EJT European Journal of Transplantation

2023;1:182-191
DOI: 10.57603/EJT-307

Review

ANIMAL ENGINEERING FOR XENOTRANSPLANTATION

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Summary

Xenotransplantation involves the transplantation of organs, tissues, or cells from animals to humans. The increasing need for organ transplantation due to longer life spans and improved medical care cannot be met by human donors. Pigs are considered suitable donors due to their physiological similarities to humans and ease of genetic modification. However, there are scientific, immunological, and ethical challenges associated with xenotransplantation. The severe immune rejection response from human recipients poses a major hurdle, which researchers are trying to or have partially overcome through genetic engineering and immunosuppression therapy. To generate the animals from genetically engineered cells somatic cell nuclear transfer is of paramount importance. Safety concerns include the transmission of infectious diseases from animals to humans, primarily porcine endogenous retroviruses (PERVs), although there have been no reports of transmission to date. Ethical considerations revolve around the welfare and rights of animals involved in research. Genome editing techniques based on programmable nucleases, such as Zinc-Fingers, TALENS and CRISPR-Cas9, have facilitated the modification of pig genomes to address immunological and physiological barriers. However, further research is needed to ensure safety, efficacy, and to develop novel immunosuppression therapies that is second pillar of xenotransplantation. Scientists are also exploring other avenues of research looking at pig-human chimeric organs, where pig embryos with human cells are generated to create organs more tolerable to the human immune system. Challenges include making cells of different species talking to each other and preventing human cells from contributing to other organs in the pig. To ensure the success and ethical implementation of xenotransplantation there is the need for rigorous scientific investigation, regulatory oversight, and public engagement.

Key words: pig, genetic engineering, CRISPR/Cas9, somatic cell nuclear transfer, xenotransplantation

Received: June 29, 2023
Accepted: September 11, 2023

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How to cite this article: Galli C. Animal engineering for xenotransplantation. EJT 2023;1:182-191. https://doi.org/10.57603/EJT-307

INTRODUCTION

Improved medical care and better living standards are increasing the life span of people around the world. Living longer however has an increased incidence of cell, tissue or organ loss of function or failure. This has opened the way to new medical disciplines, such as organ transplantation and more recently regenerative medicine. Xenotransplantation, a pioneering field at the intersection of biology, medicine, and ethics, holds the promise of revolutionizing organ transplantation and addressing the critical shortage of organs for transplantation. Xenotransplantation refers specifically to the transplantation of organs, tissues, or cells between different species, most commonly

from animals to humans. For decades, organ transplantation has been a life-saving procedure for patients suffering from end-stage organ failure. However, the demand for organs significantly exceeds the available supply, resulting in extensive waiting lists, prolonged suffering, and unfortunate deaths. Xenotransplantation presents a potential solution to this problem by utilizing organs from genetically engineered animals, primarily pigs, for transplantation into humans.

Pigs have been chosen as the most suitable donors due to their physiological similarities to humans, organ size compatibility, the ease of breeding and genetic modification [1,2], and the availability of a high resolution map of the genome [3]. Pigs are also very efficient at reproduction and have a relatively short generation interval. Other livestock species are also being used as a source of biological materials for xenotransplantation or as bioreactors for products to be used in therapeutic or biomedical applications [4-6]. Furthermore, other valid alternative options, such as cattle to produce bioprosthetic heart valves can also be envisaged [7]. Nevertheless, xenotransplantation faces numerous scientific, immunological, and ethical challenges that must be overcome to ensure its success and widespread acceptance [8,9]. One of the primary hurdles in xenotransplantation is the severe immune rejection response triggered by the human recipient's immune system against the foreign pig organs [10]. Researchers have been diligently working to develop innovative strategies such as genetic engineering techniques to produce pigs with organs that are less likely to be rejected by the human immune system and on the other side to develop more effective immunosuppression therapy [11]. These genome modifications involve the removal or alteration of specific genes responsible for the synthesis of molecules eliciting an immune response or the addition of specific human genes to make the organs more compatible with the human immune system. An example of xenotransplantation already in clinical use are bioprosthetic heart valves of animal origin, manufactured with wild type tissue [12] but they undergo calcification as a consequence of immune response [13]. Pig islet xenotransplantation from wild type animals has entered clinical trials [14]. It is likely that the clinical outcome could be significantly improved with the use of appropriately engineered animals as source materials. Life supporting solid organs transplanted into nonhuman primates, however, still do not survive long enough to warrant implementation of clinical trials [15] although heterotopic heart transplantation in a primate model has now resulted in the remarkable survival of almost 3 years [16] and in orthotopic transplantation for 195 days [17]. Several immunological hurdles have been identified (Tab. I) and these are currently being addressed at multiple levels through genetic engineering. Moreover, ensuring the safety of xenotransplantation procedures is of paramount importance. Concerns about the transmission of infectious diseases, particularly viruses, from animals to humans, such as porcine endogenous retroviruses (PERVs) [18] or porcine cytomegalovirus (PCMV) [19] have been a subject of intense scrutiny. Extensive research is being conducted to address these concerns and develop comprehensive screening and monitoring protocols to minimize the risk of cross-species infections [20]. However to date there has never been a report of PERV transmission to humans patients following tissue xenotransplantation [21-23]. Despite this context pig solid organs with several genome edits are recently being transplanted to human brain-dead decedent patients [24,25] and for the first time to a patient on a compassionate use [26]. Despite the extensive screening of the donor pig possible failure after 60 days was likely attributable to the presence of PCMV (porcine cytomegalovirus) in the heart of the donor pig [27].

Ethical considerations also surround xenotransplantation. It raises questions about the welfare and rights of animals involved in the research and potential long-term consequences for animal populations. Striking a balance between the potential benefits to human health and the ethical treatment of animals remains a critical challenge that must be addressed through robust regulations and ethical frameworks. While xenotransplantation holds tremendous promise, it is essential to approach this field with cautious optimism, ensuring rigorous scientific investigation, regulatory oversight, and public engagement. If successful, xenotransplantation has the potential to revolutionize organ transplantation, significantly alleviate human suffering, and enhance the quality of life for countless individuals in need of life-saving interventions. With the advent of somatic cell nuclear transfer [28] and now the spectacular development of highly specific synthetic programmable endonucleases [29] the generation of genetically engineered pig lines has grown exponentially in the last 5 to 10 years. Genome editing of pigs for xenotransplantation, specifically using the CRISPR-Cas9 system in various declinations [30], has been a topic of significant scientific research and discussion. The primary objective of genome editing in this context is to modify specific genes in the pig's genome to address the immunological and physiological barriers that exist between humans and pigs. By making targeted genetic modifications, scientists hope to create pigs that are more compatible with human recipients and reduce the risk of rejection or transmission of diseases. The genetic engineering of animals' genome to be realized requires the use of advanced assisted reproduction techniques to generate the animals starting from engineered cells. The enabling technique for this purpose has been SCNT (Somatic Cell Nuclear Transfer) better known as cloning [31]. Several edits are required at the same time or are added by re-cloning of already generated animals with a multi-stacking approach [32,33].

Table I. Immunological barriers to xenotransplantation that can be abrogated through genetic engineering (from Perota and Galli, 2016, mod.).

Problem	Possible cause	Possible solution
Hyperacute rejection (HAR)	Pre-formed antibodies against Galactose 1-,3-galactose and other non-Gal antigens (Neu5Gc); activation of the complement cascade.	KO of 1-3 galctosyltransferase, CMAH, B4GALNT2, iGb3S and other non-Gal antigens Expression of hCRP (CD55, CD46, CD59)
Acute humoral xenograft rejection (AHXR)	De novo antibodies against Galactose 1-,3-galactose and other non-Gal antigens (Neu5Gc); activation of the complement cascade. Endothelial cell activation; Thrombotic microangiopathy Consumptive coagulopathy	hTBM, hEPCR, hA20, TFPI, CD39,HMOX1
Immune cell-mediated rejection (ICMR)	NK and T-cell activation	hTRAIL, CTLA4Ig, HLA-E, hu 2m, CD47, SLA class I
Instant Blood-Mediated Inflammatory Reaction (IBMIR)	Surface proteins, complement mediated, innate immunity, platelets and leucocytes activation	All of the above genetic modifications

KO: Knock Out; Neu5Gc: N-Glycolylneuraminic acid; CMAH: CMP-N-acetylneuraminic acid hydroxylase; B4GALNT2: Beta-1,4-N-Acetyl-Galactosaminyl Transferase 2 ; iGb3S : isogloboside 3; hCRP: human complement regulatory proteins; hEPCR: human endothelial protein C receptor; TFPI: tissue factor pathway inhibitor; TRAIL: human tumor necrosis factor related apoptosis inducing ligand.

However for single or simple edits microinjection into zygotes is considered a viable option in some circumstances other than xenotransplantation [34]. It's important to note that genome editing for xenotransplantation is still an active area of research, and there are significant challenges and ethical considerations that need to be addressed before xenotransplantation becomes a viable clinical option. While progress has been made in modifying pig genomes, further research is required to ensure the safety and efficacy of xenotransplantation procedures and develop novel immunosuppression therapies. Further down the road another route, that is being actively explored is aimed at generating pig-human chimeric organs with greater scientific and ethical challenges [35]. If a pig organ is made up of human cells, the human immune system should better tolerate it. In this scenario, defective pig embryos for one target organ can be generated by genetic engineering and SCNT technology and then aggregated with pluripotent stem cells (PSCs) of human origin (blastocyst complementation), and thus during the development of the resulting animal the defective organ will be generated by the PSCs [36,37]. The question still to be addressed however is how to prevent that human PSC will contribute to other organs of the pig like the brain for example. This review will address the different steps and challenges that need to be addressed to generate a viable animal starting from the selection of the target gene, introduction into the genome of a somatic cell to the cloning and birth of the animals.

GENETIC ENGINEERING (GE) OF THE CELL LINE FOR SOMATIC CELL NUCLEAR TRANSFER (SCNT)

Primary cell lines have a finite lifespan *in vitro*, undergoing senescence after a certain number of population doublings. This window of time is however sufficient to introduce one round of genetic modifications.

The last twenty years have seen the advent and development of programmable nucleases for precise editing of the genome [29,38-40]. What was used in the past to achieve genetic modification essentially exploited the cell DNA repair mechanisms (NHEJ, non-homologous end-joining or HDR, homology directed repair), taking advantage of the double strand DNA breaks occurring spontaneously at a very low pace throughout the genome. With the use of current technology the frequency of DNA breaks is enhanced by a few logs times by the precise cutting ability of the programmable nucleases at selected target sequences. Amongst the programmable nucleases used today for genome editing (Tab. II), CRISPR/Cas9 is the most widely used because of its ease to use, flexibility [41] and low cost and more variants will become available in the future [30]. The full exploitation of the technologies requires accurate DNA sequencing data as well as the software tools necessary for nuclease design, target site selection and experimental validation [42-44] to ensure efficiency and avoid undesired side effects in other genomic *loci*.

These types of nucleases have all been used to successfully edit the genome in a variety of organisms

Table II. Comparison of different programmable nuclease platforms used in livestock genome editing (adapted from Cox et al., 2015, mod. with permission from the Publisher) [38].

	Zinc finger nuclease	TALEN	CRISPR/Cas9
Recognition site	Typically 9–18 bp per ZFN monomer, 18–36 bp per ZFN pair	Typically 14–20 bp per TALEN monomer, 28–40 bp per TALEN pair	22 bp (20-bp guide sequence + 2-bp protospacer adjacent motif (PAM) for *Streptococcus pyogenes* Cas9); up to 44 bp for double nicking
Specificity	Small number of positional mismatches tolerated	Small number of positional mismatches tolerated	Positional and multiple consecutive mismatches tolerated
Targeting constraints	Difficult to target non-G-rich sequences	5 targeted base must be a T for each TALEN monomer	Targeted sequence must precede a PAM
Ease of engineering	Difficult; may require substantial protein engineering	Moderate; requires complex molecular cloning methods	Easily re-targeted using standard cloning procedures and oligo synthesis
Immunogenicity	Likely low, as zinc fingers are based on human protein scaffold; FokI is derived from bacteria and may be immunogenic	Unknown; protein derived from *Xanthamonas* sp.	Unknown; protein derived from various bacterial species
Ease of *ex-vivo* delivery	Relatively easy through methods such as electroporation and viral transduction	Relatively easy through methods such as electroporation and viral transduction	Relatively easy through methods such as electroporation and viral transduction
Ease of *in vivo* delivery	Relatively easy as small size of ZFN expression cassettes allows use in a variety of viral vectors	Difficult due to the large size of each TALEN and repetitive nature of DNA encoding TALENs, leading to unwanted recombination events when packaged into lentiviral vectors	Moderate: the commonly used Cas9 from *S. pyogenes* is large and may impose packaging problems for viral vectors such as AAV, but smaller orthologs exist
Ease of multiplexing	Low	Low	High

including livestock species both for agricultural [45,46] and biomedical applications [47,48] to mention only a few. In the biomedical arena one species of long standing interest for genetic modification has been the pig for xenotransplantation research, usually targeting one specific *locus* [49] or two [50]. When inactivation of a specific endogenous gene(s) is needed then the knockout (KO) approach is required. Firstly this has been the case for the galactose 1-3 galactose epitope by genetic inactivation of the enzyme responsible for its expression (GGTA1, Alpha-1,3-galactosyltransferase) [51], followed by the KO of the enzyme CMAH (cytidine monophosphate-N-acetylneuraminic acid hydroxylase) responsible for the synthesis of Neu5Gc antigen [50,52-54] and more recently by the KO of *B4GalNT2* (1,4-N-acetylgalactosaminyltransferase) gene, done simultaneously with the KO of GGTA1 and CMAH [55]. More recently CRISPR/Cas9 has been implemented to a degree of efficiency to obtain the multiple simultaneous mutation of 3 xenoantigens in the pig [54,55]. In additional developments of the CRISPR system the cytosine base editor (CBE) has been used to convert C to T with high efficiency without causing DSBs. This technique has been developed to silence endogenous genes through directly induced nonsense mutations, which is much safer than ZFN or Cas9 [56,57]. Because this process is carried out on cells cultured in vitro, there are large margins at very limited cost to select amongst many the cell clones carrying the exact desired mutation. Then, by using SCNT, an animal originating from that genotype can be obtained in a relatively short time. Although the direct injection of CRISPR/Cas9 into zygotes can also work in creating GE animals [58,59], when a multiplexed GE is required, the efficiency of HDR mediated KI (Knock In) is low. In addition there is the risk of having a mosaic animal, due to editing taking place at later cleavage stages of the embryo, not in all blastomeres and as a consequence may not be transmitted to the offspring [60]. This possibility represents a too high risk in livestock species that have long generation

intervals compared with the direct derivation of an animal by SCNT from the selected and validated cell clone carrying the desired modification.
Moreover the possibility to target the transgenes of human origin, controlling complement activation, coagulation or inflammation, at a specific location in the pig genome, in so called "safe harbor" loci, such as ROSA-26, [61-63], or in new characterized *loci* [64], AAVS1 or CCR5 identified in the mouse and human genomes [65,66], can control undesirable side-effects, ensure single integration and sustain expression through successive generations. Indeed for xenotransplantation work the "safe harbor" can be the same target for knock out (KO) of xenoantigens like the GGTA1 [67] or the CMAH genes. The targeting at a specific permissive locus can facilitate single copy integration, allow transcription without disruption of endogenous genes. Furthermore, under circumstances of possible lethal effect on embryo development, inducible systems [68] could be used and in general more sophisticated technologies could be implemented to control gene functionality.
If the transgene, because of its biological activity, might require tissue specific expression in endothelial cells for example [69] or in insulin producing cells [70], side-effects of the transgene are reduced and GE is compatible with the life of the animal. Another approach to the control of transgene expression is the use of inducible promoters that can be activated by the administration to the animal of the required activator like tetracycline [71] or doxycycline [68]. Under these circumstances, transgene expression can be triggered when required during the lifespan of the animal or after organ harvesting and transplantation to the recipient. A third approach involves RNA interference technology. This has been used to reduce expression of porcine endogenous retroviruses (PERVs) [72,73], because they are present in multiple copies in the porcine genome, or to reduce expression of the pig Tissue Factor [74] because the KO is not compatible with the survival of animals. In these contexts, siRNA is the best option available as it can reduce the expression of a single gene by up to 95% or more but does not eliminate it completely. When using commercial line of pigs one of the problems in the long term is the size of the organ that continue to grow after xenotransplantation creating obvious consequences to the recipient. Solution to this problem can include the selection of breeds of minipigs or to edit the genome of the farmed pig to knock out the receptor for the GH (growth hormone) that together with the size reduction might unfortunately result in a diseased phenotype [75,76].

SCNT AND BIRTH OF ANIMALS

Cell line preparation and selection

Cell line source is the first key variable in the process of SCNT embryo production and still one of the black boxes responsible for success or failure. Culture conditions, doubling numbers, oxygen tension [77] etc. can all contribute to the selection during in vitro culture of a particular cell population or a sub-population that influence the status of the chromatin and most importantly its susceptibility to be reprogrammed after nuclear transfer. Therefore, from a practical point of view, the identification of cell lines with a high SCNT efficiency can lead to astonishing results as opposed to cell lines that deliver huge failures. The most used cell types are fibroblasts from skin biopsies if the animal to be cloned should be of known genotype/phenotype. If this is not the case, then fetal fibroblasts are the most used cell type especially for GE. There are many reports with claims on the most efficient cell type to be used for cloning pigs [78,79] but this choice might be conflicting with the need to use a particular cell line required for a specific project. GE of the cell line to be used for SCNT generally does not change its ability to be used successfully for generating offspring and a slight reduction was observed in the case of gene *KO* (knock-out) experiments [79],[78]. All cell lines can easily be cryopreserved at early passages before GE, ensuring that the same cells can be used repeatedly in nuclear transfer rounds while controlling for a key variable in the procedure. In the case of GE because of the clonal selection required for screening for the correct mutation, the cells undergo to a high number of population doublings bringing the cells to the limit of senescence that might reduce SCNT efficiency.

Embryo production

Over the years the technique of cloning by nuclear transfer in livestock has not changed in the basic principles pioneered by Willadsen [80] and further developed with somatic cells [28]. The first step is the preparation of a matured enucleated oocyte whereby the metaphase plate is removed from matured oocyte by micromanipulation. In a second step a nucleus coming from a somatic cell that carries the desired mutations is transferred to the enucleated oocyte. Finally in the third step, electrical or chemical activation is induced to resume the cell cycle in the oocyte. The reconstructed embryos are either transferred at one cell stage to the oviducts of synchronized recipients or cultured to the blastocyst stage that can then be transferred to the uterus. The large number of metaphase II oocytes required for embryo production in these species can easily be sourced from slaughterhouses at very low cost and in respect of the 3R principle. The procedures to mature oocytes and culture embryos are well established in the pig [81] and the same are used for SCNT as well. The micromanipulation work is still a bottleneck of the technology as it is labor intensive, it requires specialized equipment and above all experienced embryologists. The metaphase plate can effectively be visualized with Hoechst staining and UV light exposure because the cytoplasm of livestock species is

rich in lipids, making them dark compared to the mouse. A scaling up of the nuclear transfer procedure can be to some extend implemented with what is known as Handmade Cloning [82] or in its various declinations [83] by removing the zona pellucida to facilitate enucleation. However, from a practical point of view, being without zona pellucida it requires that the embryos are cultured *in vitro*, in special dish to avoid sticking them together prior to transfer until the blastocyst stage. Pre-implantation SCNT embryos do have a reduced potential to develop to term, despite having normal morphology. Developmental competence is depending on another "black box" that is cellular reprogramming, i.e how the nucleus of the donor cell is reset to direct normal embryo development. At present it is a very inefficient process that has slowly been re-winded essentially in the mouse [84] and it is only at the beginning for livestock species to be fully understood [85,86]. In mouse, significant improvements in livebirth rates have been obtained with the use of Trichostatin A (TSA), a histone deacetylase inhibitor, during the first few hours of culture of the reconstructed embryo after nuclear transfer to help demethylation of the chromatin to favor reprogramming [87]. Similar approaches with a variety of demethylating agents have been explored successfully in some laboratories in the pig [88].

Pregnancy

Upon transfer, the ability of SCNT embryos to establish pregnancies is by and large lower than that of embryos obtained by fertilization and this has an economic impact because of the cost of carrying recipients not being pregnant or loosing the pregnancy. This can be partially compensated especially in the pig by the transfer of an excess of embryos as SCNT embryo production usually is not a limiting factor. In the pig this is well tolerated, even the transfer of over 100 embryos, since this species can adjust for the number of fetuses developing by physiological reabsorption of the excessive number of embryos developing. Another issue with SCNT pregnancies is the prolonged gestation period usually requiring, induction of parturition and or caesarian C section.

Offspring

Depending on how the success rate is calculated, on the reconstructed embryos or on the transferred blastocyst, development to term can be up to 16% [89], although many variables are responsible of this rate including the pig line, the type of genetic modification introduced into the cells, etc. making comparisons impossible [79,90]. In general, SCNT offspring at birth are more fragile animals and have a higher perinatal mortality. To optimize the survival of SCNT derived offspring, special attention should be given to farrowing and neonatal care. Once the first few days or weeks are over, then the cloned animals have a normal life. They are also fertile and, most importantly, possible phenotypical deviation observed are not observed in their offspring [91-94]. This is an important aspect to be taken into consideration for the commercial application of this technology. A contributing factor to the successful generation of viable animals that can then be successfully bred by natural means is how many genome edits can be tolerated/necessary [32], if those edits are compatible with the homeostasis of a normal animal [95] and that the expression of transgenes inserted are maintained at the desired level. This requires a confirmation by a genotyping and phenotyping of the newborn animals. What exact genetic modifications do we need in the organ-source pig should be fully considered in the future and might require a more systematic approach to assess one edit at a time before going further. An example of excessive, maybe not necessary genome engineering, was the knock-out of all 64 copies of the PERVs present in the genome of one pig line [18] since there is no evidence that there has been in past xenotransplantation experiments transmission to humans [21-23], taking also into consideration that PERV might have physiological role in the genome [96,97] that has yet to be unraveled.

CONCLUSIONS

Significant progress has been made in pig genetic engineering to create pigs with organs suitable for transplantation into humans. Researchers have used gene-editing techniques such as CRISPR/Cas9 to modify pig genes associated with organ rejection, viral transmission, and immunological compatibility. This has led to the generation of many genetically modified pigs with reduced immunological barriers and increased compatibility with human recipients. One of the main challenges in xenotransplantation is the immunological response triggered by pig organs in human recipients. Pigs possess certain genetic elements that can trigger a severe immune response in humans, leading to organ rejection. Genetic engineering aims to address this barrier by modifying or eliminating these elements. While progress has been made, further research is needed to ensure long-term graft survival and prevent immune-mediated rejection. Another critical concern in xenotransplantation is the risk of transmitting porcine viruses or other pathogens to humans. However, comprehensive testing and monitoring protocols must be established to ensure the safety of xenotransplantation in terms of viral transmission. The genetic engineering of pigs for xenotransplantation raises ethical considerations regarding animal welfare and the potential consequences of modifying animal genomes. It is essential to ensure that the welfare of genetically modified pigs is safeguarded, and rigorous ethical frameworks are in place to guide their creation and use. Xenotransplantation involving

genetically modified pigs is a complex and highly regulated field. Before xenotransplantation can become a routine clinical practice, extensive preclinical studies, safety assessments, and regulatory approvals are necessary. Clinical trials are required to evaluate the safety and effectiveness of pig organ transplantation in humans. In conclusion, pig genetic engineering for xenotransplantation shows promising potential to address the organ shortage crisis. While significant progress has been made, further research is needed to overcome immunological barriers, minimize the risk of pathogen transmission, address ethical considerations, and navigate regulatory and clinical challenges. Continued scientific advancements, robust safety measures, and thoughtful ethical considerations will be crucial for the successful translation of pig genetic engineering into safe and effective xenotransplantation therapies.

Conflict of interest statement

The authors declare no conflict of interest.

Funding

This review did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical consideration

Not applicable.

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