

Overcoming Immune Rejection in Xenotransplantation via CRISPR Gene Editing Technology

Yuelin Zhang

*School of Live Science, South China Normal University, Guangzhou, China
zhang79998@163.com*

Abstract. Severe human organ donor shortages hinder the treatment of end-stage organ failure. Under this situation, the cross-species transplantation strategy of implanting porcine organs into humans has emerged as a highly promising alternative. However, intense rejection phenomena and molecular mismatches exist between different species, and these critical obstacles are difficult to overcome with conventional pharmacological immunosuppression. To address these challenges, this paper reviews recent key findings, focusing on how CRISPR tools are being used for multiplex genome modifications in donor pigs—covering both current implementation strategies and the latest breakthroughs. Furthermore, it provides an in-depth analysis of the technical challenges and future exploratory paths as this cutting-edge technology moves toward practical application. In summary, using CRISPR to achieve multiplex genetic modifications in animal donors can significantly improve their molecular expression characteristics. Early medical practices and in vivo animal experiments indicate edited organs are much better at avoiding attack from the host immune system. that the modified target organs acquire a robust capacity to evade host immune system attacks. This process effectively curtails hyperacute rejection, which carries an extremely high mortality rate, thereby substantially prolonging organ survival. Nevertheless, this technology remains subject to multiple constraints, such as the potential risk of genomic off-target effects accompanying gene modification; additionally, a comprehensive management system for delayed and chronic rejection has not yet been established, and there is an extreme lack of long-term in vivo clinical data from humans.

Keywords: Xenotransplantation, Genome Editing, Immune Rejection, Multiplex Genetic Modification, Immune Tolerance

1. Introduction

Globally, the high incidence of organ failure coupled with a severe shortage of human donor organs has created a critical public health crisis. Because pigs are highly similar to humans in terms of anatomical structure, basal metabolism, and organ size, pig-to-human xenotransplantation has become the most promising alternative. However, the the biggest problem with cross-species transplantation is immune rejection—particularly the hyperacute rejection triggered by pre-existing antibodies in the recipient's body, along with subsequent abnormal blood coagulation and cell-mediated attacks; standard anti-rejection medications cannot fundamentally overcome this immense

molecular incompatibility between species [1]. Therefore, finding foundational technological innovations capable of breaching this immune barrier has become a crucial focus for scientists.

The primary focus of this study is to explore how CRISPR gene editing technology can be used to fundamentally remodel molecules at the genomic level within donor embryos. The objective is to break the interspecies immune barriers by breeding gene-modified donor animals with human immune characteristics, so that xenotransplanted organs can survive stably in the human body over the long term. In recent years, multiplex gene modifications utilizing CRISPR technology have demonstrated immense potential. For instance, Anand and his team performed precise genomic edits at 69 loci in donor pigs, achieving a significant extension of organ survival in a non-human primate *in vivo* transplant model [2]. Furthermore, in the world's first pig-to-human cardiac xenotransplantation utilizing a genetically edited pig, the research team's 10-gene editing strategy successfully allowed the human recipient to survive for approximately two months without hyperacute rejection [3].

This study comprehensively collects and summarizes cutting-edge literature published in major international academic journals in recent years regarding CRISPR technology and xenotransplantation, profoundly analyzing the foundational mechanistic logic behind various gene modification strategies. It also comprehensively compares these genetic modification schemes using *in vitro* and *in vivo* models, as well as real-world data feedback from early clinical trials. On this basis, this paper demonstrates that CRISPR multiplex gene editing technology plays a key role in establishing cross-species immune tolerance and evaluates the shortcomings of current technologies regarding off-target risks and the management of chronic rejection. This analysis aims to build a solid theoretical foundation for the future progression of xenotransplantation toward safer, standardized, and more reliable clinical applications.

2. Fundamental mechanisms of immune rejection in xenotransplantation

The core obstacle in cross-species organ transplantation lies in the vast differences in molecular expression profiles between the donor and recipient. These discrepancies trigger intense and multi-layered immune rejection responses. Based on chronological order and pathogenic mechanisms, xenograft rejection is primarily categorized into three stages: hyperacute rejection, acute rejection, and chronic rejection. Thoroughly understanding the molecular mechanisms of these rejection responses is a theoretical prerequisite for implementing precise gene-editing interventions.

2.1. Molecular basis of hyperacute rejection

Hyperacute rejection (HAR) is the first and most destructive immune barrier encountered in xenotransplantation, typically occurring within minutes to hours after organ blood perfusion. Its molecular basis mainly stems from human natural antibodies (mainly IgM and IgG), which act on specific xenogeneic glycosylated antigens on the surface of porcine vascular endothelial cells. In the pig genome, enzymes encoded by the *GGTA1*, *CMAH*, and *B4GALNT2* genes are responsible for synthesizing three major xenogeneic polysaccharide antigens: galactose- α -1,3-galactose (α -Gal), N-glycolylneuraminic acid (Neu5Gc), and the SDa antigen [4]. When a porcine organ is transplanted into a human, circulating antibodies in the recipient's blood rapidly recognize and bind to these xenogeneic antigens on the donor's vascular endothelial cells. The massive formation of these antigen-antibody complexes leads to the cascade activation of the classical complement system, culminating in the assembly of the membrane attack complex (MAC). The MAC subsequently destroys the integrity of the porcine vascular endothelial cells, triggering severe microvascular

thrombosis, tissue ischemia, and disseminated intravascular coagulation, ultimately causing rapid and irreversible necrosis of the graft [1].

2.2. Acute and chronic immune rejection

Although existing technologies can prevent hyperacute rejection effectively, xenogeneic organs still face subsequent acute and chronic rejection. Acute rejection typically occurs within days to weeks following transplantation and can be subdivided into acute humoral xenograft rejection (AHXR) and cell-mediated rejection. AHXR is primarily driven by newly induced non-Gal xenoantibodies produced by the recipient post-transplantation. These antibodies continuously activate the complement system and endothelial cells, leading to acute vascular inflammation and thrombotic microangiopathy. At the cellular level, because major histocompatibility complexes differ greatly between pigs and humans, human T cells, NK cells, and macrophages directly recognize and attack porcine tissue cells. For example, because the CD47 protein on the surface of porcine blood vessels fails to effectively transmit immune tolerance signals to the SIRP α receptors of human macrophages, the macrophages initiate phagocytosis against the foreign cells [5]. Chronic rejection is the ultimate barrier affecting the long-term survival of grafts, usually manifesting months or years post-transplantation. Its pathological features predominantly present as ongoing low-grade alloimmune injury leading to graft vasculopathy, smooth muscle cell proliferation, luminal narrowing, and severe interstitial fibrosis. Currently, chronic rejection remains poorly understood at the molecular level, posing a long-term challenge that current gene editing and immunosuppressive strategies have yet to resolve.

3. Core applications of CRISPR gene editing technology in donor modification

Once we understand the molecular mechanisms behind immune rejection, utilizing the CRISPR-Cas9 to perform multiplexed, high-precision modifications on the porcine embryonic genome has become the most effective engineering solution to fundamentally bridge the immune gap between species. This modification process primarily encompasses the targeted elimination of xenoantigens, the site-specific knock-in of human immunomodulatory genes, and the complete eradication of potential viral sequences.

3.1. Targeted gene knockout

In the early stages of xenoantigen clearance, research largely focused on the single knockout of the GGTA1 gene (GTKO). *in vitro* experiments confirmed that a single gene knockout only reduced the binding rate of IgG in human serum by approximately 70%-80%, while the remaining antibodies targeting Neu5Gc and SDa could still trigger severe acute humoral rejection. *in vivo* animal experimental data from Adams' team clearly indicated that, compared to GTKO-only pigs, a dual-gene modified model that also knocked out CMAH led to a much later onset of acute rejection in non-human primates and complement deposition was reduced by over 60% [6]. Based on these findings, researchers moved on to a triple-gene knockout (TKO) of *GGTA1/CMAH/B4GALNT2*. In actual human environments, the superiority of the TKO strategy received decisive validation; when Montgomery's team attached a TKO pig kidney to the blood vessels of a brain-dead human, the organ instantaneously exhibited a healthy pink color upon blood perfusion, and over a 54-hour monitoring period, biopsies confirmed a 0% incidence of microthrombus formation within the

glomeruli and capillary networks. Based on these pathological data, the cornerstone role of multi-target knockouts in blocking early humoral attacks was solidified [7].

3.2. Site-specific knock-in of human genes

Gene knockouts alone remain insufficient to counter the continuous activation of the complement system and macrophage phagocytosis; therefore, it is imperative to express human immunomodulatory proteins within the pig genome through site-specific knock-ins. Utilizing CRISPR technology combined with gene-stacking strategies, Fischer's team demonstrated that the successful integration of multiple human gene cassettes could exponentially enhance cell survival *in vitro* [8]. The current optimal approach involves introducing multiple transgenic clusters encompassing human complement regulatory proteins (CD46, CD55) and coagulation regulatory genes (such as THBD). In *in vivo* clinical translation, Griffith's team transplanted a porcine heart containing 10 gene edits (3 pig antigen knockouts, 6 human gene knock-ins, and 1 growth receptor knockout) into a human recipient, enabling the recipient to survive for 60 days completely free of hyperacute rejection—a historic breakthrough in medical history [3]. In a broader exploration of genetic remodeling, Anand's team used CRISPR tools to make 69 precise edits on the pig genome. When they tested these highly humanized pig kidneys in cynomolgus macaques (a type of non-human primate), graft survival jumped from just a few days (for unedited wild-type kidneys) to up to 758 days. This clearly shows that knocking in multiple human genes is key to keeping organs alive for the long-term [2].

3.3. Eradication of endogenous retroviruses

Aside from immune rejection, another major hidden danger of xenotransplantation is the potential risk of cross-species infection by porcine endogenous retroviruses (PERVs) within the human host. Traditional breeding methods cannot eliminate these viral sequences embedded within the pig genome. In this domain, CRISPR technology has demonstrated an irreplaceable advantage in the multiplex deletion of sequences. In 2015, Yang et al. were the first to report in *Science* that CRISPR could efficiently target and inactivate these dispersed viral sequences [9]. Subsequently, breakthrough research by Niu's team utilized the CRISPR-Cas9 system to simultaneously cleave all 62 active PERV copies within the genome of a porcine cell line *in vitro*, and successfully bred the world's first batch of genetically engineered pigs lacking PERV activity via somatic cell nuclear transfer technology [10]. This operation directly reduced the *in vitro* viral infection rate of human cells by porcine cells to zero, fundamentally eliminating the biosafety risks associated with xenotransplantation.

4. Preclinical model validation and early clinical trial progress

The success of gene-editing technologies at the cellular level must be validated through *in vivo* systems. Non-human primate models and early human clinical trials provide the data we need to see how well CRISPR-edited organs really evade the immune system and fit into the body.

4.1. Long-term observation feedback from non-human primate models

Recent experimental data indicate that multiplex gene modifications have significantly surpassed previous survival bottlenecks of days or weeks. In the field of heart transplantation, Längin's group transplanted a porcine heart—depleted of α -Gal and expressing human CD46 and thrombomodulin

—into baboons. Combined with a specific CD40-blocking immunosuppressive regimen, they successfully maintained the xenogeneic heart's life-sustaining functions within the baboons for up to 195 days, without significant graft overgrowth or hyperacute rejection [11]. Research on multi-gene knockout pig models led by Lu's team further noted that high expression of CD46 effectively blocked the adherence and deposition of C3b on the endothelial surface, thereby protecting the integrity of the organ's microvascular bed [12]. Regarding kidney transplantation, Anand's team performed up to 69 CRISPR gene edits on a donor pig; after transplanting its kidney into a cynomolgus macaque, the maximum survival record reached an astounding 758 days [2]. These detailed, long-term follow-up data show that precise genetic remodeling, combined with better immunosuppressive regimens, can now help xenogeneic organs live harmoniously inside advanced mammals for a long time.

4.2. Case analysis of early human trials and compassionate use

Although NHP models have achieved significant success, subtle biological differences between species mean this technology still needs to be tested in humans. Between late 2021 and early 2022, xenotransplantation saw a historic breakthrough in human trials. In a brain-dead recipient model, Porrett's team, for the first time, transplanted a clinical-grade porcine kidney with 10 gene edits into a brain-dead patient. Results showed that the graft began producing urine within 23 minutes of vascular anastomosis and maintained normal blood perfusion throughout the 74-hour *in vivo* observation period; biopsies confirmed the complete absence of any signs of antibody-mediated hyperacute rejection within the organ [13].

In terms of *in vivo* compassionate use, the world's first transgenic pig-to-human heart transplant provided the most invaluable human immune feedback data. The term "compassionate use" refers to the use of an investigational therapy that has not yet been approved for market to treat patients with serious or life-threatening diseases when no other effective alternative therapies are available. Griffith's team transplanted a 10-gene-edited porcine heart into a 57-year-old patient with end-stage heart failure. In the first few weeks after transplant, the patient's heart looked good on echocardiograms. Over the 60 days he survived, multiple heart tissue biopsies showed no signs of typical hyperacute rejection or severe cell-mediated rejection [3]. Although the patient ultimately passed away due to a latent porcine cytomegalovirus infection and complex multi-organ failure, this two-month survival data confirmed the definitive efficacy of CRISPR technology in the human environment.

5. Conclusion

Through literature analysis, this paper finds that employing CRISPR technology to execute multiplex gene editing on animal donors can significantly improve their molecular expression characteristics. Multiple early clinical applications and animal experimental validations demonstrate that genetically modified target organs can markedly enhance immune evasion capabilities and effectively inhibit the occurrence of highly lethal hyperacute rejection, thereby substantially extending the graft's survival cycle.

Although CRISPR has solved numerous challenges for xenotransplantation, moving it into routine clinical use still means dealing with the technology's risks and regulatory issues.

While the CRISPR-Cas9 system brings efficient genetic remodeling, its inherent off-target effects are a core hidden danger hindering the long-term safe survival of organs. When cleaving targeted DNA, the Cas9 protease may induce unintended double-strand breaks at non-target sites,

leading to genomic mutations. A recent whole-genome sequencing study by Huo and their team revealed that in gene-edited pigs with a single knockout targeting the GGTA1 gene (GTKO), the genome accumulated an average of up to 1,205 off-target genetic mutations, and dozens of *de novo* mutations were also detected in their offspring [14, 15]. When the number of gene-editing loci increases to 10 or even 69, this genomic-level instability is highly likely to trigger malignant cellular proliferation or functional decline *in vivo* [16]. Looking forward to subsequent research, it is essential to introduce novel tools that do not produce double-strand breaks, combined with long-read sequencing technologies for strict screening, to ensure the foundational biological safety of donor organs.

The ultimate vision for xenotransplantation is to transition smoothly from the current compassionate use phase to formal phase I clinical trials tightly monitored by regulatory agencies like the FDA—a translational pathway accompanied by immensely complex ethical considerations. Firstly, there's the public health risk of animal-to-human infections. Unknown endogenous viruses could enter the body with the organ. So future clinical guidelines need to set up a careful, long-term biosafety tracking system that follows the recipient for their whole life [15]. Secondly, in the selection of trial subjects, a fair and ethically compliant balance must be struck between critically ill patients and those who can provide the most robust scientific follow-up data [17]. Looking ahead, as new immunosuppressants, optimized off-target screening tools, and more mature regulations advance together, CRISPR-based xenotransplantation could break through current barriers within the next decade. It may then become a standard clinical option and help end the global organ shortage.

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