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Review Article

Genetic engineering drives the breakthrough of pig models in liver disease research

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ABSTRACT

Compared with the widely used rodents, pigs are anatomically, physiologically, and genetically more similar to humans, making them high-quality models for the study of liver diseases. Here, we review the latest research progress on pigs as a model of human liver disease, including methods for establishing them and their advantages in studying cystic fibrosis liver disease, acute liver failure, liver regeneration, non-alcoholic fatty liver disease, liver tumors, and xenotransplantation. We also emphasize the importance of genetic engineering techniques, mainly the CRISPR/Cas9 system, which has greatly enhanced the utility of porcine models as a tool for substantially advancing liver disease research. Genetic engineering is expected to propel the pig as one of the irreplaceable animal models for future biomedical research.

1. Introduction

Pigs are anatomically, physiologically, immunologically, and genomically similar to humans. They have been used as representative models for experimental studies in the fields of ophthalmology, locomotor systems, reproduction and development, microbial research, brain and neurodegenerative diseases, cardiac diseases, pulmonary diseases, and tumors.[1–7] Pigs have a short gestation period, high reproductive capacity, and can be induced to exhibit a wide range of phenotypes through domestication and artificial genetic selection.[8] Furthermore, the use of pigs raises fewer ethical concerns compared to the use of nonhuman primates (NHPs). Consequently, pigs are now considered high-quality tools for biomedical research.[9]

China has among the highest burdens of liver disease globally.[10] According to recent epidemiological reports, more than one-fifth of the Chinese population is affected by some form of liver disease.[11] Viral hepatitis remains the main cause of the high burden of end-stage liver disease.[12] The rate of metabolic liver disease, mainly metabolic dysfunction-associated steatotic liver disease (MASLD), is increasing annually, and it is becoming increasingly prevalent in younger age groups.[13] This serious epidemiological background and the heterogeneity of liver diseases provide important ideas for model animal research. Although classical model organisms, such as *Drosophila*, zebrafish, and rodents, have well-established systems of scientific output in related fields, they do not closely reflect human biology. Thus, there is an urgent need to use porcine models as the main platform for model design and experiments in research on liver diseases. By using genetic engineering techniques, pig models with particular gene mutations that resemble the genetic background of liver disease in humans can be created. These models can then be used to test new medications or treatments, assisting in the assessment of their safety and efficacy. This offers researchers studying liver disease an effective tool and resource.

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In this paper, we review the latest research progress on pigs as models for human liver diseases, including cystic fibrosis liver disease (CFLD), acute liver failure (ALF), MASLD, and liver tumors, as well as liver regeneration and xenotransplantation, for which they offer significant advantages. The introduction of genetic engineering has popularized the use of porcine models, and we hope that our effort of combining currently available technologies with porcine models in liver disease research will be thought-provoking and provide researchers with some practical paradigms for their work.

2. Rationale for using pig as liver disease models

2.1. Liver features are similar in pigs and humans

Certain strains of pigs, such as Duroc, Large White, and miniature pigs, are highly suitable for preclinical studies because of some similarities between their liver and adult human livers (Table 1).[14–17] Despite differences among these pig strains, all of their livers present well-defined lobes, segments, a complete Glisson system, and polygonal lobules that constitute the basic functional unit. Unlike humans, liver lobules in pigs are irregularly pentagonal and have connective tissue intervals. In terms of liver weight, liver lobes, liver segments, liver enzymes, serum albumin and other physiological parameters, pig and human were basically similar. The porcine portal vein has substantial supplies of blood and oxygen and is crucial for testing vascular occlusion and therapeutic efficacy of drugs.[14,15] Regarding the feasibility of porcine liver xenotransplantation, it has been observed that the liver of certain strains of pigs has the capacity to synthesize proteins that are essentially equivalent to those of humans. In addition, the serum concentration of albumin in pigs is lower than that in humans, and the amino acid sequences identity of the two can be up to 65%.[18,19]

Such consistency is also reflected in the liver enzyme studies. The cytochrome P450 superfamily (CYP) has been shown to play an important role in drug metabolism and elimination, and the main P450 enzyme isoform expressed in the human liver is CYP3A4. Four isoforms, namely, CYP3A22, CYP3A29, CYP3A39, and CYP3A46, from pigs do not differ significantly from CYP3A in humans in terms of qualitative function and expression, and their amino acid sequences converge significantly.[20–23] Van Peer *et al.*[24] investigated the changes in CYP3A expression in neonatal pigs, suggesting that the pig is a suitable model for assessing the development of liver enzyme profiles in neonates.[16]

However, there are some clear differences between pigs and humans that should not be overlooked, especially in terms of the complement and coagulation systems, which exhibit significant species-specificity. Indeed, this is one of the main obstacles to the use of porcine models for xenotransplantation. Genome editing currently represents a promising approach for solving this problem and can be applied in multiple fields.

2.2. Advances in genetic engineering technology enhance the usefulness of pig models

Gene editing has become a rudder for human beings to change the trajectory of life. Since the establishment of the Swine Genome Sequencing Consortium in 2003, exhaustive whole-genome sequences and functional whole-genome annotation systems have been established,[1,25–27] which have greatly improved the utility of pigs as models. Pigs also have chromosomes homologous to those of humans,[28,29] and the size and composition of their genomes are comparable to those of humans. It has also been highlighted that the brain, liver, and lymphoid tissues have gene classifications that are highly similar between these two species.[27]

In fact, pigs have long served as one of the primary models for genetic engineering. In 1985, Hammer *et al.*[30] produced the world's first transgenic pig via microinjection. In recent years, somatic cell nuclear transfer (SCNT) technology has been applied in various large animals,[31] providing a reproductive basis for the establishment of transgenic animal models. Programmable nucleases, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system, are among the core elements of gene editing. Among these programmable nucleases, the CRISPR/Cas9 system introduces specific genomic sites for targeted modification through simple pairing with guide RNA sequences and has basically replaced ZFNs and TALENs as the main tool for gene editing since its mechanism was elaborated and implemented, lifting the bottleneck of human manipulation of the genome.[32] The synergistic use of the CRISPR/Cas9 system with SCNT allows rapid and efficient generation of target models in large animals, including NHPs.[31] The CRISPR/Cas9 system is a bacterial immune system that has evolved to combat exogenous genetic material as well as provide acquired immunity. The CRISPR/Cas9 system consists of two key components: a Cas9 protein that cleaves double-stranded DNA and a gRNA (guide RNA) that serves as a guiding signal. The CRISPR/Cas9 technology uses a gRNA complementary to the target sequence to direct the Cas9 nuclease to recognize and cut the specific target DNA, resulting in double-stranded or single-stranded DNA breaks that are then repaired using two DNA repair mechanisms, namely, nonhomologous end joining and homology-directed repair, which can achieve gene insertion and knockout.[33] Combined with a referenceable high-quality porcine genome database,[25] the CRISPR/Cas9 system has

Table 1
Liver general features comparing pigs and humans.

Attributes	Pig	Adult human
Liver weight (kg)	1.2–2.5	1.0–2.3
Liver weight to body weight (%)	2–3	Approximately 2
Liver lobes and liver segments	4–6 lobes, 8 segments	4 lobes, 8 segments
Hepatic lobules	Irregularly pentagonal hepatic lobules with connective tissue septa	Hexagonal liver lobules without leaflet intervals
Serum albumin concentration (g/L)	19–29	40–55
Serum globulin concentration (g/L)	28–41	20–30
Serum cholesterol concentration (mmol/L)	2.0–4.2	2.9–6.0
Aspartate aminotransferase (U/L)	0–125	8–40
Alanine aminotransferase (U/L)	0–103	5–40
Alkaline phosphatase (U/L)	0–300	45–125
γ-Glutamyltransferase (U/L)	0–82	11–50
Total bilirubin (μmol/L)	0–1.0	3.4–17.1

All data presented in this table are based on the findings reported in References 14–17.

been used to accurately edit a variety of human disease models, such as those of cardiovascular diseases, neurodegenerative diseases, immunodeficiencies, cystic fibrosis (CF), and tumors.[2,5] It has also achieved breakthroughs in xenotransplantation through target gene knockdown and humanized gene transfer, such as the recent landmark practice of transplanting porcine hearts and livers into human beings.[34] Against this background, genome editing in pigs has great potential in liver research and is expected to compensate for the lack of hepatology models and the distortion of information when small animals mimic humans (Table 2).[35–48]

3. Porcine models of nontumorigenic liver diseases

Non-neoplastic liver diseases often present as liver injury of varying degrees of severity, leading to a poor prognosis. Liver injury is a broad concept, referring to all kinds of liver inflammatory diseases caused by infection, immunity, drugs, metabolism, and other factors. Experimental porcine liver disease models hold significant promise for research on the pathophysiological mechanisms of liver injury, surgical technique selection, drug screening, and more. This is supported by the systematic establishment of relevant models.

3.1. CFLD

CF is an autosomal recessive disorder caused by mutations in the gene encoding CF transmembrane conductance regulator (CFTR), leading to abnormal function and chronic inflammation in organs such as the lung, pancreas, liver, and gastrointestinal tract.[49] More than two-thirds of CF cases are attributed to the deletion of the phenylalanine residue in CFTR due to the *ΔF508* mutation.[50] CFLD, a serious complication of CF, manifests as focal biliary cirrhosis in the early stage and can gradually develop into irreversible portal hypertension,[51] which is the third most common cause of death in CF cases.[52]

Mice are widely used as model animals for studying CF. Indeed, numerous heterogeneous phenotypes have been generated by targeting *CFTR* knockdown. However, most of these phenotypes do not show significant hepatic pathomorphology,[53] thereby limiting the use of mice as a model for CFLD. In 2008, Rogers *et al.*[54] used recombinant adeno-associated virus (rAAV) to disrupt *CFTR* in porcine fibroblasts and generated two piglet models through SCNT: $CFTR^{-/-}$ model with complete allelic deletion and *ΔF508* mutation model with the highest population prevalence, which ensured the degree of phenotypic reversion and population representation of CFLD, respectively. These model pigs have abnormal manifestations of focal cirrhosis, cholestasis, and biliary hyperplasia similar to those of neonatal patients.[55] Over time, inflammation spreads at the level of the portal vein and the liver disease progresses with bridging fibrosis and steatosis, which is consistent with the findings in humans.[35] In addition to effectively simulating the actual disease conditions, the CF pig models facilitate the acquisition of secretions such as pancreatic juice and bile. They also mimic the impact of nutritional differences after birth on changes in liver function,[56] playing a prominent role in alternative research aimed at developing treatments for CFLD.

3.2. ALF

In 1991, Terblanche *et al.*[57] proposed criteria for an ideal animal model of ALF; these criteria were as follows: reversibility, reproducibility, availability of a therapeutic window, a large animal model, and safety for practitioners. The large animals most commonly used as ALF models are pigs and dogs,[58] and the superiority of pigs in terms of liver anatomy and physiology makes them an important option for ALF modeling. Models are generally developed through surgery or drugs. Surgical modeling includes a total hepatectomy model,[59] partial hepatectomy model,[60] and hepatic ischemia model formed by occlusion of the hepatic artery after portal vena cava shunt surgery.[61] Meanwhile, commonly used hepatotoxic drugs include acetaminophen and galactosamine[62–64]; the use of carbon tetrachloride has also been reported.[65,66] There is also a trend for the establishment of models produced by the combination of surgery and drugs.[58]

Owing to the rapid progression and high mortality associated with ALF, models established to date have varying degrees of limitations. Although pigs are superior to other animals, the models still need to be optimized because of the difficulty of surgery, laboriousness and high cost of operation, and inability to recapitulate the inflammatory environment in ALF.[67] Other problems include the extrahepatic toxicity of drug-forming molds, poor reproducibility, and toxicity and damage to the human body. Recently, a method to induce ischemia-reperfusion injury after liver-directed radiation therapy on NHPs for the modeling of ALF was reported.[68] The model generated using this method was reliable, reproducible, and suitable for the establishment of ALF pigs.

Table 2
Summary of gene-edited porcine liver models.

Model	Gene target	Characteristic phenotype	Reference(s)
Cystic fibrosis liver disease	*CFTR* knockout	Focal biliary cirrhosis	35,36
Liver tumor	$TP53^{R167H}$/$KRAS^{G12D}$ transgene	Hepatocellular carcinoma	37
	AXIN1/ARID1A knockout	Enhancement of hepatocellular carcinoma proliferation and migration	38
Hepatocyte autophagy	*PIK3C3* transgene	Hepatocyte apoptosis and inflammatory cell infiltration	39
Immunodeficiency or hereditary tyrosinemia type 1	*RAG2/IL2Rγ/FAH* knockout	Severe immunodeficiency or hereditary tyrosinemia type 1, or as a humanized liver donor	40
	FAH/RAG1 knockout		41
	FAH knockout		42
	FAH/HPD knockout	*HPD* knockout attenuates liver injury due to hereditary tyrosinemia type 1	43
Humanized liver protein expression model	*hF7/hALB* transgene	Expression of human coagulation factor VII and ALB	44
Non-alcoholic steatohepatitis	*ALOX12* knockout	Delaying the progression of non-alcoholic steatohepatitis	45
	$PNPLA3^{I148M}$/$GIPR^{dn}$/*hIAPP* transgene	Liver inflammation, lipid infiltration, and metabolic disorders	46
	MC4R knockout	Hyperphagia, dyslipidemia, and hepatic steatosis	47
Hepatic developmental defects	*HHEX* knockout	*HHEX* knockout causes hepatic developmental defects rescued by blastocyst complementation	48

Abbreviations: ALB, albumin; ALOX12, arachidonate 12-lipoxygenase; ARID1A, AT-rich interaction domain 1A; CFTR, cystic fibrosis transmembrane conductance regulator; FAH, fumarylacetoacetate hydrolase; GIPR, glucose-dependent insulinotropic polypeptide receptor; hIAPP, human islet amyloid polypeptide; HPD, 4-hydroxyphenylpyruvate dioxygenase; KRAS, Kirsten rat sarcoma viral oncogene homologue; MC4R, melanocortin 4 receptor; PIK3C3, phosphatidylinositol 3-kinase catalytic subunit type 3; PNPLA3, patatin like phospholipase domain-containing protein 3; RAG, recombination activating gene.

Though sophisticated constructs are still lacking, we believe that genetic engineering might offer a time-switching effect to trigger liver injury at the appropriate moment, as in the mouse model of the CreERT system.

3.3. Liver regeneration models

Liver regeneration is a process that enables successful regional resection of diseased liver and partial liver transplantation.[69] In models of induced liver injury, partial hepatectomy is more controllable to perform on large animals than the administration of hepatotoxic drugs and is considered to be the event conferring the strongest stimulation of liver regeneration.[70] The possible extent of resection of porcine liver has been widely discussed, with 85% resection being highlighted as an extreme proportion, at which point the serious clinical problem of posthepatectomy liver failure (PHLF) can be easily triggered.[70,71] In contrast to that in other large animals, the regenerative response after partial hepatectomy in pigs is completely predictable,[72] with differential expression of genes involved in cell proliferation, inflammatory regulation, and molecular metabolism with increasing resection extent and elevated portal pressure.[73] Inomata *et al.*[74] reported the use of alkaloids to construct the first porcine model of hepatic regeneration inhibition, which may be used for assessing the success of liver transplantation. Recently, a potent and highly selective small-molecule liver regeneration-promoting drug, HRX215, was developed.[75] In a porcine 85% hepatectomy model, HRX215 effectively prevented the onset of ALF and maintained normal liver functions, such as metabolism and synthesis of coagulation-related proteins. HRX215-mediated hepatic regeneration effectively prevented 85% of the lethal PHLF after hepatectomy.

A better model of liver regeneration can be constructed using target gene knockout. Hereditary tyrosinemia type 1 (HT1) is a metabolic liver disease caused by loss of fumarylacetoacetate hydrolase (FAH) activity, which can lead to lethal liver injury.[43] In 2011, Hickey *et al.*[42] generated *FAH*$^{-/-}$ pigs through chimeric adeno-associated virus-mediated knockout for the expansion of high-quality human hepatocytes. Subsequently, in 2016, they successfully implemented HT1, an *ex vivo* liver-targeted gene therapy based on liver regeneration.[76] They isolated hepatocytes after partial hepatectomy in *FAH*$^{-/-}$ pigs and transduced corrected hepatocytes through lentiviral vectors to express therapeutic FAH. Almost complete hepatic regeneration was achieved and tyrosine metabolism was corrected after autologous re-infusion of FAH^{+} hepatocytes via the portal vein. Recently, Ren *et al.*[41] combined CRISPR/Cas9 with SCNT to generate a severely immunodeficient *FAH*$^{-/-}$ porcine model, which expressed high levels of human albumin 1 week after colonization of human hepatocytes.

These porcine models serve as excellent alternatives for studying human liver regeneration. They offer a liver microenvironment rich in complex signaling pathways essential for liver regeneration, closely resembling the primary hepatocyte proliferation observed in humans. This is challenging to replicate in artificial *in vitro* cultures and organoid constructs. Porcine models have significantly contributed to our understanding of the mechanisms through which the liver regenerates and to the advancement of clinical procedures. Moreover, the concept of using pigs as donors for large-scale production of humanized livers is gradually gaining traction.

3.4. MASLD

MASLD is the most common metabolic liver disease. In recent years, the global prevalence of MASLD has continuously increased, and this condition directly affects approximately 300 million people in China.[11] Metabolic dysfunction-associated steatohepatitis (MASH), the inflammatory subtype of MASLD, is clinically insidious and has become a major cause of progressive liver fibrosis and end-stage liver disease.[77] Ideal animal models of MASH possess disease phenotypes similar to those of humans, including obesity; insulin resistance; and histological features of the liver, such as macrovesicular steatosis, with varying degrees of lobular inflammation and fibrosis.[78] Pigs, although not as widely used as mice, can cover most of the clinical signs of MASH progression and have been increasingly employed experimentally.

Disease induction via dietary manipulation is a common and effective means of modeling MASH. When fed a high-fat/fructose/cholesterol diet, model pigs steadily develop a metabolic syndrome characterized by obesity, insulin resistance, and dyslipidemia, which then reverts to the hepatic manifestations of MASH.[79–81] However, this approach does not achieve consistent effects; for example, some Ossabaw pigs or those with MASH resistance show only microvesicular steatosis and do not develop hepatic fibrosis.[79,80] Furthermore, a high-fructose diet may not induce MASLD in pigs.[82,83] Unlike in humans and mice, adipose tissue is the main target tissue for fructose-induced lipogenesis in pigs, and it is often difficult to form hepatic macrovesicular steatosis via a cumulative effect.

In metabolic diseases, sex selection has a profound effect on modeling outcomes. Studies have revealed protective effects of high levels of testosterone and estradiol in metabolic male piglet models. In contrast, female piglets, with higher visceral fat mass and greater insulin resistance, may have greater potential than male piglets for diet-dependent metabolic modeling.[84,85] In contrast, in model mice, males are more sensitive to diet-induced MASH than females due to differences in estrogen levels between the two sexes.[86]

Although modeling using a high-fat diet has been reported, the modeling period is as long as 6–12 months and the outcome is unstable; therefore, the value of such models for the high-throughput, rapid, and accurate screening of drugs cannot be guaranteed. With the breakthrough provided by the establishment of the CRISPR/Cas9 system, the mining and editing of metabolism-related genes can hopefully resolve this issue. In China, Zhang *et al.*[45] used a pig model with knockout of the arachidonate 12-lipoxygenase gene (*ALOX12*) to confirm that ALOX12 is a key target to promote steatohepatitis and thus developed a new therapeutic drug targeting it. In a porcine model, ALOX12 deficiency ameliorated high-fat-diet-induced hepatic lipid deposition, inflammatory cell infiltration, and hepatic fibrosis, resulting in a broad protective effect against MASH progression. Furthermore, the transfer of the humanized metabolic disease risk gene *PNPLA3*^{I148M}-*GIPR*^{dn}-*hIAPP* was found to increase the risk of hepatic inflammation, lipid infiltration, and metabolic disorders,[46] and the knockout of the melanocortin 4 receptor gene (*MC4R*) led to hyperphagia and dyslipidemia.[47] Both of these approaches can be used to establish a porcine model of hepatic steatosis.

3.5. Others

In recent years, various porcine models of liver disease have been developed. In addition to the above models, viral hepatitis can be induced in pigs through static injection of viral strains and oral administration of immunosuppressive agents.[87] Meanwhile, folate-deficient porcine models have been shown to exhibit abnormal methionine metabolism, which promotes the progression of alcoholic liver disease.[88] Finally, the experimental use of pigs for parasitological purposes, such as hepatic schistosomiasis, has been reported.[89]

4. Porcine models of hepatic tumor

There is a significant attrition rate in the development of antitumor drugs, as only approximately 5% of new medications show sufficient efficacy in phase III clinical trials for them to be approved.[90] This is partly attributable to the absence of suitable preclinical animal models. Pigs serve as an ideal model for oncology and related drug research. In addition to the similarities in anatomical and physiological characteristics, hepatic drug enzyme metabolism, and body size between pigs and humans, the longevity of pigs permits the monitoring of preclinical drug treatment responses over longer spans and allows multistage simulation of tumorigenesis, invasion, and metastasis.

Drug induction is a conventional method of constructing models. For example, intraperitoneally injected *N*-nitrosodiethylamine (DENA) induces hepatocellular carcinoma (HCC) combined with cirrhosis in pigs, but the latency period of the tumors is up to 10 months or more.[91] The carcinogen-induced formation of HCC can be accelerated by combining with phenobarbital (PB).[92] Despite the shortening of the latency period to 5–11 months, a small proportion of pigs did not develop tumors; moreover, a valid comparison of the timeline of tumor formation was difficult due to the limitation of the number of modeled pigs.

The Oncopig Cancer Model (OCM) is a targeted model designed to study tumorigenesis at specific tissue sites and during specific timeframes through Cre recombinase-induced expression of the $TP53^{R167H}$ and $KRAS^{G12D}$ transgenes.[93] The isolation of hepatocytes from OCM livers and their exposure to an adenovirus vector encoding the Cre recombinase gene (AdCre) *in vitro* resulted in cell transformation, generating HCC cell lines that recapitulated human HCC features in terms of pathology and gene expression.[94] The obtained cell lines were injected percutaneously into porcine livers to establish a reproducible Oncopig HCC model, allowing the execution of artificial spatiotemporal regulation.[95] The Oncopig HCC cell lines were genetically manipulable, which provided the opportunity to introduce targeted mutational profiles into OCM; for example, the knockdown of *AXIN1* and/or *ARID1A* in the cell lines using the CRISPR/Cas9 system strengthened the ability of HCC to proliferate and migrate.[38] Although OCM demonstrates significant advantages in modeling, it does not generate spontaneous tumors and may not be characterized by tumor tropism and dependence on conditional vasculature. Therefore, drug induction cannot completely be replaced. Porcine oncology is a novel and promising field, and as modeling methods are refined and updated, more transferable medical value will be discovered.

5. Pigs are the protagonists in xenogeneic liver transplantation

5.1. Molecular basis of xenotransplantation

Liver transplantation is an effective treatment for end-stage liver disease. Despite the maturity and widespread availability of modern allogeneic liver transplantation,[96] there has always been a serious imbalance between the available liver supply and the number of patients awaiting transplantation. In the United States, more than 52,000 patients have died awaiting a donated liver over the last 20 years.[97] Pigs are high-quality donors for human organs, and exploration of the use of their livers for transplantation into humans has been ongoing to improve the availability of liver sources. However, one of the main obstacles in this approach is transplant rejection. Owing to interspecies differences, the presence of antibodies against donor tissue antigens in the recipient triggers activation of the complement system when these antibodies bind to antigenic epitopes on porcine endothelial cells, causing hyperacute rejection (HAR), which results in graft damage.[98] The CRISPR/Cas9 technology has overcome the limitations of genetic engineering in xenotransplantation research. The CRISPR/Cas9 system is based on a repair mechanism for double-stranded DNA breaks, and cells use repair pathways to process these breaks, leading to insertion, deletion, or precise integration of the target DNA fragment. This enables us to intervene based on effective gene editing strategies.[99] Using a porcine model as an organ repository, CRISPR/Cas9 constructs were introduced to counteract various adverse reactions, such as HAR induced by human natural anti-pig Abs and complement, as well as platelet coagulation and cellular immune response.[100,101] Fig. 1 depicts the workflow for obtaining gene-edited pigs for xenotransplantation using the CRISPR/Cas9 technology.

The major antigenic epitope that mediates HAR is alpha-galactose (αGal), which is widely present in nonhuman mammals.[102] Because of loss of function of alpha-1,3-galactosyltransferase (*α-1,3GT*), humans and some nonhuman primates lack the α-Gal epitope, but approximately 70%–90% of the antibodies against HAR produced in these species specifically target the α-Gal epitope.[103] In addition, two other non-Gal epitopes have been demonstrated: *N*-glycolylneuraminic acid (Neu5Gc) and SDa glycans,[104,105] corresponding to the key genes cytidine monophosphate-N-acetylneuraminic acid hydroxylase (*CMAH*) and beta-1,4-N-acetylg alactosaminyltransferase 2 (*β-4GALNT2*), respectively. The discovery of these antigenic epitopes is the molecular basis for prolonging graft survival. Currently, two approaches are generally used to prevent rejection: one involves targeting the removal of genes for the above antigenic epitopes to form a triple-gene-knockout (TKO) pig model and the other involves inhibiting complement activation by transducing human genes and inducing the expression of human complement-regulating proteins (hCD46 and hCD55).[106] The CRISPR/Cas9 technology has enabled advancement of the above gene editing strategies. Triple gene disruption was shown to minimize IgM and IgG binding in over 90% of humans.[107] The same results were obtained in another porcine model with the triple knockout of *α-1,3GT*, *CMAH*, and *iGb3S*, but the disruption of *iGb3S* was shown to be of little significance in later studies.[108,109] Currently, single, double, or triple knockout of *α-1,3GT*, *CMAH*, and *β-4GALNT2* has been recognized by multiple research teams and used as the basis for gene-edited pig models. Cells from transgenic piglets expressing the human complement binding inhibitors CD46, CD55, and CD59, human HO1, and A20 were completely protected against human complement-mediated cleavage and were effective in preventing damage from HAR.[110] Porcine endogenous retrovirus (PERV) can be transmitted and integrated into the genome of human cells and is a potential barrier to xenotransplantation. To reduce the risk of cross-species infection, *PERV* also needs to be knocked out in donor pigs.[111] Table 3 demonstrates common genetic modifications in xenograft pig models.

5.2. Advances in porcine xenogeneic liver transplantation

After more than 70 years of refinements since Joseph Murray's first successful kidney transplant in 1954 to today's practice of using pig hearts for human transplantation,[112] breakthroughs have been made with pig heart and kidney transplants, leading to graft survival times of up to hundreds of days.[113,114] However, efforts in liver transplantation have not led to such significant advances. In addition to innate rejection, lethal thrombocytopenia and severe coagulation disorders represent challenges. The first *in situ* transplantation of unmodified wild-type porcine liver into baboons was performed by Calne *et al.*[115] in 1968, but most baboons developed uncontrollable hemorrhage after the

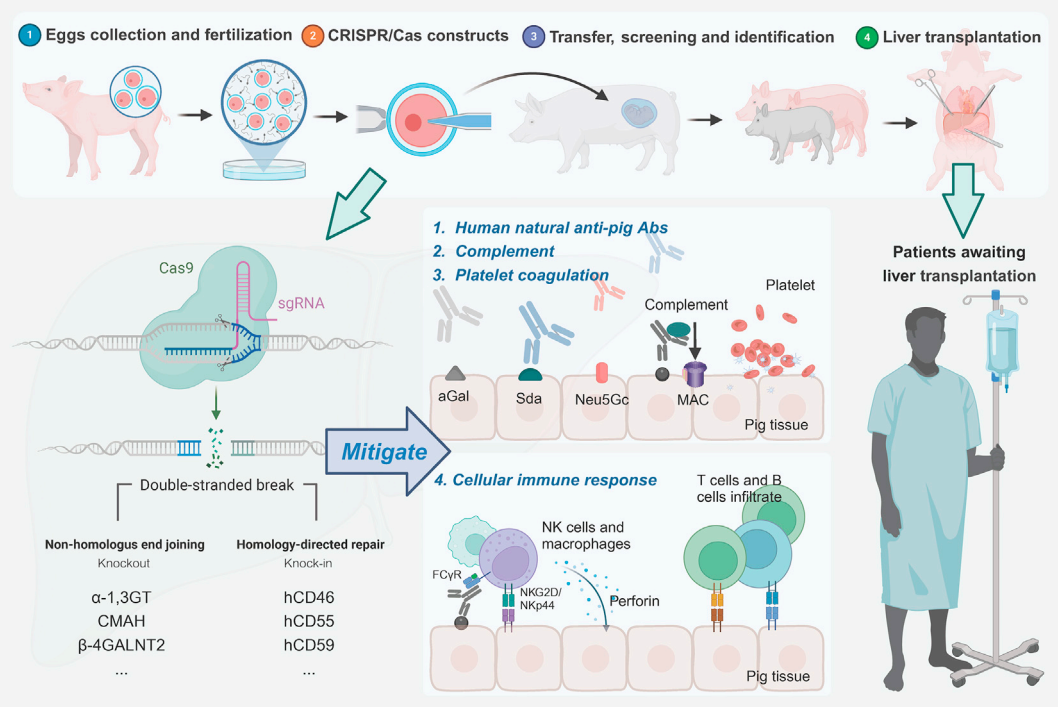

Fig. 1. The workflow for obtaining gene-edited pigs for xenotransplantation using the CRISPR/Cas9 technology. Abbreviations: α-1,3GT, alpha-1,3-galactosyltransferase; αGal, alpha-galactose; β-4GALNT2, beta-1,4-N-acetylg alactosaminyltransferase 2; CMAH, cytidine monophosphate-N-acetylneuraminic acid hydroxylase; MAC, membrane attack complex; NK, natural killer; NKG2D, natural killer group 2 member D; sgRNA, single-guide RNA.

Table 3
Common genetic modifications in xenograft porcine models.

Gene editing method	Gene target		Key gene
Knockout	alpha-1,3-galactosyltransferase		*α-1,3GT*
	N-glycolylneuraminic acid hydroxylase		*CMAH*
	The key enzyme for SDa synthesis		*β-4GALNT2*
	Porcine endogenous retrovirus		*PERV*
Knock-in	Complement regulatory protein	Human membrane cofactor protein	*hCD46*
		Human decay accelerating factor	*hCD55*
		Human membrane attack complex inhibitory factor	*hCD59*
	Coagulation regulatory protein	Human endothelial protein C receptor	*hEPCR*
		Human ectonucleoside triphosphate diphosphohydrolase-1	*hCD39*
		Thrombomodulin	*TBM*
		Human tissue factor pathway inhibitor	*hTFPI*
	Anti-inflammatory factor	Heme oxygenase-1	*HO-1*
		Protein A20	*A20*
		Human integrin-associated protein	*hCD47*
	Immune regulation	Human cytotoxic T lymphocyte-associated antigen 4 immunoglobulin	*hCTLA4-Ig*
		Human programmed cell death receptor ligand 1	*hPD-L1*
		Human leukocyte antigen E	*HLA-E*

All data presented in this table are based on the findings reported in References 97 and 106.
Abbreviations: β-4GALNT2, beta-1,4-N-acetylg alactosaminyltransferase 2; CMAH, cytidine monophosphate-N-acetylneuraminic acid hydroxylase.

procedure, with maximum survival of 3.5 days. For almost 30 years thereafter, whether rhesus monkeys or gorillas were chosen, the survival time of recipients did not improve due to the lack of methods to appropriately prevent rejection. However, the value of using gene editing to overcome interspecies rejection was gradually emphasized. In 2000, Ramirez *et al.*[116] first used porcine livers transgenically expressing human complement regulator decay accelerating factor (hCD55) for transplantation into baboons, which survived up to 4 and 8 days, with no hyperacute rejection being detected. Although they eventually died of sepsis and coagulation disorders, unmodified livers survived only 12 h after transplantation, showing the huge benefit of this approach.

In 2010, Ekser *et al.*[117] knocked down porcine *α-1,3GT* (GTKO) and transgenically expressed hCD46, which resulted in liver grafts with robust detoxification, protein synthesis, and coagulation but led to secondary severe thrombocytopenia. In 2012, Kim *et al.*[118] used Amicar to maintain post-transplant recipient platelet counts and prolong survival to 9 days. Meanwhile, in 2017, Shah *et al.*[119] continuously infused human plasminogen concentrate complex and costimulation blockers after GTKO liver transplantation to avoid coagulation disorders and achieve spontaneous platelet recovery, but this increased the risk of thrombosis. The maximum survival of this regimen reached 29 days. Encouragingly, some research teams from China have recently made major breakthroughs in clinical research on xenogeneic liver transplantation.

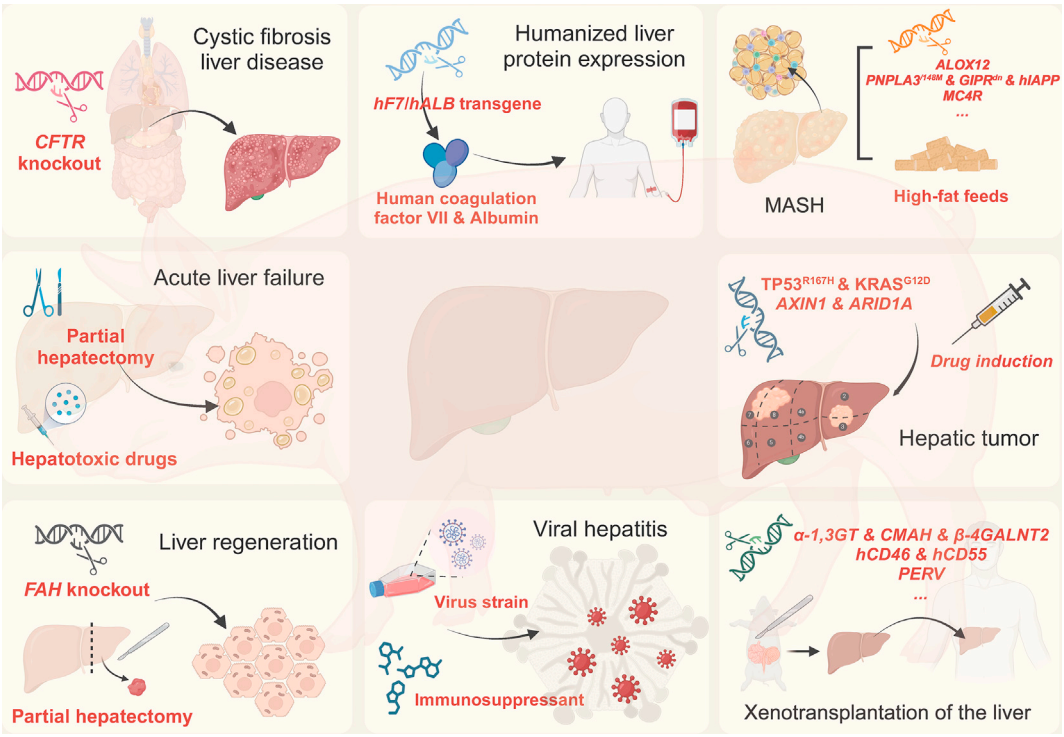

Fig. 2. Summary of liver disease models in pigs. Abbreviations: α-1,3GT, alpha-1,3-galactosyltransferase; ALB, albumin; ALOX12, arachidonate 12-lipoxygenase; ARID1A, AT-rich interaction domain 1A; β-4GALNT2, beta-1,4-N-acetylg alactosaminyltransferase 2; CFTR, cystic fibrosis transmembrane conductance regulator; CMAH, cytidine monophosphate-N-acetylneuraminic acid hydroxylase; FAH, fumarylacetoacetate hydrolase; GIPR, glucose-dependent insulinotropic polypeptide receptor; hIAPP, human islet amyloid polypeptide; KRAS, Kirsten rat sarcoma viral oncogene homologue; MASH, metabolic dysfunction-associated steatohepatitis; MC4R, melanocortin 4 receptor; PERV, porcine endogenous retrovirus; PNPLA3, patatin like phospholipase domain-containing protein 3.

For example, Kefeng Dou's team successfully transplanted a whole liver from a multigene-edited pig in an assisted manner into a brain-dead patient. During the operation, the transplanted liver secreted bile immediately after blood flow was restored without hyperacute rejection and maintained its function for 10 days. These exciting findings demonstrate that xenotransplantation using gene-edited pigs as donors is a promising way to address the shortage of transplantable organs. In addition to living organs, liver cell xenotransplantation from genetically engineered pigs has been conducted.[120]

Currently, several limitations exist in xenotransplantation, and substantial efforts are still needed in the discovery and optimal combination of genetic modifications, screening of immunosuppressive agents, and improvement of post-transplantation adjuvant supportive therapy.

6. Summary and outlook

This article summarizes recent research progress on pigs as a model for human liver disease (Fig. 2). Biological similarities between pigs and humans provide significant advantages in using pigs for treating CFLD, ALF, MASLD, and liver tumors as well as for liver regeneration and xenotransplantation. Genetic engineering has greatly enhanced the utility of porcine models, providing more opportunities to customize target forms while broadening the spectrum of model-forming diseases. However, pigs require more space and involve higher maintenance costs than rodents. In addition, some operations may require separate housing. Moreover, to avoid stress, operators need to be highly trained to perform treatments, such as injections.[4] Considering their long reproductive cycle, pigs are also not suitable for large-scale experiments. Furthermore, some porcine models have not developed stable disease phenotypes. A wide variation exists in modeling results across strains, and most experimental results are difficult to translate to a clinical context. These factors are both limiting and promising, and they show potential avenues for future exploration. In the future, it is anticipated that porcine models will be increasingly accepted as a model of choice for biomedical research.

Authors' contributions

Chenhao Xu: Writing – original draft. **Xixi Fang:** Visualization, Investigation. **Xiao Xu:** Supervision. **Xuyong Wei:** Supervision. All authors read and approved the final version of the manuscript.

Declaration of competing interest

The authors declare that there is no conflicts of interest. All figures were created with BioRender.com.

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References

1. Groenen MA, Archibald AL, Uenishi H, et al. Analyses of pig genomes provide insight into porcine demography and evolution. *Nature*. 2012;491:393–398. https://doi.org/10.1038/nature11622.

2. Lunney JK, Van Goor A, Walker KE, Hailstock T, Franklin J, Dai C. Importance of the pig as a human biomedical model. *Sci Transl Med*. 2021;13:eabd5758. https://doi.org/10.1126/scitranslmed.abd5758.
3. Meurens F, Summerfield A, Nauwynck H, Saif L, Gerdts V. The pig: a model for human infectious diseases. *Trends Microbiol*. 2012;20:50–57. https://doi.org/10.1016/j.tim.2011.11.002.
4. Pabst R. The pig as a model for immunology research. *Cell Tissue Res*. 2020;380:287–304. https://doi.org/10.1007/s00441-020-03206-9.
5. Perleberg C, Kind A, Schnieke A. Genetically engineered pigs as models for human disease. *Dis Model Mech*. 2018;11:dmm030783. https://doi.org/10.1242/dmm.030783.
6. Schook LB, Collares TV, Darfour-Oduro KA, et al. Unraveling the swine genome: implications for human health. *Annu Rev Anim Biosci*. 2015;3:219–244. https://doi.org/10.1146/annurev-animal-022114-110815.
7. Hou N, Du X, Wu S. Advances in pig models of human diseases. *Animal Model Exp Med*. 2022;5:141–152. https://doi.org/10.1002/ame2.12223.
8. Li WT, Zhang MM, Li QG, et al. Whole-genome resequencing reveals candidate mutations for pig prolificacy. *Proc Biol Sci*. 2017;284:20172437. https://doi.org/10.1098/rspb.2017.2437.
9. Carvalho C, Gaspar A, Knight A, Vicente L. Ethical and scientific pitfalls concerning laboratory research with non-human primates, and possible solutions. *Animals (Basel)*. 2018;9:12. https://doi.org/10.3390/ani9010012.
10. Li Q, Cao M, Lei L, et al. Burden of liver cancer: from epidemiology to prevention. *Chin J Cancer Res*. 2022;34:554–566. https://doi.org/10.21147/j.issn.1000-9604.2022.06.02.
11. Xiao J, Wang F, Wong NK, et al. Global liver disease burdens and research trends: analysis from a Chinese perspective. *J Hepatol*. 2019;71:212–221. https://doi.org/10.1016/j.jhep.2019.03.004.
12. Shi JF, Cao M, Wang Y, et al. Is it possible to halve the incidence of liver cancer in China by 2050? *Int J Cancer*. 2021;148:1051–1065. https://doi.org/10.1002/ijc.33313.
13. Zhou J, Zhou F, Wang W, et al. Epidemiological features of NAFLD from 1999 to 2018 in China. *Hepatology*. 2020;71:1851–1864. https://doi.org/10.1002/hep.31150.
14. Lada E, Anna M, Patrik M, et al. Porcine liver anatomy applied to biomedicine. *J Surg Res*. 2020;250:70–79. https://doi.org/10.1016/j.jss.2019.12.038.
15. Lossi L, D'Angelo L, De Girolamo P, Merighi A. Anatomical features for an adequate choice of experimental animal model in biomedicine: II. Small laboratory rodents, rabbit, and pig. *Ann Anat*. 2016;204:11–28. https://doi.org/10.1016/j.aanat.2015.10.002.
16. Gasthuys E, Vandecasteele T, De Bruyne P, et al. The potential use of piglets as human pediatric surrogate for preclinical pharmacokinetic and pharmacodynamic drug testing. *Curr Pharm Des*. 2016;22:4069–4085. https://doi.org/10.2174/1381612822666160303111031.
17. Klem TB, Bleken E, Morberg H, Thoresen SI, Framstad T. Hematologic and biochemical reference intervals for Norwegian crossbreed grower pigs. *Vet Clin Pathol*. 2010;39:221–226. https://doi.org/10.1111/j.1939-165X.2009.00199.x.
18. Hammer C. Physiological obstacles after xenotransplantation. *Ann N Y Acad Sci*. 1998;862:19–27. https://doi.org/10.1111/j.1749-6632.1998.tb09113.x.
19. Ibrahim Z, Busch J, Awwad M, Wagner R, Wells K, Cooper DK. Selected physiologic compatibilities and incompatibilities between human and porcine organ systems. *Xenotransplantation*. 2006;13:488–499. https://doi.org/10.1111/j.1399-3089.2006.00346.x.
20. Swindle MM, Makin A, Herron AJ, Clubb Jr FJ, Frazier KS. Swine as models in biomedical research and toxicology testing. *Vet Pathol*. 2012;49:344–356. https://doi.org/10.1177/0300985811402846.
21. Shang H, Guo K, Liu Y, Yang J, Wei H. Constitutive expression of CYP3A mRNA in Bama miniature pig tissues. *Gene*. 2013;524:261–267. https://doi.org/10.1016/j.gene.2013.04.007.
22. Anzenbacherova E, Baranova J, Zuber R, et al. Model systems based on experimental animals for studies on drug metabolism in man: (mini)pig cytochromes P450 3A29 and 2E1. *Basic Clin Pharmacol Toxicol*. 2005;96:244–245. https://doi.org/10.1111/j.1742-7843.2005.pto960316.x.
23. Wright WC, Chenge J, Chen T. Structural perspectives of the CYP3A family and their small molecule modulators in drug metabolism. *Liver Res*. 2019;3:132–142. https://doi.org/10.1016/j.livres.2019.08.001.
24. Van Peer E, Verbueken E, Saad M, Casteleyn C, Van Ginneken C, Van Cruchten S. Ontogeny of CYP3A and P-glycoprotein in the liver and the small intestine of the Göttingen minipig: an immunohistochemical evaluation. *Basic Clin Pharmacol Toxicol*. 2014;114:387–394. https://doi.org/10.1111/bcpt.12173.
25. Warr A, Affara N, Aken B, et al. An improved pig reference genome sequence to enable pig genetics and genomics research. *Gigascience*. 2020;9:giaa051. https://doi.org/10.1093/gigascience/giaa051.
26. Pan Z, Yao Y, Yin H, et al. Pig genome functional annotation enhances the biological interpretation of complex traits and human disease. *Nat Commun*. 2021;12:5848. https://doi.org/10.1038/s41467-021-26153-7.
27. Karlsson M, Sjostedt E, Oksvold P, et al. Genome-wide annotation of protein-coding genes in pig. *BMC Biol*. 2022;20:25. https://doi.org/10.1186/s12915-022-01229-y.
28. Sun HS, Yerle M, Pinton P, et al. Physical assignments of human chromosome 13 genes on pig chromosome 11 demonstrate extensive synteny and gene order conservation between pig and human. *Anim Genet*. 1999;30:304–308. https://doi.org/10.1046/j.1365-2052.1999.00474.x.
29. Sun HF, Ernst CW, Yerle M, et al. Human chromosome 3 and pig chromosome 13 show complete synteny conservation but extensive gene-order differences. *Cytogenet Cell Genet*. 1999;85:273–278. https://doi.org/10.1159/000015312.
30. Hammer RE, Pursel VG, Rexroad Jr CE, et al. Production of transgenic rabbits, sheep and pigs by microinjection. *Nature*. 1985;315:680–683. https://doi.org/10.1038/315680a0.
31. Matoba S, Zhang Y. Somatic cell nuclear transfer reprogramming: mechanisms and applications. *Cell Stem Cell*. 2018;23:471–485. https://doi.org/10.1016/j.stem.2018.06.018.
32. Doudna JA, Charpentier E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science*. 2014;346:1258096. https://doi.org/10.1126/science.1258096.
33. Knott GJ, Doudna JA. CRISPR-Cas guides the future of genetic engineering. *Science*. 2018;361:866–869. https://doi.org/10.1126/science.aat5011.
34. Griffith BP, Goerlich CE, Singh AK, et al. Genetically modified porcine-to-human cardiac xenotransplantation. *N Engl J Med*. 2022;387:35–44. https://doi.org/10.1056/NEJMoa2201422.
35. Fiorotto R, Amenduni M, Mariotti V, et al. Animal models for cystic fibrosis liver disease (CFLD). *Biochim Biophys Acta Mol Basis Dis*. 2019;1865:965–969. https://doi.org/10.1016/j.bbadis.2018.07.026.
36. Rogers CS, Stoltz DA, Meyerholz DK, et al. Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. *Science*. 2008;321:1837–1841. https://doi.org/10.1126/science.1163600.
37. Elkhadragy L, Regan MR, M Totura W, et al. Generation of genetically tailored porcine liver cancer cells by CRISPR/Cas9 editing. *Biotechniques*. 2021;70:37–48. https://doi.org/10.2144/btn-2020-0119.
38. Elkhadragy L, Dasteh Goli K, Totura WM, et al. Effect of CRISPR knockout of AXIN1 or ARID1A on proliferation and migration of porcine hepatocellular carcinoma. *Front Oncol*. 2022;12:904031. https://doi.org/10.3389/fonc.2022.904031.
39. Wang J, Khan SU, Cao P, et al. Construction of PIK3C3 transgenic pig and its pathogenesis of liver damage. *Life (Basel)*. 2022;12:630. https://doi.org/10.3390/life12050630.
40. Zhao H, Ye W, Guo J, et al. Development of RAG2 (-/-) IL2Rgamma (-/Y) immune deficient FAH-knockout miniature pig. *Front Immunol*. 2022;13:950194. https://doi.org/10.3389/fimmu.2022.950194.
41. Ren J, Yu D, Wang J, et al. Generation of immunodeficient pig with hereditary tyrosinemia type 1 and their preliminary application for humanized liver. *Cell Biosci*. 2022;12:26. https://doi.org/10.1186/s13578-022-00760-3.
42. Hickey RD, Lillegard JB, Fisher JE, et al. Efficient production of Fah-null heterozygote pigs by chimeric adeno-associated virus-mediated gene knockout and somatic cell nuclear transfer. *Hepatology*. 2011;54:1351–1359. https://doi.org/10.1002/hep.24490.
43. Gu P, Yang Q, Chen B, et al. Genetically blocking HPD via CRISPR-Cas9 protects against lethal liver injury in a pig model of tyrosinemia type I. *Mol Ther Methods Clin Dev*. 2021;21:530–547. https://doi.org/10.1016/j.omtm.2021.04.002.
44. Li L, Meng H, Zou Q, et al. Establishment of gene-edited pigs expressing human blood-coagulation factor VII and albumin for bioartificial liver use. *J Gastroenterol Hepatol*. 2019;34:1851–1859. https://doi.org/10.1111/jgh.14666.
45. Zhang XJ, She ZG, Wang J, et al. Multiple omics study identifies an interspecies conserved driver for nonalcoholic steatohepatitis. *Sci Transl Med*. 2021;13:eabg8117. https://doi.org/10.1126/scitranslmed.abg8117.
46. Zhang K, Tao C, Xu J, et al. CD8(+) T cells involved in metabolic inflammation in visceral adipose tissue and liver of transgenic pigs. *Front Immunol*. 2021;12:690069. https://doi.org/10.3389/fimmu.2021.690069.
47. Hao H, Lin R, Li Z, et al. MC4R deficiency in pigs results in hyperphagia and ultimately hepatic steatosis without high-fat diet. *Biochem Biophys Res Commun*. 2019;520:651–656. https://doi.org/10.1016/j.bbrc.2019.08.016.
48. Ruiz-Estevez M, Crane AT, Rodriguez-Villamil P, et al. Liver development is restored by blastocyst complementation of HHEX knockout in mice and pigs. *Stem Cell Res Ther*. 2021;12:292. https://doi.org/10.1186/s13287-021-02348-z.
49. Shteinberg M, Haq IJ, Polineni D, Davies JC. Cystic fibrosis. *Lancet*. 2021;397:2195–2211. https://doi.org/10.1016/s0140-6736(20)32542-3.
50. Fiorotto R, Strazzabosco M. Pathophysiology of cystic fibrosis liver disease: a channelopathy leading to alterations in innate immunity and in microbiota. *Cell Mol Gastroenterol Hepatol*. 2019;8:197–207. https://doi.org/10.1016/j.jcmgh.2019.04.013.
51. Dana J, Debray D, Beaufrere A, et al. Cystic fibrosis-related liver disease: clinical presentations, diagnostic and monitoring approaches in the era of CFTR modulator therapies. *J Hepatol*. 2022;76:420–434. https://doi.org/10.1016/j.jhep.2021.09.042.
52. Toledano MB, Mukherjee SK, Howell J, et al. The emerging burden of liver disease in cystic fibrosis patients: a UK nationwide study. *PLoS One*. 2019;14:e0212779. https://doi.org/10.1371/journal.pone.0212779.
53. Guilbault C, Saeed Z, Downey GP, Radzioch D. Cystic fibrosis mouse models. *Am J Respir Cell Mol Biol*. 2007;36:1–7. https://doi.org/10.1165/rcmb.2006-0184TR.
54. Rogers CS, Hao Y, Rokhlina T, et al. Production of CFTR-null and CFTR-DeltaF508 heterozygous pigs by adeno-associated virus-mediated gene targeting and somatic cell nuclear transfer. *J Clin Invest*. 2008;118:1571–1577: 10.1111/xen.12289. https://doi.org/10.1172/JCI34773.

55. Meyerholz DK, Stoltz DA, Pezzulo AA, Welsh MJ. Pathology of gastrointestinal organs in a porcine model of cystic fibrosis. *Am J Pathol*. 2010;176:1377–1389. https://doi.org/10.2353/ajpath.2010.090849.
56. Uc A, Giriyappa R, Meyerholz DK, et al. Pancreatic and biliary secretion are both altered in cystic fibrosis pigs. *Am J Physiol Gastrointest Liver Physiol*. 2012;303:G961–G968. https://doi.org/10.1152/ajpgi.00030.2012.
57. Terblanche J, Hickman R. Animal models of fulminant hepatic failure. *Dig Dis Sci*. 1991;36:770–774. https://doi.org/10.1007/BF01311235.
58. van de Kerkhove MP, Hoekstra R, van Gulik TM, Chamuleau RAFM. Large animal models of fulminant hepatic failure in artificial and bioartificial liver support research. *Biomaterials*. 2004;25:1613–1625. https://doi.org/10.1016/s0142-9612(03)00509-x.
59. Filipponi F, Boggi U, Meacci L, et al. A new technique for total hepatectomy in the pig for testing liver support devices. *Surgery*. 1999;125:448–455.
60. Filipponi F, Fabbri LP, Marsili M, et al. A new surgical model of acute liver failure in the pig: experimental procedure and analysis of liver injury. *Eur Surg Res*. 1991;23:58–64. https://doi.org/10.1159/000129137.
61. Xue W, Fu Y, Zhang H, et al. A novel, simplified, and reproducible porcine model of acute ischemic liver failure with portal vein preservation. *Exp Anim*. 2022;71:60–70. https://doi.org/10.1538/expanim.21-0076.
62. Lee KC, Palacios Jimenez C, Alibhai H, et al. A reproducible, clinically relevant, intensively managed, pig model of acute liver failure for testing of therapies aimed to prolong survival. *Liver Int*. 2013;33:544–551. https://doi.org/10.1111/liv.12042.
63. Sun H, Ni HM, McCracken JM, et al. Liver-specific deletion of mechanistic target of rapamycin does not protect against acetaminophen-induced liver injury in mice. *Liver Res*. 2021;5:79–87. https://doi.org/10.1016/j.livres.2021.03.001.
64. Wang J, Sun Z, Jiang J, et al. Proteomic signature of acute liver failure: from discovery and verification in a pig model to confirmation in humans. *Mol Cell Proteomics*. 2017;16:1188–1199. https://doi.org/10.1074/mcp.M117.067397.
65. Yuasa T, Yamamoto T, Rivas-Carrillo JD, et al. Laparoscopy-assisted creation of a liver failure model in pigs. *Cell Transplant*. 2008;17:187–193. https://doi.org/10.3727/000000008783906973.
66. Abbasnezhad A, Salami F, Mohebbati R. A review: systematic research approach on toxicity model of liver and kidney in laboratory animals. *Animal Model Exp Med*. 2022;5:436–444. https://doi.org/10.1002/ame2.12230.
67. Newsome PN, Plevris JN, Nelson LJ, Hayes PC. Animal models of fulminant hepatic failure: a critical evaluation. *Liver Transpl*. 2000;6:21–31. https://doi.org/10.1002/lt.500060110.
68. Kalsi RS, Ostrowska A, Olson A, et al. A non-human primate model of acute liver failure suitable for testing liver support systems. *Front Med (Lausanne)*. 2022;9:964448. https://doi.org/10.3389/fmed.2022.964448.
69. Yagi S, Hirata M, Miyachi Y, Uemoto S. Liver regeneration after hepatectomy and partial liver transplantation. *Int J Mol Sci*. 2020;21:8414. https://doi.org/10.3390/ijms21218414.
70. Court FG, Laws PE, Morrison CP, et al. Subtotal hepatectomy: a porcine model for the study of liver regeneration. *J Surg Res*. 2004;116:181–186. https://doi.org/10.1016/j.jss.2003.08.007.
71. Golriz M, Ashrafi M, Khajeh E, Majlesara A, Flechtenmacher C, Mehrabi A. Establishing a porcine model of small for size syndrome following liver resection. *Can J Gastroenterol Hepatol*. 2017;2017:5127178. https://doi.org/10.1155/2017/5127178.
72. Kahn D, Hickman R, Terblanche J. A porcine model for the study of liver regeneration. *J Invest Surg*. 1988;1:139–142. https://doi.org/10.3109/08941938809141086.
73. Mortensen KE, Conley LN, Hedegaard J, et al. Regenerative response in the pig liver remnant varies with the degree of resection and rise in portal pressure. *Am J Physiol Gastrointest Liver Physiol*. 2008;294:G819–G830. https://doi.org/10.1152/ajpgi.00179.2007.
74. Inomata K, Tajima K, Yagi H, et al. A pre-clinical large animal model of sustained liver injury and regeneration stimulus. *Sci Rep*. 2018;8:14987. https://doi.org/10.1038/s41598-018-32889-y.
75. Zwirner S, Abu Rmilah AA, Klotz S, et al. First-in-class MKK4 inhibitors enhance liver regeneration and prevent liver failure. *Cell*. 2024;187:1666–1684.e26. https://doi.org/10.1016/j.cell.2024.02.023.
76. Hickey RD, Mao SA, Glorioso J, et al. Curative ex vivo liver-directed gene therapy in a pig model of hereditary tyrosinemia type 1. *Sci Transl Med*. 2016;8:349ra99. https://doi.org/10.1126/scitranslmed.aaf3838.
77. Sheka AC, Adeyi O, Thompson J, Hameed B, Crawford PA, Ikramuddin S. Nonalcoholic steatohepatitis: a review. *JAMA*. 2020;323:1175–1183. https://doi.org/10.1001/jama.2020.2298.
78. Ibrahim SH, Hirsova P, Malhi H, Gores GJ. Animal models of nonalcoholic steatohepatitis: eat, delete, and inflame. *Dig Dis Sci*. 2016;61:1325–1336. https://doi.org/10.1007/s10620-015-3977-1.
79. Lee L, Alloosh M, Saxena R, et al. Nutritional model of steatohepatitis and metabolic syndrome in the Ossabaw miniature swine. *Hepatology*. 2009;50:56–67. https://doi.org/10.1002/hep.22904.
80. Liang T, Alloosh M, Bell LN, et al. Liver injury and fibrosis induced by dietary challenge in the Ossabaw miniature Swine. *PLoS One*. 2015;10:e0124173. https://doi.org/10.1371/journal.pone.0124173.
81. Schumacher-Petersen C, Christoffersen BO, Kirk RK, et al. Experimental non-alcoholic steatohepatitis in Göttingen Minipigs: consequences of high fat-fructose-cholesterol diet and diabetes. *J Transl Med*. 2019;17:110. https://doi.org/10.1186/s12967-019-1854-y.
82. Maj M, Harbottle B, Thomas PA, et al. Consumption of high-fructose corn syrup compared with sucrose promotes adiposity and increased triglyceridemia but comparable NAFLD severity in juvenile iberian pigs. *J Nutr*. 2021;151:1139–1149. https://doi.org/10.1093/jn/nxaa441.
83. Schmidt NH, Svendsen P, Albarran-Juarez J, Moestrup SK, Bentzon JF. High-fructose feeding does not induce steatosis or non-alcoholic fatty liver disease in pigs. *Sci Rep*. 2021;11:2807. https://doi.org/10.1038/s41598-021-82208-1.
84. Christoffersen B, Golozoubova V, Pacini G, Svendsen O, Raun K. The young Gottingen minipig as a model of childhood and adolescent obesity: influence of diet and gender. *Obesity (Silver Spring)*. 2013;21:149–158. https://doi.org/10.1002/oby.20249.
85. Christoffersen BO, Grand N, Golozoubova V, Svendsen O, Raun K. Gender-associated differences in metabolic syndrome-related parameters in Gottingen minipigs. *Comp Med*. 2007;57:493–504.
86. Hewitt KN, Pratis K, Jones ME, Simpson ER. Estrogen replacement reverses the hepatic steatosis phenotype in the male aromatase knockout mouse. *Endocrinology*. 2004;145:1842–1848. https://doi.org/10.1210/en.2003-1369.
87. Cao D, Cao QM, Subramaniam S, et al. Pig model mimicking chronic hepatitis E virus infection in immunocompromised patients to assess immune correlates during chronicity. *Proc Natl Acad Sci U S A*. 2017;114:6914–6923. https://doi.org/10.1073/pnas.1705446114.
88. Halsted CH, Villanueva JA, Devlin AM. Folate deficiency, methionine metabolism, and alcoholic liver disease. *Alcohol*. 2002;27:169–172. https://doi.org/10.1016/s0741-8329(02)00225-2.
89. Johansen MV, Bogh HO, Nansen P, Christensen NO. Schistosoma japonicum infection in the pig as a model for human schistosomiasis japonica. *Acta Trop*. 2000;76:85–99. https://doi.org/10.1016/s0001-706x(00)00103-0.
90. Hutchinson L, Kirk R. High drug attrition rates–where are we going wrong? *Nat Rev Clin Oncol*. 2011;8:189–190. https://doi.org/10.1038/nrclinonc.2011.34.
91. Li X, Zhou X, Guan Y, Wang YX, Scutt D, Gong QY. N-nitrosodiethylamine-induced pig liver hepatocellular carcinoma model: radiological and histopathological studies. *Cardiovasc Intervent Radiol*. 2006;29:420–428. https://doi.org/10.1007/s00270-005-0099-8.
92. Ho J, Ware M, Law J, et al. Improved, Shorter-latency carcinogen-induced hepatocellular carcinoma model in pigs. *Oncology*. 2018;95:360–369. https://doi.org/10.1159/000491092.
93. Robertson N, Schook LB, Schachtschneider KM. Porcine cancer models: potential tools to enhance cancer drug trials. *Expet Opin Drug Discov*. 2020;15:893–902. https://doi.org/10.1080/17460441.2020.1757644.
94. Schachtschneider KM, Schwind RM, Darfour-Oduro KA, et al. A validated, transitional and translational porcine model of hepatocellular carcinoma. *Oncotarget*. 2017;8:63620–63634. https://doi.org/10.18632/oncotarget.18872.
95. Gaba RC, Elkhadragy L, Boas FE, et al. Development and comprehensive characterization of porcine hepatocellular carcinoma for translational liver cancer investigation. *Oncotarget*. 2020;11:2686–2701. https://doi.org/10.18632/oncotarget.27647.
96. Ling S, Jiang G, Que Q, Xu S, Chen J, Xu X. Liver transplantation in patients with liver failure: twenty years of experience from China. *Liver Int*. 2022;42:2110–2116. https://doi.org/10.1111/liv.15288.
97. Lamm V, Ekser B, Vagefi PA, Cooper DKC. Bridging to allotransplantation-is pig liver xenotransplantation the best option? *Transplantation*. 2022;106:26–36. https://doi.org/10.1097/TP.0000000000003722.
98. Ladowski JM, Houp J, Hauptfeld-Dolejsek V, Javed M, Hara H, Cooper DKC. Aspects of histocompatibility testing in xenotransplantation. *Transpl Immunol*. 2021;67:101409. https://doi.org/10.1016/j.trim.2021.101409.
99. Ryczek N, Hryhorowicz M, Zeyland J, Lipinski D, Slomski R. CRISPR/Cas technology in pig-to-human xenotransplantation research. *Int J Mol Sci*. 2021;22:3196. https://doi.org/10.3390/ijms22063196.
100. Ekser B, Burlak C, Waldman JP, et al. Immunobiology of liver xenotransplantation. *Expert Rev Clin Immunol*. 2012;8:621–634. https://doi.org/10.1586/eci.12.56.
101. Zhang X, Li X, Yang Z, et al. A review of pig liver xenotransplantation: current problems and recent progress. *Xenotransplantation*. 2019;26:e12497. https://doi.org/10.1111/xen.12497.
102. Chen G, Qian H, Starzl T, et al. Acute rejection is associated with antibodies to non-Gal antigens in baboons using Gal-knockout pig kidneys. *Nat Med*. 2005;11:1295–1298. https://doi.org/10.1038/nm1330.
103. Zhou Q, Li T, Wang K, et al. Current status of xenotransplantation research and the strategies for preventing xenograft rejection. *Front Immunol*. 2022;13:928173. https://doi.org/10.3389/fimmu.2022.928173.
104. Song KH, Kang YJ, Jin UH, et al. Cloning and functional characterization of pig CMP-N-acetylneuraminic acid hydroxylase for the synthesis of N-glycolylneuraminic acid as the xenoantigenic determinant in pig-human xenotransplantation. *Biochem J*. 2010;427:179–188. https://doi.org/10.1042/BJ20090835.
105. Byrne G, Ahmad-Villiers S, Du Z, McGregor C. B4GALNT2 and xenotransplantation: a newly appreciated xenogeneic antigen. *Xenotransplantation*. 2018;25:e12394. https://doi.org/10.1111/xen.12394.
106. Cooper DKC, Hara H, Iwase H, et al. Justification of specific genetic modifications in pigs for clinical organ xenotransplantation. *Xenotransplantation*. 2019;26:e12516. https://doi.org/10.1111/xen.12516.
107. Estrada JL, Martens G, Li P, et al. Evaluation of human and non-human primate antibody binding to pig cells lacking GGTA1/CMAH/beta4GalNT2 genes. *Xenotransplantation*. 2015;22:194–202. https://doi.org/10.1111/xen.12161.

108. Butler JR, Skill NJ, Priestman DL, et al. Silencing the porcine iGb3s gene does not affect Galalpha3Gal levels or measures of anticipated pig-to-human and pig-to-primate acute rejection. *Xenotransplantation*. 2016;23:106–116. https://doi.org/10.1111/xen.12217.
109. Li P, Estrada JL, Burlak C, et al. Efficient generation of genetically distinct pigs in a single pregnancy using multiplexed single-guide RNA and carbohydrate selection. *Xenotransplantation*. 2015;22:20–31. https://doi.org/10.1111/xen.12131.
110. Fischer K, Kraner-Scheiber S, Petersen B, et al. Efficient production of multi-modified pigs for xenotransplantation by 'combineering', gene stacking and gene editing. *Sci Rep*. 2016;6:29081. https://doi.org/10.1038/srep29081.
111. Niu D, Wei HJ, Lin L, et al. Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9. *Science*. 2017;357:1303–1307. https://doi.org/10.1126/science.aan4187.
112. Hume DM, Merrill JP, Miller BF, Thorn GW. Experiences with renal homotransplantation in the human: report of nine cases. *J Clin Invest*. 1955;34:327–382. https://doi.org/10.1172/JCI103085.
113. Adams AB, Kim SC, Martens GR, et al. Xenoantigen deletion and chemical immunosuppression can prolong renal xenograft survival. *Ann Surg*. 2018;268:564–573. https://doi.org/10.1097/SLA.0000000000002977.
114. Langin M, Mayr T, Reichart B, et al. Consistent success in life-supporting porcine cardiac xenotransplantation. *Nature*. 2018;564:430–433. https://doi.org/10.1038/s41586-018-0765-z.
115. Calne RY, White HJ, Herbertson BM, et al. Pig-to-baboon liver xenografts. *Lancet*. 1968;1:1176–1188. https://doi.org/10.1016/s0140-6736(68)91869-2.
116. Ramirez P, Chavez R, Majado M, et al. Life-supporting human complement regulator decay accelerating factor transgenic pig liver xenograft maintains the metabolic function and coagulation in the nonhuman primate for up to 8 days. *Transplantation*. 2000;70:989–998. https://doi.org/10.1097/00007890-200010150-00001.
117. Ekser B, Long C, Echeverri GJ, et al. Impact of thrombocytopenia on survival of baboons with genetically modified pig liver transplants: clinical relevance. *Am J Transplant*. 2010;10:273–385. https://doi.org/10.1111/j.1600-6143.2009.02945.x.
118. Kim K, Schuetz C, Elias N, et al. Up to 9-day survival and control of thrombocytopenia following alpha1,3-galactosyl transferase knockout swine liver xenotransplantation in baboons. *Xenotransplantation*. 2012;19:256–264. https://doi.org/10.1111/j.1399-3089.2012.00717.x.
119. Shah JA, Patel MS, Elias N, et al. Prolonged survival following pig-to-primate liver xenotransplantation utilizing exogenous coagulation factors and costimulation blockade. *Am J Transplant*. 2017;17:2178–2185. https://doi.org/10.1111/ajt.14341.
120. Iwase H, Liu H, Schmelzer E, et al. Transplantation of hepatocytes from genetically engineered pigs into baboons. *Xenotransplantation*. 2017;24:10.1111/xen.12289. https://doi.org/10.1111/xen.12289.