Hum Genet (2016) 135:1093–1105
DOI 10.1007/s00439-016-1710-6

REVIEW

Genome editing revolutionize the creation of genetically modified pigs for modeling human diseases

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Received: 29 March 2016 / Accepted: 6 July 2016 / Published online: 18 July 2016
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Abstract Pigs have anatomical, physiological and genomic characteristics that make them highly suitable for modeling human diseases. Genetically modified (GM) pig models of human diseases are critical for studying pathogenesis, treatment, and prevention. The emergence of nuclease-mediated genome editing technology has been successfully employed for engineering of the pig genome, which has revolutionize the creation of GM pig models with highly complex pathophysiologies and comorbidities. In this review, we summarize the progress of recently developed genome editing technologies, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9), which enable highly efficient and precise introduction of genome modifications into pigs, and tailored disease models that have been generated in various disciplines via genome editing technology. We also summarize the GM pig models that have been generated by conventional transgenic strategies. Additionally, perspectives regarding the application of GM pigs in biomedical research are discussed.

Electronic supplementary material The online version of this article (doi:10.1007/s00439-016-1710-6) contains supplementary material, which is available to authorized users.

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Introduction

Pigs have been extensively used in biomedical research due to the similarities between pigs and humans in terms of organ size, anatomy, physiology, metabolism, neurobiology and genome. Pigs are excellent models of human diseases, such as cardiovascular diseases and neurodegenerative diseases. Furthermore, they are considered ideal organ donors for xenotransplantation. However, the most dominant strategy to create genetic modifications in pigs via homologous recombination and somatic cell nuclear transfer (SCNT) is time-consuming and inefficient, thus impairing the creation of disease models. Recently, great progress has been made in nuclease-mediated precise genome editing (ZFNs, TALENs or CRISPR/Cas9) in mammalian organisms, which has enabled the modeling of highly complex pathophysiologies and comorbidities in pigs. Utilizing these tools, genetically modified pigs can be more easily and quickly produced and are sparking a new revolution in the application of pigs in biomedical research. Here, we review current progress in genome editing technologies and porcine modeling for human diseases.

Overview of genome editing technology in pigs

With the development of reproductive biology and foreign gene transfer methods, particularly nuclease-mediated gene targeting, the ability to edit the porcine genome (delete, insert and modify DNA sequences) has opened a new era for the introduction of pigs into biomedical research. Since the first report of genetically modified pigs in 1985 (Hammer et al. 1985), several strategies have been successfully developed to introduce gene modifications into the porcine genome, including pronuclear microinjection (Hammer

et al. 1985; Nottle et al. 2001), virus-mediated gene transfer (such as retrovirus and lentivirus) (Cabot et al. 2001; Hofmann et al. 2003), sperm-mediated gene transfer (SMGT) (Chang et al. 2002; Lavitrano et al. 1997), intracytoplasmic sperm injection (ICSI) (Kurome et al. 2006), transposon-mediated transgenesis (Garrels et al. 2011; Wu et al. 2013), RNA interference (RNAi) (Li et al. 2009, 2014; Ramsoondar et al. 2009), and somatic cell nuclear transfer (SCNT). Although large numbers of GM pigs have been created, some disadvantages and safety concerns regarding these strategies may compromise their application. Pronuclear microinjection, virus-mediated gene transfer, SMGT, ICSI, and transposon-mediated transgenesis can only introduce random transgenes; furthermore, SMGT and ICSI are not reproducible. The differences in copy numbers and uncertainty of the integration site often make it difficult to perfectly mimic human diseases in transgenic animals and study gene function. Although RNAi can specifically inhibit the expression of target genes, the efficiency of RNAi cannot be precisely controlled, thus limiting its wide application. Since the birth of Dolly by SCNT using donor cells derived from adult animals (Wilmut et al. 1997), various animal species were created through SCNT, such as mice (Wakayama et al. 1998), cattle (Cibelli et al. 1998) and goat (Baguisi et al. 1999), etc. SCNT was successfully applied in pigs (Lai et al. 2002; Polejaeva et al. 2000) in 2000. Since then, because no characterized embryonic stem cells (ESCs) that can be used for gene targeting have been generated in pigs, homologous recombination (HR) in somatic cells followed by SCNT is the primary strategy for creating knockout (KO) animals in pigs. However, HR-mediated gene targeting in pig somatic cells has extremely low efficiency, and SCNT also exhibits low efficiency for the production of healthy offspring given our poor understanding of somatic epigenetic reprogramming. These limitations have impaired the production of gene targeting pigs for biomedical research; only 10 KO pig models have been created through HR and SCNT since 2002 prior to the successful application of nucleases in mammalian genome editing (Table S1). Furthermore, the targeting of multiple genes has not been reported by HR in pigs. However, with the development of nuclease-mediated gene editing tools, the efficiency of gene editing has improved dramatically and bi-allelic mutant animals can be created in one step, representing immense promise for the creation of genetic modifications in pigs. Since 2011, 35 publications have reported gene-disrupted pigs generated via nuclease-mediated gene editing, whereas only seven models were produced by HR (Table S1).

Nuclease-mediated gene editing

The emergence of nuclease-mediated gene editing technologies, including ZFNs, TALENs, and the CRISPR/Cas9 system, has introduced a new era for gene targeting, especially in large animals. Nuclease-mediated gene editing technologies possess various advantages compared with conventional HR-mediated gene targeting (Table 1). In addition to the difficulties associated with the use of large fragments as homologous templates, conventional HR can normally achieve only mono-allelic gene targeting at an extremely low efficiency. Comparatively, ZFNs, TALENs and CRISPR/Cas9-mediated gene targeting are considerably more efficient and can achieve bi-allelic KO in one step, which remarkably reduces the duration required to generate homozygous mutant offspring in farm animals. Precise genome editing and the knockin of large fragments can also be achieved in pigs via nuclease-mediated technology, which meets the various requirements for the introduction of pigs into biomedical research. Given our focus on porcine genome editing in this review, the sophisticated application of nuclease-mediated genome editing in model species and cells, such as gene repression, activation and large scale screening, is not discussed here. Instead, the applications of three nuclease-mediated gene editing systems in pigs will be discussed, respectively.

ZFNs

The mutation of an eGFP transgene and two endogenous genes (*IgM* and *Rab38*) using ZFNs with a high efficiency (5–75 %) in rats stimulated a new generation of genome engineering in mammals (Geurts et al. 2009). ZFNs consist of two domains: a DNA recognition domain and a non-specific DNA cleavage domain of *Fok*I endonuclease (Kim et al. 1996). The DNA recognition domain contains three or

Table 1 Comparison of HR and nuclease-mediated gene editing in pigs

	HR	ZFNs	TALENs	CRISPR/Cas9
Design and assembly duration	3–4 weeks	2–3 weeks	2–3 weeks	Within 1 week
Efficiency	Low (~10^{-6})	High (~10 %)	High (~20 %)	High (35–100 %)
Bi-allelic KO	Serial cloning/breeding, 3–5 years	One step, ~1 year	One-step, ~1 year	One-step, 6–12 months
Selection-free	No	Yes	Yes	Yes
SCNT-dependent	Yes	No	No	No

more Cys_2His_2 zinc fingers, and each finger interacts with three consecutive DNA base pairs. The *Fok*I endonuclease is active only as a dimer (Smith et al. 2000). Therefore, two individual ZFNs heterodimerized at a particular genomic locus in an inverted orientation with appropriate spacing can produce double-strand breaks (DSBs) in the target DNA and induce DSB repair pathways, including nonhomologous end-joining (NHEJ), which is the direct ligation of two DNA DSB ends, and homology-directed repair (HDR), in the presence of exogenous DNA fragments. In 2001, Bibikova et al. (2001) first reported ZFNs-mediated gene targeting in living cells by injecting both ZFN plasmids and exogenous DNA fragments into *Xenopus laevis* oocyte nuclei. Soon after, ZFNs were applied in gene targeting in *Drosophila*. NHEJ-mediated gene targeting occurred in the absence of homologous donor DNA, resulting in small deletions and/or small insertions (Bibikova et al. 2002). In contrast, homologous recombination events were identified in the presence of homologous donor DNA, which prompted precise gene targeting applications (Bibikova et al. 2003).

Soon after that, ZFNs have been widely used for gene deletion in many mammalian species, such as rats (Geurts et al. 2009) and cattle (Yu et al. 2011); targeted gene insertion into human cells (Moehle et al. 2007; Uddin et al. 2015; Wang et al. 2015a); and the generation of conditional KO rats (Brown et al. 2013). ZFNs-mediated gene targeting in pigs were first exploited in 2011, Whyte et al. (2011b) combined ZFNs and SCNT to generate enhanced green fluorescent protein (eGFP) transgene KO pigs, opened a new era of efficient gene editing in pigs. Almost at the same time, Yang et al. (2011) reported the creation of monoallelic peroxisome proliferator-activated receptor gamma (*PPAR*γ) KO pigs by ZFNs. Since then, many ZFNs-mediated genetically modified pigs were generated (Table S1). Although the efficiency of gene targeting via ZFNs has been greatly improved, some disadvantages (i.e., off-target cleavage, cytotoxicity, and limited target sites) (Carlson et al. 2012a; Cornu et al. 2008; Radecke et al. 2010) limit their applications.

TALENs

TALENs were first reported as a new type of sequence-specific nuclease in 2010 (Christian et al. 2010). Similar to ZFNs, TALENs are composed of a TALE DNA binding domain and a *Fok*I endonuclease. The specificity of TALE binding to the target DNA depends on its central domain, which is composed of tandem repeats of 34 amino acids. The amino acid repeat units are largely invariant with the exception of the 12th and 13th amino acids, which are called repeat variant di-residues (RVDs). One RVD in a repeat corresponds to one base pair in the target DNA, and the tandem repeat recognizes a consecutive DNA sequence. The straightforward cipher between TALEs and the target DNA makes it simple to design TALENs of any length (Boch et al. 2009; Moscou and Bogdanove 2009). Fusion of the *Fok*I endonuclease to TALE proteins constitutes TALENs, which can theoretically target any DNA sequence. Dimerized *Fok*I cuts TALE-binding DNA sequences, thereby producing DSBs and inducing NHEJ or HDR-mediated gene repair as described for the ZFNs.

TALENs present several advantages over ZFNs. First, zinc finger proteins (ZFPs) only recognize three continuous base pairs, whereas each repeat in TALEs binds to a single base pair. Thus, TALENs can theoretically target any DNA sequence. Second, TALENs possess comparable or higher efficiency to ZFNs (Hockemeyer et al. 2011; Moore et al. 2012). Third, the off-target effects and cytotoxicity are very low. Although they have advantages over ZFNs, the assembly of TALENs is laborious due to their highly repetitive modules, which may compromise their adoption.

TALENs-mediated gene editing, including gene deletion and targeted gene insertion, has been successfully used in a variety of organisms, such as zebrafish (Bedell et al. 2012; Sander et al. 2011), rats (Tesson et al. 2011), human cells (Hockemeyer et al. 2011; Sun et al. 2012), rice (Li et al. 2012), mice (Sung et al. 2013; Wefers et al. 2013), rabbits (Song et al. 2013) and monkeys (Liu et al. 2014). Carlson et al. (2012b) first evaluated the targeting efficiency of different scaffolds of TALENs in porcine embryos and primary cells, and then generated mono-allelic and bi-allelic *LDLR* mutant pigs combined with SCNT as models of familial hypercholesterolemia. TALENs has been shown to be easier to obtain the bi-allelic mutation compared to ZFNs, which greatly shorten the time for creating homologous mutant in large animals. Thus, it is not surprising that many TALENs mediated genetically modified pigs were sprouting up in such a short time (Table S1). The scientific society was inspired by its versatile applications in various disciplines of biology and rated TALENs as one of the top ten scientific breakthroughs by *Science* (2012).

CRISPR/Cas9

In 2013, a novel RNA-guided endonuclease named the CRISPR/Cas9 system was introduced into genome engineering and sparked a new revolution in biomedical research (Mussolino and Cathomen 2013). Ishino et al. (1987) first discovered short regularly spaced repeats in the 3′ flanking region of *iap* in *Escherichia coli*. Then, these repeat regions were identified in most archaea and bacteria (Mojica et al. 2005). In 2002, these short repeats were named clustered regularly interspaced short palindromic repeats (CRISPR) (Jansen et al. 2002). CRISPR spacers have homology with foreign genetic elements, which may

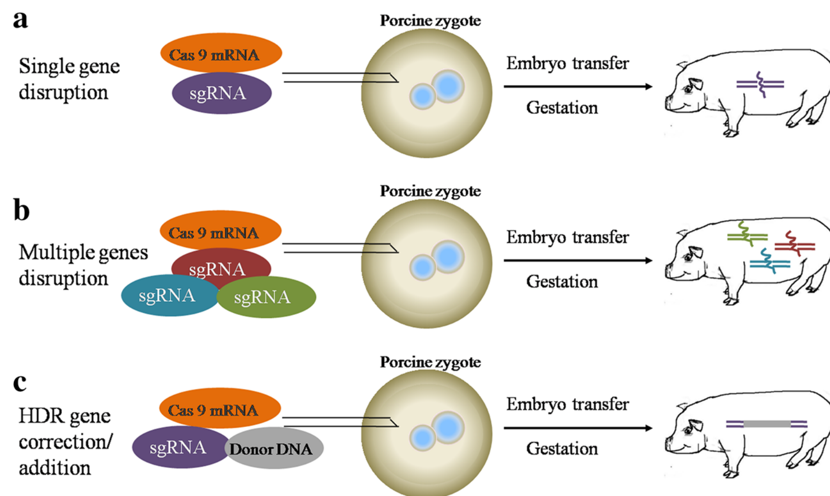

Fig. 1 Schematic diagram of different types of gene modifications mediated by the direct injection of the CRISPR/Cas9 system into embryos. **a** In vitro transcribed Cas9 mRNA and sgRNA targeting specific sequences are co-injected into the cytoplasm of porcine zygotes. The injected embryos are transferred into surrogates to produce piglets harboring the targeted gene modification. **b** Multiplexed sgRNAs are co-injected with in vitro transcribed Cas9 mRNA into the cytoplasm of porcine zygotes to produce multiple gene-targeting piglets. **c** Donor DNA is co-injected with the Cas9 mRNA and sgRNA, resulting in pigs carrying targeted gene corrections or additions

protect cells against phage infection (Bolotin et al. 2005; Mojica et al. 2005). In 2007, Barrangou et al. (2007) found that CRISPR, together with CRISPR-associated (Cas) genes that were typically located adjacent to the CRISPR locus in the genome, provided specific resistance against phages. Subsequently, CRISPR RNA (crRNA), trans-activating crRNA (tracrRNA), protospacer adjacent motif (PAM) and other details of the mechanisms underlying CRISPR-Cas systems were reported (Barrangou and Marraffini 2014; Brouns et al. 2008; Haurwitz et al. 2010; van der Oost et al. 2014). Based on the diversity of Cas proteins, CRISPR-Cas systems were classified into three categories: type I, II and III. The type II system requires only one Cas protein (Cas9) to recognize and cleave the target DNA sequence (Makarova et al. 2011). In 2012, Jinek et al. (2012) reported that crRNA-tracrRNA formed a two-RNA structure that directed the Cas9 protein to cleave the double strands in the target DNA. When dual-tracrRNA:crRNA was engineered as a single RNA chimera, it could also guide Cas9 to perform site-specific cleavage of dsDNA, suggesting that the Cas9-crRNA-tracrRNA complex could be a powerful gene editing tool via the induction of DSBs at target DNA sites. Since then, the CRISPR/Cas9 system has emerged as a novel tool for gene KO and site-specific knockin in numerous species, such as human and mouse cells (Cong et al. 2013), zebrafish (Auer et al. 2014; Chang et al. 2013; Hwang et al. 2013; Jao et al. 2013), mice (Wang et al. 2013; Zhang et al. 2015), cynomolgus monkeys (Niu et al. 2014) and rhesus monkeys (Chen et al. 2015). Hai et al. (2014) first generated *vWF* KO pigs by zygote injection of CRISPR/Cas9 system at the targeting efficiency of 68.8 % (11/16). The successful application and high efficiency of CRISPR/Cas9 system in porcine gene editing led to the wide usage in replacement of TALENs and ZFNs, and multiple genes KO, site-specific gene knockin and single nucleotide correction were successfully achieved in pigs (Table S1). Besides the high efficient genome editing, off-target cleavage might be a major challenge for CRISPR/Cas9-mediated precise gene editing. One of our studies showed that the CRISPR/Cas9 system did not introduce significant off-target cleavages in the pig genome, despite the fact that a high number of sgRNAs were simultaneously injected into the cytoplasm (Wang et al. 2016). Furthermore, several strategies have been reported to enhance the specificity of Cas9 that satisfy the demand for certain genome editing applications requiring a high level of specificity (Slaymaker et al. 2016).

Another merit of nuclease-mediated gene modifications in large animals is that gene targeting can be achieved by the direct injection of one-cell embryos (Hai et al. 2014; Lillico et al. 2013; Wang et al. 2015b, c, 2016; Whitworth et al. 2014; Zhou et al. 2016). With this procedure, SCNT is not necessary for gene targeting or even precise gene editing in pigs, which makes the need for ESCs less urgent. To date, gene disruption, multiple gene disruption and HDR-directed gene correction or addition have been achieved in pigs via the direct injection of the Cas9 mRNA, gRNA and donor fragments (Fig. 1).

Genetically modified pigs modeling human diseases and biomedical research

Xenotransplantation

The use of organs from other species is an alternative to overcome the shortage of human organs for transplantation. Considering the high degree of similarity between humans and pigs, pigs are considered the most suitable animals to supply organs to humans. The enzyme alpha1,3-galactosyltransferase (GGTA1) synthesizes Gal epitopes on the surface of pig cells; Gal is the major antigen in pig-to-human transplantation and causes hyperacute rejection. To repress hyperacute rejection in pig-to-human transplantation, one allele of the *GGTA1* gene was first deleted in pigs through HR (Dai et al. 2002; Lai et al. 2002). Then, bi-allelic KO pigs were obtained via toxin A selection and consecutive rounds of cloning (Phelps et al. 2003) and nuclear transfer with heterozygous fibroblasts underwent spontaneous loss of the WT allele (Kolber-Simonds et al. 2004). Recently, with the development of nuclease-mediated gene targeting, *GGTA1* was deleted in pigs using ZFNs (Bao et al. 2014; Hauschild et al. 2011) and TALENs (Kang et al. 2016; Xin et al. 2013), which offered a promising approach for sophisticated genome engineering in the pig genome for xenotransplantation. Transplantation of hearts from *GGTA1* KO pigs into baboons extended the survival of pig hearts in baboons to as long as 179 days, which was associated with the prevention of hyperacute rejection (Kuwaki et al. 2005). However, before pig organs can be transplanted into humans, several other hurdles must be overcome, including post-hyperacute rejection, cell-mediated rejection, nonvascular rejection, coagulation dysregulation, inflammation and porcine endogenous retroviruses. Transplanted kidneys from *GGTA1* KO pigs only survived for 8–16 days in baboons because of severe acute humoral xenograft rejection, which was caused by antibodies induced against non-Gal antigens (Chen et al. 2005), indicating that the deletion of non-Gal antigens is necessary for xenotransplantation. *N*-glycolylneuraminic acid (Neu5Gc), synthesized by cytidine monophosphate-*N*-acetylneuraminic acid hydroxylase (*CMAH*) and expressed in all mammals except humans, was identified as another xenoantigen (Song et al. 2010). Complement-dependent cytotoxicity against *GGTA1*/*CMAH* double KO mouse thymocytes was significantly reduced compared with *GGTA1* KO mouse thymocytes (Basnet et al. 2010), indicating that simultaneous deletion of the *GGTA1* and *CMAH* genes in pigs may further reduce xenoantigenicity in humans. Thus, *GGTA1*/*CMAH* double KO pigs were created using ZFNs, and the xenoantigenicity was further reduced compared with that of the *GGTA1* KO pigs (Lutz et al. 2013). Other xenoantigens were recently targeted with the aid of CRISPR/Cas9, and triple KO pigs were generated to further reduce the xenoantigenicity (Estrada et al. 2015; Li et al. 2015). Sixty-two copies of porcine endogenous retroviruses (PERVs) were simultaneously disrupted by the CRISPR/Cas9 system in PK15 cells to eliminate the risk of in vitro transmission of PERVs to human cells, which is another admirable application of Cas9 technology in modifying the pig genome for xenotransplantation (Yang et al. 2015).

Beyond the strategy of genetically modifying pigs to resemble humans at the genome level, Matsunari et al. reported that a functional pancreas can be regenerated via complementing pancreatogenesis-disabled embryos with allogenic normal blastomeres, indicating the promise of generating functional organs from xenogenic pluripotent stem cells (PSCs) in pigs, including human induced pluripotent stem cells (iPSCs) or ES cells. Their study provides a novel strategy for the production of functional human organs in pigs and is predicted to overcome the difficulties of using pig organs for xenotransplantation more efficiently (Matsunari et al. 2013). For a comprehensive review of the use of gene-modified pigs for xenotransplantation, readers should refer to Cooper's review (Cooper et al. 2016).

Cardiovascular disease

Cardiovascular disease is a major cause of morbidity and mortality in contemporary societies. Cardiac and vascular complications are complex multifactorial pathologies that are affected by both genetic and environmental factors (Zaragoza et al. 2011). The development of animal models would help us understand pathologies in humans and develop protocols for clinical translation. Although many rodent models are involved in cardiovascular disease research, their small size and disparity from humans in terms of physiological characteristics, such as the plasma lipoprotein profile, immune system (Kapourchali et al. 2014), heart architecture, and heart contractility (Zaragoza et al. 2011), compromise the translation of basic research into the clinic. In contrast, the heart anatomy, blood vessels and supply, coronary artery system anatomy and function, and cholesterol and lipoprotein metabolism in pigs are very similar to those of humans (Swindle et al. 2012; Vilahur et al. 2011), which makes pigs an excellent model of human cardiovascular diseases.

To study the functional regulation of the cardiac and vascular systems, endothelial cell nitric oxide synthase (eNOS) (Hao et al. 2006), catalase (Whyte et al. 2011a), and human CD39 transgenic pigs (Wheeler et al. 2012) have been produced. Transgenic human CD39 protected pigs from myocardial ischemia–reperfusion injury and significantly reduced infarct size, which validated the potential application of CD39 in clinical cardiac protection

(Wheeler et al. 2012). Human apolipoprotein (APO) CIII, apo (a) and D374Y-proprotein convertase subtilisin/kexin type 9 (*PCSK9*) transgenic pigs were created to study hypertriglyceridemia and atherosclerosis (Al-Mashhadi et al. 2013; Shimatsu et al. 2016; Wei et al. 2012). Human *APOCIII* transgenic pigs exhibited increased plasma triglyceride levels, and D374Y-*PCSK9* transgenic pigs exhibited severe hypercholesterolemia and the spontaneous development of progressive atherosclerotic lesions, thus providing important information for translational research in atherosclerosis (Al-Mashhadi et al. 2013; Wei et al. 2012).

The creation of genetically engineered pigs to model human cardiovascular diseases has accelerated with the development of nuclease-mediated gene editing technology. Yang et al. (2011) used ZFNs technology combined with SCNT to generate peroxisome proliferator-activated receptor gamma (*PPARγ*) KO pigs, which could be used to study the role of *PPARγ* in cardiovascular disease in humans.

Low-density lipoprotein receptor (*LDLR*) is the major pathogenic gene for familial hypercholesterolemia (FH), which is characterized by elevated serum low-density lipoprotein (LDL) cholesterol levels that lead to accelerated atherosclerosis and a higher risk of premature coronary heart disease (Soutar and Naoumova 2007). Carlson et al. (2012b) created *LDLR* mono-allelic and bi-allelic KO Ossabaw minipigs using TALENs combined with SCNT. Davis et al. (2014) reported that $LDLR^{+/-}$ pigs exhibited moderate hypercholesterolemia, while $LDLR^{-/-}$ pigs exhibited severe hypercholesterolemia and developed atherosclerotic lesions in the coronary arteries and abdominal aortas. These pathogenic symptoms were accelerated when the genetically modified pigs were fed a high fat and high cholesterol diet.

Niemann-Pick C1-Like 1 (*Npc1l1*), essential for dietary cholesterol absorption and biliary cholesterol reabsorption, was bi-allelically knocked out via the direct injection of one-cell embryos with Cas9 mRNA and sgRNA. This pig model will provide new information to understand how *Npc1l1* influences cardiovascular and metabolic diseases in humans (Wang et al. 2015c).

Neurodegenerative diseases

Neurodegenerative diseases (NDs) affect a large number of people of all ages and are characterized by the loss of specific neurons and the accumulation of abnormal protein aggregates [amyloid beta and tau for Alzheimer's disease (AD), alpha-synuclein for Parkinson's disease (PD), polyglutamine for Huntington's disease (HD), and TDP-43 and FUS for amyotrophic lateral sclerosis (ALS)] in the affected neurons. Thus far, there are no effective therapies for the majority of NDs because of their complicated pathologies and poor understanding.

Pigs have a long life span (12–15 years), which is advantageous for studying age-related neurodegenerative diseases. Importantly, the gross anatomy of the pig brain is very similar to that of the human brain and can be diagnosed with clinical imaging instruments, such as magnetic resonance imaging (MRI) and positron emission tomography (PET), to identify cortical and subcortical structures. Recently, Holm et al. (2016) presented a comprehensive review of the current status of pig models of NDs, including AD, HD, PD, ALS, spinal muscular atrophy (SMA), and ataxia-telangiectasia (A-T). The authors proposed a multiplex genome editing and preterm recloning (MAP) approach with the hopes that it would improve the efficiency and success rate of the creation of multiplex gene-modified pig models. A novel porcine model of A-T was generated and characterized by Purkinje cells (PCs) loss and altered cytoarchitecture of the cerebellum from birth, growth retardation and motor deficits. This is the first animal model to resemble some of the neuropathological and motor features of human A-T patients, and the quantifiable cerebellar lesions (PC loss and altered cytoarchitecture) and motor deficits could be used to evaluate the progression of A-T and development of early therapies to intervene in the disease in humans (Beraldi et al. 2015). Recently, we generated *Parkin*/*DJ-1*/*PINK1* triple gene-targeted pigs using the CRISPR/Cas9 system through direct zygote injection in one step. Both resulting piglets harbored site-specific modifications in the target sites, and one piglet showed bi-allelic mutations in all three genes. Although no abnormalities were observed at 10 months of age in these two pigs, some stress defense genes were disrupted in their fibroblast cells, indicating that they would be a valuable large animal model to study PD in humans (Wang et al. 2016).

Cystic fibrosis

One of the most successful pig models of human disease is the model of cystic fibrosis (CF). Cystic fibrosis is a chronic disease that primarily affects the lungs, but it also affects other organs, including the liver, the intestines and the pancreas. CF is an autosomal recessive disorder caused by a mutation in the CF transmembrane conductance receptor (*CFTR*) gene (Riordan et al. 1989). *CFTR* mutant mice did not recapitulate the symptoms of human CF (Guilbault et al. 2007). However, either the deletion of *CFTR* or the introduction of the most common CF-associated mutation (ΔF508) resulted in defects in pigs that were similar to those observed in human CF patients, such as defective chloride transport, meconium ileus, pancreatic destruction, and focal biliary cirrhosis (Klymiuk et al. 2012; Rogers et al. 2008a, b; Welsh et al. 2009). These pig models

are being used to decipher the pathophysiology of CF and as preclinical models for gene therapy trials in CF-related diseases (Griffin et al. 2014; Guo et al. 2014; Potash et al. 2013; Reznikov et al. 2013; Stoltz et al. 2013).

Metabolic diseases

Pigs are suitable animals for modeling metabolic diseases in humans as they share many similarities with humans in terms of anatomy, physiology and metabolism, including the fact that they are both omnivores and they have similar anatomies and functions of the pancreas and islets.

Diabetes mellitus is a group of chronic metabolic disorders characterized by hyperglycemia due to defects in insulin secretion, insulin action, or both (American Diabetes Association 2013). The first gene-modified pig model of type 2 diabetes mellitus was transgenic pigs expressing a dominant-negative GIP receptor ($GIPR^{dn}$) in their pancreatic islets via lentiviral transgenesis. These pigs exhibited significantly reduced oral glucose tolerance and reduced β-cell proliferation at 11 weeks of age and progressive deterioration of glucose control and a reduced pancreatic β-cell mass as they aged (Renner et al. 2010). Metabolomic analysis of the $GIPR^{dn}$ pigs revealed that the concentrations of seven amino acids (Phe, Orn, Val, xLeu, His, Arg, and Tyr) in the plasma were altered compared with control pigs. Specific sphingomyelins, diacylglycerols, and ether phospholipids were decreased in the plasma of the $GIPR^{(dn)}$ transgenic pigs at 5 months. These effects significantly correlated with the β-cell masses of the transgenic pigs. These metabolites represent candidate biomarkers that may serve as indicators of the early stages of prediabetes (Renner et al. 2012). The authors also generated INS^{C94Y} transgenic pigs to establish a pig model of permanent neonatal diabetes mellitus (PNDM). Compared with their littermate controls, these INS^{C94Y} transgenic pigs exhibited reduced body weights, decreased β-cell masses, and lower fasting insulin levels. Additionally, the β-cells of 4.5-month-old transgenic pigs exhibited reduced insulin secretory granules and severe dilation of the endoplasmic reticulum. Cataracts were visible in 8-day-old transgenic pigs and became more severe as the pigs aged (Renner et al. 2013). Another porcine model of diabetes mellitus was created by expressing a mutant human *HNF1α* gene through intracytoplasmic sperm injection-mediated gene transfer and SCNT. The *HNF 1αP291fsinsC* transgenic pigs that lived for a longer time (20–196 days) developed diabetes mellitus with non-fasting blood glucose levels greater than 200 mg/dl and exhibited abnormal pancreas morphologies and functions (Umeyama et al. 2009).

Hereditary tyrosinemia type I (HT1) is a severe, autosomal recessive disease caused by deficiency of a metabolic enzyme called fumarylacetoacetate hydrolase (FAH), which catalyzes the last step of tyrosine metabolism. HT1 results in hepatic failure, cirrhosis, and hepatocellular carcinoma early in childhood. *Fah* mutant mice were generated to study HT1, and although some phenotypes were observed in these mutant mice, including the formation of liver cancer, they did not recapitulate all aspects of the disease, such as cirrhosis. *Fah* heterozygous KO pigs were then generated by HR and SCNT and were phenotypically normal and exhibited normal tyrosine metabolism and histologically normal livers but demonstrated reduced Fah transcript, protein, and FAH enzyme activity compared to wild type controls (Hickey et al. 2011). These $Fah^{+/-}$ pigs were outbred and crossed to generate $Fah^{-/-}$ pigs. A lethal defect of $Fah^{-/-}$ pigs in utero was observed, but the administration of 2-(2-nitro-4-trifluoromethylbenzyol)-1,3 cyclohexanedione (NTBC) throughout pregnancy corrected this defect, and piglets were phenotypically normal at birth. However, NTBC withdrawal in these $Fah^{-/-}$ piglets resulted in failure to thrive and complications of acute liver failure including hypoglycemia, coagulopathy, encephalopathy and infection. Biochemical and histological analysis confirmed the diagnosis of severe liver injury. These $Fah^{-/-}$pigs represent a valuable large animal model of a metabolic liver disease that could be applied in preclinical studies of regenerative therapies for metabolic liver disorder and preclinical efficacy testing of liver cell therapies (Hickey et al. 2014).

Eye diseases

Pigs are excellent models of human eye diseases; their eyes are similar to human eyes in terms of anatomy, size, and retinal and optic nerve structures. Retinitis pigmentosa (RP) is an inherited retinal disease that leads to vision loss. RP causes night blindness early in the disease course due to the loss of rod photoreceptors followed by the progressive loss of cone photoreceptors, resulting in peripheral vision loss and eventually central vision loss (Phelan and Bok 2000). Numerous mutations are reported to cause RP, including the *RHO* (rhodopsin) gene. Transgenic pigs carrying mutant porcine *RHO* mutations (P347L, P347L/P347S, and P23H) were created. These pigs exhibit early and severe loss of rod photoreceptors and progressive degeneration of cone photoreceptors (Kraft et al. 2005; Petters et al. 1997; Ross et al. 2012). The phenotypes of these transgenic pigs resemble human RP patients, and the P23H mutant RHO transgenic NIH miniature pigs provide additional advantages because they were highly inbred, and thus histocompatibility disparities are avoided. The use of this model would facilitate cell therapy by minimizing immunological rejection (Ross et al. 2012).

Stargardt-like macular dystrophy type 3 (STGD3) is an autosomal dominant juvenile-onset form of macular

degeneration that is characterized by decreased visual acuity and macular atrophy (Edwards et al. 1999). Truncation mutations in the elongation of the very long chain fatty acids-4 (*ELOVL4*) gene cause STGD3. Two different transgenic pigs carrying a 5-bp deletion and a 270 stop codon mutation with an N-terminal EYFP fusion (Y270terEYFP) in the *ELOVL4* gene were generated by pronuclear DNA microinjection and SCNT. These transgenic pigs exhibited photoreceptor loss, protein mislocalization and a diminished electroretinography response (Sommer et al. 2011).

Cone rod dystrophies (CRDs) comprise a retinal disease characterized by cone loss preceding rod degeneration in contrast to RP described above. The symptoms of CRD are the early onset of decreased visual acuity in the central visual field with the progressive loss of peripheral vision and night blindness occurring later. Guanylate cyclase 2D (*GUCY2D*) is responsible for many cases of autosomal dominant CRD (Hamel 2007). A cohort of *GUCY2D*$^{E837D/R838S}$ transgenic pigs was generated via lentiviral transgenesis. These pigs exhibited abnormal retinal morphology and progressive vision impairment. Moreover, these pigs exhibited a range of phenotype severity, which reflected the heterogeneity observed in human patients (Kostic et al. 2013). All of these transgenic pigs provided valuable information for studies on the pathogenesis of RP, STGD3 and CRD as well as the pre-clinical testing of new therapies.

Immunodeficiency

Severe combined immunodeficiency (SCID) comprises a group of primary immunodeficiencies with impaired T and B lymphocyte development, function or both. According to the immunological phenotype, SCID defects are categorized as those with B lymphocytes (T^-B^+ SCID) or without B lymphocytes (T^-B^- SCID). X-linked SCID (SCIDX1) accounts for 40 % of all SCID cases and is caused by IL-2 receptor γ gene (*IL2RG*) mutations. Recombinase-activating gene 1 (*RAG1*) and *RAG2* encode the enzymes that catalyze V(D)J recombination during B and T cell development. Mutations in *RAG1* or *RAG2* in humans result in T^-B^- SCID or Omenn syndrome (Huang et al. 2014; Notarangelo 2010). Considering the differences between the mouse and human immune systems (Mestas and Hughes 2004), SCID mice are not suitable to simulate human genetic and physiological states. In contrast, pigs are excellent animal models to represent human diseases due to their immunological similarities with humans.

IL2RG was disrupted by two groups to obtain SCID pigs. Suzuki et al. used conventional HR technology to target the porcine *IL2RG* gene in fetal fibroblasts followed by a serial cloning strategy and further breeding. *IL2RG*$^{-/Y}$ males were obtained that had undetectable thymi and significant reductions in T and NK cells. Allogeneic bone marrow transplantation reconstituted the lymphoid lineage of the *IL2RG*$^{-/Y}$ SCID pigs (Suzuki et al. 2012). Watanabe et al. combined ZFNs with SCNT to generate *IL2RG* KO pigs. The mutant pigs exhibited phenotypes similar to Suzuki's (Watanabe et al. 2013).

RAG1/2 were also targeted using TALENs technology by two groups. Huang et al. (2014) used TALENs to create bi-allelic *RAG1* or *RAG2* mutant pigs that exhibited hypoplasia of the immune organs, a loss of mature B and T lymphocytes and a lack of V(D)J rearrangement. Lee et al. generated *RAG2* mono- and bi-allelic mutant pigs via TALENs. Of the 4 bi-allelic mutant pigs generated, three lacked a thymus, whereas the thymus of the fourth pig was extremely underdeveloped. Moreover, the white pulp of the pigs' spleens lacked B and T cells. Following injection with human iPSCs, the *RAG2*$^{-/-}$ pigs rapidly formed teratomas representing all three germ layers and could accept allogeneic porcine cells (Lee et al. 2014a). All of these SCID pigs resembled human SCID and could serve as valuable models for regenerative medicine, xenogeneic transplantation studies of pluripotent stem cells, cancer research and therapy development for human SCID patients.

Pigs as bioreactors

The production of recombinant proteins through genetic engineering has enabled the generation of transgenic animals for pharmaceutical protein production. The mammary gland has been widely used as a bioreactor for the production of various recombinant proteins since ovine β-lactoglobulin (Simons et al. 1987) and human tissue plasminogen activator (Gordon et al. 1992) were obtained from the milk of transgenic mice. Recombinant human antithrombin III (ATryn, GTC Biotherapeutics Inc.) expressed in transgenic goat milk (Kling 2009; Schmidt 2006) and recombinant C1 esterase inhibitor (Ruconest, Pharming Group NV) expressed in rabbit milk (Kling 2011) have been approved by the US FDA and European Medicines Agency (EMA) for clinical applications. The approval of these two drugs demonstrated the great potential of the mammary gland in the production of pharmaceutical proteins.

Pig mammary gland has been shown to be more suitable for the completion of post-translational modifications (PTMs) than Baby Hamster Kidney cells and mammary epithelial cells of mice as well as ruminant dairy livestock such as sheep. Compared with cell culture systems, mammary gland is better for therapeutic proteins production since it harbors higher cell density and it can perform the PTMs processing which are important for the native biological activity and circulating half-life of the recombinant proteins (Morcol et al. 1994; Van Cott et al. 1999). For example, a comparison

Fig. 2 Biomedical applications for which genetically modified pigs have been created. Eight-Precepts Pig, one of the main characters in the most famous Chinese fairy tale, "Journey to the West," has the ability to undergo 36 polymorphic transformations. However, with the help of current nuclease-mediated genome technologies, pigs can undergo more transformations than Eight-Precepts Pig

of rFIX biosynthesis in Baby Hamster Kidney Cells and pig mammary epithelial cells illustrated the cell specific limitations in PTM processing needed for vitamin K-dependent (VKD) protein functionality (Zhao et al. 2015). Furthermore, the mammary gland of mice and sheep made much lower levels of functional rFIX and other VKD proteins when compared with pigs (Clark et al. 1989; Lisauskas et al. 2008; Morcol et al. 1994; Velander et al. 1992a, b; Zhao et al. 2015).

The first pig bioreactor was reported in 1991 when Wall et al. introduced the mouse whey acidic protein (WAP) gene into the porcine genome and detected foreign protein expression in milk from all lactating females at levels similar to those found in mouse milk (Wall et al. 1991). Recombinant human protein C (Velander et al. 1992a), factor VIII (Paleyanda et al. 1997), von Willebrand factor (rhvWF) (Lee et al. 2009), factor IX (rhFIX) (Lee et al. 2014b), and human lysozyme (rhLZ) (Lu et al. 2015) were successfully obtained from the milk of transgenic pigs. Recently, bio-engineering of the pig mammary gland to co-express rFIX and rFurin largely improved the expression levels of rFIX with biological activity, which further supported the usage of pigs as bioreactors for the large-scale production of therapeutic human proteins (Zhao et al. 2015).

Perspectives and conclusions

The above descriptions summarize the most significant developments involving gene-modified pigs for modeling human diseases using conventional transgenesis and gene editing, especially nuclease-mediated gene editing (Fig. 2). At present, a total of 35 gene-disrupted pig models have been created using nuclease-mediated technologies (to February 2016). The rapid development of gene editing technology has revolutionized the production of genetically modified cells or animals for biomedical and translational research.

During the revision of this manuscript, a DNA-guided endonuclease, *Natronobacterium gregoryi* Argonaute (NgAgo), was reported to be able to cleave genomic DNA in mammalian cells. It could bind a 5′ phosphorylated single-stranded guide DNA (gDNA) and create site-specific DSBs, resulting in site-specific genome editing. The NgAgo-gDNA system did not require a PAM sequence, and could target GC-rich regions. Most of all, it had a low tolerance to guide-target mismatch, which indicated the high specificity of the NgAgo-gDNA system (Gao et al. 2016). By improving Cas9 and reprogramming novel enzymes, genome editing tools represent immense potential for gene modifications.

Although major technical hurdles have been overcome and many pig models have been established, most of the pig models are in their infancy and limited data are available now because of the following restricts: the long generation interval, the limited number of individuals of GM pigs, no truly inbred pig breeds and the immature platforms for systematic phenotyping. The broad applications of GM pigs were also hampered by limitations described above. Further, the increasing understanding of the genetic basis of human diseases and the annotation of the pig genome will facilitate a more directed approach to create human disease models in pigs. These precious animal models will finally contribute to better understanding the pathologies of specific human diseases and accelerating the movement toward the clinic as therapeutics and drug development.

Acknowledgments This study was supported by the National High Technology Research and Development Program of China (2012AA020602), the National Natural Science Foundation of China (31172281, 31272440), and the National Basic Research Program of China (2011CBA01005 and 2011CB944100).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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