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Review Article

Recent advances in genetically modified large-animal models of human diseases

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Abstract

Large-animal models show greater advantages than rodents in recapitulating human genetic diseases, primarily because of their higher similarity to humans in terms of anatomy, physiology and genetics. Notably, as genome-editing technologies have rapidly improved, particularly transcription activator-like effector nuclease (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 (CRISPR-associated protein 9) systems, their application in biomedical research has accelerated. A variety of genetically modified large-animal models, including non-human primates, pigs, dogs, bovines and sheep, have been produced to recapitulate human inherited disorders, thus providing novel biological and translational insights. Here, we review recent progress in the generation of large-animal models over the past 5 years and summarize their use in studying human genetic diseases, focusing on the nervous system, cardiovascular and metabolic systems, the immune system, xenotransplantation, the reproductive system and embryonic development.

Keywords

CRISPR/Cas9, human inherited diseases, large animal models, TALEN, translational medicine.

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Introduction

Animal models, which are essential in biological and medical research, greatly promote advances in genetic research on human diseases. Among them, large-animal models have advantages because of their greater similarity to humans in terms of anatomy and physiology (Table S1) [1]. In addition, large-animal models show higher heterogeneity in genetic backgrounds, thus mirroring the genetic diversity of humans, and are genetically closer to humans than rodent models. Non-human primates (NHPs) are the model most closely resembling humans in evolution, genetics, physiology, the aging process, behavioral symptoms and pathological changes. Other large-animal models, including pigs, dogs, bovines and sheep, have been extensively studied to mimic human genetic diseases. However, their wide use in studies has been limited by a lack of efficient genetic engineering tools in these large animals. In recent years, new gene-editing technologies, mainly TALEN [2], CRISPR [3] and base editing [4], have made rapid progress and provided highly efficient tools for developing genetically modified large-animal models. Large-animal models are increasingly used to study genetic diseases, owing to their high efficiency and simplicity and the flexibility of CRISPR/Cas9 systems [5]. To date, two conventional pipelines have been established for the generation of genetically modified large-animal models [6]. One method is based on somatic-cell nuclear transfer (SCNT) combined with a genome-editing system [7]. The other involves generating gene-modified animals in a single step via microinjection of the CRISPR/Cas9 genome-editing system into zygotes, without a need for gene editing of somatic cells *in vitro* [8]. The ability to introduce genes or variants into animal genomes has allowed for mechanistic investigation into the genetic contributions of specific genes to human diseases. Here, we review the recent development of large-animal models and their applications in human genetic diseases over the past 5 years, focusing on disorders associated with the nervous, cardiovascular and metabolic, immune and reproductive systems.

Nervous system diseases

Nervous system diseases are a group of disorders associated with impairment of

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the central and peripheral nervous system. Research attention has focused on neurodegenerative disorders, which are accompanied by several representative symptoms in patients, including extrapyramidal and pyramidal movement disorders, as well as cognitive or behavioral disorders [9]. Human diseases associated with neurodegenerative disorders mainly include Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD) and infantile neuronal ceroid lipofuscinosis (CLN1), each of which has specific clinical symptoms and etiologies. In PD, studies have shown that both genetic and environmental factors are associated with pathogenesis. Patients with PD mainly present with muscle stiffness, tremors, unsteady gait and balance and coordination difficulties [10]. AD is the most common type of dementia affecting the older population, and patients with AD present a progressive decline in cognition, memory and behavioral skills, which is probably driven by β-amyloid protein deposition and intracellular accumulation of hyperphosphorylated tau protein [11]. HD is a rare inherited neurodegenerative disorder caused by a trinucleotide repeat (CAG) expansion in the *HTT* gene. Patients with HD exhibit uncontrolled choreatic movements, behavioral and psychiatric problems, and dementia [12]. CLN1 is a rare genetic disease caused by genetic changes in the *PPT1* gene, which contribute to a deficiency in the soluble lysosomal enzyme palmitoyl protein thioesterase-1 (PPT1). Clinically, patients with CLN1 show severe neuronal degeneration, cortical thinning and overall brain atrophy [13]. Importantly, neurodegenerative diseases are age dependent [14], and previous studies have shown that mouse PD models do not fully replicate human pathological manifestations, probably because rodents lack major anatomical features found in humans, such as distinguishable subdivisions of the globus pallidus and a subthalamic nucleus [15, 16]. Therefore, the establishment of large-animal models for reproducing neurodegenerative lesions has become an attractive choice. Recently, Li et al. have built the first rhesus monkey model of etiological PD by co-editing the *PINK1* and *DJ-1* genes in the substantia nigra region of the monkey brain with the CRISPR/Cas9 system delivered by adeno-associated virus serotype 9 (AAV9). This transgenic monkey model simulates PD phenotypes well, such as bradykinesia, tremor and postural instability, which are accompanied by the key pathological characteristics of PD, including severe loss of nigral dopaminergic neurons and the presence of α-synuclein pathology within the gene-edited substantia nigra [17]. In 2019, Yang et al. generated a CRISPR/Cas9-mediated *PINK1*-deleted monkey model and observed robust early-onset neurodegeneration in various brain regions; this model should provide important information for studying the function of *PINK1* and progressive neurodegeneration [18]. In addition, this group has also demonstrated that PINK1 kinase activity rather than its mitochondrial function is a selective requirement for neuronal survival in primate brains, and have further suggested that PINK1 kinase dysfunction might be associated with human PD and other pathological conditions [19]. Furthermore, to create an ideal inherited PD minipig model, Zhu et al. have produced Bama minipig models by introducing three PD-causing missense variants in the *SCNA* gene (E46K, H50Q and G51D) by using CRISPR/Cas9-mediated gene editing combined with SCNT. Owing to the absence of α-synuclein-immunopositive pathology at 3 months of age, these pig models still must be developed to investigate the presence of PD-like pathological features [20]. In contrast to PD, AD is associated with clinical memory and cognition deficits as well as pathologically neurofibrillary tangles and amyloid plaques [21]. Recently, an AD transgenic pig model bearing mutations in h*APP* (K670N/M671L, I716V and V717I), h*Tau* (P301L) and h*PS1* (M146V and L286P) has been developed. This model shows high expression of target genes in tissues, particularly in the brain, and exhibits hallmarks of damaged neurons consisting of Aβ-40/42, total Tau and GFAP; therefore, it may serve as an ideal model for studying AD pathogenesis [22]. A knock-in (KI) pig model carrying the mutant huntingtin (*HTT*) gene shows consistent movement and behavioral abnormalities, which are accompanied by striking and selective degeneration of striatal medium spiny neurons [15]. This work first demonstrated that the overt and selective neurodegeneration seen in patients with HD can be reproduced by endogenously expressed mutant proteins in large mammals. Additionally, $SURF1^{-/-}$ pig models have been generated with TALENs and CRISPRs to recapitulate Leigh syndrome associated with cytochrome c oxidase (COX) deficiency. $SURF^{-/-}$ pigs show failure to thrive, a short life span and muscle weakness; in newborn piglets, delayed central nervous system development is observed in the absence of clear COX deficiency [23]. A CLN1 sheep model with CRISPR/Cas9-mediated insertion of human *PPT1* (R151X) has been successfully constructed, which exhibits behavioral and motor deficits as well as hallmarks of brain atrophy, thus providing substantial opportunities for further revealing the mechanisms and discovering a potential treatment for this form of neurodegenerative disease [24].

Moreover, several large-animal models have recently been created for modeling other nervous system diseases, such as autism spectrum disorder (ASD), Rett syndrome (RTT) and tuberous sclerosis (TSC). In 2019, Zhou et al. reported that *SHANK*-mutant monkeys exhibit sleep disturbances, motor deficits and repetitive behaviors, as well as social and learning impairments, which resemble characteristics of ASD and Phelan–McDermid syndrome [25]. Likewise, Tu et al. have developed a cynomolgus monkey model with *SHANK3* gene disruption, which exhibits the core disease phenotypes of ASD. Furthermore, their results have indicated that treatment with the antidepressant fluoxetine alleviates the abnormal behaviors and brain activity, thus indicating the advantages of using NHPs for ASD modeling [26, 27]. In addition, Qin et al., concentrating on non-syndromic ASD, have found that specific knockout of giant *ANK2* in monkeys does not generate nonsyndromic ASD-like behaviors, but gives rise to pronounced brain structural alterations [28]. Of interest, TALEN-edited *MECP2* cynomolgus monkey models show major phenotypic similarities to human patients with RTT, thus suggesting that *MECP2* gene-edited mutant monkeys will be valuable for dissecting disease pathogenesis and developing potential therapeutic strategies for RTT [29]. Moreover, *Tph2* knockout (KO) pig models have been generated and provided important insights into behavioral abnormalities induced by 5-HT

deficiency [30]. In addition, a recent study has developed a pig model by introducing a monoallelic mutation in the *TSC1* gene with the CRISPR system. *TSC1*$^{+/-}$ pigs develop the clinical features observed in patients with TSC, including cardiac rhabdomyoma and subependymal nodules, which are absent in mouse TSC models [31]. STXBP1 is essential for neurotransmitter release, and *STXBP1* (R292H)-mutated monkeys created through base editing show core symptoms of STXBP1 encephalopathy, thus providing a suitable animal model for STXBP1 encephalopathy [32]. Furthermore, Beraldi et al. have established an *ATM*$^{-/-}$ pig model for modeling ataxia telangiectasia. Interestingly, *ATM*$^{-/-}$ pigs not only simulate the neurological phenotype but also show other pathological features of patients with ataxia telangiectasia, including altered thymus structure, dysregulation of the immune system and sterility [33].

Cardiovascular and metabolic diseases

Cardiovascular diseases refer to a group of disorders affecting the heart and blood vessels [34]. Metabolic diseases are conditions in which abnormal metabolic processes occur, primarily including dyslipidemia and perturbation of amino acid metabolism [35]. In studies of inherited cardiovascular and metabolic diseases, pigs have received the greatest attention among large-animal models. Notably, pigs present many advantages over other animals, such as similar cardiovascular anatomy and cardiac physiology to those in humans [36], as well as a similar heart size to that in humans [37]. Therefore, they provide an ideal model for cardiovascular disease research. For example, pigs and humans express β-MHC in ventricles, which play important roles in regulating heart rates and maintaining the cardiac output. However, fast α-MHC, instead of β-MHC, has been found to be expressed in mouse ventricles—a characteristic notably different from those in humans and pigs [38]. In 2021, Gabriel et al. generated a CRISPR-edited pig model with *SAP130* mutation for modeling human congenital heart disease, which is rare in pigs. This pig model, which manifests coronary heart disease with tricuspid dysplasia and tricuspid atresia associated with early embryonic lethality, provides opportunities for research in surgical operation and testing ventricular assist devices [39]. In addition, Montag et al., using the TALEN system, have successfully constructed an *MYH7* (R723G)-mutant pig model mimicking human familial hypertrophic cardiomyopathy [40]. A TALEN-induced SGCD KO pig mimicking human genetic cardiomyopathy has been generated and found to exhibit symptoms of systolic dysfunction, myocardial tissue degeneration and sudden death, thus potentially enabling the development of preclinical therapies [41]. Chen et al. have found that transgenic p*F9* KO pigs carrying the human coagulation factor IX show partial amelioration of bleeding; this model may be used to explore the pathological process of hemophilia [42]. Moreover, Zhang et al. have produced *DUOX2*$^{D409G/D409G}$ mutant pigs through ENU mutagenesis and demonstrated that the TR-KLF9 axis is responsible for the blood cell development in hypothyroidism [43].

Furthermore, many groups have chosen pigs or dogs for modeling human metabolic diseases. Studies have shown that, compared with those in mice, the lipoprotein profiles and metabolism patterns of pigs are overall more similar to those in humans [44]. However, a large proportion of cholesterol transport is mediated by high-density lipoprotein in mice—an aspect clearly different from the low-density-lipoprotein delivery in humans and pigs [45]. Therefore, several groups have produced pig mutants via the CRISPR/Cas9 system for modeling human diabetes by targeting the *INS* [46], *NGN3* [47] and h*IAPP* [48] genes, whose functions are associated with pancreatic development. Furthermore, Wang et al. first generated permanent neonatal diabetes mellitus (PNDM) dog models carrying *GCK* point mutations by using the BE3 system. These models exhibit similar features to those in patients with GCK-PNDM and thus may serve as ideal animal models to study this disease [49]. Moreover, *ASGR1*-deficient [50], *ApoE* KO [51] and *ApoE/LDLR* dKO [52] pigs have been found to be ideal models for human cardiovascular diseases associated with lipid metabolism, particularly high cholesterol. Likewise, *ApoE* KO dog models produced with CRISPR/Cas9 also show advanced severe hypercholesterolemia and atherosclerosis characterized by stenosis and occlusion of arteries, together with stroke and gangrene [53]. These pig and dog models will be invaluable in developing and evaluating new therapies, including endovascular procedures, to treat atherosclerosis and related disorders. Yao et al. have found that *OSBPL2* KO pigs, generated through a combination of CRISPR/Cas9 and SCNT techniques, show hypercholesterolemia and progressive hearing loss, thus confirming the roles of *OSBPL2* gene in nonsyndromic hearing loss and providing opportunities to unravel the potential relationships between auditory dysfunction and dyslipidemia/hypercholesterolemia [54]. Yin et al. have found that *MC3R* KO pigs, exhibiting increased body weight and body fat, can be used to reveal the physiological roles of MC3R in energy homeostasis [55]. In addition, chronic inflammation has been demonstrated to contribute to obesity and metabolic diseases, particularly metaflammation [56]. Zhang et al. have generated triple transgenic pigs through CRISPR/Cas9-mediated KI of *GIPR*^{dn}, *hIAPP* and *PNPLA3*^{I148M} and found that the model develops metabolic disorders accompanied by inflammation activation; thus, this model may be ideal for investigating metabolic inflammation [57]. Interestingly, Zheng et al., in adipose-specific *UCP1* KI pigs, have uncovered crucial roles of UCP1 in protecting the cardiovascular system through inhibiting tissue inflammatory responses [58].

Using *FAH*$^{-/-}$ pigs created by CRISPR/Cas9, an ideal animal model of hereditary tyrosinemia type 1 (HT1), Gu et al. have found that, before intrauterine death, direct intracytoplasmic delivery of CRISPR-Cas9 targeting the *HPD* gene reprograms the tyrosine metabolism pathway and protects pigs against FAH-deficiency-induced lethal liver injury, thus providing a therapeutic option for the treatment of HT1 [59]. Additionally, in 2020, Koppes et al. successfully produced a CRISPR/Cas9-mediated *PAH*-null pig model recapitulating human phenylalanine hydroxylase–deficient

phenylketonuria (PKU) and enabling investigation of therapeutic interventions [60]. In addition, in 2021, Kaiser et al. generated a *PAH*$^{hR408W/hR408W}$ PKU pig model by using a TALEN system and found that this model mimics human phenotypes and responds well to dietary phenylalanine restriction [61]. Importantly, these pig models for human PKU have introduced perspectives in the development of therapeutic interventions and have unique value in gene-therapy studies.

Immune system diseases

Previous studies have indicated that rodent models have shortcomings in immunology research [62], and the differences in immune responses between rodents and humans might be attributable to genetics, lifespan, living environment and specific species-pathogen relationships [63]. Large-animal models can offer unique biological advantages in understanding human immunology and may be able to address questions that rodent models cannot answer [64]. Remarkably, in contrast with rodents, large animals show greater similarities to humans in terms of immune system development and response, including immune cell development, innate immunity, regional immunity and infectious immunity. The pig immune system has been demonstrated to resemble that in humans for more than 80% of parameters, whereas the mouse immune system has similarity for approximately 10% of parameters [65]. Swine models have been widely used in studies of autoimmune and immune-mediated inflammatory diseases. Interestingly, transgenic pigs with *leptin* overexpression show symptoms of systemic lupus erythematosus, including anemia, leukopenia and thrombocytopenia, along with kidney and liver impairment. However, glucocorticoid therapies have been found to partially relieve the autoimmune symptoms. The *leptin* transgenic pig model is valuable for investigating the roles of adipocytokines in the modulation of immune responses [66]. Zhang et al. have successfully established complement protein *C3* KO pigs, which can be used to delineate the roles of C3 in pathology and physiology [67]. In addition, Li et al. have found that pigs carrying *NLRP3* (R259W) homozygous mutations mimic aspects of human cryopyrin-associated periodic syndrome, such as early mortality, poor growth and spontaneous systemic inflammation symptoms [68]. Song et al. have built a pig model of human familial acne inversa, an inflammatory skin condition, by introducing an *NCSTN*$^{+/R117X}$ heterozygous point mutation, and have further elucidated the mechanism underlying the development of this condition [69]. Large-animal models are also increasingly being developed and used in studies on infectious diseases. Yugo et al. have successfully established $J_H^{-/-}$ gnotobiotic pigs with knockout of the immunoglobulin heavy chain. Compared with wild-type pigs, $J_H^{-/-}$ pigs show lower levels of HEV replication and enlarged livers after HEV infection, thus suggesting that $J_H^{-/-}$ pigs may provide an efficient animal model to mimic HEV-specific lesions and dissect the mechanisms of HEV pathogenesis [70]. Notably, the emergence of the novel virus SARS-CoV-2 has greatly affected human life worldwide. ACE2, the major entry receptor for this virus, acts on the kinin-kallikrein, renin-angiotensin and coagulation systems, which have been implicated in the pathogenesis of severe cases of the related disease, COVID-19 [71]. To reproduce severe cases of COVID-19, Du et al. have successfully established *hACE2* KI pigs, and have detected higher expression of hACE2 protein in the lungs, kidneys, testes and intestines, similarly to the conditions observed in humans [72].

Xenotransplantation

Xenotransplantation research in large-animal models has made major advances. The current research focus in this field includes immune rejection, physiological incompatibilities and the risk of microbial transmission in conducting transplantation [73]. Pigs have received substantial attention, owing to their similarities with humans in terms of biological features. The enzyme 1,3-galactosyltransferase (Galα (1,3) Gal), encoded by *GGTA1*, acts as a key factor in xenograft rejection, by catalyzing the synthesis of αGal. In 2017, TALEN modified *CMAH*KO/*GT*KO/*sh-TNFRI-Fc/hHO-1* quadruply modified pigs have been found to overcome ultra-acute and acute anti-inflammatory rejection of xenografts [74]. In addition, Adams et al. have found that the kidneys transplanted from CRISPR/Cas9-mediated double-xenoantigen *Gal-Sd*$_a$ KO pigs into chemical immunosuppression rhesus monkeys show prolonged xenogeneic survival times as long as 435 days; they have also revealed that early graft rejection is mediated by IgM antibody, but the 435-day graft loss might nonetheless have resulted from IgG-antibody-mediated rejection [75]. In addition, Kim et al. have performed renal transplantation from *αGal* KO/*CD55* transgenic pigs into rhesus macaques, and have found that early xenograft rejection was induced by abundant CD4$^+$ cell infiltration, and later rejection is mediated by antibodies [76]. In addition, Fu et al. have demonstrated that skin grafts from *GGTA1*$^{-/-}$*β2M*$^{-/-}$*CIITA*$^{-/-}$ triple knockout (GBC-3KO) pigs show significantly prolonged survival in mice, thus indicating that GBC-3KO effectively decreases xenogeneic immune responses [77]. Rao et al. have developed *HLA-G1*$^+$*/GGTA1* KO pigs through transgenic expression of HLA-G1$^+$ in *GGTA1* KO pigs, and have demonstrated that these donors suppress the activation and proliferation of monkey and human T, B and natural killer (NK) cells [78]. In 2019, Watanabe et al. transplanted pig lung xenografts expressing human-CD47 (*hCD47*) into baboons and found that the xenografts had a prolonged survival time for 8 weeks [79]. In addition, two reported cases of pig-to-human kidney xenotransplantation have shown that genetically modified kidney xenografts from GGTA1$^{-/-}$ pigs are viable and can function in brain-dead human recipients for 54 hours, without signs of hyperacute rejection [80]. In regard to the xeno-organ overgrowth problem, Hinrichs et al. have eliminated the growth hormone receptor in *GGTA1* KO/*hCD46*$^+$*/hTHBD*$^+$ pigs and found that *GHR* knockout decreases the intrinsic growth potential of pig xeno-organs [81].

In addition to being an organ-transplant donor, immunodeficient pigs are also favorable recipient research models.

Choi et al. have reported that *RAG2*$^{-/-}$ pigs representing severe combined immunodeficiency (SCID) show advantages over *Rag2*$^{-/-}$ SCID mice, because the mice show intense, infrequent and mild clusters of CD3^{+}, CD4^{+} and CD8^{+} signals. The gene expression of T, B or NK cell maturation in *RAG2*$^{-/-}$ SCID pigs is less than that in *Rag2*$^{-/-}$ SCID mice [82]. Nelson et al. have found that *RAG2*$^{-/-}$*FAH*$^{-/-}$ immunodeficient pigs can receive infusions of human liver cells, although the NK cells are a barrier to the expansion of hepatocytes [83]. Furthermore, Ren et al. have demonstrated that *IL2RG*$^{-/Y}$ pigs allow for the development of human melanoma-derived tumors and thus may serve as hosts for human cancer [84]. In addition, Hendricks-Wenger et al. have found that *RAG2/IL2RG* double KO pigs permit growth of human pancreatic adenocarcinomas, whose electrical properties and responses to irreversible electroporation are similar to those of excised human pancreatic cancer tumors, thus suggesting a key step in the development of immune humanized SCID pig models [85]. Dong et al. have successfully generated porcine endogenous-retrovirus-inactivated pigs by using CRISPR/Cas9, thus addressing the safety concerns in clinical xenotransplantation [86].

Reproductive system and embryonic development

Large-animal models also have advantages over rodent models in human reproduction studies. For example, large animals provide valuable resources for investigating folliculogenesis, which is challenging in rodents. Shi et al. have generated bone morphogenesis protein 15 (*BMP15*) KO pigs with the CRISPR/Cas9 system and found that this model shows an infertility phenotype. In detail, BMP15 depletion obstructs follicle development at preantral stages, thus resulting in the development of many structurally abnormal follicles and consequently leading to streaky ovaries and lack of a pronounced estrus cycle [87]. In addition, large animals are ideal models for studies on mammalian embryonic development. Recently, two groups have found that *SRY* KO pigs generated by CRISPR/Cas9 [6] and *SRY* KI bovines generated by TALEN [88] show sex reversal, thus further demonstrating the conserved roles of SRY in mammalian sex determination and differentiation. Zhou et al. have constructed a pig parthenogenetic embryo model by targeting the *PDHA1* gene with CRISPR/Cas9 at the four-cell stage and found that early embryonic development is blocked, and histone acetylation significantly decreases, thereby demonstrating the critical roles of *PADH1* in zygotic genome activation in porcine embryos [89]. Kilian et al. have constructed *OCT4* KO bovine embryos by using CRISPR and SCNT; their results have indicated that, as in human early embryonic development, *OCT4* is necessary for maintaining NANOG-positive epiblast cells in the inner cell mass of bovine blastocysts, in contrast to findings in mice [90]. Likewise, Daigneault et al. have shown that disruption of *POU5F1* in bovine embryos by the CRISPR/Cas9 system contributes to embryonic arrest at the morula stage, thereby preventing blastocyst formation. Moreover, conservation of POU5F1 functions in embryonic development has been observed in bovines and humans, in contrast to mice [91]. Thus, these studies highlight that bovine embryogenesis provides an outstanding model for understanding human early development.

Other diseases

In 2019, Dorado et al. established a Yucatan minipig model of Hutchinson-Gilford progeria syndrome through a heterozygous *LMNA* c.1824 C>T mutation. This model shows severe growth retardation, lipodystrophy, skin and bone alterations, cardiovascular disease and death around puberty, in agreement with symptoms in humans [92]. Furthermore, in 2020, monkey models bearing *LMNA* c.1824 C>T, created by base editing, were also found to show the typical symptoms of Hutchinson-Gilford progeria syndrome [93]. To investigate the biological function of longevity protein SIRT6 in primates, Zhang et al. have generated *SIRT6*-null cynomolgus monkey models with the CRISPR/Cas9 system. The KO monkeys died shortly after birth and exhibited severe prenatal developmental retardation, thus mimicking human perinatal lethality syndrome [94]. In addition, Tsukiyama et al. have introduced mutations in *PKD1* and produced a cynomolgus macaque model of autosomal dominant polycystic kidney disease. *PKD1* depletion in heterozygous monkeys leads to severe cyst formation, primarily in the collecting ducts, and cyst formation perinatally in distal tubules, thus somewhat reflecting the initial pathology in humans [95].

Duchenne muscular dystrophy (DMD) is a common hereditary childhood myopathy caused by *DMD* gene mutations [96]. Notably, dogs spontaneously produce DMD [97]. Multiple canine DMD models have also been established with genome-editing technologies, thus providing opportunities for *in vivo* gene-therapy trials. In 2018, Amoasii et al. performed *in vivo* CRISPR gene editing in a deltaE50-MD dog model of DMD by using adeno-associated viruses (AAVs); this gene-editing treatment restores the dystrophin in skeletal muscle and cardiac muscle [98]. Furthermore, Moretti et al. have demonstrated that intramuscular injection of AAV9-Cas9-gE51 in a deltaE50-MD swine model induces expression of a shortened dystrophin (DMDΔ51–52), thereby improving skeletal muscle function [99]. In addition, Li et al. have successfully established *FSI-I-I* KI pigs by using CRISPR/Cas9. The myofiber sizes in *FSI-I-I* KI pigs were significantly greater than those in wild-type pigs, thus indicating great promise for treatment of human muscular dystrophy [100]. However, although dystrophin expression was restored, Hakim et al. have found that AAV-CRISPR treatment leads to a Cas9-specific immune response in multiple dystrophic canine models, thus posing major challenges for CRISPR gene-editing therapies [101].

In 2019, Gao et al. used CRISPR/Cas9 and SCNT to knock out the *HR* gene in pigs; piglets with mutations exhibit a lack of hair on the eyelids, and abnormalities in the thymus and peripheral blood [102]. Furthermore, genome editing of the *FGF5* [103], *EDAR* [104] and *VEGF* [105] genes

in goats and sheep has been found to significantly increase hair growth and hair-follicle density, thus suggesting potential roles of these genes in follicle diseases. Han et al. have produced *HOXC13* KO pigs with CRISPR/Cas9. This model shows a diminished number of follicles and disarray in hair follicle cables, thus mimicking human ectodermal dysplasia-9 [106]. In addition, a pig model bearing the deep intronic mutation IVS49-727 A>G in *ABCA12* shows hyperkeratotic skin and a response to systemic retinoid treatment, thereby recapitulating human harlequin ichthyosis [107]. In 2018, Li et al. successfully generated pigs carrying the *TWIST2* E75K mutation and *TYR* Q68Stop variant by using BE3 and SCNT; the phenotypes were consistent with those of human ablepharon macrostomia syndrome and oculocutaneous albinism type 1 (OCA1), respectively [108]. Moreover, Zhang et al. have established a COL2A1 KO pig model exhibiting severe skeletal dysplasia and tracheal collapse, to investigate the pathogenesis of early skeletal developmental defects [109]. Likewise, Williams et al. have created a sheep hypophosphatasia model through CRISPR/Cas9-mediated KI of an *ALPL* gene mutation (1077 C>G). The KI sheep exhibit diminished serum alkaline phosphatase activity, tail vertebral bone size and metaphyseal faring, thus providing a unique platform for bone research [110]. Additionally, Watanabe et al. have created a *SALL1*-null pig model displaying a nephrogenic phenotype, thus potentially offering a nephrogenic niche for human kidney regeneration [111]. In 2018, FGFR2-IIIb was found to play a role in lung branching morphogenesis in pigs overexpressing dominant-negative FGFR2-IIIb made by SCNT [112]. Hai et al. have produced a pig model carrying the c.740 T>C (L247S) mutation in the *MITF* gene for modeling human Waardenburg syndrome type 2A [113]. The group has further performed CRISPR-Cas9-mediated gene therapy to correct phenotypes including anophthalmia and hearing loss [114]. Moreover, Engevik et al. have generated a pig model by introducing the *MYO5B* P663L mutation with TALENs; the pigs mimic human microvilli inclusion body disease [115].

Conclusions and future prospects

In conclusion, large-animal models have been widely used to mimic human genetic diseases, particularly disorders of the nervous system, cardiovascular and metabolic systems, immune system, reproductive system and embryonic development. Since 2017, more than 80 research articles regarding the construction of large-animal models for human diseases have been published (in NHPs, pigs, dogs, cattle, sheep and goats, on the basis of PubMed searches). The development of genome-editing tools has greatly revolutionized the field, thus making genetic engineering and genome editing of large-animal genomes simpler, and more precise and efficient. Moreover, large-animal models provide major advantages in modeling specific human diseases that rodent models may fail to faithfully recapitulate. Importantly, large-animal models may also provide unique or unexpected insights for better understanding of human diseases.

The increasing number of large-animal models has heralded a new phase of understanding of the complex conditions of human inherited disease. These advances have further offered prospects for therapeutic applications of gene therapy in large animals. Many large animals, such as pigs [116], dogs [117] and NHPs [118], have been used for the assessment of gene-transfer techniques or gene-therapy treatment trials. Notably, owing to their relatively longer lifespan and size, large animals have substantial advantages in addressing concerns regarding the long-term efficacy and safety of gene-therapy approaches. Large-animal models also present a unique translational framework for validating and testing novel therapeutic tools, such as new genome-editing or gene-delivery systems. For example, in 2021, Musunuru et al. delivered a CRISPR base-editing system by using lipid nanoparticles and generated durable low-density-lipoprotein cholesterol for 8 months in monkey livers, thus providing a promising strategy to target *in vivo* treatment for liver diseases [119]. Currently, omics technologies, including genomics, transcriptomics, proteomics and metabolomics, have been used extensively in biomedical studies and yielded valuable new findings. Similarly, increasing amounts of omics data have been obtained in large-animal models; these data may be used to choose suitable animal models according to different scenarios. Specially, recent technological advances have enabled omics investigations of single cells or restricted spatial areas, thus revealing information on gene expression within individual cells and also capturing spatial gene expression profiles. Prominently, four studies have built a single-cell atlas including 33 human organs [120–123]. Han et al. completed a mouse cell atlas including more than 40 mouse organs and tissues [124]. Recently, an adult monkey cell atlas covering 45 different tissues has been created [125]. Furthermore, the BodyMap transcriptome containing approximately 31 adult pig tissues has been reported [126]. In addition, single-cell RNA sequencing of various tissues or organs derived from dog lung immune cell populations [127], sheep germ cells [128] and bovine sperm cells [129] have been obtained. Theoretically, comparison of these omics data between human and animal models should provide systemic information for estimating the biological relevance or similarity of the model to humans, thus helping researchers select the right model according to a broad perspective.

In 1990, the first gene-therapy trial for a rare inherited disease known as SCID was initiated [130]. Owing to great advances in genome-editing and gene-delivery systems, gene therapy has become a major research field that holds great promise for treating human genetic diseases. Importantly, gene therapy has been clinically used in human genetic disorders, such as thalassemia, DMD, cystic fibrosis, eye disorders, metabolic disorders and blood coagulation disorders. Trials of gene therapy for infectious diseases, including acquired immunodeficiency syndrome and COVID-19, have also been reported recently [131]. In fact, large-animal models have substantial advantages in gene-therapy studies. Similarly to humans, large animals commonly have a heterogeneous genetic background, unlike inbred mice; have long lifespans enabling investigation of long-term effects; and have relevant organs and body sizes matching those of neonates or children, thus providing unique opportunities to

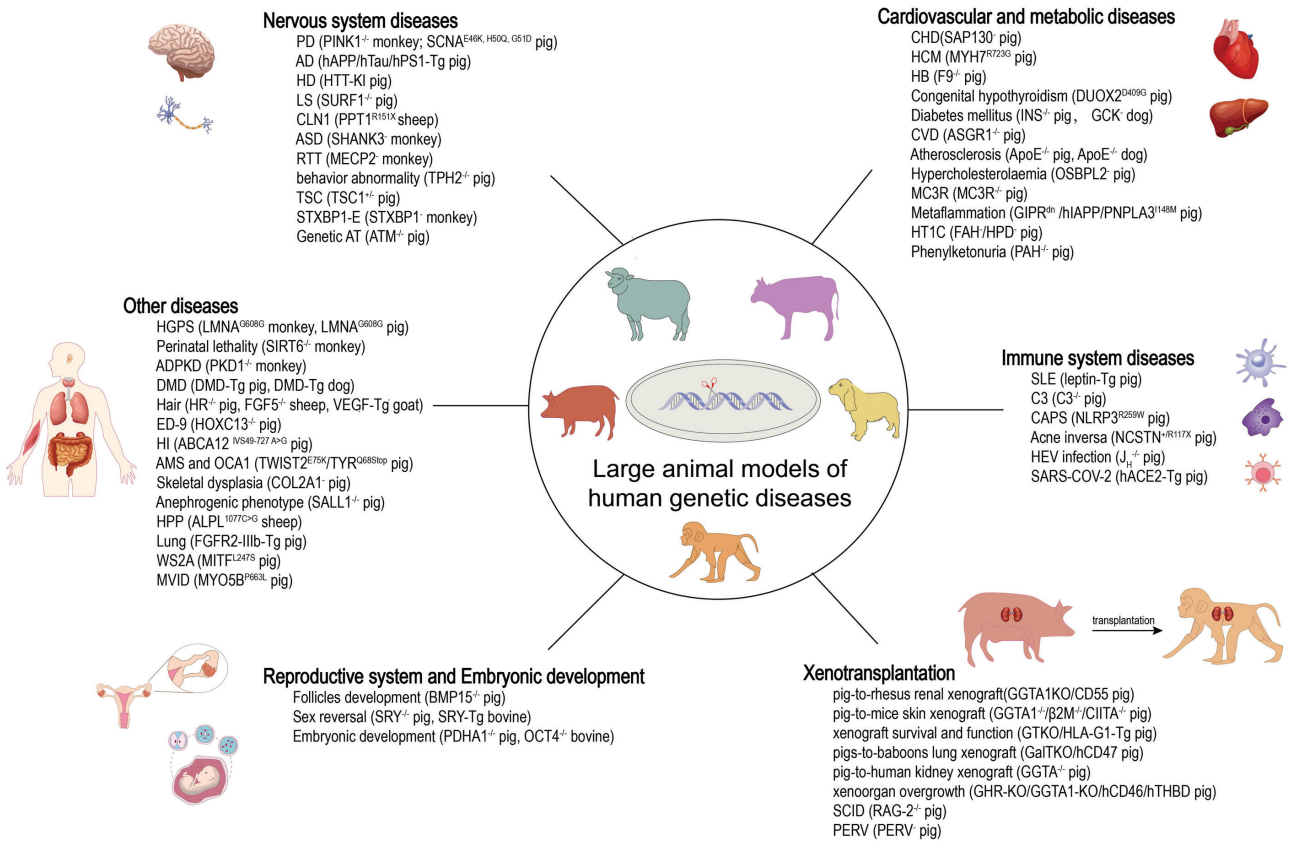

Figure 1 The genetically modified large animal models of human diseases. –/– represents konckout. Tg means transgenic. – means mutation.

address issues associated with gene therapy [132]. Specially, large animals have been considered suitable for modeling human neurodegenerative diseases, largely because large animals have similar brain sizes to those in humans [133] and have a sulcated cortex, which is not observed in rodents [134]. Because of the advantages of their long life span, gene therapy targeting cystic fibrosis in large animals has been successfully demonstrated [135]. Large animals have also been used for developing and assessing novel gene-delivery techniques. For example, the clinically well established and catheter-based antegrade delivery methods, as evaluated in large-animal models, are safer than other invasive delivery techniques, which is a critical aspect for treating patients with cardiac disease [136]. Furthermore, numerous gene-delivery systems, including viral and non-viral gene-delivery systems, have recently been developed for use in gene therapy. Indeed, large animals are ideal models for evaluating the specificity, safety and efficiency of these gene-delivery systems.

Collectively, the production of large-animal models for human inherited diseases has made considerable advances resulting from the rapid progress in genome editing in large animals (**Figure 1**; Table S2). Further development of genetic manipulation tools with higher gene modification efficiency, as well as better gene-delivery efficiency and specificity, will support broader application of large-animal models in translational biomedical research.

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