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REVIEW

Transgenic pigs as models for translational biomedical research

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Abstract The translation of novel discoveries from basic research to clinical application is a long, often inefficient, and thus costly process. Accordingly, the process of drug development requires optimization both for economic and for ethical reasons, in order to provide patients with appropriate treatments in a reasonable time frame. Consequently, "*Translational Medicine*" became a top priority in national and international roadmaps of human health research. Appropriate animal models for the evaluation of efficacy and safety of new drugs or therapeutic concepts are critical for the success of translational research. In this context rodent models are most widely used. At present, transgenic pigs are increasingly being established as large animal models for selected human diseases. The first pig whole genome sequence and many other genomic resources will be available in the near future. Importantly, efficient and precise techniques for the genetic modification of pigs have been established, facilitating the generation of tailored disease models. This article provides an overview of the current techniques for genetic modification of pigs and the transgenic pig models established for neurodegenerative diseases, cardiovascular diseases, cystic fibrosis, and diabetes mellitus.

Keywords Pig · Genetic engineering · Animal model · Translational medicine

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Introduction

The term "*Translational Medicine*" is being increasingly used to describe strategies of developing discoveries in basic research into clinically applicable novel therapies [1]. Despite increased efforts and investments into research and development, the output of novel pharmaceuticals has declined dramatically over the past years. The phenomenon of a retarded entry of new drugs and diagnostics to the market in spite of increased scientific discoveries and major financial investments is often addressed as "*pipeline problem*" [2]. This is attributed to the fact that currently used in vitro models, animal models, and early human trials do not reflect the patient situation well enough to reliably predict efficacy and safety of a novel compound or device. Advanced insights into the molecular pathogenesis of diseases lead to a plethora of innovative therapeutic concepts which address defined molecular targets. However, the translation of these concepts into clinical application requires a serial and systematic evaluation of efficacy and safety all the way through from discovery, preclinical science to the phases of clinical testing.

The "*Critical Path Initiative*" of the US Food and Drug Administration (http://www.fda.gov/oc/initiatives/criticalpath/) focuses on the scientific developments that are necessary to realize the required systematic processes and mechanisms of evaluation. One of the leading topics is "Biomarker Development", since biomarkers play major roles both in early (e.g., testing of efficacy and safety in animal models) and late phases of drug development (e.g., establishment of dose–response profiles, evaluation of side-effects). Therefore, biomarker discovery and validation are also central themes in the "*Innovative Medicine Initiative (IMI)*" of the European Union (http://www.imi-europe.org/).

Biomarkers are objective and quantitative parameters that may serve as indicators of physiological processes, pathological changes as well as reactions to therapeutic intervention. The development of qualified biomarkers requires an integrated network of technology platforms. State-of-the-art technologies for molecular profiling at various levels (genome, transcriptome, proteome, metabolome, etc.) are connected with advanced techniques of bioimaging. Quantitative data from the different levels of information are integrated using the fast growing tools of bioinformatics and quantitative biology to optimize the prediction of efficacy and safety of new drugs and biomarkers. Suitable animal models play a pivotal role in this process. Rodent models are most widely used due to the possibility for genetic and environmental standardization, a broad spectrum of strains tailored to specific scientific problems, and their acceptance by the regulatory authorities. At present, transgenic pigs are increasingly established as additional large animal models for selected human diseases.

Pigs as models for translational research

Livestock pig breeds and miniature pigs are relevant models in many fields of medical research [3]. The omnivores human and pig have a large number of similarities in anatomy, physiology, metabolism, and pathology, e.g., they have a very similar gastrointestinal anatomy and function, pancreas morphology, and metabolic regulation. Moreover, pigs as large animal models are highly reproductive displaying early sexual maturity (with 5–8 months), a short generation interval (of 12 months), parturition of multiple offspring (an average of 10–12 piglets per litter), and all season breeding [4]. Standardization of the environment, i.e., pig housing, feeding, and hygiene management, is well developed [5]. Reproductive technology and techniques of genetic modification have considerably advanced in the last years (see below).

Intense breeding efforts have provided pig breeds differing substantially in important traits such as size, metabolic characteristics, and behavior. If livestock pig breeds are employed for experimentation, the genetic background is mostly not defined. In contrast, minipig outbred stocks with full pedigree are delivered from commercial suppliers (http://www.minipigs.com/). In addition, inbred minipigs are available [6, 7]. Some pig breeds such as the Göttingen minipig® are used as non-rodent models for pharmacological and toxicological studies and are fully accepted by regulatory authorities worldwide (http://www.minipigs.com/).

As a member of the artiodactyls (cloven-hoofed mammals), the pig is evolutionarily distinct from the primates and rodents [8]. An initial evolutionary analysis based on ~3.84 million shotgun sequences (0.66× coverage of the pig genome) and the available human and mouse genome data revealed that for each of the types of orthologous sequences investigated (e.g., exonic, intronic, intergenic, 5′ UTR, 3′ UTR, and miRNA), the pig is closer to human than mouse [9]. This was confirmed by the comparative analysis of protein coding sequences using full-length cDNA alignments comprising more than 700 kb from human, mouse, and pig where most gene trees favored a topology with rodents as outgroup to primates and artiodactyls [10]. A draft sequence of the whole pig genome is expected to be completed in the near future. The sequence data are being released through Ensembl (http://www.ensembl.org/Sus_scrofa/Info/Index) as sequencing progresses. In addition to the genome-sequencing project, efforts were made in several groups to identify single nucleotide polymorphisms (SNP) through a substantial amount of shallow sequencing of additional breeds, resulting in a high-density (60 k) SNP chip distributed by Illumina, Inc. [11]. Recently, the so far largest collection of more than one million porcine-expressed sequence tags (ESTs) from 35 different tissues and three developmental stages was analyzed. This EST collection represents an essential resource for annotation, comparative genomics, assembly of the pig genome sequence, and further porcine transcriptome studies [12].

Genetic engineering of pigs

Importantly, pigs can be genetically modified to recapitulate the genetic and/or functional basis of a particular human disease, resulting in refined and tailored animal models for translational biomedical research. Current techniques for the genetic modification of pigs include DNA microinjection into the pronuclei of fertilized oocytes (DNA-MI), sperm-mediated gene transfer (SMGT), lentiviral transgenesis (LV-GT), and somatic cell nuclear transfer using genetically modified nuclear donor cells (SCNT; Fig. 1).

Other large non-primate animal models for human diseases include dogs and rabbits. Reproductive as well as transgenic techniques are poorly developed for dogs. Transgenic rabbits were produced using additive gene transfer, but no targeted mutations were introduced in the rabbit genome to date. In addition, the rabbit genome is sequenced only to a low coverage (http://www.ensembl.org).

Pronuclear DNA microinjection

The first technique successfully used to produce transgenic pigs was DNA microinjection into pronuclei of zygotes [13, 14]. Generally, the efficiency of DNA microinjection is low. In addition, pronuclear DNA microinjection suffers from the fact that it may yield founder animals that are

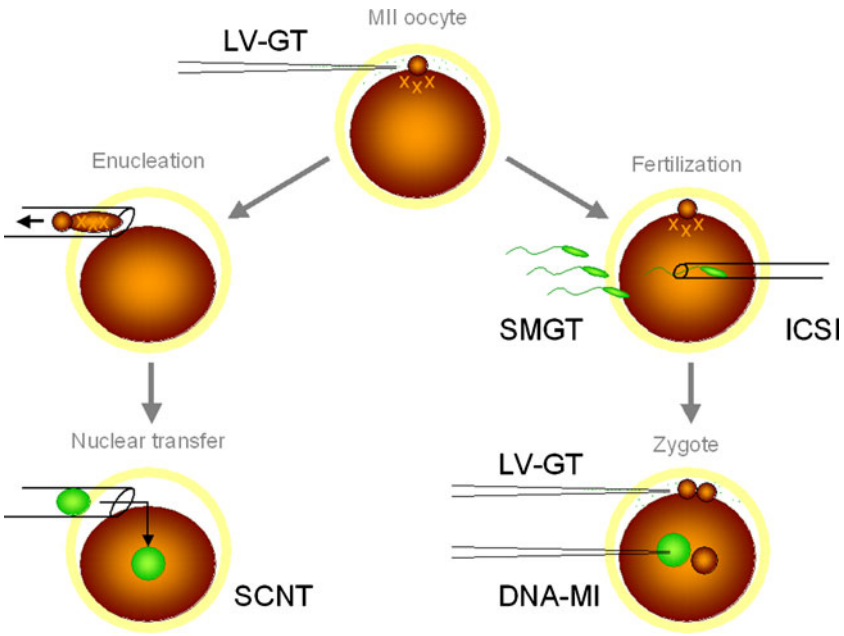

Fig. 1 Current techniques for the genetic modification of pigs include DNA microinjection into the pronuclei of fertilized oocytes (*DNA-MI*), sperm-mediated gene transfer (*SMGT*), lentiviral transgenesis (*LV-GT*), and somatic cell nuclear transfer using genetically modified nuclear donor cells (*SCNT*). LV-GT can be performed by subzonal injection of viral particles into oocytes before or after fertilization. A modification of SMGT is intracytoplasmic injection (*ICSI*) of frozen-thawed sperm after incubation with DNA (see text for further details)

mosaic, and that random integration of the injected DNA fragments may cause varying expression levels due to position effects of the neighboring DNA or may disrupt functional endogenous sequences (insertional mutagenesis; reviewed in [4]). In spite of the overall low efficiency, probably most of the transgenic pig lines existing so far have been established by the pronuclear microinjection technique.

Sperm-mediated gene transfer

SMGT is based on the intrinsic ability of sperm to bind and internalize exogenous DNA and to transfer it into the egg during fertilization (reviewed in [15]). Although the efficiency of SMGT was discussed controversially after its first description in the mouse, SMGT in the pig was achieved by collection of sperm, incubation of sperm with exogenous DNA, and artificial insemination of gilts with DNA-loaded sperm. An important factor for the success of this method seems to be the selection of suitable sperm donor animals [16]. Linker-based sperm-mediated gene transfer is a variant of the procedure where the uptake of exogenous DNA by sperm cells is improved by receptor-mediated endocytosis of DNA–antibody complexes [17]. Another modification of SMGT is intracytoplasmic sperm injection-mediated gene transfer. The first step is the induction of sperm membrane damage (e.g., by freeze-thawing), followed by incubation with exogenous DNA, and finally intracytoplasmic injection of sperm with bound DNA into oocytes [18].

Lentiviral gene transfer

Lentiviruses belong to the family *Retroviridae* and transfer their RNA genome into infected cells, where it is reverse transcribed to DNA and integrated into the host genome as a so-called provirus which is transmitted in Mendelian manner to the offspring. Lentiviruses can transduce non-dividing cells which allows immediate integration of the vector genome into the early embryo, reducing the risk of mosaic formation [19]. Lentiviral gene transfer was adapted to pigs [20, 21] and resulted in high proportions of transgenic offspring. Although lentiviral vector systems can only carry <10 kb exogenous DNA, this is considered to be enough for transfer of expression vectors for cDNAs and small interfering RNAs. As prokaryotic vector sequences are often subject to epigenetic silencing by DNA methylation, it was important to investigate this phenomenon in transgenic pigs harboring lentiviral integrants. Our studies revealed that—after segregation to the G1 generation—one third of lentiviral integrants exhibited low expression levels and hypermethylation [22], whereas two thirds of the lentiviral integrants were expressed faithfully through subsequent generations. Thus, lentiviral transgenesis is clearly an attractive alternative to the pronuclear microinjection technique.

Somatic cell nuclear transfer

Since successful SCNT protocols are available for the pig [23–25], this technology is an attractive route for genetic

modification of this species. In general, transgenesis by SCNT involves the following steps: (1) genetic modification and selection of donor cells in culture; (2) recovery and enucleation of in vivo or in vitro matured oocytes (metaphase II); (3) nuclear transfer by electrofusion or piezo-actuated microinjection (less common) and activation; (4) in vitro culture of the reconstructed embryos; and (5) embryo transfer to synchronized recipients. Various modifications like "handmade cloning" were developed to simplify SCNT in pigs [26]. SCNT is so far the only route to introduce targeted mutations into the pig genome. Via homologous recombination in nuclear donor cells, mutations have been introduced in the alpha-1,3-galactosyltransferase (*GGTA1*) and the cystic fibrosis transmembrane conductance regulator (*CFTR*) genes, and live offspring have been born following SCNT using these cells [27, 28]. Other attractive characteristics comprise: no generation of mosaic phenotypes and the possibility of pre-selection of donor cells with regard to transgene expression or gender. Furthermore, SCNT from genetically modified pools of donor cells followed by selection of suitable donor fetuses or offspring can be used to speed up transgenesis in the pig (Fig. 2). The efficiency of cloning in pig is still relatively low, ranging between 0.5% and 5% offspring per transferred SCNT embryos. As in other species the low efficiency of SCNT is attributed to failures in epigenetic reprogramming (reviewed in [29]).

Transgenic pigs as models for human diseases

Compared to laboratory rodents, experimental standardization of large animal models is low, and cost and labor are high. Therefore, transgenic pig models have been primarily developed for important disease areas where translational research in the available rodent models is limited by their small size and short life span or where rodent models do not adequately reflect the respective disease phenotypes. A summary of published transgenic pig models is provided in Table 1.

Referring to the genes described in Table 1, protein data of amyloid precursor protein (APP), huntingtin (HTT), rhodopsin (RHO), endothelial cell nitric oxide synthase (eNOS), CFTR, and hepatocyte nuclear factor 1 alpha (HNF1A) are available for human, pig, and mouse (http://www.uniprot.org; http://www.ensembl.org). Species comparison of orthologous proteins was done by alignment and manual adaptation of the amino acid sequences in BioEdit [30]. For APP, RHO, eNOS, CFTR, and HNF1A, the human proteins are more similar to the pig orthologs. For HTT, the human protein is more similar to the mouse ortholog. However, analysis of the repeat number of intragenic trinucleotide repeats associated with inherited human neurodegenerative diseases showed that the trinucleotide repeat regions are more conserved in terms of repeat length between humans and pigs than between humans and rodents [31].

Neurodegenerative diseases

Alzheimer's disease is a multifactorial neural disease and occurs in some families as an autosomal dominant disorder showing the onset of the disease after 40 years. Causative mutations were identified in the amyloid precursor protein gene (*APP*) leading to the increased production of distinct protein fragments which in turn results in neuropathy. To develop a pig model for Alzheimer's disease, transgenic pigs were produced using the human dominant mutant allele APP^{sw} harboring two amino acid exchanges due to two neighboring nucleotide exchanges which was found to cause Alzheimer's disease. According to previous transgenic mouse studies, a 7.5 kb transgene was constructed with a 1-kb platelet-derived growth factor-beta promoter, intronic and exonic sequences of the beta-globin gene, the cDNA encoding the mutant allele APP^{sw}, and SV40 polyadenylation sequences. After stable genetic modification of fibroblasts of the Göttingen minipig breed, one transgenic cell clone was used for SCNT to produce seven healthy transgenic cloned pigs with normal weight gain. The transgenic pigs harbored a single full-length copy of the transgene in their genome and showed strong, promoter-specific expression of the transgenic protein in brain tissues. Accumulation of the pathogenic protein and subsequent appearance of clinical consequences were estimated to develop with increasing age [32].

Huntington's disease is an autosomal dominant, progressive neurodegenerative disorder involving the premature loss of specific neurons. It is associated with an expansion of a CAG trinucleotide repeat in the 5′ region of the huntingtin gene (*HTT*) which results in a lengthened polyglutamine tract of the protein. The CAG repeat number is polymorphic, ranging from 6 to 35 units in normal alleles and from 36 to 120 units in alleles associated with Huntington's disease. The 12.8-kb *HTT* transcript of Göttingen minipigs codes for a 345-kDa protein (3,139 amino acids) [33]. The 8.2-kb transgene used for DNA microinjection into minipig embryos consisted of the 4-kb rat neuron-specific enolase (*Nse*) promoter, a 3.3-kb 5′ minipig huntingtin cDNA which was mutated by insertion of 75 CAG repeats into the triplet region of exon 1, and a 0.9-kb SV40 polyadenylation signal. Five transgenic founder pigs were produced each harboring one to three different integration sites with variable copy numbers and indication of genetic mosaicism [34]. To date, no follow-up publication describing mutant phenotypes appeared.

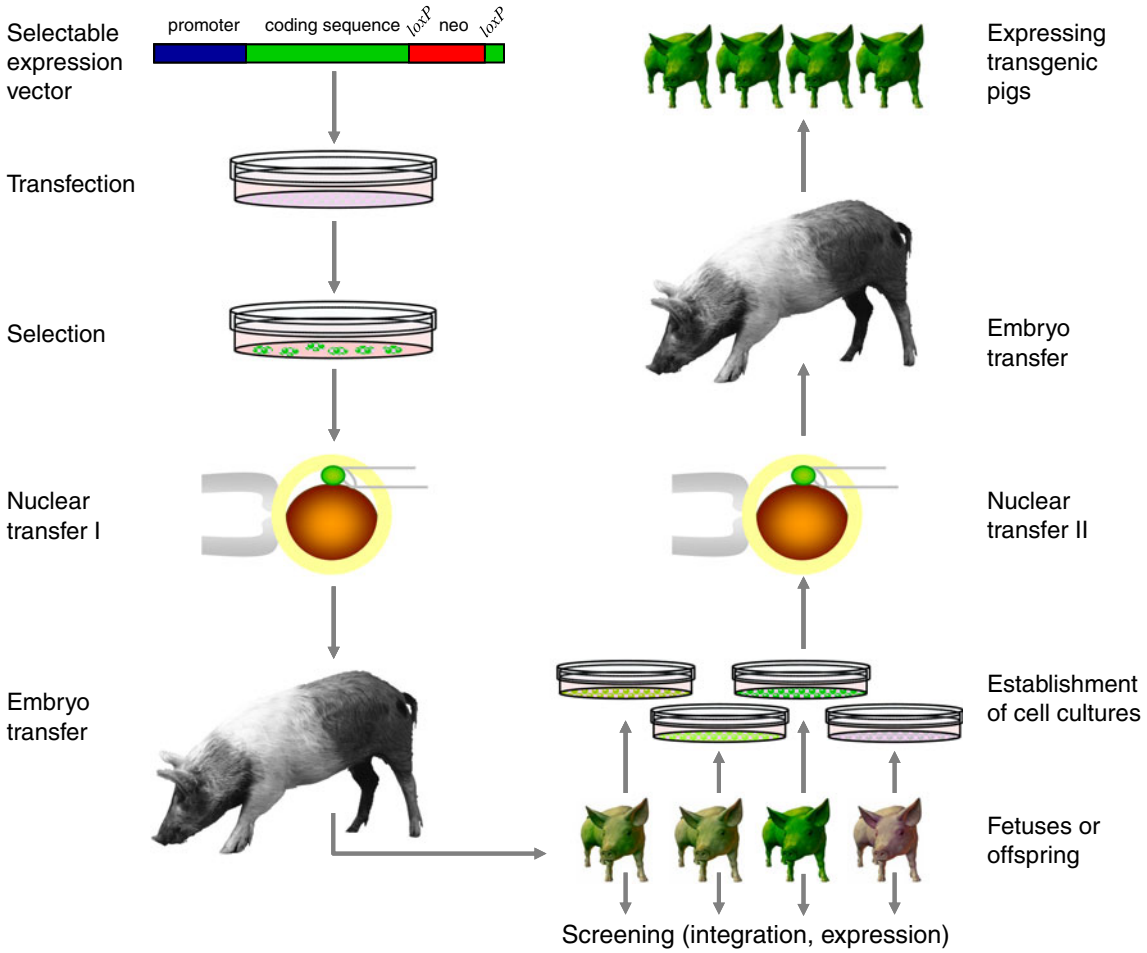

Fig. 2 Efficient production of transgenic pigs by using somatic cell nuclear transfer. An expression vector carrying a removable selection cassette is transfected into nuclear donor cells. After selection, the resulting transgenic cells are pooled and used for nuclear transfer. Pooling of cell colonies reduces the time in culture and allows the generation of independent founder fetuses/offspring in one litter. Cloned embryos are transferred to synchronized recipients. Depending on the expected onset and tissue specificity of transgene expression, pregnancies may be terminated to recover fetuses, or birth and early development of offspring is awaited. Fetuses or tissues from born offspring are processed for transgene integration and expression studies, while individual cell cultures are established for re-cloning of the fetuses/offspring with the most suitable integration/expression pattern

Retinitis pigmentosa (RP) typically causes night blindness early in life due to loss of rod photoreceptors. The remaining cone photoreceptors slowly degenerate leading ultimately to blindness. Various genes and loci are associated with the disease. Both transgenic and knockout rodent models of retinal dystrophy contributed to the analysis of the disease. Compared to humans, rodent models are limited by two disadvantages, the small number and different distribution of

Table 1 Transgenic pigs as disease models

Human disease	Transgene expression	References
Alzheimer's disease	Expression of mutant human *APP*^{sw} in the brain	Kragh PM et al. 2009 [32]
Huntington's disease	Transgenic animals with mutant pig *HTT*	Uchida M et al. 2001 [34]
Retinitis pigmentosa	Retinal expression of mutant pig *RHO*^{P347L} or *RHO*^{P347S}	Petters RM et al. 1997, Kraft TW et al. 2005 [36, 37]
Cardiovascular disease	Endothelial over-expression of pig *eNOS*	Hao YH et al. 2006 [40]
Cystic fibrosis	*CFTR* knockout or mutant pig *CFTR*^{deltaF508} knockin	Rogers CS et al. 2008 [28, 44]
Type 2 diabetes mellitus	Beta-cell expression of mutant dominant-negative human *GIPR*^{dn}	Renner S et al. 2010 [56]
Type 3 of maturity-onset diabetes of the young	Beta-cell expression of mutant dominant-negative human *HNF1A*^{P291fsinsC}	Umeyama K et al. 2009 [59]

photoreceptors in the retina and the small eyes [35]. Therefore, transgenic pigs expressing a mutant porcine rhodopsin (*RHO*; P347L or P347S) were produced by additive gene transfer. A 12.5-kb porcine genomic DNA containing 4-kb 5′ flanking sequences, the coding sequences for the 348 amino acid protein and 2.9-kb 3′ flanking sequences of the porcine *RHO* gene was used for the introduction of the mutation CCA (Pro) → CTA (Leu) or TCA (Ser) in codon 347. DNA microinjection of the expression vectors for mutant rhodopsin resulted in the generation of transgenic lines. Retinal RNA expression of the mutant transgene exceeded the expression of the wild-type endogenous gene. Like human patients with the same mutation, the transgenic pigs showed early and severe loss of rod photoreceptors, and the surviving cone photoreceptors slowly degenerated. The phenotypes of mutant *RHO* transgenic pigs and of RP patients are comparable. Therefore, this novel animal model is intensely used for studying the pathogenesis of retinitis pigmentosa as well as for preclinical treatment trials [36, 37].

Furthermore, production of a transgenic pig model for spinal muscular atrophy, an autosomal recessive disorder characterized by the degeneration of motor neurons of the spinal cord leading to muscle atrophy, has been announced [38].

Cardiovascular diseases

Endothelial cell nitric oxide synthase (eNOS) regulates vascular function by releasing nitric oxide [39]. Transgenic pigs were produced for the analysis of the cardiovascular regulation by eNOS. The 7.3 kb transgene consisted of the 3.6-kb Yucatan pig *eNOS* cDNA and a V5 epitope and polyhistidine tag (V5-His tag) to discriminate between endogenous and transgenic eNOS that was cloned between the 2-kb *TIE2* promoter and 1.7-kb *TIE2* intron/enhancer elements for the endothelial cell-specific expression. The transgene was used for co-electroporation with a neomycin resistance gene expression cassette into Yucatan pig fetal fibroblasts for additive gene transfer. Four cloned transgenic pigs derived by SCNT of transgene-positive cells expressed the fusion protein which was localized to the endothelial cells of placental vasculature from the conceptuses as did the endogenous eNOS. The predicted size of the recombinant eNOS (1,242 amino acids) was 138 kDa, compared to 133 kDa of endogenous eNOS (1,205 amino acids). Localization of endogenous and transgenic eNOS revealed the expression in the endothelium. The transgenic pigs are further used to analyze the function of eNOS in regulating muscle metabolism and in the cardiorespiratory system [40]. In addition, a complementary knockout model of *eNOS* has been announced [41].

Cystic fibrosis

Alterations of the cystic fibrosis transmembrane conductance regulator (1,480 amino acids) were identified to cause the autosomal recessive cystic fibrosis which still remains incurable. Mice with a disrupted *Cftr* gene failed to develop the lung and pancreatic disease causing most of the morbidity and mortality in human patients [42]. Porcine lungs share many anatomical, histological, biochemical, and physiological features with human lungs [43]. Mutant pigs were produced using SCNT and fetal fibroblasts with the *CFTR* gene either disrupted or containing the most common cystic fibrosis-associated mutation (deltaF508). Therefore, recombinant adeno-associated virus (rAAV) vectors were used to target *CFTR* in male fetal fibroblasts of outbred domestic pigs. The 4.5-kb knockout gene construct disrupted exon 10 encoding a portion of nucleotide-binding domain 1 with a stop codon at position 508 (F508X) followed by a floxed neomycin resistance gene driven by the phosphoglycerate kinase (*PGK*) promoter, whereas the deltaF508 knockin gene construct harbors the three nt deletion in exon 10 leading to deltaF508 followed by a floxed neomycin resistance gene driven by the *PGK* promoter in the downstream intronic region. Using successfully targeted cells without viral vector sequences for SCNT, heterozygous mutant male piglets were generated with each mutation [28, 44]. Newborn piglets with a targeted disruption of both *CFTR* alleles exhibited similar defects as seen in newborn human patients, i.e., meconium ileus, exocrine pancreatic destruction, and focal biliary cirrhosis. Thus, the novel disease models may improve the analysis of the pathogenesis as well as the development of treatment strategies for cystic fibrosis [28, 44]. In addition, preliminary conference reports announced the production of transgenic pigs for the suppression of *CFTR* expression by RNA interference.

Diabetes mellitus

Rodent models for diabetes mellitus have been developed by the structural and/or functional modification of candidate genes [45] or by random mutagenesis programs [46]. Transgenic pigs were recently established as large animal models. In the context of type 2 diabetes mellitus, a chronic metabolic disorder of multiple etiologies characterized by uncontrolled hyperglycemia caused by both insulin resistance and progressive pancreatic beta-cell dysfunction [47], the two incretin hormones glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) attracted particular attention. GIP and GLP-1 are secreted by enteroendocrine cells in response to nutrients like fat and glucose and enhance glucose-induced insulin secretion [48]. In type 2 diabetic patients, the insulinotropic action of GIP is

highly impaired [49]. Nearly sustained insulinotropic action of GLP-1 in type 2 diabetic patients revealed its therapeutic potential to compensate for the loss of GIP function and initiated the development of incretin-based therapeutics [50]. The reasons for the reduced response to GIP in type 2 diabetes are unclear, but it was suggested that impaired GIP action might be involved in the early pathogenesis of type 2 diabetes mellitus [51]. Recently, a meta-analysis of nine genome-wide association studies in humans revealed that variation in the GIP-receptor (*GIPR*) gene influences the glucose and insulin responses to an oral glucose challenge [52].

To clarify the role of the GIP/GIPR axis, a mouse model lacking expression of a functional GIPR was generated by gene targeting [53]. *Gipr*$^{-/-}$ mice displayed only slightly impaired glucose tolerance and did not develop diabetes mellitus. Compensatory regulation of the GLP-1 system or other compensatory mechanisms were discussed as possible explanations for this relatively mild phenotype (reviewed in [54]). In contrast, two independent lines of transgenic mice overexpressing a dominant-negative GIPR (GIPR^{dn}) under the control of the rat *Ins2* promoter (RIP II) in the pancreatic islets displayed early-onset diabetes mellitus and loss of beta-cells associated with extensive structural alterations of the pancreatic islets [55]. However, it could not be excluded that the severe phenotype observed in the pancreatic islets of this model was due to effects other than impaired GIPR signaling (e.g., squelching of G-proteins, hence impairment/inhibition of other signaling pathways). To address the question of whether GIPR signaling plays a role in maintaining pancreatic islet function and structure, a large animal model has been generated [56]. Efficient lentiviral vectors were used to generate transgenic pigs expressing a GIPR^{dn} under the control of the rat *Ins2* promoter in the pancreatic islets (Fig. 3a). The mutant *GIPR*^{dn} cDNA harbored two mutations in the third cytoplasmic domain which is essential for signal transduction [55]. *GIPR*^{dn} transcription was detected in isolated islets of Langerhans. Young, 11-week-old GIPR^{dn} transgenic pigs exhibited reduced oral glucose tolerance due to delayed insulin secretion, whereas intravenous glucose tolerance was found to be unaltered compared to controls. Also, both groups showed similar beta-cell mass at this age. With increasing age glucose control deteriorated so that 5-month-old GIPR^{dn} transgenic pigs showed reduced oral glucose tolerance due to reduced insulin secretion (Fig. 3c, d). At the age of 11 months, intravenous glucose tolerance was also impaired and insulin secretion diminished (Fig. 3e, f). Quantitative-stereological analyses of the pancreas of 5-month-old transgenic and control pigs revealed a reduction of 35% of the total beta-cell volume in GIPR^{dn} transgenic pigs while an even more pronounced reduction of 58% of the total beta-cell volume was detected in 1–1.4-year-old GIPR^{dn} transgenic pigs compared to controls (Fig. 3g). GIPR^{dn} transgenic pigs showed a reduced increase of beta-cell mass from the age of 11 weeks to the age of 5 months compared to control pigs while there was almost no further augmentation of the total beta-cell volume from the age of 5 months to the age of 1–1.4-years, demonstrating an important role of the GIP/GIPR axis for beta-cell expansion. The reduction of the total beta-cell volume in GIPR^{dn} transgenic pigs could be traced back to a highly diminished beta-cell proliferation rate in 11-week-old transgenic pigs compared to age-matched controls (Fig. 3h). Additionally, a trend of increased apoptosis of beta-cells observed in 1–1.4-year-old GIPR^{dn} transgenic pigs may also have contributed to the lack of expansion of the total beta-cell volume.

In conclusion, a large animal model has been generated that mimics important aspects of human type 2 diabetes mellitus: reduced GIP action, impaired oral and intravenous glucose tolerance and reduced pancreatic beta-cell mass. Furthermore, these findings point to an essential role of GIP in beta-cell expansion [56]. GIPR^{dn} transgenic pigs appear to be a useful animal model for further applications including analysis of the mechanisms by which GIP supports islet maintenance in vivo, the development and preclinical evaluation of incretin-based therapeutic strategies, as well as the development of novel techniques for dynamic in vivo monitoring of pancreatic islet mass [57].

In addition, a mutant mouse line showing diabetes which was caused by a point mutation in the insulin 2 (*Ins2*) gene has been established previously. The point mutation leads to the amino acid exchange C95S and the loss of the A6-A11 intrachain disulfide bond of the insulin. Male heterozygous *Ins2*^{C95S} mutant mice develop progressive diabetes mellitus with strong reduction of the total pancreatic islet volume and the total beta-cell volume together with severe alterations of the beta-cell structure [58]. Therefore, we established a transgenic pig model expressing a mutant porcine insulin analogous to the mutant mouse insulin by additive gene transfer for the subsequent study of beta-cell dysfunction in diabetes mellitus. Using SCNT, transgenic founder pigs were established with normal development and unaltered fasting blood glucose levels, but disturbed intravenous glucose tolerance and reduced insulin secretion (unpublished data).

Another transgenic porcine diabetes model was produced for type 3 of maturity-onset diabetes of the young (MODY3) which is caused by dominant mutations of the hepatocyte nuclear factor 1 alpha (*HNF1A*) gene. The effect of the dominant-negative mutation used was previously verified in transgenic mice. The transgene consisted of the 1.2 kb chicken beta-globin insulator, the 0.4 kb enhancer for the immediate early gene of the cytomegalovirus followed by the 0.7 kb porcine insulin promoter, a 2.3-kb

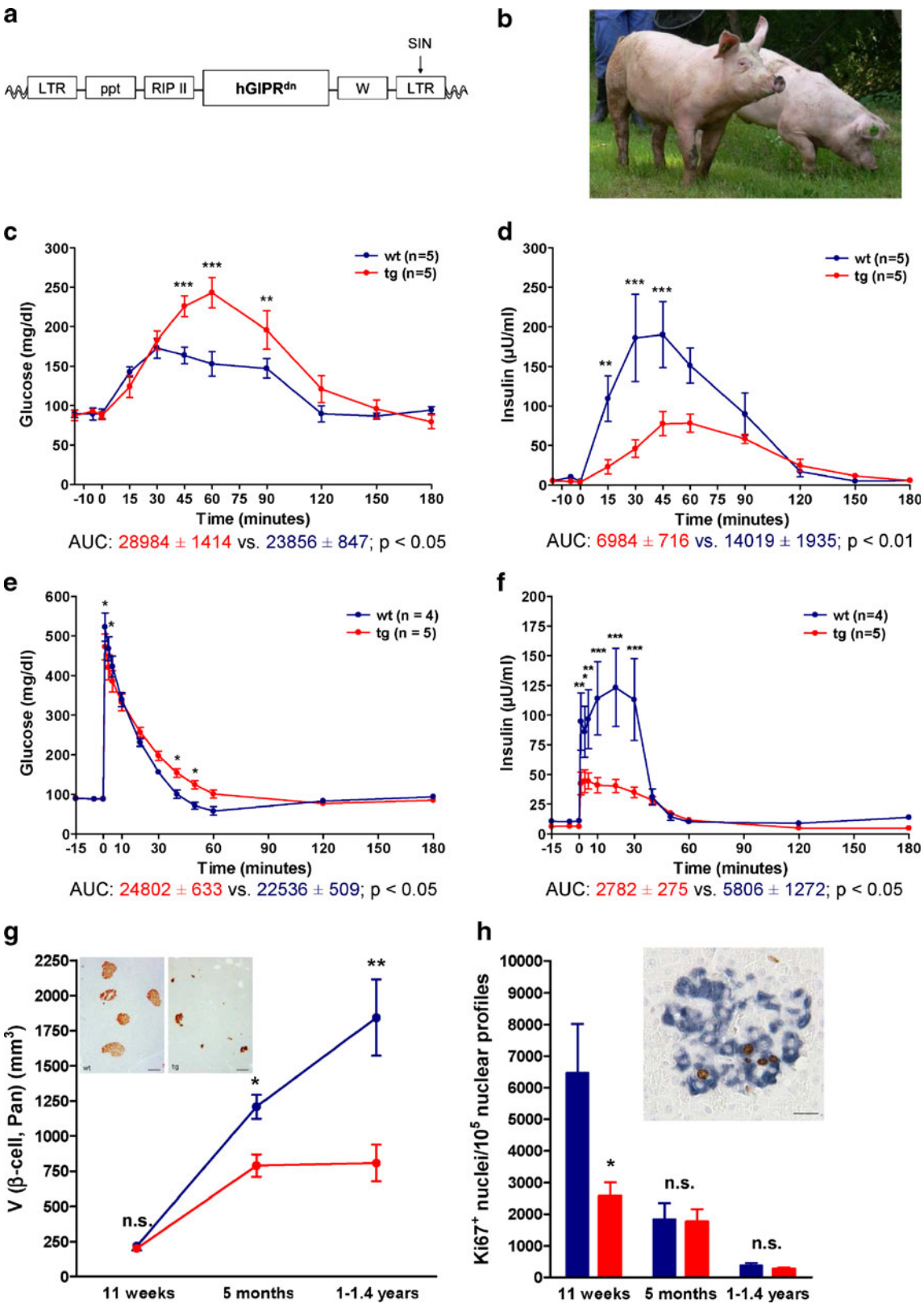
a
SIN
LTR
ppt
RIP II
hGIPR^dn
W
LTR
b
c
wt (n=5)
tg (n=5)
Glucose (mg/dl)
Time (minutes)
AUC: 28984 ± 1414 vs. 23856 ± 847; p < 0.05
d
wt (n=5)
tg (n=5)
Insulin (µU/ml)
Time (minutes)
AUC: 6984 ± 716 vs. 14019 ± 1935; p < 0.01
e
wt (n = 4)
tg (n = 5)
Glucose (mg/dl)
Time (minutes)
AUC: 24802 ± 633 vs. 22536 ± 509; p < 0.05
f
wt (n=4)
tg (n=5)
Insulin (µU/ml)
Time (minutes)
AUC: 2782 ± 275 vs. 5806 ± 1272; p < 0.05
g
V (β-cell, Pan) (mm³)
n.s.
11 weeks
5 months
1-1.4 years
h
Ki67⁺ nuclei/10⁵ nuclear profiles
n.s.
n.s.
11 weeks
5 months
1-1.4 years

Fig. 3 GIPR^{dn} transgenic pigs show impaired oral/intravenous glucose tolerance and reduced insulin secretion with increasing age, reduced total beta-cell volume as well as reduced beta-cell proliferation. **a** The lentiviral vector (LV-GIPR^{dn}) consisting of the cDNA of the dominant-negative GIP-receptor (GIPR^{dn}) under the control of the rat insulin 2 gene promoter (RIP II) and the lentiviral backbone (*LTR* long terminal repeat, *ppt* polypurine tract, *W* woodchuck hepatitis posttranscriptional regulatory element), *wavy lines* pig genome, *SIN* self-inactivating mutation. **b** GIPR^{dn} transgenic founder pigs. **c**, **d** Oral glucose tolerance in 5-month-old GIPR^{dn} transgenic pigs (*tg*) compared to non-transgenic littermates (*wt*). **c** Serum glucose levels; 0 min = point of glucose administration; **d** serum insulin levels. *AUC* area under the glucose/insulin curve for tg pigs (*red*) and wt pigs (*blue*). Data are means±SEM; $^{**}p<0.01$ vs. control; $^{***}p<0.001$ vs. control. **e**, **f** Intravenous glucose tolerance in 11-month-old GIPR^{dn} transgenic pigs (*tg*) compared to non-transgenic controls (*wt*). **e** Serum glucose levels; 0 min = point of glucose administration; **f** serum insulin levels. *AUC* area under the glucose/insulin curve for tg pigs (*red*) and wt pigs (*blue*). Data are means±SEM; $^{*}p<0.05$ vs. control; $^{**}p<0.01$ vs. control; $^{***}p<0.001$ vs. control. **g** Total beta-cell volume of 11-week-old ($n=5$ per group), 5-month-old ($n=4$ per group), and 1–1.4-year-old ($n=5$ per group) GIPR^{dn} transgenic (*red*) and control (*blue*) pigs; *insert* representative histological sections of pancreatic tissue from a 1-year-old control (*wt*) and a GIPR^{dn} transgenic pig (*tg*) stained for insulin; *scale bar*=200 µm. **h** Beta-cell proliferation of 11-week-old ($n=5$ per group), 5-month-old ($n=4$ per group), and 1–1.4-year-old ($n=5$ per group) GIPR^{dn} transgenic (*red*) and control (*blue*) pigs; *insert* representative histological sections double-stained for insulin (*blue*) and the proliferation marker Ki67 (*brown*); *scale bar*=20 µm. Data are means±SEM; $^{*}p<0.05$ vs. control; *n.s.* not significant [56]

mutant human hepatocyte nuclear factor 1 alpha cDNA with the most common mutation (P291fsinsC), the 0.1-kb SV40 polyadenylation signal, and the 1.2-kb chicken beta-globin insulator. A transgenic cell clone with ten copies of the transgene was used for SCNT. Twenty-two live transgenic cloned pigs were produced. Most of them died within 2 weeks after birth. The transgenic protein was detected in pancreas, heart, and kidney. Persistent diabetes with non-fasting blood glucose levels over 200 mg/dl was observed in four transgenic pigs with longer living time. Histological analysis revealed abnormal pancreatic islet morphogenesis and pathological alterations of the kidneys, such as glomerular hypertrophy and sclerosis [59].

Conclusions and future perspectives

Recent progress in techniques for the genetic modification of pigs facilitates the generation of tailored models for translational research. For the development of advanced preclinical animal models, target genes and mechanisms for the development of novel therapies are revealed by genome-wide association studies and pathophysiological investigations of human patient cohorts. In addition, forward and reverse genetics approaches in model organisms as well as cellular systems may contribute to the target discovery pipeline. Mouse models can be precisely designed or obtained from the large archive of mutants. Based on the findings in mouse models, large animal models such as genetically modified pigs can be designed for selected human diseases (Fig. 4).

Further refinements of transgenic technology in pigs can be expected in the near future. These include inducible transgene expression [60], the *Cre/loxP* system for conditional transgenic modifications [61] and nonviral episomal expression systems that replicate autonomously in mammalian cells [62]. Zinc finger nuclease (ZFN) technology,

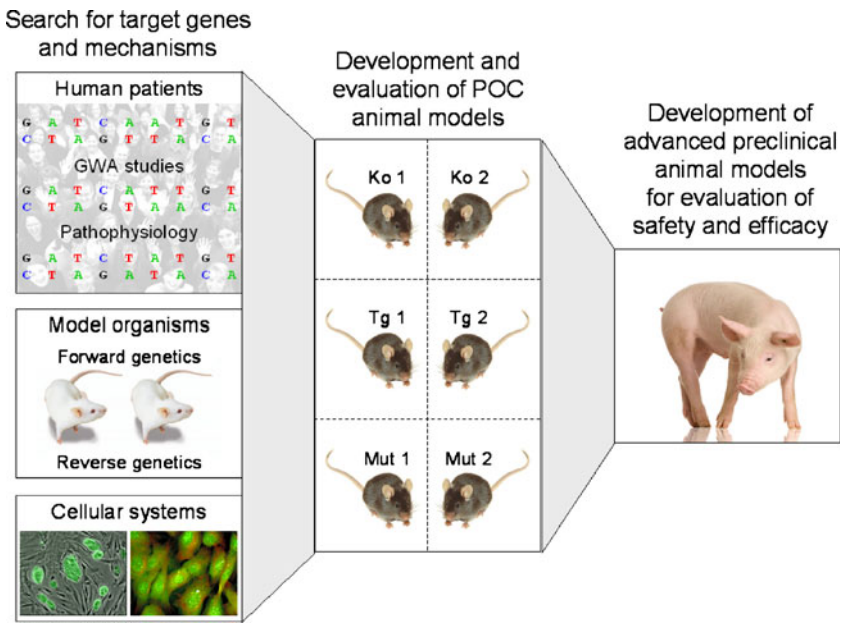

Fig. 4 Development of advanced preclinical animal models. For target genes and mechanisms identified in various discovery pipelines (*left*), mouse models can be precisely designed or obtained from the large archive of mutants in order to facilitate proof of concept (*POC*) studies. Based on the findings in mouse models, advanced preclinical animal models such as genetically tailored pigs can be designed for predictive efficacy and safety studies

which facilitates sequence-specific double-strand breaks of DNA, has recently been successfully used in the rat [63], and will, in the very near future, also be used to mutate specific genes in other mammalian species including pigs. This approach does not even require the technically demanding SCNT, but should work via cytoplasmic injection of DNA or RNA coding for the respective ZFN into zygotes. While repair of double-strand breaks by non-homologous end joining (NHEJ) frequently leads to mutations, ZFN technology is also expected to increase the rate of homologous recombination (HR) if a targeting vector is simultaneously introduced. Recently, attempts have been made to favor HR vs. NHEJ by transient downregulation of integral NHEJ proteins [64]. Furthermore, rAAV has been successfully used for efficient gene targeting in mammalian cells [65].

The refinement of techniques for the generation of tailored transgenic pigs is expected to widen the spectrum of potential applications. In addition to the disease areas covered by this article, future applications of genetically modified pig models may include cancer research [66] and regenerative medicine. Gene targeting will allow recapitulating causative mutations of human tumors in pig models, which can be used to investigate the multi-step process of tumorigenesis and to develop novel strategies for early diagnosis and therapy. In addition, pig models may be particularly important for regenerative medicine, since the "Guidelines for the Clinical Translation of Stem Cells" developed by the International Society for Stem Cell Research (http://www.isscr.org) recommend investigators to develop preclinical cell therapy protocols in small animal models, as well as in large animal models. These studies may involve allotransplantation of porcine stem cells in pig models (e.g., [67]). Importantly, recent studies described the derivation of porcine-induced pluripotent stem cells [68–70], and protocols for the derivation and differentiation of porcine mesenchymal stem cells are also available [71]. Transgenic pigs expressing marker genes, such as the green fluorescent protein, ubiquitously or in specific tissues or cell types [72] will be important to monitor the safety and efficacy of cell therapies. Alternatively, human stem cells or their derivatives may be tested in pig models, which would require immunosuppression or the development of genetically immunodeficient pigs.

The plethora of potential applications of pig models require infrastructures which are able to generate, archive, and distribute pig models in an international framework. Furthermore, platforms and protocols for systematic phenotyping are required to fully exploit the potential of pig models for translational research.

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