

CRISPR-Cas9-Mediated Genetic Modifications in Pig-to-Human Xenotransplantation

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Abstract. Donor shortage remained a major challenge in organ transplantation that may continue to deteriorate as life expectancy increases. Therefore, xenotransplantation is a promising and effective alternative approach to narrow the imbalance of organ supply and demand. Pigs are the major xenograft donor. While organs without genetic modifications may trigger several immune rejection and PERV infection, CRISPR-Cas9 has brought unprecedented progress to recent xenotransplant research with high efficiency and broad applications. This paper analyzes the principal mechanism and components of CRISPR-Cas9. In addition, this paper analyzes recent achievements of CRISPR-Cas9 in gene modification, including inactivating three major glycan absent in human from porcine cells to prevent immune rejection, insertion of a human gene, and inactivating PERVs completely from the porcine primary cell line. Finally, this paper analyzes recent pig-to-human xenotransplant and summarizes their shared commonalities. Most pig-to-human transplantations succeed without acute rejection or PERV infection after gene modification on the donor. This paper is intended to analyse achievements and progress of CRISPR-Cas9 in pig-to-human xenotransplantation. Though current xenotransplantation has just entered the clinical stage and is more suitable for bridge therapy instead of complete substitution for human organs. Future research can improve xenotransplantation for temporary treatment for patients who suffer from severe organ dysfunction, and use more living human recipients for more valuable data to continue editing xenografts for long-term survival.

Keywords: CRISPR-Cas9, Xenotransplantation, Gene modification

1. Introduction

In the United States alone, over 100,000 patients [1] are on the waiting list for organ transplant as of 2024, which number has increased 180% from 1994, with the disparity between organ supply and demand widening more than 200%. (35,751 patients [2] waiting in 1994 18,299 transplants [2] 48,149 transplants in 2024 17452 to 51851). The organ shortage is worsening over time, indicating an urgent need for substitutes. A promising alternative is xenotransplantation. Xenotransplantation involves the transplantation between different species of living cells, tissues, or organs, is considered a feasible substitute for scarce human donors [3,4]. Recent attempts for xenotransplants started in the 1920s with mainly nonhuman primate (NHP) donors. However, NHP was phased out due to express high risk of zoonosis infections, inefficient breeding, existing ethical concerns, and

other limitations. Since the 1990s, donors of xenotransplant switched from NHP to pigs. Pigs demonstrate an advantage in a similar size of organ to human adults, are fast-breeding species, and, as abundant domesticated animals, generate less ethical concerns [5]. Despite its obvious merits, the pigs and humans' genetic dissimilarities still triggered obstacles of several xenotransplant rejections and the concerning discovery of porcine endogenous retrovirus (PERVs) in pigs. Even though whether PERVs infect humans in vivo is still unproven, research indicates that human cells infected by PERV can pass PERVs onto human cells that are previously unexposed [6].

Gene modification is crucial to prolong graft survival, as in many attempts through the 20th century, recipients could die within hours to days due to possible rejections and inflammation [7]. Involvement of gene engineering and especially CRISPR-Cas9 revolutionized the field of xenotransplantation, leading to advanced progress in recent studies with more efficiency requiring redesign of only the single guide RNA to target various genomes for cutting and insertion [8]. In order to be human-compatible and alleviate infection, the organs of pigs need to be genetically modified. First, three glycans present in pigs ought to be removed in order to correspond with humans' lack of these glycans. Respectively, they are the α -Gal (galactose- α -1,3-galactose) and Sd(a) (Sia- α 2.3- [GalNAc- β 1.4]Gal- β 1.4-GlcNAc) glycans, and Neu5Gc (N-glycolylneuraminic acid) glycan [9]. Several immune responses, including hyperacute xenograft rejection and acute humoral xenograft rejection, may be triggered due to antibodies and incompatibility towards the three glycans mentioned. Second, insertion of complement regulatory proteins (CRPs) in humans was also suggested to prevent porcine cells from complement-mediated injury [10]. Thirdly, inactivation of PERV diminishes the chance of infection.

Though xenotransplantations are still in the experimental stage. Success in non-human primates recipients for up to 2 years (758 days) gives the possibility that xenotransplant can extend human patients' lives [9]. Recent attempts of pig organs to human recipients were also successful and can be used as a promising bridge therapy to buy time for organ transplant [11].

2. The principle of CRISPR-Cas9 technology

2.1. Structure of CRISPR-Cas9

Gene editing system widely adopted before the introduction of the CRISPR system were mainly two, Zinc finger nucleases (ZFN) and Transcription activator-like effector nucleases (TALENs) [8]. They share limiting factors of inefficiency, being complex to engineer by recoding long DNA segments, and being expensive. The finding of CRISPR and the CRISPR-Cas9 system revolutionized gene modification with higher flexibility, efficiency, and friendly operation for non-experts [8,12]. There are two primary categories into which the CRISPR-Cas systems may be divided, Class I and Class II, each with subtypes. Class I contains multi-subunit effector complexes, whereas Class II mechanised under a single Cas protein, like Cas-9. Specifically, the Class II systems have been widely applied in gene editing due to their simpler formation [8].

Cas-9 is composed by two parts: the nuclease (NUC) lobe and the recognition (REC) lobe. The REC lobe is composed of REC1 and REC2 domains and has a distinct structure, revealing it is a Cas9-specific domain, and the NUC lobe involves three components of Protospacer Adjacent Motif (PAM) interacting RuvC, and HNH, domains. The later two domains are both responsible for cleaving one single-stranded DNA, which together cleave a double-strand break (DSBs). PAM-interacting domain acts to start the binding to target DNA [13].

The endogenous CRISPR system contains two RNAs of CRISPR RNAs (crRNA) and a trans-activating crRNA (tracrRNA). The crRNA binds with the tracrRNA to form a crRNA:tracrRNA

duplex, which is required to guide Cas9 to cut of DNA sequences with DSBs at specific locations. Adding on that, the Cas9 protein extracted from *Streptococcus pyogenes* can be guided by a single chimeric RNA formed by the synthetic fusion of tracrRNA and crRNA, called single guide RNA (sgRNA) [13]. The sgRNA after programmable researchers to cut any sequence preceding a 5'-NGG-3' PAM sequence in mammalian cells. This triggers the broad usage of CRISPR technology as NGG appears in genomes of most organisms [14]. Numerous uses and applications have been made possible by this unparalleled adaptability.

2.2. Process of CRISPR-Cas9 gene editing

CRISPR/Cas-9 gene edit consist three basic phases that can be concluded as: recognition, cleavage, and repair. sgRNA will be designed to recognize a specific target where adjustment is needed. It can lead the cutting Cas9 protein to the exact part of target genome. The Cas9 protein is then prepared for cleavage, it will then create DSBs approximately 3 base pairs upstream of the PAM. The HNH domain cleaves the complementary strand of the target region of DNA, as the RuvC domain cleaves the non-complementary strand, result in blunt-ended DSBs. In the final phase, the cell identified that the strands were damaged, so the DSB is mended by the host cell [8]. Utilizing the repair mechanism, researchers can create changes to the existing genome by deleting, inserting, or modifying.

3. How CRISPR-Cas9 makes pig organs more human-compatible

3.1. Removing glycans absent in humans

Three glycans mentioned (α -Gal, Sd(a), and Neu5Gc) in pigs should be cleaved to be unified with their nonpresence in human body. These are generated by these glycan synthesis genes respectively, glycoprotein α -galactosyltransferase 1 (GGTA1), cytidine monophospho-N-acetylneuraminic acid hydroxylase (CMAH), and β -1,4-N-acetyl-galactosaminyltransferase 2 (B4GALNT2)/B4GALNT2-like (B4GALNT2L) [9].

The incompatibility between pig and human can trigger several immune responses, leading to three major classes of rejection which can appear in sequence: (i) hyperacute xenograft rejection, (ii) acute humoral xenograft rejection, and (iii) acute cellular rejection. Hyperacute rejection (HAR) can happen within minutes to hours when a xenograft transferred from unmodified pigs to humans is destroyed. The major xenoantigen to HAR is α -Gal. As much as ~1% of all circulating antibodies in human blood act against α -Gal epitopes. The binding of preformed antibodies-naturally produced antibodies after birth in human recipients, to the xenoantigenic epitopes on porcine endothelial cells activates of complement proteins. Complement after activation triggers deeper activation and lysis of endothelial cells. Eventually leads to graft vasculature defunction followed by transplantation failure.

Even though HAR is avoided, the reascend concentration of antibody may still trigger pause in organ functioning, with the name acute humoral xenograft rejection (AHXR), also known as “acute vascular rejection”. NHP received organs after GGTA1 knockout though were still rejected by antibody-mediated rejection over days, suggesting that Gal antigens are not the only cause to AHXR, and non-Gal antigens present additional obstacles to the transplantation of organs from pigs to humans [10]. The three glycan synthesis genes can be disrupted by using CRISPR-Cas9 to alleviate rejection and prolong xenograft survival.

3.2. Expression of human genes

Complement activation is an obvious drawback in contributing to xenograft failure. An attempt was made to apply an inhibiting agent, though it only functioned for a short time and even increased the risk of infection. An alternative solution is genetic engineering. Though close to CRPs in humans, pig CRPs are incapable to prevent pig epithelial cells against human complement-mediated damage. Thus, inserting human CRPs (hCRPs) into pig cells was recommended. By inserting human CD47, it is possible for genetically engineered pigs organs to remedy rejection. In vitro, porcine cells with human CD47 are able to decrease phagocytosis and are also an anti-inflammatory protein [10]. In vivo, hCD47 expression was reported to extend the porcine skin grafts' survival in baboons [15]. Other CRPs, like CD46 and CD55, acting as complement inhibitors, also decrease hyperacute rejection [11]. Through gene modification and promisingly by CRISPR-Cas9, inserting the human genome into the donor enhances the compatibility of xenograft and alleviates the risk of rejection.

3.3. PERV inactivation

Another serious concern in the realm of xenotransplantation is the spread of PERVs to human patients from xenografts. PERVs cannot be destroyed by biosecure breeding, which is different from other zoonotic pathogens [16]. PERV mRNAs are expressed in the genomes of all breeds of pig tested, which presents an unacceptable risk. PERVs consist of three subtypes: PERV-A, PERV-B, and PERV-C. Every pig breeds consist PERV-A and PERV-B, whereas PERV-C is present only in certain pigs [10]. There are many copies of PERVs in genome, though just the few of human-tropic infectious viruses exist as key focus. The copy number can differ in organs in a single pig, demonstrating that PERVs are active in living organisms. A perfect solution to inactivate PERVs is gene editing. Though, as recorded by using ZFN, there is a chance that the multiple edits could ruin the genome and defunction the cell completely because there could be more than 60 integrated proviruses [17]. CRISPR-Cas9 has been proven to be a more successful tool in terms of PERV inactivation.

In 2017, Yang et al., with help from the tool CRISPR-Cas9, inactivated all of the PERVs in a porcine primary cell line and successfully produced PERVs free pigs.

They first planned to map and characterize the present of PERVs in a primary porcine fetal fibroblast cell line (hereafter denoted as FFF3) to inactivate PERV in FFF3. They found 25 copies of functioning PERVs using droplet digital polymerase chain reaction (ddPCR) on the reverse transcriptase (pol) gene. The team designed two CRISPR guide RNAs to the catalytic core of the PERV pol gene, allowing them to trace detected PERVs. 37 % PERV pol inactivation was observed at the end of 12 days treating a group of FFF3 cells with CRISPR-Cas9 and the two gRNAs. The single cells the team directly genotyped include 100% PERV-inactivated FFF3 cells, though for cells after single cell expansion, only those with efficiency $\leq 39\%$ remained.

Fortunately, another discovery is, treatment of FFF3 clones using both a p53 inhibitor, pifithrin alpha (PFT α), and a basic fibroblast growth factor, bFGF, considerably increases the average targeting efficiency by alleviating stress of multiple DNA cleavages and avoiding DNA damage. By applying PFT α and bFGF, average targeting efficiency surged, and 100% PERV-inactivated FFF3 cells were received from FFF3 populations after using CRISPR-Cas9. Off-target effects of CRISPR-Cas9 in 100% PERV-inactivated FFF3 were tested. The result of karyotyping for eight 100% PERV-inactivated FFF3 clones has only three carrying normal chromosomal structures. Further examination of these three FFF3 clones proved there was no potential deletion between DSBs performed by CRISPR-Cas9, with all 21 junctions undamaged. PERV-inactivated pigs were

successfully produced by using modified porcine fibroblasts to generate embryos through somatic cell nuclear transfer. Embryos created were then transferred into surrogate pigs [6]. CRISPR-Cas9 clearly demonstrated potential in broad application and made a great leap to produce human human-compatible organ for xenotransplantation. Though, as indicated in the research by Yang et al., it is not without drawbacks of off-target and may require mixed applications like growth factor to enhance the success rate when applied to multiple genome engineering.

4. Analysis of the current xenotransplant

As xenotransplantation moved on to the clinical stage, several researchers started to transplant porcine organs into human recipients. Several porcine organs including hearts and kidneys have been progressively transplanted into humans. Though xenotransplantation can't support patients for a long period, recent research could eliminate most acute rejections and PERV infection by using gene engineering [18,19].

One example is the first successful heterotopic auxiliary liver transplantation used a donor of a six-gene-modified Bama miniature pig to an adult recipient diagnosed with brain death. The donor has the three glycan synthesis genes GGTA1, B4GALNT2, and CMAH mostly inactivated to alleviate hyperacute rejection and extend xenograft survival. Human CRPs of CD46 (membrane cofactor protein) and CD55 (decay-accelerating factor) were overexpressed in the donor and increased after gene modification to decrease hyperacute rejection and extend organ survival time. In order to activate antithrombotic protein C and inhibit clot generating, human thrombomodulin was expressed. The xenograft persist functional till the research end without signs of hyperacute rejection. Due to the enormous merit of gene modification, humoral immunity is removed from having major impact on graft survival length. Though the xenograft secretes bile two hours after portal vein reperfusion and produces porcine albumin in the study, the team suggested the quantity of bile and porcine albumin was only sufficient to maintain the human body function for a limited length of time. The authors admit several limitations. First, the study only lasted 10 days due to the recipient's families request ; thus there is not enough data to analyse potential changes in xenograft function. Second, the researchers could detect only the few fundamental functions of the liver. The researchers believe current auxiliary liver transplantation is more suitable as a promising bridge therapy available for patients with deteriorating liver function, as it is not hard to remove the porcine liver and rebuild the inferior vena cava when the original liver restarts to function or prolongs survival time to wait for a suitable donor liver [11].

5. Conclusion

The application of the CRISPR/Cas system in genetic engineering has created unprecedented development in recent pig-to-human xenotransplantation research. Simplicity and ease for non-experts to design short sgRNA make the development of genetic engineering more efficient.

So far, three glycans absent in human α -Gal, Sd(a) and Neu5Gc can be inactivated from porcine donors, along with insertion of human CRPs, effectively eliminating most acute rejections arising from incompatibility between pig genomes and human in recent pig-to-human xenotransplant attempts. Successful removal of 100% PERVs from the primary cell line enabled the generation of PERV-inactivated pigs. These indicate the feasibility and encourage future research to keep developing CRISPR-Cas9 for future xenotransplantation, as by utilizing gene engineering, most recent researches report no evidence of HAR and AHXR and PERV infections, which were major factors of xenotransplant failure back in 20 century. Though CRISPR-Cas9 is not without limitations

of off-target effect and destruction of the cell when multiple edits are made, this could be alleviated by the assistance of other chemicals and improvement in the CRISPR-Cas9 system. Xenotransplantations are still in the experimental stage, with limited human recipients willing and available for long-term experiments. Human recipients in current research died months after xenotransplantation, as recent reports, though avoiding rejection, appeared to have unclear side effects. Current xenotransplantation research may only fulfill the temporary need for bridge therapy instead of completely substituting human organs. Though even the bridge therapy is still a very promising tool to extend the waiting time of most patients if well developed in the future, and xenograft survival time can be prolonged gradually until it's suitable for long period of transplantation as human organs.

References

- [1] Organ Procurement and Transplantation Network. (n.d.). National data. Retrieved August 15, 2025, from <https://optn.transplant.hrsa.gov/data/view-data-reports/national-data/>
- [2] Organ Procurement and Transplantation Network. (2005, March 16). Report identifies transplant trends for 2004. <https://optn.transplant.hrsa.gov/news/report-identifies-transplant-trends-for-2004/>
- [3] U.S. Food and Drug Administration. (2021, March 3). Xenotransplantation. <https://www.fda.gov/vaccines-blood-biologics/xenotransplantation>
- [4] Cooper, D. K. C., Gaston, R., Eckhoff, D., Ladowski, J., Yamamoto, T., Wang, L., Iwase, H., Hara, H., Tector, M., & Tector, A. J. (2018). Xenotransplantation—the current status and prospects. *British Medical Bulletin*, 125(1), 5–14.
- [5] Adli, M. (2018). The CRISPR tool kit for genome editing and beyond. *Nature Communications*, 9(1), 1911.
- [6] Niu, D., Wei, H. J., Lin, L., George, H., Wang, T., Lee, I. H., ... & Yang, L. (2017). Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9. *Science*, 357(6357), 1303–1307.
- [7] Cooper, D. K. C., Ekser, B., & Tector, A. J. (2015). A brief history of clinical xenotransplantation. *International Journal of Surgery*, 23(Pt B), 205–210.
- [8] Asmamaw, M., & Zawdie, B. (2021). Mechanism and applications of CRISPR/Cas-9-mediated genome editing. *Biologics: Targets and Therapy*, 353–361.
- [9] Anand, R. P., Layer, J. V., Heja, D., Hirose, T., Lassiter, G., Firl, D. J., ... & Qin, W. (2023). Design and testing of a humanized porcine donor for xenotransplantation. *Nature*, 622(7982), 393–401.
- [10] Lu, T., Yang, B., Wang, R., & Qin, C. (2020). Xenotransplantation: Current status in preclinical research. *Frontiers in Immunology*, 10, 3060.
- [11] Tao, K. S., Yang, Z. X., Zhang, X., Zhang, H. T., Yue, S. Q., Yang, Y. L., ... & Dou, K. F. (2025). Gene-modified pig-to-human liver xenotransplantation. *Nature*, 1–8.
- [12] Gupta, R. M., & Musunuru, K. (2014). Expanding the genetic editing tool kit: ZFNs, TALENs, and CRISPR-Cas9. *The Journal of Clinical Investigation*, 124(10), 4154–4161.
- [13] Nishimasu, H., Ran, F. A., Hsu, P. D., Konermann, S., Shehata, S. I., Dohmae, N., Ishitani, R., Zhang, F., & Nureki, O. (2014). Crystal structure of Cas9 in complex with guide RNA and target DNA. *Cell*, 156(5), 935–949.
- [14] Li, T., Yang, Y., Qi, H., Cui, W., Zhang, L., Fu, X., ... & Yu, T. (2023). CRISPR/Cas9 therapeutics: Progress and prospects. *Signal Transduction and Targeted Therapy*, 8(1), 36.
- [15] Tena, A. A., Sachs, D. H., Mallard, C., Yang, Y. G., Tasaki, M., Farkash, E., ... & Hawley, R. J. (2017). Prolonged survival of pig skin on baboons after administration of pig cells expressing human CD47. *Transplantation*, 101(2), 316–321.
- [16] Yang, L., Güell, M., Niu, D., George, H., Lesha, E., Grishin, D., ... & Church, G. (2015). Genome-wide inactivation of porcine endogenous retroviruses (PERVs). *Science*, 350(6264), 1101–1104.
- [17] Denner, J. (2022). Virus safety of xenotransplantation. *Viruses*, 14(9), 1926.
- [18] Griffith, B. P., Goerlich, C. E., Singh, A. K., Rothblatt, M., Lau, C. L., Shah, A., ... & Mohiuddin, M. M. (2022). Genetically modified porcine-to-human cardiac xenotransplantation. *New England Journal of Medicine*, 387(1), 35–44.
- [19] Montgomery, R. A., Stern, J. M., Lonze, B. E., Tatapudi, V. S., Mangiola, M., Wu, M., ... & Stewart, Z. A. (2022). Results of two cases of pig-to-human kidney xenotransplantation. *New England Journal of Medicine*, 386(20), 1889–1898.