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Gene editing advance re-ignites debate on the merits and risks of animal to human transplantation

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- xenotransplantation
- gene editing
- xenozoonosis
- emerging biotechnologies
- tissue engineering

- regenerative medicine

Abstract

In Australia and internationally, the shortage of organ and tissue donors significantly limits the number of patients with critical organ or tissue failure who are able to receive a transplant each year. The rationale for xenotransplantation – the transplantation of living cells, tissues or organs from one species to another – is to meet this shortfall in human donor material. While early clinical trials showed promise, particularly in patients with Type I diabetes whose insulin dependence could be temporarily reversed by the transplantation of porcine islet cells, these benefits have been balanced with scientific, clinical and ethical concerns revolving around the risks of immune rejection and the potential transmission of porcine endogenous retroviruses (PERV) or other infectious agents from porcine grafts to human recipients. However, the advent of CRISPR/Cas9, a revolutionary gene editing technology, has re-ignited interest in the field with the possibility of genetically engineering porcine organs and tissues that are less immunogenic and have virtually no risk of PERV transmission. At the same time, CRISPR/Cas9 may also open up myriad possibilities for tissue engineering and stem cell research which may complement xenotransplantation research by providing an additional source of donor cells, tissues and organs for transplantation into patients. The recent international symposium on gene editing, organised by the US National Academy of Sciences, highlights both the enormous therapeutic potential of CRISPR/Cas9 and the raft of ethical and regulatory challenges that may follow its utilisation in transplantation and in medicine more generally.

Due to demand far outstripping supply in terms of the number of patients with critical organ or tissue failure who are able to receive a transplant each year, governments in Australia and elsewhere around the world have adopted a range of different policies to increase the number of organs available for transplantation, including ‘presumed consent’ or ‘opt out’ systems of consent for organ donation and donation after cardio-circulatory death (DCD) pathways to supplement the donation after brain death (DBD) pool. For the most part, however, the impact of these policies has been modest, and unable to meet the shortfall in human donor material.¹ This situation has provided the impetus for scientific research into alternative means of procuring donor cells, tissues and organs, including in tissue engineering, stem cell biology and xenotransplantation. While distinct, these areas of research and biotechnological enquiry are not mutually exclusive. Rather, as is the case with science more generally, it is anticipated that cross-fertilisation of knowledge across these fields would accelerate progress and facilitate translation of therapeutic applications from bench to bedside.

The recent development of CRISPR/Cas9, a novel gene editing technology which allows custom modification of almost any part of any genome with unmatched precision and accuracy (compared to previous gene editing technologies such as zinc finger nucleases and transcription activator-like effector nucleases) has created enormous excitement in each of these fields of research.² The reason is that one of the major challenges that is shared across tissue engineering, stem cell and xenotransplantation research is the ability to produce donor cells, tissues or organs that faithfully recapitulate the phenotype of the cells, tissues or organs they are designed to replace. And as genotype strongly determines phenotype, and CRISPR/Cas9 technology provides a groundbreaking and potent means of altering genotype, it is not surprising that the advent of CRISPR/Cas9 technology has created great excitement

amongst stakeholders in tissue engineering, stem cell and xenotransplantation research as well as assisted reproductive medicine.³

CRISPR/Cas9 is an enzyme complex derived from bacteria that can be harnessed to bind to and cleave DNA sequences at specific locations, whether in cells of animal or human origin, thereby providing seemingly endless possibilities for gene ‘therapy’.² Not surprisingly, much of the public attention and controversy has revolved around its implications for germline gene editing.⁴ Should, for example, scientists be given free rein to correct gene mutations in human gametes or embryos to prevent hereditary diseases like cystic fibrosis from being passed on to offspring? However, given the moral contest surrounding such questions (and indeed the entire field of assisted reproduction) and the fact that many of the putative ‘benefits’ of CRISPR/Cas9 in reproductive medicine may be achieved through pre-implantation genetic diagnosis, it seems likely that the more immediate impact of CRISPR/Cas9 may lie in its application to genetically modify patient-derived induced pluripotent stem cells, or cells and tissues of porcine origin, for example, which are destined for transplantation into humans.

Potential applications of CRISPR/Cas9 gene editing technology

i) Tissue engineering and 3D printing

CRISPR/Cas9 technology has already been applied to tissue engineering research, opening up a range of exciting possibilities in regenerative medicine.⁵ One of the potential applications of tissue engineering is the transplantation of human endothelial cells to promote revascularisation in patients with ischemic injury. For this to be possible, however, one must overcome the fact that allogeneic endothelial cells undergo cell-mediated immunologic rejection.⁶ Recently, Abrahami and colleagues used CRISPR/Cas9 to ablate the expression of major histocompatibility complex class II molecules in human endothelial cells thereby blocking the activation of allogeneic CD4+ T cells in a co-culture environment, whilst preserving the endothelial cells’ ability to self-assemble into blood vessels.⁵ While this is promising, 3D printing faces other challenges including the difficulty of generating biological constructs that have sufficient structural integrity for surgical implantation, and that comprise a complex microarchitecture of extracellular matrix components and various cell types to reproduce the phenotypic properties of the tissues they are intended to replace.^{7,8}

ii) Stem cell biology

One of the areas of focus of stem cell-based regenerative medicine is the derivation of patient-specific induced pluripotent stem (iPS) cells and their differentiation into specific types of adult cells as a form of autologous cell replacement therapy for patients with various genetic diseases. Examples include the transplantation of healthy myocytes to treat Duchenne’s muscular dystrophy or healthy lung epithelial cells to treat cystic fibrosis. However, for autologous iPS cell therapy to be feasible, the genetic mutations that are present in the patient-derived iPS cells (which are causative for the patient’s genetic condition) must first be corrected before the iPS cells are harnessed for downstream applications. To this end, researchers have already had some success in utilising CRISPR/Cas9 to correct gene mutations in iPS cells derived from patients with Duchenne muscular dystrophy⁹ and patients with cystic fibrosis.¹⁰

Xenotransplantation

While stem cell biology shows significant therapeutic potential, there are many diseases for which it may not be as readily applicable. In these cases, alternative sciences and approaches to transplantation may prove to be more apposite. Since the 1960s, xenotransplantation has been championed as a potential solution to the shortage of human donor material to treat patients with various types of organ and tissue failure.¹¹ Transplanting a patient with acute hepatic failure with a pig liver, for example, could

theoretically restore physiological function or at least ‘buy time’ while the patient waits for a human donor liver. However, the transplantation of animal cells and tissues rather than whole organs has shown the most promise, with phase II studies demonstrating that porcine pancreatic islet transplantation can reverse insulin dependence and reduce hypoglycaemic unawareness in a proportion of patients with Type I diabetes.¹² And despite claims that xenotransplantation may soon be superseded by advances in tissue engineering and stem cell biology,¹³ it seems much more likely that progress in each of these fields will benefit from CRISPR/Cas9 technology, and that any one (or all) of these research avenues may ultimately prove to have significant therapeutic benefits in different contexts.

i) Ethics of xenotransplantation

But despite the therapeutic promise of xenotransplantation, progress in xenotransplantation research has historically been slow. Firstly, the ethical and regulatory questions that it poses – questions that resonate with, but are qualitatively different to those raised by other emerging biotechnologies, such as synthetic biology and stem cell biology – are invariably complex. On the one hand, there is the fundamental question of whether the pursuit of xenotransplantation *in any form* should be permitted or whether it is simply ethically untenable, because it undermines both the ‘natural order’ and the moral status of animals. Indeed, for some, because xenotransplantation entails hybridising humans with animal organs or tissues, this inevitably compromises species boundaries, violates the reverence due to human beings and erodes human dignity.¹⁴ Animal welfare groups, on the other hand, have generally opposed xenotransplantation on the grounds that non-human animals are creatures of moral ‘worth’ and should not be treated as ‘re-designable systems’ that can be manipulated for human use.¹⁵

Moreover, progress in xenotransplantation, in both research and clinical settings, requires that its potential benefits are balanced with the potential risks – not only to the patient (in terms of the risk of immune rejection) but also to public health (particularly regarding the transfer of infectious agents from animal tissues and organs to human recipients). These risks are not insignificant and are inordinately difficult to quantify.

ii) Immune rejection

While immune rejection in (human to human) allotransplantation may be mitigated to some extent by HLA matching between donor and recipient, the significant genetic divide that exists between non-human animals (like pigs) and humans means that a human recipient's immune system is strongly primed to recognise a porcine graft as foreign and to attack it. The major mechanism of immune rejection in porcine islet transplantation is instant blood-mediated inflammatory reaction (IBMIR) which is triggered by preformed antibodies in the human recipient's serum against antigens like galactose- α -1,3-galactose (α Gal) – an oligosaccharide that is not present in humans but is expressed highly by porcine islets – and leads to activation of the host coagulation and complement systems, recruitment of leukocytes and ultimately the destruction of islet structure and function.¹⁶ Following the acute IBMIR response, the islet xenograft may also undergo cellular rejection, either by direct T cell lysis or indirect T cell-mediated actions.¹⁷ A number of strategies have been developed to overcome immune rejection such as gene knockout of the α -1,3-galactosyltransferase enzyme to produce α Gal-deficient islets; transgenic overexpression of human complement regulatory proteins; and encapsulation of transplanted porcine islets to physically shield them from influx of host immune cells.^{18, 19} However, these strategies have had limited success in achieving long-term graft survival and have not removed the need to administer lifelong high-dose immunosuppressant medications to xenotransplant recipients, which in itself causes accelerated vascular disease and increased rates of cancer.²⁰

iii) Xenozoonosis

Xenozoonosis – the transmission of both known and unknown pathogens from donor animals to immunosuppressed transplant recipients, their close contacts and the broader community – also presents a very real clinical and ethical challenge to xenotransplantation. Given the emphasis on porcine cellular or tissue transplantation, most interest has focused on porcine endogenous retroviruses (PERV) which are repetitive, latent retrovirus fragments found in the genome of all pigs and cannot be eliminated by biosecure breeding, and which once activated could potentially give rise to a serious infection in human hosts. PERV virions released from porcine cells have been shown to infect primary human cells *in vitro*, although no cases have been recorded of PERV infection in nonhuman primate or human recipients of porcine grafts.²¹ Various techniques such as small interfering RNAs and zinc finger nucleases have been used to suppress the expression of PERV in porcine tissues, but these have limited efficiency, are labour-intensive and expensive.^{22, 23} Furthermore, data on the transmissibility of PERV from porcine graft to host tissue compartments in small animal and nonhuman primate models cannot readily be extrapolated to humans, as inter-species differences in the PERV receptor mean that the retrovirus is not tropic for rat or murine cells, while it is tropic for cells from baboons and macaques at a reduced efficiency compared to human cells.²⁴ As such, the uncertain risk of xenozoonosis heightens the ethical and epistemic challenge of determining when it is appropriate to cross the translational gap and to move from preclinical to first-in-human trials.

The uncertain and potentially serious risk of xenozoonosis is not, however, borne solely by the research subject or patient but also by their close contacts. This fact alone means that – unlike most other ‘clinical’ interventions – adopting xenotransplantation may have significant public health implications. For these reasons, researchers and regulators have proposed that xenograft recipients (and potentially also their close contacts) would need to comply with mandatory lifelong surveillance and to submit to quarantine if an infection emerged, obligations that, to some, pose unreasonable restrictions on personal liberties and privacy.²⁵

iv) *Regulation of xenotransplantation research in Australia*

In light of both the therapeutic promise of xenotransplantation and the ethicolegal and regulatory concerns raised by this area of research, in 2002/3 the National Health and Medical Research Council (NHMRC) conducted an extensive public consultation process to determine whether and how xenotransplantation research should proceed in Australia.²⁶ Following an initial five year moratorium on all clinical xenotransplantation trials on the basis of the unresolved public health risks, in 2009, the NHMRC decided to allow clinical xenotransplantation research to proceed in Australia provided that there was a robust regulatory framework in place as implemented by the Therapeutic Goods Administration; a robust standard of oversight and monitoring including a surveillance strategy and patient register were established; and researchers and ethics committees had access to clear NHMRC guidance on the conduct of xenotransplantation research.²⁷ Despite the ongoing uncertainty about the risk of xenozoonosis and the lack of available tools to significantly modify this risk at the time, the NHMRC decided to adopt a risk minimisation approach in allowing clinical xenotransplantation research to cautiously proceed. After a lengthy delay, in April 2016, the NHMRC released draft guidelines specific to xenotransplantation research which are proposed for inclusion in the *National Statement on Ethical Conduct in Human Research*.²⁸

v) *Application of CRISPR/Cas9 technology in xenotransplantation*

The recent development of CRISPR/Cas9 is particularly significant and has created such excitement amongst stakeholders in xenotransplantation research because of its potential to mitigate against and perhaps even eliminate the risk of xenozoonosis. Yang and colleagues recently reported using CRISPR/Cas9 to target and inactivate all 62 copies of PERV in the genome of a porcine kidney epithelial

cell line, resulting in a greater than 1000 fold reduction of PERV transmission to human cells *in vitro*.²⁹ Moreover, Yang and colleagues and another group, Sato and colleagues, used CRISPR/Cas9 to target genes encoding immunogenic proteins like α Gal that are expressed on the surface of porcine cells.^{29, 30} These results provide, for the first time, the genuine possibility that porcine tissues and organs could be modified by gene editing techniques and transplanted into human recipients without the need for immunosuppressant medication and with virtually no risk of PERV transmission.

Scientific, ethical and regulatory challenges raised by CRISPR/Cas9 technology

CRISPR/Cas9 technology is not, however, unproblematic, and there remain a series of scientific and ethical concerns. Foremost is the uncertain safety profile of CRISPR/Cas9 gene editing. While Yang and colleagues found that CRISPR/Cas9 was highly specific and did not cause any unintended mutations in the porcine genome apart from at PERV sequences, the use of CRISPR/Cas9 in non-viable human embryos to modify the β -globin gene (causative in the haemoglobin disorder, β -thalassaemia) resulted in cleavage of DNA segments at multiple *off-target* sites, thereby raising the possibility that CRISPR/Cas9 gene editing may lead to unintended oncogenic mutations.³¹ Nevertheless, the safety profile of CRISPR/Cas9 technology will likely become more favourable as it continues to be refined.¹ For example, Slaymaker and colleagues recently demonstrated that structure-guided protein engineering can improve the specificity of Cas9 enzyme derived from the bacterium *Streptococcus pyogenes*, thereby reducing *off-target* effects whilst maintaining robust *on-target* cleavage.³²

CRISPR/Cas9 is also directed to *known* genetic sequences such as PERV, and one of the great challenges of xenotransplantation is that porcine grafts may harbour *unknown* pathogens. While it is true that the risk of zoonoses due to unknown pathogens is low, the potential risks of zoonoses are profound and this fact alone should warn us against scientific hubris in our enthusiasm for harnessing CRISPR/Cas9 technology for xenotransplantation.

CRISPR/Cas9 also raises biosecurity and regulatory concerns – in part because, in contrast with traditional genetic engineering techniques, it is efficacious, cheap and easy to use. Indeed, although it may sound far-fetched, it seems likely that as biosciences become less tied to highly regulated and organised laboratories and more ‘democratised’, technologies like CRISPR/Cas9 may become increasingly accessible both to ‘do-it-yourself biohackers’ outside regulated research environments and, more worryingly, to individuals or groups with mal-intent who may use it to synthesise virulent organisms in order to inflict harm on others.³³

The application of CRISPR/Cas9 in xenotransplantation may also increase pressure for it to be used more widely in other settings that are currently tightly restricted, such as gene editing or ‘repair’ of human germline cells. However, there are concerns that its use in these situations raises the spectre of eugenics and human enhancement for non-medical purposes, may introduce mutations into ‘healthy’ genes and poses transgenerational risks associated with *off-target* germline genomic modification.³

These scientific, ethical and regulatory concerns were explored during the International Summit on gene editing convened by the US National Academy of Sciences in Washington in December 2015. There was general consensus amongst summit participants that basic and preclinical research on CRISPR/Cas9 technology should be pursued in order to better understand the potential benefits and risks of proposed clinical applications.³ In terms of clinical research utilising CRISPR/Cas9 technology, there was widespread support for carrying out somatic cell editing – whether it involve correcting mutations causing sickle cell anemia in blood cells or improving the ability of immune cells to target cancer – under existing regulatory frameworks for traditional gene therapy. In regards to gene editing of human

germline cells in the setting of assisted reproductive therapies (ART) – which was the focus of the summit – it was concluded that clinical germline editing should not proceed until "the relevant safety and efficacy issues have been resolved, based on appropriate understanding and balancing of risks, potential benefits and alternatives...and there is broad societal consensus about the appropriateness of the proposed application".³ Despite the fact that the ethical concerns differ and are more circumscribed, the same could be concluded with regards to gene editing of cells and tissues for the purpose of clinical xenotransplantation, or for tissue engineering and stem cell therapies for that matter.

Conclusion

CRISPR/Cas9 technology has the potential to revolutionise not only xenotransplantation research, but also tissue engineering and stem cell biology, and bring these distinct but complementary avenues of scientific endeavour closer to the clinic. While the fundamental ethical concerns surrounding xenotransplantation will always remain a source of ongoing debate, the development of CRISPR/Cas9 technology provides an impetus for enabling research into xenotransplantation within a regulatory framework that is robust enough to ensure that it proceeds in an ethically defensible manner but flexible enough to provide at least the possibility that xenotransplantation may realise its therapeutic potential.

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