells. This defect results in reduced epithelial fluid transportation and thus creates abnormal fluid secretion of the airway mucous glands that are the major cause for CF (cystic fibrosis) pathology (Widdicombe 2010). Mouse airways contain only few mucous glands, consequently the mouse model of CF shows little airway pathology (Widdicome 2010). The production of viable pigs carrying a homozygous knockout of the CFTR gene led to the creation of a cystic fibrosis model in domestic pigs (Rogers *et al.* 2008). The pigs were produced via SCNT using somatic cells with a homozygous knockout of the CFTR gene locus. The CFTR gene was disrupted by inserting an antibiotic resistance cassette in exon 10 of the CFTR gene (Rogers *et al.* 2008). Pigs lacking the CFTR showed defective chloride transport, developed meconium ileus, displayed exocrine pancreatic destruction and focal biliary cirrhosis, thus showing the same abnormalities observed in human CF patients (Rogers *et al.* 2008). These pigs provide a new source to study CF pathology and to develop novel therapies for CF patients.

The CFTR$^{-/-}$ pigs developed the full hallmark of the CF lung disease, including airway inflammation, remodeling mucus accumulation, and infection. Their lungs contained multiple bacterial species as consequence of the defect in the bacterial defense system (Stoltz *et al.*, 2010). It was further found that the ΔF508 mutation induced a CF-like disease in the CFTR$^{-/-}$ pigs. This is an important step towards unraveling the molecular pathogenesis of common CF disease (Ostedgaard *et al.*, 2011). The lack of functional CFTR was shown to reduce bacterial killing on the airways and to change of the pH towards a more acidic situation. This reduced the antimicrobial activity of airways surface liquid (Pezzulo *et al.* 2012). Airways surface liquid was thus identified as critical factor in the lung defense system and is directly linked with the initial host defense defect (Pezzulo *et al.*, 2012). In a further attempt to unravel the etiology of CF, it was shown that pigs and humans with CF have reduced insulin-like growth factor 1 (IGF1) levels at birth (Rogan *et al.*, 2010). IGF levels might thus serve as biomarker to predict disease severity or the response to specific therapeutics. These findings also raise the possibility that IGF1 supplementation early in development might be beneficial for CF patients (Rogan *et al.*, 2010).

Hereditary tyrosinemia type 1 (HT1) is a human disease that is caused by deficiency in the enzyme fumary lacetoacetate hydrolase (FAH), causing hepatic failure, cirrhosis and hepatocellular carcinoma already early in childhood. The FAH gene was knocked out in porcine fibroblasts that in turn were used in SCNT. Several viable FAH$^{+/-}$ pigs were produced that showed a normal phenotype, but had decreased FAH transcriptional and enzymatic activity compared with wild-type pigs (Hickey *et al.*, 2011).

Transgenic pigs for human eye diseases

Patients with Retinitis Pigmentosa (RP) develop night blindness early in life due to the loss of rod photoreceptors. Transgenic pigs were created by injecting a mutated porcine rhodopsin gene into pronuclei of zygotes and transgenic pigs showed a similar pathology as RP patients (Petters *et al.* 1997). Subsequently lensectomy and vitrectomy were applied which delayed photoreceptor degeneration in rhodopsin transgenic pigs (Mahmoud *et al.* 2003). Further research revealed that oxidative damage is a potential cause of cone cell death in a RP situation (Shen *et al.* 2005). These data are in line with the hypothesis that death of rods is associated with decreased oxidant consumption and hyperoxia in the outer retina followed by gradual cone cell death. In an attempt to develop a treatment for RP patients, fetal neuroretinal cells were transplanted into pig eyes with severe retina degeneration. However, graft and host retinal neurons did not form proper connections leading to reduced retinal function in the host (Ghosh *et al.* 2007). Ribozyme based gene therapy was tested to treat autosomal dominant RP in a transgenic pig

model. However, the allele specific ribozyme used for the human sequence was not successful, whereas the hammerhead ribozyme had beneficial effects (Shaw *et al*. 2001). Further study of the RP transgenic pigs revealed that the rhodopsis PSD-95 is nearly completely lost from most rod terminals in transgenic swine. But an early postnatal PSD-95 expression continues in cone terminals even in 10 months old transgenic swine when all the rods have disappeared. This indicates that the loss of PSD-95 is not the consequence of the deteriorating cells (Blackmon *et al*. 2000). In transgenic porcine retina, the ectopic synapses formed between cones and rod bipolar cells were altered with impaired processing of inner retinal neurons (Ng *et al*. 2008).

SCNT was used to create a transgenic miniature pig model expressing a specific rhodopsin mutation. The founder animals showed abnormal full-field electroretinography and the offspring inherited the transgene with the autosomal dominant mutation. The miniature pig carrying the P23H RHO mutation is a new model to study morphology and treatment of RP (Ross *et al* 2012).

Obviously, these models display key features of human RP; however the time course of disease progression makes this model costly, time consuming and difficult to study. Treatment with iodoacetic acid emerged as an alternative rod dominant model of retinal damage which shared many features with the transgenic RP pig model (Wang *et al*. 2011).

Another model for human eye disease was recently reported. Transgenic pigs expressing the human disease causing ELOVL4 mutation revealed photoreceptor loss and disorganized inner and outer segments. These pigs are promising as new model to examine macular degeneration and STGD3 pathogenesis (Sommer *et al*. 2011).

Transgenic pigs in diabetes research

Transgenic pigs expressing a dominant negative receptor for the incretin hormone glucose-dependent insulinotropic polypeptide (GIP) revealed a crucial role of the GIP system for age related expansion of pancreatic β-cell mass. This model shared important characteristics with type 2 diabetes mellitus patients, including reduced glucose tolerance, insulin secretion and progressive reduction of β-cells (Renner *et al*. 2010). Metabolic signatures of specific amino acids and lipids were investigated in this model and several potential biomarkers of early phases of β-cell dysfunction and mass reduction were identified (Renner *et al*. 2012 a). Transgenic pigs with permanent diabetes were created by SCNT with a mutated insulin gene. These pigs show typical features of progressive diabetes, including cataract development and pathology of kidneys and the nervous system (Renner *et al*. 2012 b).

Transgenic pigs with β-cell specific expression of LEA29Y served as donors in a xenotransplantation model. Xenograft islet cell clusters from these pigs rescued diabetes and prevented rejection in a humanized mouse model (Klymiuk *et al*. 2012). Transgenic cloned pigs have also been produced carrying a dominant negative mutant for a hepatocyte nuclear factor 1α mutation showing obvious diabetic symptoms (Umeyama *et al*. 2009). The use of islets from pigs transgenic for a fluorogenic protein GFP (green fluorescent protein or Kusabira-Orange) may facilitate development of islet cell xenotransplantation (Teratani *et al*. 2012).

Pigs as models for neurological diseases

The survival motor neuron (SMN1) gene was mutated in fibroblasts to create a transgenic swine model for spinal muscular atrophy (SMA); but pigs have not yet been produced with this mutation (Lorson *et al*. 2008). In an effort to develop porcine models for Alzheimer disease, Göttingen miniature pigs were produced that carry a random integration of the Alzheimer disease causing dominant mutation APPsw. Pigs were produced by handmade cloning in cells that had a single copy of the transgene inserted in the GLIS locus. The transgene was consistently expressed and

the accumulation of Alzheimer proteins is expected to occur in the brain at the age of 1 – 2 years (Kragh *et al*. 2008). A construct harboring the Huntington cDNA was injected into pronuclei of zygotes and transgenic minipigs were produced. However, a phenotype was not reported (Uchida *et al*. 2001). More recently, pigs transgenic for Huntington's disease were produced via SCNT. These pigs expressed a mutant Huntington gene with an expanded polyglutamine tract (Yang *et al*. 2010). Severe postnatal death, dyskinesia and chorea-like movement were observed in some transgenic pigs with expression of the mutant huntingtin. The typical apoptotic neurons with DNA fragmentation were found in the brains of transgenic pigs (Yang *et al*. 2010).

Porcine cancer models

Pigs have been produced with a knockout of the breast cancer associated gene 1 (BRCA1) which predisposes for breast cancer and accounts for the majority of the cases of familial breast and ovarian cancer. Cells with a knockout of BRCA1 mediated via recombinant adeno-associated virus were produced and used in SCNT. Targeting efficiency was high, however, all BRCA1 hemizygous transgenic piglets died shortly after birth (Luo *et al*. 2011). The reasons of this postnatal mortality remain unclear; it illustrates the difficulty to produce a meaningful large animal model for specific diseases.

Gene targeted cloned pigs carrying a mutation in the APC (adenomatous polyposis coli) gene displayed a similar pathology in the intestine as human patients with familial adenomatous polyposis (Flisikowska *et al*., 2012). This large animal model is promising for the development of novel diagnostic and therapeutic strategies for colorectal cancer. Live pigs with a mutation in the tumor suppressor p53 gene were produced, that is orthologous to the oncogenic human mutant TP53^{R175H} and mouse Trp53^{R172H} mutation. Gene targeted MSCs were successfully employed in SCNT and viable piglets were produced with the TP53^{R167H} mutant allele in a heterozygous form. This is the first pig model that demonstrates the feasibility of an inactivation mutation of the gatekeeper tumor suppressor gene p53 in a non-rodent mammal (Leuchs *et al*. 2012).

Immunodeficient pigs

A porcine model of severe combined immunodeficiency (SCID) has been produced which is largely similar to the well-known mouse model. Fibroblasts were targeted for disruption of the X-linked interleukin 2 receptor gamma chain gene (Il2rg) and were employed as donor cells to produce cloned pigs. Viable heterozygous Il2rg females were produced, whereas the IL2rg heterozygous males were athymic and showed significantly reduced immunoglobulin and T and NK cell production, clearly mimicking the human SCID situation (Suzuki *et al*. 2012). Moreover, allogeneic bone marrow transplantation was compatible with stable integration of heterozygous Il2rg-IY and reconstituted the Il2rg-IY lymphoid lineage. These pigs are a very important step towards the creation of pig models for the evaluation of cell based regenerative treatments. These significant advances may complement the already existing arsenal of genomically humanized mice that are a valuable tool for gaining a better understanding of basic immunological activity (Devoy *et al*. 2012).

Porcine atherosclerosis models

Modeling atherosclerosis in pigs has been difficult because rapid atherosclerosis could not be induced in normal pigs by high-fat feeding regimens. Promising transgenic approaches have been reported recently. Transgenic Yucatan minipigs were produced that over-expressed human catalase on the endothelial cells. The transgene was transfected into fibroblasts and transgenic

fibroblasts were used in SCNT. Transgenic pigs showed increased activity of catalase and reduced levels of H_2O_2 in culture (Whyte *et al.* 2011). Using Sleeping Beauty DNA transposition and SCNT, Yucatan minipigs were created with liver specific expression of the human D374Y-PCSK9 gain of function mutation. The PCSK9 gene encodes human proprotein convertase subtilisin/kexin type 9 that is critically involved in cholesterol metabolism. D374Y-PCSK9 transgenic pigs displayed the typical pathology of human atherosclerosis, including reduced hepatic low-density lipoprotein (LDL) receptor levels, impaired LDL clearance, severe hypercholesterolemia and atherosclerotic lesions in the vasculatory system (Al-Mashhadi *et al.*, 2013). Moreover, pigs with a mutated LDL receptor were created using specific TALEN, but the phenotype has not yet been reported (Carlson *et al.* 2012).

Porcine models of human skin diseases

The keratinocyte-specific human transgene K5-hGli2ΔN was expressed in transgenic pigs produced by SCNT. This gene is critically involved in the development of basal cell carcinomas. The transgenic pigs developed the typical skin lesions that could not be treated by antibiotics leading to an early death of the animals. This pathology has not been observed in the corresponding mouse model (McCalla-Martin *et al.* 2010). A model for cutaneous inflammation was produced in Göttingen miniature pigs, expressing the human β1 or α2 integrin genes under control of a keratinocyte specific promoter (Staunstrup *et al.* 2012). Transgenic pigs showed ectopic expression of human integrins and localization within the keratinocyte plasma membrane. This indicates that regulation of integrins β1/α2 by over-expression of the transgenes occurred via different cellular signaling pathways. Several markers of perturbed skin homeostasis were identified. These pigs are the first model with molecular markers of skin inflammation (Staunstrup *et al.* 2012).

Xenotransplantation of porcine organs to human patients

Today more than 250,000 people owe their lives a successful human organ transplantation (allotransplantation). Ironically, the success of organ transplantation technology has led to an acute shortage of appropriate organs, because cadaveric and live organ donation falls far short of meeting the demand in western societies. To close the growing gap between demand and availability of appropriate organs, transplant surgeons have long considered the possibility of using xenografts from domesticated pigs (Bach 1998; Platt *et al.* 1998; Kues and Niemann 2004). Essential prerequisites for successful xenotransplantation are: (i) overcoming the immunological hurdles, (ii) preventing the transmission of pathogens from the donor animal to the human recipient, and (iii) compatibility of the donor organs with human physiology. This requires a series of critical steps and can be time, labor and cost expensive (Figure 2).

An important advantage of xenotransplantation is the opportunity to modify the genome of the donor animals. Two modifications are required for preventing the first immunological hurdle, the hyperacute rejection (HAR), in pig-to-human transplantation: i) Elimination of antigenic sugar residues via genetic knockout of the porcine α1,3-galactosyltransferase (GGAT-1)(homozygous Gal kO), and/or ii) Suppression of the recipient's complement system by the introduction of one or more regulators of complement activation (RCA), such as CD55, 46, or 59 (Figure 3). A tremendous amount of research focused on the production of genetically engineered pigs expressing inhibitors of the human complement cascade. The validity of this approach has been convincingly demonstrated by several groups (Schuurman *et al.*, 2002; McCurry *et al.*, 1995; Bhatti *et al.*, 1999; Chen, *et al.* 1999). Most importantly, it has been demonstrated that organs from genetically engineered pigs lacking functional α1,3-galactosyltransferase and thus lacking

1. Production and propagation of transgenic pigs (SCNT)
2. Determination of transgenic expression patterns
   - mRNA
   - Protein (intensity, tissue specificity, etc.)
3. Selective breeding of transgenic lines
4. In-vitro-testing to determine the protective properties against HAR and/or AVR (eventually safety testing for PERV, etc.)
5. **Perfusion of isolated porcine organs with human blood (Modelling the human situation)**
6. In-vivo-studies in primates
   - Physiological compatibility
   - Protection against immune reactions
   - Testing for transmission of pathogens

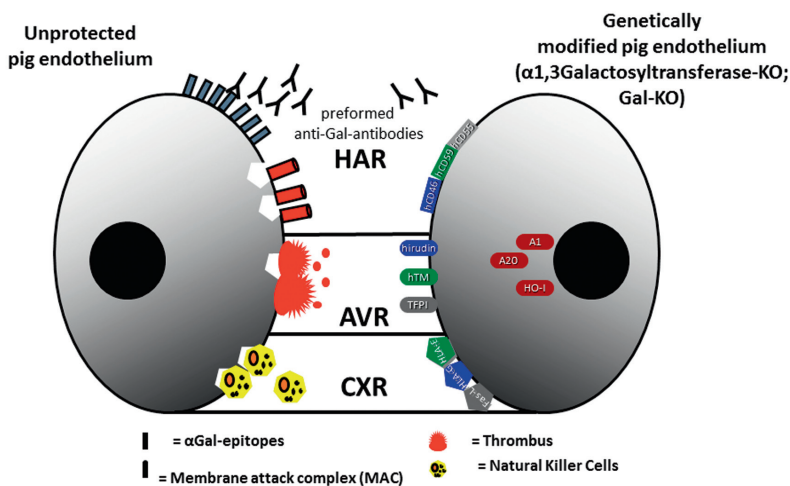**Fig. 2** Steps of the evaluation of transgenic pigs for suitability in xenotransplantation

Fig. 3 Schematic diagram of the rejection responses in a xenotransplantation setting and molecules that are involved in these processes. (for details see text)

expression of α-Gal epitopes (GGTA1-KO pigs), do not undergo HAR once transplanted into primates (Yamada *et al.*, 2005; Kuwaki *et al.*, 2005).

While induction of a knockout of a specific gene is extremely difficult and inefficient in somatic cells used in SCNT, novel approaches have been successfully explored to overcome this bottleneck in the production of pigs with inactivation of epitopes critically involved in the immunological rejection after porcine-to-primate xenotransplantation. Zinc finger nucleases (ZFNs) are a class of engineered DNA-binding proteins that facilitate editing of the genome by creating double-strand breaks in DNA at targeted loci. The ZFN mediated knock-out makes the integration of an antibiotic selection cassette superfluous that are used in conventional HR strategies for selection of the targeted cells. The first pigs with a homozygous GGTA-1KO induced with the aid of specific ZFNs were recently reported (Hauschild *et al.* 2011).
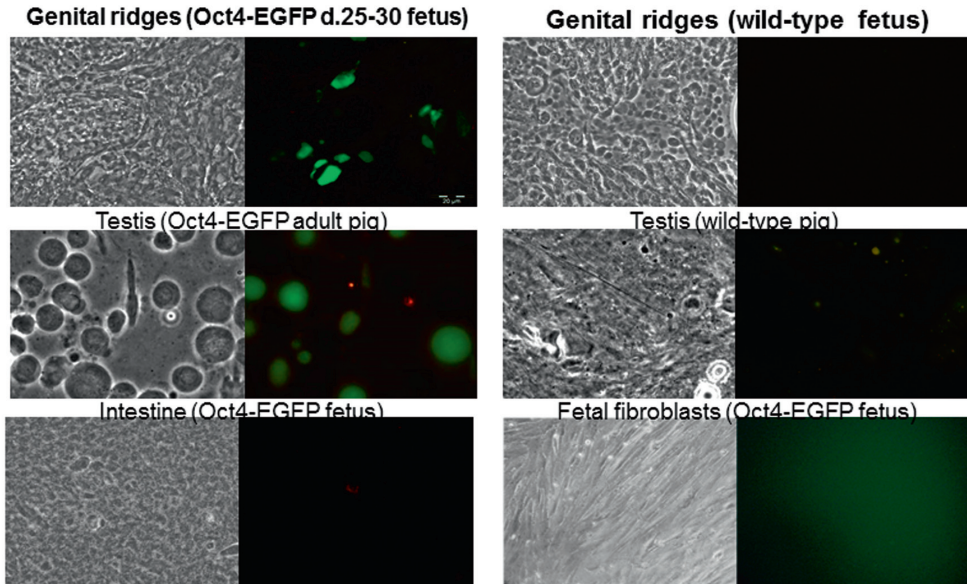
Even in the case of GGTA1-KO pigs, porcine xenografts eventually failed as a consequence of the acute humoral (AHXR) or delayed (DXR) xenograft rejection, also called acute vascular rejection (AVR) (Platt *et al.*, 1998; Bach *et al.*, 1996). Several factors have been implicated in the pathogenesis of AHXR and pathology is primarily characterised by vascular thrombosis, blood extravasation and oedema (Platt *et al.*, 1991). Cellular infiltrates include neutrophils, macrophages, CD8+ T cells and few NK cells (Vega *et al.*, 2002). AVR is characterised by the progressive deposition of antibodies and complement and is associated with apoptosis and necrosis of endothelial cells, contributing to platelet aggregation and thrombosis in the graft. The current view is that long term survival of xenografts after transplantation into primates requires a specifically tailored immunosuppression regimen compliant with current clinical standards, and additional modifications of the pig genome. Several candidate genes, incl. human thrombomodulin (hTM), human heme-oxygenase 1 (hHO-1), human A20 (hA20), or CTLAIg (soluble CD28 receptor analog), have been explored in their ability to improve long term survival of porcine xenografts after transplantation into nonhuman primates (Petersen *et al.*, 2009; Petersen *et al.*, 2011; Oropeza *et al.*, 2009; Phelps *et al.*, 2009) (Figure 3).

Extensive research has revealed that the risk of porcine endogenous retrovirus (PERV) transmission to human patients is low, paving the way for preclinical testing of xenografts (Switzer *et al.* 2001; Irgang *et al.* 2003). RNA interference (RNAi) is a promising method for knocking down PERV expression in porcine somatic cells. Using RNAi mediated knockdown, PERV expression has been significantly reduced in porcine somatic cells for 4-6 months, these cells were successfully used in SCNT and gave normal piglets with long-term suppression of PERV (Dieckhoff *et al.*, 2008; Semaan *et al.*, 2012). RNAi knockdown thus provides an additional level of safety for porcine-to-human xenotransplantation.

Although additional refinements will always be possible, it is expected that appropriate lines of transgenic pigs will be available as organ donors within the next five to ten years. Guidelines for the clinical application of porcine xenografts already exist in the USA and are being developed in other countries. The general consensus of a worldwide debate is that the technology is ethically acceptable provided that the individual`s well-being does not compromise public health (e.g. the risk of PERV recombination). The improvement in quality of life for patients receiving conventional allotransplants is dramatic, but xenotransplantation is also economically attractive because the long term costs of maintaining patients with severe kidney disease on dialysis or treating patients with chronic heart disease can be greater than the cost of a successful transplant. Preliminary functional data on porcine kidneys and hearts in non-human primates is promising although the long term interaction between porcine organs and human physiology is to a great extent unexplored (Ibrahim *et al.* 2006).

Reprogramming of somatic cells and development of cell based therapies

To facilitate the use of domesticated pigs as a tool for preclinical testing of novel therapies and to facilitate the derivation of germ line competent pluripotent stem cells, Oct4-GFP transgenic pigs were recently produced from our laboratory (Nowak-Imialek *et al.*, 2011). The transcription factor Oct4 is essential for the maintenance of pluripotency and for reprogramming somatic cells into a pluripotent state. Using a18 kb genomic sequence of the murine Oct4 gene fused to the enhanced green fluorescent (eGFP) cDNA, pluripotent cells could unequivocally identified by the green fluorescence. Expression of the EGFP reporter was confined to germ line cells in live pigs, the inner cell mass and trophectoderm in blastocysts and in testicular germ cells (Figure 4). Reprogramming of fibroblasts from these animals by fusion with pluripotent murine embryonic stem cells or viral transduction using human Oct4, Sox2, KLF4 and cMYCcDNAs revealed Oct4-EGFP reactivation (Figure 5) clearly showing the usefulness of this approach.

Genital ridges (Oct4-EGFP d.25-30 fetus)

Genital ridges (wild-type fetus)

Testis (Oct4-EGFP adult pig)

Testis (wild-type pig)

Intestine (Oct4-EGFP fetus)

Fetal fibroblasts (Oct4-EGFP fetus)

(Analysis of 12 transgenic fetuses on days 25-30)

Fig. 4 Germ line specific expression of the Oct4/eGFP transgene, the green fluorescence indicates the presence of Oct4 expression in porcine testicular tissue, which is a marker for pluripotency.
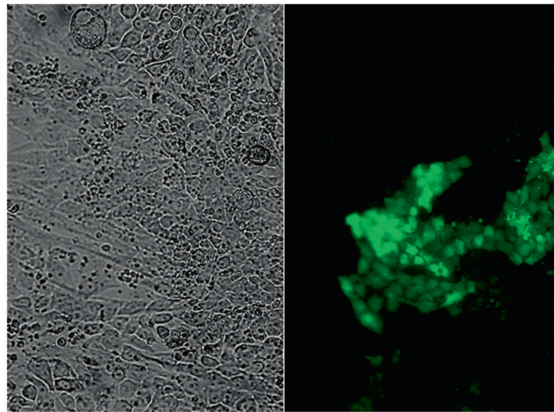

Fig. 5 Activation of the reporter transgene Oct4-eGFP in porcine fibroblasts by over-expression of the 4 reprogramming factors. The green fluorescence indicates expression of the pluripotency marker Oct4 which in turn indicates reprogramming of the somatic cells into a pluripotent state.

These cells have been used for studies to isolate and characterize pluripotent stem cells in the pig (Kues *et al.*, 2013; Petkov *et al.*, 2013).

Transgenic pigs were also produced that expressed mitochondria localized enhanced yellow fluorescent protein under the control of the germ cell specific stimulated by retinoic acid (Stra8) promoter in the testicular tissue. Expression of the Stra EYFP transgene in spermatogenic cells could serve as a useful model for germ cell transplantation and *in vitro* spermatogenic studies

(Sommer *et al.*, 2011). Chimeric pigs derived from induced pluripotent stem cells with germ line transmission in the absence of tumor formation, were recently reported. This is a major step forward towards the establishment of a translational model to study effects and safety of stem cell therapies (West *et al.*, 2011).

Concluding remarks and perspectives

The pig has a long and successful history in biomedical research and has thus benefitted significantly human health and well-being. The number of non-transgenic pig models is already large and the potential of the pig in biomedical research will be further enhanced with the recent availability of the porcine genome and molecular tools needed for targeted genetic modification similar to the laboratory mouse. Pig models will not replace the already existing mouse models but can provide significant novel insight into a variety of diseases, as mouse models frequently do not mimic the human situation. Transgenic pigs will also play an increasing role in the development of novel therapies based on stem cell technology. The biomedical use of pigs will also facilitate transgenic pig production for agricultural production. This will be stimulated by novel genomic knowledge and tools emerging from ongoing research.

Acknowledgements

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