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Chapter

Genome Engineering for Xenotransplantation

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Abstract

Xenotransplantation, the transfer of cells, tissues or organs between species, has the potential to overcome the critical need for organs to treat patients. One major barrier in the widespread application of xenotransplantation in the clinic is the overwhelming rejection response that occurs when non-human organs encounter the human immune system. Recent progress in developing new and better genome engineering tools now allows the genetic engineering of genes and pathways in non-human animals to overcome the human rejection response and provide an unlimited supply of rejection-free organs. In this review, the benefits and drawbacks of various genome engineering protocols, and examples of their application in xenotransplantation, are discussed.

Keywords: xenotransplantation, xenoantigen, transgene, gene targeting, gene editing, homologous recombination

1. Introduction

According to the Organ Procurement and Transplantation Network, there are currently over 110,000 patients on the waiting list for organ transplants. Over the past 50 years, therapeutic advances and improvements in surgical techniques have increased the number of patients who could survive and benefit from organ transplantation. Unfortunately, the number of organs available through donation has not changed significantly. Thus, there is a growing disparity between organ supply and demand. Although efforts to enlarge the human donor pool have improved organ availability, even a massive expansion in organ donation would not ensure that a compatible organ would be available when and where necessary for a patient in need. Therefore, other alternatives besides expanded human donation are required.

A variety of efforts are making substantial progress in addressing the lack of organs. One area of research that is rapidly approaching clinical reality is xenotransplantation, the use of animal tissues and organs to treat patients [1]. Aside from the potential for creating an unlimited supply of organs, recent advancements in genome engineering technologies allows the genetic modification of animals to produce donor organs which are less prone to rejection for xenotransplantation in human patients.

2. History

Xenotransplantation experiments were described as early as the seventeenth-century [2], with sporadic attempts made to transplant a variety of animal tissues and

organs into patients throughout the nineteenth and early twentieth centuries [3–7]. After World War II, organ transplantation from living humans was considered too high risk, and cadaveric organs were insufficient in both quality and number to meet clinical needs. Development of immunosuppressive drugs suggested the possibility that organs from more closely-related mammals could potentially be used in humans. As a consequence, xenotransplantation efforts shifted to the use of organs from primates in human patients. In the 1960s, experiments by Reemstma et al. [8, 9], Hardy et al. [10], and Starzl et al. [11, 12] showed that while it was technically possible to transplant animal organs into humans, there were still too many clinical challenges at that time for the approach to be viable. More research was required to understand and overcome the barriers to the practical application of xenotransplantation in humans.

One of the major advances in xenotransplantation research in the past few decades has been the focus on the use of pigs as donors [13]. This was based, in part, on purely practical considerations. Unlike primates, pigs are an agricultural species for which large scale breeding is well-established. In addition, the evolutionary distance between humans and pigs reduces the risk of transmission of zoonoses from pig organs to patients compared with primate organs. Most importantly, the use of porcine organs does not present the same ethical barriers as the use of non-human primate organs [14].

Although the anatomy and physiology of pig organs is closely analogous to that of humans, the advantages of porcine organ production and availability do not address the critical issue of incompatibilities of non-human tissues and organs with the human immune system [15]. Significant advancements have been made in recent years in understanding the molecular mechanisms of xenorejection responses, and a variety of genetic modifications have been made to overcome these mechanisms. Experiments transplanting pig organs into non-human primates have demonstrated a progressive improvement in organ survival and function as new genetics and drug regimens have been implemented [16]. The FDA is currently developing guidelines for clinical xenotransplantation [17], and efforts to initiate clinical trials in the near term have been announced [18].

3. Immunity and xenotransplantation

The immune system is designed to recognize and eliminate harmful pathogens, while remaining unresponsive to host cells and beneficial microbes. The immune system can be divided into innate and adaptive responses, an interdependent set of activities which both contribute to immunity. The innate response is more immediate, broadly recognizing conserved microbial elements, such as cell wall polysaccharides, and activating a variety of cell types which attack the invading pathogens [19]. The adaptive immune response, which is typically initiated by innate response mechanisms, leads to more precise antigen-specific antibodies and immune cells that continue to control and eliminate pathogens. In addition, the adaptive response creates long-lasting immune "memory" for rapid and specific protection against future infections, as demonstrated by vaccines [20].

Despite being described as separate systems, the innate and adaptive immune responses are highly interdependent and create a layered set of defenses with increasing specificity for pathogens over time [21]. Under normal circumstances, any individual function may not eliminate a given target with 100% efficiency, but when used together in a redundant fashion can prevent nearly all infection. Although the specificity of the immune response indirectly helps to avoid recognition of host tissues, additional tolerance mechanisms are required to restrain the immune system to prevent autoreactivity. Disruptions of the balance between

immunity and tolerance can lead to the immune system destroying host tissues (autoimmunity) or allowing repeated severe infections (immunodeficiency) [22].

The transplantation of foreign cells or tissues into a human host can trigger a hostile response from the immune system, leading to immune rejection. The extraordinary precision of the immune system can distinguish even minor differences between donor and recipient, so that even organs from closely related donors may be rejected. Although immunosuppressive drugs can reduce the chance of rejection of human donor organs, the massive amount of immunogenic material found in a whole organ presents an ongoing risk which requires monitoring. Because of the greater genetic differences between pigs and humans, the vigor of the rejection response is much stronger than occurs between human donors and recipients, requiring more and different solutions.

4. Genome engineering and xenotransplantation

A major advantage in using pigs for xenotransplantation is the potential to manipulate the porcine genome to create donor organs that are more compatible for human patients. However, the scope of the engineering challenge in xenotransplantation is extremely large, involving a variety of genes and pathways. With so many potential targets for genetic modification, an assortment of different genome engineering strategies have been applied, including editing or deletion of porcine genes and insertion of human or engineered genes. Because of the great diversity of genome modification efforts being carried out in xenotransplantation research, representative approaches will be highlighted here as examples of the general types of the engineering strategies being employed.

Historically, mice have been subject to more and different genetic modifications than any other mammalian species, and many of the protocols described here were first developed in mice. Aside from their well-established and convenient husbandry, small size, and rapid generation times, mice also have a variety of technological advantages for genome manipulation and production. Although genetic modification has been demonstrated for multiple agricultural species, including pigs, the scale and complexity possible with mice has, until recently, not been available for pigs [23].

One advantage for the creation of mice with multiple genetic modifications is the availability of embryonic stem (ES) cells, which can be cultured *in vitro* for many generations and subject to repeated transfections and selections without loss of competence for production of viable mice [24]. By contrast, pig ES cells have been much more difficult to create, and have not been routinely used for genetic manipulation and production of animals [25]. Cloning of genetically-modified pigs has required use of primary cells, typically fetal, which can be passaged only a short time *in vitro* before losing their competence to produce viable embryos [26]. Therefore, the complex multi-site modifications and selections used in mice are not accessible for use in pigs.

Mouse ES cells not only allow more straightforward and efficient genome engineering, but also facilitate large scale production of cloned mice. The mouse ES cells typically employed for genetic modification can be injected into very early stage embryos (blastocysts) and will aggressively populate the inner cell mass, creating viable chimeric mice which are almost entirely ES-cell derived. Since the ES cells will also contribute to the germ cells of the chimeric mice, the progeny will be highly likely to receive the genetic modifications made to the ES cells [27]. Without readily available porcine ES cells, pig cloning instead relies upon somatic cell nuclear transfer (SCNT), similar to the protocols used to create the sheep

"Dolly". In this approach, pig oocyte nuclei are replaced with nuclei from the modified primary pig cells, and embryonic development stimulated electrochemically. The embryos are transferred to female surrogates and allowed to develop. The level of complexity and effort involved leads to lower efficiencies and higher costs for porcine SCNT relative to mouse ES cell cloning. Additionally, the size and scale of the facilities required for pig cloning is significantly greater compared with mouse cloning, further limiting availability [28].

In the following sections, different types of gene modifications are described with examples of their application in porcine genome engineering for xenotransplantation.

5. Gene deletions

As mentioned above, porcine cells produce molecules which are rapidly recognized by the human immune system and rejected. One straightforward approach to engineering the pig would be to simply eliminate the genes encoding reactive genes by either disrupting or removing the coding sequence. Several of the technical routes which can be employed to accomplish this are discussed below.

5.1 Gene knockout

Gene knockout (KO) approaches developed for use in mouse ES cells generally rely upon homologous recombination to replace a region of genomic DNA with a heterologous DNA sequence, which interrupts the function of the target gene [29]. To accomplish this, a DNA vector is generated with the heterologous DNA flanked on either side by sequences identical to regions flanking the genomic region to be eliminated. When introduced into mouse ES cells, the flanking sequences of the DNA vector first align with the cognate regions of the genome on either side of the target gene, after which the homologous recombination machinery replaces the genomic target with the heterologous DNA vector sequence found between the flanking sequences.

Because homologous recombination occurs at a relatively low rate, in order to identify properly targeted cells within the larger cell population, it is common to include a gene in the heterologous DNA to be inserted into the genome, which, once properly inserted, allows selection of the desired cells. For example, genes which confer resistance to drugs which kill mammalian cells (neomycin hygromycin or puromycin resistance), or genes encoding molecules that enable cells to be isolated via flow cytometry (green fluorescent protein or novel cell surface markers), allow isolation of even extremely rare targeted cells from a large mixed population [30].

The use of gene KO approaches was one of the earliest successes in pig genetic modification for xenotransplantation [31]. The porcine genome encodes proteins that can be substantially different from their human counterparts, or that carry additional modifications which are not present in humans and can induce immune responses. These molecules are collectively referred to as "xenoantigens" [32]. Some of the most reactive of these targets are carbohydrate molecules found as post-translational modifications to proteins observed in pig, but not human, cells. Human serum can contain high levels of pre-existing antibodies specific for these porcine-specific glycan epitopes, leading to the destruction of pig cells expressing these molecules through antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) mechanisms [33]. It is not entirely clear why human serum carries antibodies to these particular carbohydrates; one proposal is that the xenoantigens are related to glycans found in the cell walls of pathogens, others suggest that the human dietary consumption of pork causes

antibody generation to the porcine-specific molecules [34]. Because the novel carbohydrate structures are created by specific glycosylation enzymes, it is possible to eliminate the gene responsible for the enzymatic activity and prevent the xenoantigen from being expressed by pig cells.

The GGTA1 gene encodes the enzyme responsible for creating the highly reactive glycan Gal alpha (1,3) Gal epitope in pigs [35]. The KO of the GGTA1 gene is one of the earliest genetic modifications of pigs for application in xenotransplantation, and resulted in greatly reduced human antibody recognition of porcine cells [36, 37]. However, ablation of the GGTA1 gene alone did not completely eliminate porcine cell recognition by human serum antibodies. The enzymes responsible for other xenoantigens, such as CMAH (cytidine monophosphate-N-acetylneuraminic acid hydroxylase critical for Neu5Gc biosynthesis) and B4GALNT2 (beta 1,4 N-acetylgalactosaminyltransferase), have been identified as sources of porcine-specific epitopes bound by antibodies found in human serum. In each case, the deletion of the gene responsible for creating the specific glycan leads to greatly decreased recognition of porcine cells by antibodies in human serum, and reduction in complement-mediated destruction [38, 39].

Another subset of xenoantigens is the swine leukocyte antigens (SLA), the physical and functional equivalent of the human leukocyte antigens (HLA) [40]. Much like the case for human HLA, the SLA genes are highly diverse and individual patients will have a variable level of cross-reactive antibodies in their serum for a given set of SLA genes [41]. Although typing of patients and porcine donors to find the best HLA-SLA matches would be similar to the current system used for determining allotransplant cross-reactivity [42], use of gene targeting or editing technologies could easily eliminate the genes encoding SLA entirely. However, unlike the glycan epitopes described above, the SLA have a critical role in antigen presentation as part of the immune response, and thus the deletion of SLA could create risks of immune deficiencies that outweigh their risks as xenoantigens. Instead, alternate approaches seek to create engineered SLA proteins lacking the epitopes responsible for the immunogenicity while maintaining their antigen presentation functions [43].

5.2 Gene editing

The ease and efficiency of creating gene KO has improved recently through the use of engineered molecules to create genome disruptions in a process referred to as "gene editing". These novel molecules can be designed to generate double-strand DNA breaks at virtually any chosen genomic site *in situ*. Cellular machinery closely surveils the genome for double-strand breaks which are then recognized and often repaired by non-homologous end joining (NHEJ). Because NHEJ relies upon small single-strand overlaps at the ends of a break, the repair may be imprecise and, if within a coding region, can lead to frame shift mutations which inactivate the gene [44].

The most prominent of these novel tools for gene editing are Zinc Finger Nucleases (ZFN), Transcription Activator-Like Effector Nuclease (TALEN) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), each of which consists of two regions: a sequence-specific DNA binding domain and an enzymatic function that creates a double-strand break in the target DNA [45–47]. For ZFN and TALEN, the synthetic DNA binding domain is created by repetitive protein modules which can be joined combinatorially to recognize a particular DNA sequence. Both approaches, while successful, require a significant investment of time and resources to identify functional molecules. CRISPR, like ZFN and TALEN, has the ability to generate double-strand DNA breaks, however, the DNA binding domain relies upon RNA base-pairing with target DNA for its precision. The use of an RNA to guide specificity greatly improves the speed and efficiency in identifying optimal molecules at a much lower cost, which has led to its rapid adoption in genome engineering [48, 49].

The use of CRISPR for the rapid modification of the pig genome was recently demonstrated with the ablation of porcine endogenous retroviral (PERV) sequences. The pig genome carries 25 or more copies of these gamma retroviral sequences, which are transmitted from parent to offspring through inheritance. Application of CRISPR was able to eliminate the PERV sequences from the genome of porcine cells [50]. Although the potential risk of infectious disease from porcine organs caused by PERV sequences in xenotransplantation is debatable, the results show the ability of CRISPR to target multiple, homologous loci throughout the genome. A key question that has arisen regarding large scale CRISPR targeting at multiple genomic sites is whether significant numbers of off-target double-stranded breaks were introduced, which may create unexpected mutations in the resulting pigs [51]. Nonetheless, the ease of use of CRISPR has resulted in widespread adoption for genome engineering in xenotransplantation.

Gene deletion has been instrumental in the advancement of xenotransplantation, however, there are limitations to its application; the genes of interest must be non-essential to pig viability, development, fertility and, most importantly, organ function. The number of distinct loci to be targeted is also a serious consideration, since independently-assorting alleles will be challenging to breed together in a reasonable timeframe. To address these concerns, additional engineering strategies are required as discussed below.

6. Gene insertions

Gene KO and editing techniques have been used for ablation of xenoantigen genes but do not address the need to express human or synthetic genes in pig cells and organs. Unlike gene deletions, gene insertions require heterologous DNA to be introduced into the genome in a manner that allows subsequent expression of the gene(s) encoded by the inserted DNA. Because they are being transferred into the genome from another source, these novel genes are referred to as "transgenes" (TG), whether they are derived from natural or synthetic sequences. The general approaches to introduce TG into the genome are detailed below.

6.1 Random integrant TG

One of the earliest types of genetic modification described in mammals was insertion of DNA into the target genome by random integration. After transfection of DNA into nearly all mammalian cells, some portion of the heterologous DNA can be found incorporated at random sites in the genome [52]. The precise process for this is unclear, but presumably is a result of aberrant repair mechanisms. One hypothesis is that endogenous NHEJ machinery recognizes breaks in the genome and fortuitously utilizes the relatively higher concentration of the heterologous DNA vector sequences to repair the break [53], resulting in the insertion of the TG into the genome.

Agricultural species, including pig, were some of the earliest TG animals described, establishing the utility of this approach [54]. For xenotransplantation, several of the initial TG approaches focused on inhibiting human antibody-mediated damage of porcine organs. The binding of human antibodies to porcine cells leads to complement pathway activation and subsequent cell ablation [55]. The complement function is controlled by several proteins, such as CD46, CD55 and CD59, referred to as complement regulatory proteins (CRPs). The CRPs are broadly expressed on many different cell types to prevent harm from complement activity by raising the threshold of antibody binding required for complement pathway

induction [56]. The transgenic expression of human CRPs in pigs appears to overcome human complement activity, and may have the potential to reduce, or even eliminate, the need for xenoantigen KOs. By placing the human CRP TGs under the control of strong gene expression elements, the CRP protein levels on the porcine cells can be much higher than CRP levels on normal human cells, further increasing resistance to complement-mediated destruction [57].

Pig lineages developed by multiple labs have been engineered to express human CRPs, individually or in combination. In most cases, the porcine cells appeared to be more resistant to complement-mediated destruction, and organs from TG animals survived longer in xenotransplant experiments in non-human primates [58–60]. Because each of the CRPs control a different part of the complement pathway, the use of multiple human TGs was more effective in protecting cells from complement-mediated destruction than individual TGs [61]. Together with the removal of key xenoantigens, the expression of human CRPs by porcine cells has greatly reduced the effects of human serum antibodies on xenografts.

Because random insertion of DNA does not require homologous recombination, it is relatively rapid and efficient to produce transgenic animals [62]. The process is so efficient that the selection methods that are critical for gene KO described above are often unnecessary for TG. Despite the speed and ease of generation, random integration of TGs has several drawbacks. Variabilities in genome structure can affect the expression level of genes inserted at distinct chromosomal regions, such that identical TGs may express at very different levels depending upon their specific location [63]. Furthermore, multiple copies of a given TG may be inserted into the genome at a single site, creating concatenated repeats which can be unstable and yield variable expression levels [64]. Random TG insertion may occur within or near endogenous genes and alter or inactivate their function, leading to tumorigenesis, instability or even lethality [65].

6.2 Homologous recombination

The development of techniques for precise gene KO by homologous recombination has been adapted for site-specific gene insertion or gene knock-in (KI). Similar to the KO vectors described above, the gene to be introduced is flanked by DNA sequences that are identical to regions of the genome to be targeted. After introduction of the heterologous DNA vector, the regions of DNA sequence identity are aligned with the target genome sequence, after which the homologous recombination machinery catalyzes reactions which swap the endogenous genomic DNA with the heterologous DNA within the construct. If the recombination event occurs with high fidelity, the gene of interest will functionally replace the gene that was removed [24]. Similar to gene KO, this approach is much less efficient than random TG integration. Therefore, vectors carrying the TG are often designed to incorporate selectable markers, similar to those used for gene KO, to allow the identification of cells carrying the desired TG in the genome. In this case, both targeted and randomly integrated TGs may be selected, requiring additional assays, such as PCR or Southern blotting, to distinguish between sequence-specific and random insertion events [30].

As described above for gene KO, specific targeting is more efficient in murine ES cells, which express the enzymatic machinery necessary for homologous recombination, than is currently possible for pig primary cells. Insertion of heterologous DNA into the mammalian genome is believed to be driven by endogenous DNA repair mechanisms, presumably in response to DNA breaks, whether randomly via NHEJ, or specifically via homologous recombination [66]. The deliberate introduction of double-stranded DNA breaks at the desired integration site should therefore improve the efficiency of heterologous DNA insertion by activating and recruiting the cellular

repair machinery. Application of ZFN, TALEN and CRISPR technologies have shown that homologous recombination efficiencies are improved when one or more double-strand DNA breaks are introduced into the genome at the desired site of insertion [67] with CRISPR exhibiting bi-allelic targeting rates as high as 90% [68]. The use of these more advanced genome engineering tools has greatly improved the rates and specificity of both gene deletion and gene insertion in genomes.

Gene insertion by homologous recombination for xenotransplantation has not advanced as rapidly as other approaches, in part due to the challenges of using primary porcine cells, for which the efficiencies can be extremely low, particularly with large DNA constructs. Use of improved genome engineering tools with increased targeting efficiencies have already been applied in pig and will continue to grow in impact [69]. However, even with improved efficiencies of gene insertion, breeding pigs with multiple, independently segregating loci is challenging. The number of litters required to produce animals bearing all of the genetic modifications greatly increases with each additional locus, which can be impractical for large animals such as pigs. Therefore, the ability to insert the maximal amount of genetic information into the genome in the minimal number of steps, as discussed below, is highly valuable.

6.3 Multigenic insertion

The most straightforward example of multigenic targeting at a single site takes advantage of the observation that multiple DNA vectors co-transfected into cells will tend to insert together at a given genome site. This approach was used successfully to simultaneously introduce as many as five large transgenes in a single step into porcine cells [70]. Although difficulties in producing mature cloned pigs limited the study to animals with fewer integrated genes, the study demonstrated the feasibility of rapidly making animals with multiple TG.

Another, relatively less complicated, way to introduce multiple TG is to generate large DNA constructs bearing multiple TGs for integration at random into the genome [71]. This greatly reduces the complexity of screening, while increasing the efficiency of insertion, but still relies upon random integrants which can have variable TG copy numbers and expression levels.

Multiple genomic regions have been defined, such as Rosa26, which allow expression of heterologous genes at similar levels regardless of cell type. These "safe harbor" regions are believed to have a chromatin structure that is more easily accessible to the gene expression machinery, regardless of cell type. Targeting at porcine homologs of murine safe harbor sites such as Rosa26 has been described and demonstrates the utility of this approach [70, 72]. On a practical level, the use of safe harbor sites yields more reproducible gene expression than random TG insertions, so fewer lineages are required to select animals with desired TG levels. Furthermore, the defined location and copy number of TG inserted at a safe harbor site makes breeding and genotyping more straightforward, and is expected to provide a less complicated regulatory pathway for clinical use.

As DNA synthesis and assembly has improved, increasingly large DNA constructs encoding a variety of TG are possible, however, as the size of the DNA increases, the rate of insertion decreases. Considering the lower insertion rates observed for large animals such as pig, alternate approaches are necessary to incorporate larger DNAs into the genome.

6.4 Site-specific recombination

Bacteriophage- and yeast-derived site-specific recombinases are, as the name suggests, proteins which catalyze recombination between two specific DNA recognition sites, small (<50 base pair) sequences that are unique to the recombinase being

used. The recombination event is highly efficient, in some cases eliminating the need for selection genes, and allowing large DNA constructs to be inserted at a much higher frequency than possible for homologous recombination [73].

One limitation for the use of site-specific recombination is the need for a recognition sequence to be present in the target genome at the desired locus. This requires a preceding step in which the recognition site is engineered into the genome using less efficient homologous recombination. Therefore, the gain in efficiency for introduction of large DNA constructs may be offset by the need for insertion of the recombinase recognition site into the genome. Despite this constraint, the potential for site-specific recombination into a defined locus has been demonstrated in pigs [70] and provides a route for more rapid complex genetic modifications.

7. Future needs

The advancements in genome engineering, both in general and in their application to xenotransplantation, have been significant, but many needs remain to be addressed. As new genome engineering tools are identified and further refined, improvement of targeting efficiencies will allow more sophisticated modifications of the pig genome. Ideally, the pig genome will become as readily manipulated as the mouse, allowing researchers to further leverage approaches shown to be effective in murine models.

One major technological difference in the genetic modification of mice and pigs (and many other mammalian species) is the lack of ES cells possessing significant rates of homologous recombination that can be grown in culture for extended periods and subjected to multiple manipulations without losing the ability to produce viable pigs. Efforts to identify natural or induced pluripotent stem cells (iPSC) suitable for these purposes have been described, but have yet to demonstrate practical application for porcine genome engineering [74]. Ongoing work will be required to identify and validate cells which meet these needs.

The function of the TGs themselves can also be further improved. The majority of TG constructs used in pigs have used constitutive promoters to drive high level expression of the proteins encoded by the TGs. In some cases, such as CRPs, this approach may be useful, however, overexpression of TGs which inhibit critical immune processes may create risks of immune deficiency and infections. For this reason, use of expression control elements which can turn on and off TG activity is of increasing interest in xenotransplantation. There are multiple examples of inducible promoters employed in mice which can be controlled by exogenously applied small molecules (such as the tetracycline repressor system), or by endogenous signals (such as promoters for innate immune response genes) [75]. Advanced DNA synthesis and assembly methods also allow synthetic biology approaches to create novel signaling pathways and networks not present in nature.

Immune tolerance is another very active area in xenotransplantation research. As the molecular mechanisms controlling the balance between immunity and tolerance are further elucidated, manipulation of the human immune system itself to specifically reduce or eliminate responses to porcine targets, while leaving intact immunity to infectious diseases, will help overcome xenorejection. Multiple approaches are currently being tested and genome targets identified to encourage human immune tolerance of porcine cells and tissues [76, 77].

8. Conclusions

The speed and ease of genome engineering technologies has helped to overcome many of the limitations for the use of pig organs for xenotransplantation. Despite recent

achievements, a key question remains: which combination of genetic modifications is most critical to make a pig organ useful for xenotransplantation? Ongoing experiments seek to address this question, but the answers are likely to be complex and dependent upon the type of organ, the specific immune mechanisms involved, and perhaps other factors that are not yet defined. It is very likely that the first set of genetic modifications of pigs used for xenotransplantation in humans will not be the final set, as the understanding of the mechanisms of xenorejection increases and better strategies developed to influence the human immune response. Continuing progress in genome engineering technologies of pigs will allow the creation of the more complex modifications necessary to meet these demands. Although much remains to be done, it is clear that given the current rate of progress, overcoming the crisis of human organ shortage with unlimited rejection-free porcine organs is rapidly growing closer to reality.

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