Chapter 1

# Introductory Chapter: The State of Xenotransplantation

Shuji Miyagawa, Akira Maeda, Hiroshi Eguchi, Rieko Sakai, Pei-Chi Lo, Hantang Wang, Chihiro Takakura, Tasuku Kodama, Rei Matsuura and Hiroomi Okuyama

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.69563

## 1. Basic research

Studies of discordant xenografts, such as guinea-pig to rat and pig to human, were started more than 30 years ago. The first subject to be addressed was the mechanism of discordant xenograft rejection [1], i.e., hyperacute rejection. After verification of the reaction of the complement system, xeno-specific glycoantigens in pig-to-human xenotransplantation, such as the $\alpha$-gal epitope, were then studied [2, 3], followed by the other immune systems.

Therefore, the first gene modification on pigs was focused on issues related to complement regulatory proteins (CRPs) such as Membrane Cofactor Protein (MCP, CD46), Decay Accelerating Factor (DAF, CD55), and CD59 [4]. DAF (CD55)-transgenic pigs were then first produced in 1994 [5, 6], followed by other CRP-transgenic pigs [7, 8]. On the other hand, different from the mouse system, pig embryonic stem (ES) cells had not yet been established. Therefore, other methods for reducing the $\alpha$-gal epitope, such as the overexpression of $\alpha$1,2 fucosyltransferase [9], End-β-GalC [10], and GnT-III [11, 12], were examined [13].

Fortunately, the gene targeting technique was combined with (fetus) fibroblasts and the nuclear transfer techniques, resulting in the successful development of $\alpha$-gal knockout (KO) pigs in 2002 [14].

Many kinds of CRP and glycoantigens [15] are now being nominated for transgenic and knockout, respectively, based on improved genetic engineering (GE) techniques. The next obstacle to xenograft is cellular rejection by the innate immune system, which comprises natural killer (NK) cells and monocytes/macrophages.

Strategies for suppressing NK function on the pig cells have been extensively examined. HLA-class Ia molecules, such as HLA-C, but also class Ib, HLA-G1 [16, 17] and –E [18, 19], has been considered in the case of the transgenic pig. In addition, changing the pattern of glycosylation on the surfaces of pig cells is also a reasonable strategy [20–24].

The issue of how to regulate monocytes/macrophages, it is now known that only CD47 [25] binds to SIRP$\alpha$ on the surface of monocytes/macrophages that contains the immune receptor tyrosine–based inhibition motif (ITIM). Therefore, until quite recently, other routes to the downregulation of monocytes/macrophages have not been not well studied.

However, especially in these past 5 years, additional key molecules for suppressing monocytes/macrophages have clearly been identified. Thus, for example, HLA class Ib, HLA-G1 [26], and –E [27] were identified as having a suppressive function not only for NK cells but also for monocytes/macrophages as well. Monocytes/macrophages actually have common receptors in common with NK cells. In addition, changes in glycoantigens, such as the overexpression of the $\alpha$2,6-sialic acids, as well as other methods [28], also function to downregulate monocytes/macrophages [29].

In addition, meanwhile, the many strategies for suppressing the movement of T cells have been proposed, such as class II dominant negative (CIIDN) [30, 31], HLA class I-KO [32], FasL, and tumor necrosis factor receptor I IgG-Fc (TNFRI-Fc) [33]. In addition to immunological studies, studies of coagulation systems, such as thrombomodulin (TM), the tissue factor pathway inhibitor (TFPI), the endothelial cell protein C receptor (EPCR), CD39 and CD73, and anti-apoptotic and anti-inflammatory genes, such as heme oxygenase 1 (HO-1) and A20, have also progressed.

## 2. Genetic engineering

The most progress during these past 5 years involves gene targeting technology. One involves zinc-finger nucleases (ZFN) [34] and is continued by the transcription activator-like effector nuclease (TALEN) [35] method, and finally the clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9 (CRISPR/CAS) [36]. These methods had brought about a revolution in certain aspects of gene-targeting technology. Therefore, the KO of a special gene became extremely easier than in the past. Not only $\alpha$-gal KO but also cytidine monophospho-*N*-acetylneuraminic acid hydroxylase (CMAH, the gene for producing the Hanganutziu-Deicher antigen; NeuGc)-KO, SLA class I-KO, $\beta$4GalNT2-KO [37], etc. have been established in many institutes, combined with transgenic human genes. In addition, the 2A system is now popular in our field and also was a great help in producing transgenic pigs with multigenes [38, 39].

In addition, as a new strategy, attempts are being made to retain the fixed expression of transgenes, because the transgenic expression of each gene was sometimes not stable over generations. Knockin (KI) human genes to the ROSA locus of the pig genome became of interest [40].

## 3. Preclinical study

During these 5 years since the first version, remarkable progress has been made in the area of preclinical xenotransplantation experiments [41–43].

Surprisingly, heterotopic hearts from the GE-pigs continued to beat for almost 2.5 years, when implanted in the monkey abdomen [44], and pig life-supporting kidney could function for nearly 1 year in monkeys [45].

Concerning islet cells, trials in which islet cells from GE-pigs are transplanted in monkeys have been reported. Several groups have reported survival periods of more than 1 year, using adult pig islets (APIs) [46, 47]. Generally speaking, results using neonatal porcine islet-like cell clusters (NPCCs) were worse than those using APIs. It is noteworthy that one group reported a survival of over 600 days using API from wild-type pigs [48], suggesting that the combination of API from GE-pigs and excellent drug therapy may permit islets to survive for more than 2–3 years.

## 4. Porcine endogenous retrovirus (PERV)

Concerning the problems associated with the porcine endogenous retrovirus (PERV) [49], new studies have appeared during these past 5 years, trials to knockout all PERV genes from the pig genome were done using the new techniques, ZFN and CRISPR [50, 51]. However, success has not yet been achieved.

However, in spite of hundreds of patients undergoing transplantation of pig organs or tissue, no reports have appeared of suffering [52]. The controversy associated with the risks of PERV has already been minimized.

## 5. For clinic

In Japan, in 2014, a law related to the pig cell (islets) transplants was passed. In 2016, the guidelines for xenotransplantation were revised. At this moment, clinical detection systems for identifying infectious diseases from pig tissue are being improved. Thus, it has already become possible to start clinical pig islets transplantation. In addition, in the USA, the councilors of the International Xenotransplantation Association (IXA) will be holding meetings with FDA-staff concerning the start of clinical trials in this September at IXA2017 in Baltimore. We are hoping for positive results from this meeting.

On the other hand, regarding clinical trials, many trials have completed and some are ongoing, such as in Sweden [53], China [54], Mexico [55], Argentina, Russia, the USA, and New Zealand [56].

In the near future, possibly within 1 or 2 years, in Japan, the USA, and Europe, some clinical trials involving the use of genetic-modified pigs or microencapsulation pancreatic islets in xenotransplantation will start.

## Author details

Shuji Miyagawa*, Akira Maeda, Hiroshi Eguchi, Rieko Sakai, Pei-Chi Lo, Hantang Wang, Chihiro Takakura, Tasuku Kodama, Rei Matsuura and Hiroomi Okuyama

*Address all correspondence to: miyagawa@orgtrp.med.osaka-u.ac.jp

Department of Surgery, Graduate School of Medicine, Osaka University, Japan

## References

[1] Miyagawa S, Hirose H, Shirakura R, et al. The mechanism of discordant xenograft rejection. Transplantation. 1988;**46**:825-830

[2] Galili U, Swanson K. Gene sequences suggest inactivation of alpha-1,3-galactosyltransferase in catarrhines after the divergence of apes from monkeys. Proceedings of the National Academy of Sciences of the United States. 1991;**88**:7401-7404

[3] Cooper DKC. Depletion of natural antibodies in non-human primates – A step towards successful discordant xenografting in humans. Clinical Transplantation. 1992;**6**:178-183

[4] Miyagawa S, Yamamoto A, Matsunami K, et al. Complement regulation in the GalT KO era. Xenotransplantation. 2010;**17**:11-25

[5] Dalmasso AP, Vercellotti GM, Platt JL, et al. Inhibition of complement-mediated endothelial cell cytotoxicity by decay-accelerating factor. Potential for prevention of xenograft hyperacute rejection. Transplantation. 1991;**52**:530-533

[6] Cozzi E, White DJG. The generation of transgenic pigs as potential organ donors for humans. Nature Medicine. 1995;**1**:964-966

[7] Loveland BE, Milland J, Kyriakou P, et al. Characterization of a CD46 transgenic pig and protection of transgenic kidneys against hyperacute rejection in non-immunosuppressed baboons. Xenotransplantation. 2004;**11**:171-183

[8] Takahagi Y, Fujimura T, Miyagawa S, et al. Production of alpha-1,3-galactosyltransferase gene knockout pigs expressing both human decay-accelerating factor and N-acetylglucosaminyltransferase III. Molecular Reproduction and Development. 2005;**71**:331-338

[9] Sandrin MS, Fodor WL, Mouhtouris E, et al. Enzymatic remodelling of the carbohydrate surface of a xenogenic cell substantially reduces human antibody binding and complement-mediated cytolysis. Nature Medicine. 1995;**1**:1261-1267

[10] Ogawa H, Muramatsu H, Kobayashi T, et al. Molecular cloning of endo-beta-galactosidase C and its application in removing alpha-galactosyl xenoantigen from blood vessels in the pig kidney. Journal of Biological Chemistry. 2000;**275**:19368-19374

[11] Tanemura M, Miyagawa S, Ihara Y, et al. Significant downregulation of the major swine xenoantigen by N-acetylglucosaminyltransferase III gene transfection. Biochemical and Biophysical Research Communications. 1997; **235**:359-364

[12] Miyagawa S, Murakami H, Takahagi Y, et al. Remodeling of the major pig xenoantigen by N-acetylglucosaminyltransferase III in transgenic pig. Journal of Biological Chemistry. 2001;**276**:39310-39319

[13] Byrne GW, Du Z, Stalboerger P, et al. Cloning and expression of porcine β1,4 N-acetylgalactosaminyl transferase encoding a new xenoreactive antigen. Xenotransplantation. 2014;**21**:543-554

[14] Dai Y, Vaught TD, Boone J, et al. Targeted disruption of the alpha-1,3-galactosyltransferase gene in cloned pigs. Nature Biotechnology. 2002;**20**:251-255

[15] Miyagawa S. Xenotransplantation and glycomedicine. In: Kamerling JP, editor. Comprehensive Glycoscience. Elsevier Press; 2007. pp. 533-553.

[16] Sasaki H, Xu XC, Mohanakumar T. HLA-E and HLA-G expression on porcine endothelial cells inhibit xenoreactive human NK cells through CD94/NKG2-dependent and -independent pathways. Journal of Immunology. 1999;**163**:6301-6305

[17] Matsunami K, Miyagawa S, Nakai R, et al. The possible use of HLA-G1 and G3 in the inhibition of NK cell-mediated swine endothelial cell lysis. Clinical & Experimental Immunology. 2001;**126**:165-172

[18] Matsunami K, Miyagawa S, Nakai R, et al. Modulation of the leader peptide sequence of the HLA-E gene up-regulates its expression and down-regulates natural killer cell-mediated swine endothelial cell lysis. Transplantation. 2002;**73**:1582-1589

[19] Matsunami K, Kusama T, Okura E, et al. Involvement of position-147 for HLA-E expression. Biochemical and Biophysical Research Communications. 2006;**347**:692-697

[20] Miyagawa S, Nakai R, Yamada M, et al. Regulation of natural killer cell-mediated swine endothelial cell lysis through genetic remodeling of a glycoantigen. Journal of Biochemical. 1999;**126**:1067-1073

[21] Inverardi L, Clissi B, Stolzer AL, et al. Human natural killer lymphocytes directly recognize evolutionarily conserved oligosaccharide ligands expressed by xenogeneic tissues. Transplantation. 1997;**63**:1318-1330

[22] Artrip JH, Kwiatkowski P, Michler RE, et al. Target cell susceptibility to lysis by human natural killer cells is augmented by alpha-(1,3)-galactosyltransferase and reduced by alpha-(1,2)-fucosyltransferase. Journal of Biological Chemistry. 1999;**274**:10717-10722

[23] Baumann BC, Schneider MK, Lilienfeld BG, et al. Endothelial cells derived from pigs lacking Gal alpha-(1,3)-gal: No reduction of human leukocyte adhesion and natural killer cell cytotoxicity. Transplantation. 2005;**79**:1067-1072

[24] Christiansen D, Mouhtouris E, Milland J, et al. Recognition of a carbohydrate xenoepitope by human NKRP1A (CD161). Xenotransplantation. 2006;**13**:440-446

[25] Ide K, Wang H, Tahara H, et al. Role for CD47-SIRP$\alpha$ signaling in xenograft rejection by macrophages. Proceedings of the National Academy of Sciences of the United States of America. 2007;**104**:5062-5066

[26] Esquivel EL, Maeda A, Eguchi H, et al. Suppression of human macrophage-mediated cytotoxicity by transgenic swine endothelial cell expression of HLA-G. Transplant Immunology. 2015;**32**:109-115

[27] Maeda A, Kawamura T, Ueno T, et al. The suppression of inflammatory macrophage-mediated cytotoxicity and proinflammatory cytokine production by transgenic expression of HLA-E. Transplant Immunology. 2013;**29**:76-81

[28] Sakai R, Maeda A, Choi T-V, et al. Human CD200 suppresses macrophage-mediated xenogeneic cytotoxicity and phagocytosis. Surgery Today. 2017. DOI: 10.1007/s00595-017-1546-2

[29] Maeda A, Kawamura T, Nakahata K, et al. Regulation of macrophage-mediated xenocytotoxicity by overexpression of alpha-2,6-sialyltransferase in swine endothelial cells. Transplantation Proceedings. 2014;**46**:1256-1258

[30] Hara H, Witt W, Crossley T, et al. Human dominant-negative class II transactivator transgenic pigs – effect on the human anti-pig T-cell immune response and immune status. Immunology. 2013;**140**:39-46

[31] Yun S, Gustafsson K, Fabre JW. Suppression of human anti-porcine T-cell immune responses by major histocompati bility complex class II transactivator constructs lacking the amino terminal domain. Transplantation. 1998;**66**:103-111

[32] Reyes LM, Estrada JL, Wang ZY, et al. Creating class I MHC-null pigs using guide RNA and the Cas9 endonuclease. Journal of Immunology. 2014;**193**:5751-5757

[33] Park SJ, Cho B, Koo OJ, et al. Production and characterization of soluble human TNFRI-Fc and human HO-1(HMOX1) transgenic pigs by using the F2A peptide. Transgenic Research. 2014;**23**:407-419

[34] Hauschild J, Petersen B, Santiago Y, et al. Efficient generation of a biallelic knockout in pigs using zinc-finger nucleases. Proceedings of the National Academy of Sciences of the United States of America. 2011;**108**:12013-12017

[35] Miyagawa S, Matsunari H, Watanabe M, et al. Generation of $\alpha$1,3-galactosyltransferase and cytidine monophospho-N-acetylneuraminic acid hydroxylase gene double-knockout pigs. Journal of Reproduction and Development. 2015;**61**:449-457

[36] Kwon DJ, Kim DH, Hwang IS, et al. Generation of $\alpha$-1,3-galactosyltransferase knocked-out transgenic cloned pigs with knocked-in five human genes. Transgenic Research. 2017;**26**:153-163

[37] Martens GR, Reyes LM, Butler JR, et al. Humoral reactivity of renal transplant-wait-listed patients to cells from GGTA1/CMAH/B4GalNT2, and SLA class I knockout pigs. Transplantation. 2017;**101**:e86-e92

[38] Szymczak AL, Workman CJ, Wang Y, et al. Correction of multi-gene deficiency in vivo using a single 'self-cleaving' 2A peptide-based retroviral vector. Nature Biotechnology. 2004;**22**:589-594

[39] Fisicaro N, Londrigan SL, Brady JL, et al. Versatile co-expression of graft-protective proteins using 2A-linked cassettes. Xenotransplantation. 2011;**18**:121-1130

[40] Li S, Flisikowska T, Kurome M, et al. Dual fluorescent reporter pig for Cre recombination: Transgene placement at the ROSA26 locus. PLoS One. 2014;**9**:e102455

[41] Cooper DK, Satyananda V, Ekser B, et al. Progress in pig-to-non-human primate transplantation models (1998-2013): A comprehensive review of the literature. Xenotransplantation. 2014;**21**:397-419

[42] Cooper DK, Bottino R. Recent advances in understanding xenotransplantation: Implications for the clinic. Expert Review of Clinical Immunology. 2015;**11**:1379-1390

[43] Puga Yung GL, Rieben R, Bühler L, et al. Xenotransplantation: Where do we stand in 2016? Swiss Medical Weekly. 2017;**147**:w14403

[44] Mohiuddin MM, Singh AK, Corcoran PC, et al. Chimeric 2C10R4 anti-CD40 antibody therapy is critical for long-term survival of GTKO.hCD46.hTBM pig-to-primate cardiac xenograft. Nature Communications. 2016;**7**:11138

[45] Iwase H, Hara H, Ezzelarab M, et al. Immunological and physiological observations in baboons with life-supporting genetically engineered pig kidney grafts. Xenotransplantation. 2017. Mar;**24**(2). DOI: 10.1111/xen.12293. Epub 2017 Mar 17

[46] Bottino R, Wijkstrom M, van der Windt DJ, et al. Pig-to-monkey islet xenotransplantation using multi-transgenic pigs. American Journal of Transplantation. 2014;**14**:2275-2287

[47] Hawthorne WJ, Salvaris EJ, Phillips P, et al. Control of IBMIR in neonatal porcine islet xenotransplantation in baboons. American Journal of Transplantation. 2014;**14**: 1300-1309

[48] Shin JS, Kim JM, Kim JS, et al. Long-term control of diabetes in immunosuppressed non-human primates (NHP) by the transplantation of adult porcine islets. American Journal of Transplantation. 2015;**15**:2837-2850

[49] Patience C, Takeuchi Y, Weiss RA. Infection of human cells by an endogenous retrovirus of pigs. Nature Medicine 1997;**3**:282-286

[50] Semaan M, Ivanusic D, Denner J, et al. Cytotoxic effects during knock out of multiple porcine endogenous retrovirus (PERV) sequences in the pig genome by zinc finger nucleases (ZFN). PLoS One. 2015;**10**:e0122059

[51] Yang L, Güell M, Niu D, et al. Genome-wide inactivation of porcine endogenous retroviruses (PERVs). Science. 2015;**350**:1101-1104

[52] Morozov VA, Wynyard S, Matsumoto S, et al. No PERV transmission during a clinical trial of pig islet cell transplantation. Virus Research. 2017;**227**:34-40

[53] Groth CG, Korsgren O, Tibell A, et al. Transplantation of porcine fetal pancreas to diabetic patients. Lancet. 1994;**344**:1402-1404

[54] Wang W, Mo Z, Ye B, et al. A clinical trial of xenotransplantation of neonatal pig islets for diabetic patients. J Cent South Univ Med Sci. 2011;**36**:1134-1140

[55] Valdés-González RA, Dorantes LM, Garibay GN, et al. Xenotransplantation of porcine neonatal islets of Langerhans and Sertoli cells: A 4-year study. European Journal of Endocrinology. 2005;**153**:419-427

[56] Elliott RB, Escobar L, Tan PL, et al. Live encapsulated porcine islets from a type 1 diabetic patient 9.5 yr after xenotransplantation. Xenotransplantation. 2007;**14**:157-161

Chapter 2

# Current Status in Peripheral Nerve Xenotransplantation

Jose M. Lasso and Esther Deleyto

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.69143

**Abstract**

Nerve grafts are used to repair segmental defects in peripheral nerves. However, autografts and even allografts are limited for clinical use. Xenotransplantation offers a potentially unlimited source for tissue transplantation. We have conducted a systematic review of the literature, aiming to clarify the latest and more appealing proposals and discoveries in nerve xenotransplantation. A total of 22 articles were retrieved, all of them experimentally controlled studies in animals. There are no current studies in humans. Fresh xenografts provoke an immune response that leads to graft rejection. Immunosuppressive drugs or pretreatment of the grafts are the preferred methods against immune rejection. Recently, investigative groups have proposed the use of acellular nerve xenografts, which do not elicit immune rejection while they do allow and promote axonal regeneration. The addition of human stem cells increases nerve growth. Limits to the analyzed studies are the absence of trials in humans and the short length of the nerve defects that have been successfully repaired. Further investigations and clinical trials are needed before nerve xenografting is accepted as a valid method of nerve repair.

**Keywords:** heterologous transplantation, immune tolerance, nerve repair, peripheral nerves, stem cells, xenografts

## 1. Introduction

Nerve grafts are used to bridge defects in peripheral nerves that cannot be repaired by direct suturing. However, autografts and even allografts are limited for clinical use. The ready availability of xenografts has put them in the center of clinical surgery research as an alternative graft strategy.

Xenotransplantation offers a potentially unlimited source for tissue transplantation, but with the obvious drawback of immune rejection. Many groups are investigating the molecular, immunologic, biologic, and cellular aspects of xenotransplantation and have proposed various

techniques and approaches to perfect the composition of the transplanted tissue and to module the immune response, in an attempt to find the perfect nerve xenograft.

We have conducted a systematic review of the literature, aiming to clarify the latest and more appealing proposals and discoveries in nerve xenotransplantation, which we detail in the following text.

## 2. Systematic review

We searched PubMed and Embase databases, using the combined search terms "xenotransplantation" or "heterologous transplantation" and "peripheral nerve." We screened titles and abstracts and decided which articles to retrieve. Articles were also identified by a manual search of bibliographies from all retrieved articles. Studies were eligible for inclusion if they addressed both heterologous transplantation and peripheral nervous system. Only articles with English language abstracts were included. For those articles that were not available in English, only the content of the abstracts was analyzed. Excluded studies were those addressing the central nervous system. No limits were placed on publication date or study design.

## 3. Results

A total of 22 articles were retrieved, all of them experimentally controlled studies in animals. Most studies used rats as host species [1–15]. Four studies used mice [16–19]. The most commonly used nerve for the nerve defect was the sciatic nerve [2, 3, 5, 8–10, 12–19]. As for donor species, New Zealand rabbits [1, 3, 6, 12] and Sprague-Dawley rats [16–19] were the most commonly used. One study compared the outcomes using different species [9]. One study used human nerves (sural nerve) [9]. Six studies used human mesenchymal stem cells laden in autologous or synthetic conduits [8, 10, 13–15, 20]. There have been no studies with humans as recipients for xenografts.

**Table 1** shows the details of the species and nerve defects used in each study.

Sample size ranged from 6 to 96. Follow-up time ranged from 2 to 360 days. **Table 2** shows details of the sample size, follow-up time, and type of graft used.

### 3.1. Type of graft

Of the 22 retrieved articles, 7 described a study in which nerve defects were repaired with fresh nerve xenografts [4, 7, 16–19]. Six studies used acellular nerve xenografts [1–3, 6, 9]. Two used both fresh xenografts and acellular xenografts [11, 12]. Six studies used biological or synthetic conduits seeded with xenogeneic cells [8, 10, 13–15, 20]. One of the articles does not specify the type of graft used [21].

Among the studies that used acellular nerve xenografts, different extraction procedures were used. Two studies compare results with the extraction procedure as a variable [9,12].

| | Reference | Host | Donor | Gap (mm) |
|---|---|---|---|---|
| 1 | Hebebrand et al. [5] | Lewis rat (sciatic n) | Golden Syrian hamster (sciatic n) | 5 |
| 2 | Hebebrand et al. [21] | ? | ? | ? |
| 3 | De Vaconcellos et al. [11] | Sprague-Dawley rat (median n) | Beagles dog (antebrachial cutaneous n) | 10 |
| 4 | Li et al. [12] | Sprague-Dawley rat (sciatic n) | Rabbit (tibial n) | 15 |
| 5 | Udina et al. [19] | OF1 mouse (sciatic n) | Sprague-Dawley rat (peroneal n) | 6 |
| 6 | Choi and Raisman [4] | AS strain rat (facial n) | Balb-C mouse (sciatic n) | 7–8 versus 15–20 |
| 7 | Lu et al. [7] | Sprague-Dawley rat (peroneal n) | Balb-C mouse (sciatic n) | 10 |
| 8 | Zhang et al. [2] | Sprague-Dawley rat (sciatic n) | York pig (intercostal n) | 10 |
| 9 | Kvist et al. [9] | Wistar rat (sciatic n) | Frog *Rana temporaria*, NRMI mouse (sciatic n), human (sural n), pig *Suidae Yorkshire* (tibial n) | 7 |
| 10 | Jia et al. [3] | Wistar rat (sciatic n) | New Zealand rabbit (?) | 10 |
| 11 | Yu et al. [16] | Balb-C mouse (sciatic n) | Sprague-Dawley rat (sciatic n) | 5 |
| 12 | Huang et al. [22] | Rhesus monkey (radial n) | Landrace pig (tibial n) | 25 |
| 13 | Zhu and Lou [1] | Wistar rat (facial n) | New Zealand rabbit (facial n) | 6 |
| 14 | Sakar et al. [14] | Sprague-Dawley rat (sciatic n) | Human cells | 10 |
| 15 | Gärtner et al. [13] | Sprague-Dawley rat (sciatic n) | Human cells | – |
| 16 | Chai et al. [18] | C57 BL6 mouse (sciatic n) | Sprague-Dawley rat (sciatic n) | 20 |
| 17 | Tremp et al. [8] | Sprague-Dawley rat (sciatic n) | Human cells | 10 |
| 18 | Huang et al. [6] | Wistar rat (facial n) | New Zealand white rabbit (facial n) | 10 |
| 19 | Lasso et al. [20] | New Zealand rabbit (peroneal n) | Human cells | 40 |
| 20 | Zarbakhsh et al. [10] | Wistar rat (sciatic n) | Human cells | 10 |
| 21 | Yu et al. [17] | Balb-C mouse (sciatic n) | Sprague-Dawley rat (sciatic n) | 5 |
| 22 | Masgutov et al. [15] | Rat (sciatic n) | Human cells | 10 |

**Table 1.** Detail of the species and nerve defects used in each study.

In general, the consensus is that fresh xenografts provoke an immune response [5, 16–18] that leads to graft rejection [7, 11, 12, 19]. Choi and Raisman [4] conclude that in short nerve gaps of 7–8 mm, regeneration can occur in spite of the immune rejection, without the need for immunosuppressant drugs, but longer defects of 15–20 mm require immunosuppression to

| | Reference | Sample | Follow up (days) | Type xenograft |
|---|---|---|---|---|
| 1 | Hebebrand et al. [5] | ? | ? | Fresh xenograft ± FK506/RS61443 |
| 2 | Hebebrand et al. [21] | ? | ? | ? |
| 3 | De Vaconcellos et al. [11] | 60 | 360 | ANX versus fresh xenograft |
| 4 | Li et al. [12] | 30 | 180 | ANX versus fresh xenograft |
| 5 | Udina et al. [19] | 35 | 21 | Fresh xenograft ± FK506 |
| 6 | Choi and Raisman [4] | 96 | 84 | Fresh xenograft ± cyclosporine |
| 7 | Lu et al. [7] | 30(?) | 56 | Fresh xenograft |
| 8 | Zhang et al. [2] | 6 | 90 | AXN + autoADSC |
| 9 | Kvist et al. [9] | 53 | 10 | ANX |
| 10 | Jia et al. [3] | 50 | 56 | ANX + BMSC |
| 11 | Yu et al. [16] | 48 (?) | 30 | Fresh xenograft |
| 12 | Huang et al. [22] | 10 | 150 | ANX + autoADSC |
| 13 | Zhu and Lou [1] | 40 | 140 | ANX |
| 14 | Sakar et al. [14] | 27 | 56 | hMSC |
| 15 | Gärtner et al. [13] | | 140 | hUCSC |
| 16 | Chai et al. [18] | 200(?) | 28 | Fresh xenograft |
| 17 | Tremp et al. [8] | 13 | 28 | Fibrin conduit + hADSC or hSVF |
| 18 | Huang et al. [6] | 18 | 84 | ANX |
| 19 | Lasso et al. [20] | 60 | 90 | Vein graft ± Cyclosporine ± hADSC |
| 20 | Zarbakhsh et al. [10] | 24 | 84 | Silicone conduit ± autoBMSC ± hUCSC |
| 21 | Yu et al. [17] | ? | 3 | Fresh xenograft + BDNF |
| 22 | Masgutov et al. [15] | 29 | 65 | hADSC |

ANX, acellular nerve xenograft; hADSC, human adipose-derived stem cells; ADSC, adipose-derived stem cells; BMSC, bone marrow stem cells; BDNF, brain-derived neurotrophic factor; hSVF, human stromal vascular fraction; hUCSC, human umbilical cord stem cells; hMSC, human mesenchymal stem cells.

**Table 2.** Detail of sample size, follow-up time, and type of graft used.

achieve nerve growth. Acellular nerve xenografts do not elicit an immune response [22] and can therefore be used to bridge nerve defects without immunosuppressant drugs with good results [1–3, 6, 9, 11, 12, 22].

Nerve conduits are useful for nerve restoration. Xenogeneic stem cell-laden conduits prove an increased regenerative ability [8, 10, 13–15, 20].

### 3.2. Immunosuppression

There is a total of four studies that compare the outcomes with or without the use of immunosuppressive drugs (two use Cyclosporine A, two use FK506) [4, 5, 19, 20]. Immunosuppressant

treatment with cyclosporine A, FK506, or RS61443 can reduce acute graft rejection and allow nerve regeneration [4, 5, 19].

Two studies propose that specific antibodies against interleukins could also be useful in decreasing graft rejection[16, 18].

Among the studies that use xenogeneic cells only, one compares outcomes with and without immunosuppressive therapy [20]. Tremp et al. [8] and Zarbakhsh et al. [10] suggest that human stem cells act as immunosuppressants, with an ability to induce the production of anti-inflammatory cytokines, and they therefore do not use immunosuppressive drugs.

### 3.3. Defect length

The nerve defect ranged from 5 to 25 mm in the studies that used xenografts and up to 40 mm in one study that used vein grafts laden with human adipose-derived stem cells (hADSC) [2].

The length of the gap that has been successfully bridged is 7–8 mm with unprocessed xenografts [4], 25 mm with acellular nerve grafts [5], and 40 mm with vein conduits seeded with hADSC [20]. The consensus is that only short gaps can reach complete regeneration with a xenograft, and further studies are required to find a viable conduit that bridges longer nerve gaps with a tolerable immune response.

## 4. Discussion

Nerve grafting was first reported by Philipeaux and Vulpian in 1870. The first human nerve graft was reported by Albert in 1878. For decades, research has advanced in favor of autografts, with progress being made in the understanding of nerve biology and chemical mechanisms involved in nerve repair and the perfecting of suture and surgical techniques. But although autologous nerve grafting is ideal, it has some obvious disadvantages, such as lack of availability and donor-site morbidity. For this reason, investigations turned to nerve allografts. Attempts to reduce the rejection of nerve allografts have focused on either nerve graft pretreatment or host immunosuppression [23–25]. The results have not reached those of autografting, and even allografts are a limited source. The ready availability of xenografts has recently put them in the center of clinical surgery research as an alternative graft strategy.

Much of the current research is focused on the study of host immune response to xenografts, as well as the genetics and biochemical reactions involved in graft integration. The immune response to nerve xenotransplantation is poorly understood; most of the research is based on the existing knowledge of nerve allografts.

Peripheral nerves are composed of nerve axons, fibroblasts, Schwann cells, and extracellular matrix. Host Schwann cells are critical for nerve regeneration and production of neurotrophic factors and, Schwann cells of long nerve grafts are also involved in the regenerative process [26]. But donor Schwann cells are one of the most immunogenic components of nerve allografts [27, 28] and it is immune rejection and the scar tissue that is formed due to the immune response that inhibits axon regeneration [29]. To reduce this reaction, allografts have been pretreated to

decrease their antigenicity, but these treatments also reduce Schwann cell viability [30]. Recent studies are moving away from nerve graft pretreatment and toward investigating other mechanisms of immune response suppression.

Lu et al. [7] described the importance of cellular immune responses in xenograft rejection. They also described the limitations in xenografting of cold preservation of the grafts as a way to decrease rejection, a method frequently used in allografting.

Of the same group, Yu et al. [16] proved that xenograft rejection is mediated especially by interferon-gamma (IFγ)-producing Th1 cells and IL17-producing Th17 cells. They suggested that the rejection of a xenograft can be prevented after treatment with IL17 and IFγ-neutralizing antibodies. In a recent study [17], they proposed brain-derived neurotrophic factor as a promising inhibitor of peripheral nerve xenograft rejection. Chai et al. [18] studied the significance of Th22 and Treg cells interaction in the regulation of xenograft rejection.

Based on these studies of immune response, trials have been made using different types of immunosuppressive drugs. Choi and Raisman [4] propose that there is a limit distance that nerve regeneration through a xenograft is able to cover against acute host rejection, but to grow further it requires the assistance of immunosuppression (their experiments are carried out on facial nerve grafts from mouse to rat).

Hebebrand [5, 21] proved increased nerve regeneration through xenografts with immunosuppression with FK506 and RS61443 based on the knowledge that they have on neuroregenerative and neuroprotective effects independent of their immunosuppressive activity. Udina et al. [19] proved that a 5-mg/kg/day dose of FK506 is necessary to achieve nerve regeneration in rat to mice xenografts, as opposed to a 2-mg/kg/day dose for allografts.

There are no clinical studies in humans. Magnusson et al. [31] proposed to begin the study of pig to human xenotransplantation by describing the xenoantigenic pattern on porcine peripheral nerve.

A different line of research regarding peripheral nerve repair has focused on the application of biologic or synthetic nerve conduits [32]. Donor-site morbidity is reduced, as is surgery time, and the problem of rejection is avoided. The ideal properties of a nerve conduit are biocompatibility, biodegradability, neuroinductivity, and neuroconductivity. The last two properties can be enhanced by adding host or xenogeneic multipotent stem cells with the ability to produce the necessary growth factors. Bone marrow stromal cells (BMSCs), human umbilical cord stromal cells (HUCSCs), undifferentiated, and adipose-derived stem cells have been studied, with different results [8, 10, 13–15, 20, 33]. Zarbakhsh et al. [10] conducted a study with 24 Wistar rats, where 10-mm gaps in the sciatic nerve were bridged with a silicone conduit with added bone marrow stromal cells, human umbilical cord stromal cells or no cells. He concluded that both auto-BMSCs and xeno-UCSC have the potential to regenerate peripheral nerve injury and that BMSCs are more effective than HCUCSCs in rat. As opposed to other xenogeneic cells, stem cells did not seem to provoke an immune response in the host after transplantation [34, 35].

Silicone or fibrin scaffolds, or even veins, only provide a physical conduit for nerve regeneration. But the goal is to provide a conduit that is also able to produce the adequate molecular signals that promote cell differentiation, migration, and axonal elongation. This can only be achieved using peripheral nerves as nerve grafts. Therefore, the aim of investigators has been to find or create a non-immunogenic xenograft. Acellular xenografts are created chemically eliminating the cellular constituents that cause immunogenic reactions but preserving the native extracellular matrix, which retains sufficient bioactivity to promote axon regeneration [36].

Huang et al. [22] used acellular xenografts with allogenic adipose-derived stem cells in rhesus monkey, obtaining no immune response to the grafts. They later conducted a study in rat facial nerve defects, achieving similar results to those obtained with allografts [6]. Similar results were reported by Zhu et al. [1].

In 2010, Zhang et al. [2] reported that acellular nerve xenografts, similar to acellular nerve allografts (ANAs), are immunocompatible. He also proposed that short defects can regenerate along acellular scaffolds but that longer defects might require certain cellular impulses, which should be provided by added autologous stem cells.

Li et al. [12] repaired rat sciatic nerve gaps with acellular xenogeneic scaffolds, with good results.

Jia et al. [3] transplanted acellular nerve allografts and rabbit xenografts (ANX), with and without BMSC enhancement, into rat sciatic nerve gaps, comparing the different groups with autografts. They concluded that ANX implanted with BMSCs had a functional rehabilitation efficacy comparable to autografting.

De Vaconcellos et al. [11] repaired 2 cm median nerve gaps in rats with Beagle dog acellular frozen xenografts, managing a correct but slow regeneration, and thus suggesting that freezing suppresses the immune reaction but produces a deficient environment.

Kvist et al. [9] studied the differences in acellular xenografts from different species (frog, mice, human, and pig) transplanted into rat sciatic nerve gaps, proposing differences in axonal outgrowth which should be further studied before clinical use.

All existing studies have a clear limitation regarding the species in which the experiments are carried out on. No studies used humans as hosts, and only one study included human sural nerves as donor for xenografting. Unlike organ transplantation, peripheral nerve grafting does not usually occur in a scenario of urgency, and nerve injury is not life-threatening. Thus, nerve xenografts can only be considered in real clinical situations when benefits are heavier than the risks associated to immunosuppression and even cross-species disease transmission.

The future moves toward a xenograft that is immunocompatible—probably acellular, seeded with xenogeneic stem cells or similar growth factor-producing elements—with no need of immunosuppressive therapy. Also, advance has to be made in the way of creating longer grafts or ways to make the process of regeneration occur fast enough to achieve a complete axonal growth in longer defects before scarring and inflammation block nerve advancement.

## 5. Conclusions

Most of the existing studies on nerve xenografting concur in their results of peripheral nerve xenotransplantation, which are found to be similar to those reached with nerve allografts and acceptable, though lower, compared to the results of autografting. The scenario in which these results can be reached are in all cases similar, defects of 5–25 mm in peripheral nerves, of rats or rabbits mostly, repaired with either fresh xenografts—supplemented with immunosuppressive therapy—or acellular grafts. The direction in which all investigations move is toward adding stem cells or other sources of growth factors that might improve the reach of axonal growth. A long way still separates us from creating a graft that will work in humans.

## Author details

Jose M. Lasso* and Esther Deleyto

*Address all correspondence to: lasso.jm@gmail.com

Hospital Gregorio Marañón, Madrid, Spain

## References

[1] Zhu G, Lou W. Regeneration of facial nerve defects with xenogeneic acellular nerve grafts in a rat model. Head & Neck. 2014;**36**(4):481-486. DOI: 10.1002/hed.23321

[2] Zhang Y, Luo H, Zhang Z, Lu Y, Huang X, et al. A nerve graft constructed with xenogeneic acellular nerve matrix and autologous adipose-derived mesenchymal stem cells. Biomaterials. 2010;**31**(20):5312-5324. DOI: 10.1016/j.biomaterials.2010.03.029

[3] Jia H, Wang Y, Tong XJ, Liu GB, Li Q, et al. Sciatic nerve repair by acellular nerve xenografts implanted with BMSCs in rats xenograft combined with BMSCs. Synapse. 2012;**66**(3):256-269. DOI: 10.1002/syn.21508

[4] Choi D, Raisman G. Immune rejection of a facial nerve xenograft does not prevent regeneration and the return of function: An experimental study. Neuroscience. 2003;**121**(2):501-507. DOI: 10.1016/S0306-4522(03)00433-0

[5] Hebebrand D, Zohman G, Jones NF. Nerve xenograft transplantation: Immunosuppression with FK-506 and RS-61443. Journal of Hand Surgery (British and European volume). 1997;**22**(3):304-307

[6] Huang H, Xiao H, Liu H, Niu Y, Yan R, Hu M. A comparative study of acellular nerve xenografts and allografts in repairing rat facial nerve defects. Molecular Medicine Reports. 2015;**12**(4):6330-6336. DOI: 10.3892/mmr.2015.4123

[7] Lu LJ, Sun JB, Liu ZG, Gong X, Cui JL, Sun XG. Immune responses following peripheral nerve xenotransplantation in rats. J Biomed Biotechnol. 2009;**2009**:412598. DOI: 10.1155/2009/412598. Epub 2009 Oct 12.

[8] Tremp M, Meyer zu Schwabedissen M, Kappos EA, Eangels PE, Fischmann A, et al. The regeneration potential after human and autologous stem cell transplantation in a rat sciatic nerve injury model can be monitored by MRI. Cell Transplantation. 2015;**24**(2):203-211. DOI: 10.3727/096368913X676934

[9] Kvist M, Sondell M, Kanje M, Dahlin LB. Regeneration in, and properties of, extracted peripheral nerve allografts and xenografts. Journal of Plastic Surgery and Hand Surgery. 2011;**45**(3):122-128. DOI: 10.3109/2000656X.2011.571847

[10] Zarbakhsh S, Goudarzi N, Shirmohammadi M, Safari M. Histological study of bone marrow and umbilical cord stromal stem cell transplantation in regenerating rat peripheral nerve. Cell Journal (Yakhteh). 2016;**17**(4):668

[11] De Vaconcellos ZA, Duchossoy Y, Kassar-Duchossoy L, Mira JC. Experimental median nerve repair by fresh or frozen nerve autografts and xenografts. Annales de Chirurgie de la Main et du Membre Supérieur. 1999;**18**(1):74-84

[12] Li YJ, Chen SZ, Qu H. Repair of peripheral nerve defect by xenogeneic acellular nerve basal lamina scaffolds. Zhongguo xiu fu Chong jian wai ke za zhi = Chinese Journal of Reparative and Reconstructive Surgery. 2002;**16**(3):161-165

[13] Gärtner A, Pereira T, Amrmada-da-Silva P, Amado S, Veloso A, et al. Effects of umbilical cord tissue mesenchymal stem cells (UCX) on rat sciatic nerve regeneration after neurotmesis injuries. Journal of Stem Cells and Regenerative Medicine. 2014;**10**(1):14-26

[14] Sakar M, Korkusuz P, Demirbilek M, Çetinkaya DU, Arslan S, et al. The effect of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHNHHx) and human mesenchymal stem cell (hMSC) on axonal regeneration in experimental sciatic nerve damage. International Journal of Neuroscience. 2014;**124**(9):685-696. DOI: 10.3109/00207454.2013.876636

[15] Masgutov RF, Masgutova GA, Zhuravleva N, Salafutdinov II, Mukhametshina RT, et al. Human adipose-derived stem cells stimulate neuroregeneration. Clinical and Experimental Medicine. 2016;**16**:451-461. DOI: 1 0.1007/s10238-015-0364-3

[16] Yu X, Jian Y, Lu L, Gong X, Sun X, et al. A crucial role of IL-17 and INF-y during acute rejection of peripheral nerve xenotransplantation in mice. PloS One. 2012;**7**(3):e34419. DOI: 10.1371/journal.pone.0034419

[17] Yu X, Lu L, Liu Z, Yang T, Gong X, et al. Brain-derived neurotrophic factor modulates immune reaction in mice with peripheral nerve xenotransplantation. Neuropsychiatric Disease and Treatment. 2016;**12**:685. DOI: 10.2147/NDT.S98387

[18] Chai H, Yang L, Gao L, Guo Y, Li H, et al. Decreased percentages of regulatory T cells are necessary to activate Th1-Th2-Th22 responses during acute rejection of the peripheral

nerve xenotransplantation in mice. Transplantation. 2014;**98**(7):729-737. DOI: 10.1097/TP.0000000000000319

[19] Udina E, Voda J, Gold BG, Navarro X. Comparative dose-dependence study of FK506 on transected mouse sciatic nerve repaired by allograft or xenograft. Journal of the Peripheral Nervous System. 2003;**8**(3):145-154

[20] Lasso JM, Cano RP, Castro Y, Arenas L, Garcia J, Fernández-Santos ME. Xenotransplantation of human adipose-derived stem cells in the regeneration of a rabbit peripheral nerve. Journal of Plastic, Reconstructive & Aesthetic Surgery. 2015;**68**(12):e189-197. DOI: 10.1016/j.bjps.2015.07.005

[21] Hebebrand D, Wagner D, Jones NF, Steinau HU. Genetic methods in experimental xenogeneic nerve transplantation. Langenbecks Arch Chir Suppl Kongressbd. 1998;**115**:1279-1281

[22] Huang X, Zhu Q, Jiang L, Zheng C, Zhu Z, et al. Study on immune response after repair of nerve defect with acellular nerve xenograft laden with allogenic adipose-derived stem cells in Rhesus monkey. Zhongguo xiu fu Chong jian wai ke za zhi = Chinese Journal of Reparative and Reconstructive Surgery. 2012;**26**(8):993-1000

[23] Siemionow M, Sonmez E. Nerve allograft transplantation: A review. Journal of Reconstructive Microsurgery. 2007;**23**(8):511-520. DOI: 10.1055/s-2007-1022694

[24] Brown DL, Bishop DK, Wood SY, Cederna PS. Short-term anti-CD40 ligand costimulatory blockade induces tolerance to peripheral nerve allografts, resulting in improved skeletal muscle function. Plastic and Reconstructive Surgery. 2006;**117**:2250-2258. DOI: 10.1097/01.prs.0000219341.73134.82

[25] Hess JR, Brenner MJ, Fox IK, et al. Use of cold-preserved allografts seeded with autologous Schwann cells in the treatment of a long-gap peripheral nerve injury. Plastic and Reconstructive Surgery. 2007;**119**:246-259. DOI: 10.1097/01.prs.0000245341.71666.97

[26] Hall S. Axonal regeneration through acellular muscle grafts. Journal of Anatomy. 1997;**190**(1):57-71. DOI: 10.1046/j.1469-7580.1997.19010057.x

[27] Aguayo AJ, Bray GM, Perkis CS, Duncan ID. Axon–sheath cell interactions in peripheral and central nervous system transplants. Social Neuroscience Symposium. 1979;**4**:361-383

[28] Rovak JM, Bishop DK, Boxer LK, Wood SC, Mungara AK, Cederna PS. Peripheral nerve transplantation: The role of chemical acellularization in eliminating allograft antigenicity. Journal of Reconstructive Microsurgery. 2005;**21**(3):207-213. DOI: 10.1055/s-2005-869828

[29] Evans PJ, Midha R, Mackinnon SE. The peripheral nerve allograft: A comprehensive review of regeneration and neuroimmunology. Progress in Neurobiology. 1994;**43**:187-233

[30] Gulati AK. Evaluation of acellular and cellular nerve graft in repair of rat peripheral nerve. Journal of Neurosurgery. 1988;**68**:117-123

[31] Magnusson S, Strokan V, Svenson L, Mansson JE, Rydberg L, Breimer LE. Expression of carbohydrate xenoantigens on porcine peripheral nerve. Xenotransplantation. 2005;**12**(1):49-58. DOI: 10.1111/j.1399-3089.2004.00192.x

[32] Arslantunali D, Tursun T, Yucel D, Hasirci N, Hasirci V. Peripheral nerve conduits: Technology update. Medical Devices: Evidence and Research. 2014;**7**:405-424. DOI: 10.2147/MDER.S59124

[33] Kalbermatten DF, Kingham PJ, Mahay D, et al. Fibrin matrix for suspension of regenerative cells in an artificial nerve conduit. Journal of Plastic, Reconstructive & Aesthetic Surgery. 2008;**61**(6):669-675. DOI: 10.1016/j.bjps.2007.12.015

[34] Kalinina NI, Sysoeva VY, Rubina KA, Parfenova YV, Tkachuk VA. Mesenchymal stem cells in tissue growth and repair. Acta Naturae. 2011;**3**(4):30-37

[35] Santos JM, Barcia RN, Simoes SI, Gaspar MM, Calado S, et al. The role of human umbilical cord tissue-derived mesenchymal stromal cells (UCX(R)) in the treatment of inflammatory arthritis. Journal of Translational Medicine. 2013;**11**:18. DOI: 10.1186/1479-5876-11-18

[36] Hudson TW, Liu SY, Schmidt CE. Engineering an improved acellular nerve graft via optimized chemical processing. Tissue Engineering. 2004;**10**:1346-1358. DOI: 10.1089/ten.2004.10.1641

Chapter 3

# Current Progress in Corneal Xenotransplantation

Mee Kum Kim

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.69144

**Abstract**

Blindness is a devastating situation, and one of the common causes is corneal blindness. Corneal transplantation is the standard treatment for the corneal blindness. The lack of human donors demands the exploration of alternative treatments such as corneal xenotransplantation and bioengineered corneas. We review the researches regarding immunological and physio-anatomical barriers of corneal xenotransplantation, recent progress of corneal xenotransplantation in nonhuman primate studies, and updates of regulatory guidelines to conduct clinical trials for corneal xenotransplantation. The current development of genetically-engineered and gene-editing technologies suggests that the promise much for the field of xenotransplantation. A clinical trial of xenotransplantation using a cellular porcine corneal stroma has already been conducted; however, safety concerns have not been reported so far. With regard to the regulatory aspects and preclinical efficacies, corneal xenotransplantation has become one of the clinically realistic options as human substitutes and progress in recent research is promising to advance corneal xenotransplantation field.

**Keywords:** cornea, clinical trial, nonhuman primate, regulatory guidelines, transplantation, xenotransplantation

## 1. Introduction

Blindness is a devastating situation with an estimated 39 million cases worldwide, and one of the common causes is corneal blindness [1]. Corneal transplantation is the standard treatment for the corneal blindness. According to "Cost-benefit analysis of corneal transplant," which had been reported by Eye Bank Association of America and the Lewin group in 2013, the net lifetime benefit from the transplantation was estimated at $118,000, whereas the medical cost of the transplant was $16,500 [2]. However, supply of the donor cornea cannot meet the demand in developing countries, and in near future, the number of the eligible cornea

will be reduced in the aged societies of the developed countries [1, 3, 4]. Another reason to seek a substitute for allograft is that ethical concerns about organ trafficking [2, 5]. The lack of human donors and the ethical concerns regarding the human organ trafficking drive the need to explore alternative treatments such as corneal xenotransplantation and bioengineered corneas [2, 6–12]. When a survey was conducted through a telephonic interview to assess how corneal xenotransplantation will be perceived by the society, 42.4% of the individuals in the wait-list for corneal allotransplantation expressed favorable views on corneal xenotransplantation [13].

Cornea is considered applicable as a xenograft, because the eye is regarded as an immune-privileged site. Surprisingly, Dr. Kissam was the first one who conducted pig-to-human corneal xenotransplantation in 1844, although the pig cornea did not survive [14]. Current progress in genetically engineered (GE) pigs and development in gene editing made by clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 technology have made xenotransplantation a possible option for human application [15–21]. Recent advances in corneal xenotransplantation through the success in primate studies and the establishment of international regulatory guidelines have brought us a step closer to apply xenograft in clinical trials [22–25]. In fact, clinical trial of lamellar corneal transplantation using a decellularized porcine graft had been already conducted in human subjects in China to treat fungal ulcers [26].

This chapter reviews the current knowledge of immunological and physiological barriers of corneal xenotransplantation, recent progress of corneal xenotransplantation in animal studies, and updates of regulatory guidelines in order to conduct clinical trials of corneal xenotransplantation.

## 2. Anatomy and physiology in corneal transplantation

A cornea is an avascular and transparent collagenous tissue with a critical role in vision by transmitting and refracting a light in order to focus the light on the macula. Adult human cornea measures 11–12 mm horizontally and 9–11 mm vertically [27]. It is approximately 500–550 μm thick in the center and 700 μm thick in the periphery [27]. The refractive power of the cornea is 40–44 diopters [27].

The cornea consists of three different cellular layers and two interfaces; the epithelial cell layer, Bowman's layer (interface), the stroma containing keratocytes (fibroblasts), Descemet's membrane (interface), and the endothelial cell layer (**Figure 1**) [27]. The thickness of the corneal epithelial layer is approximately 50 μm. Stem cells of the epithelium reside in the limbus, which is located in the peripheral junction between the cornea and the conjunctiva [27]. The stroma constitutes the largest portion, accounting for more than 90% of the total corneal thickness [27]. The uniform arrangement and continuous slow turn-over of the collagen fibers by keratocytes are essential for corneal transparency [27]. A single layer of corneal endothelial cells covers the posterior surface of Descemet's membrane, and it keeps the cornea transparent by actively pumping out the water from the stroma using $Na^+$- and $K^+$-dependent ATPase against imbibition pressure [27].

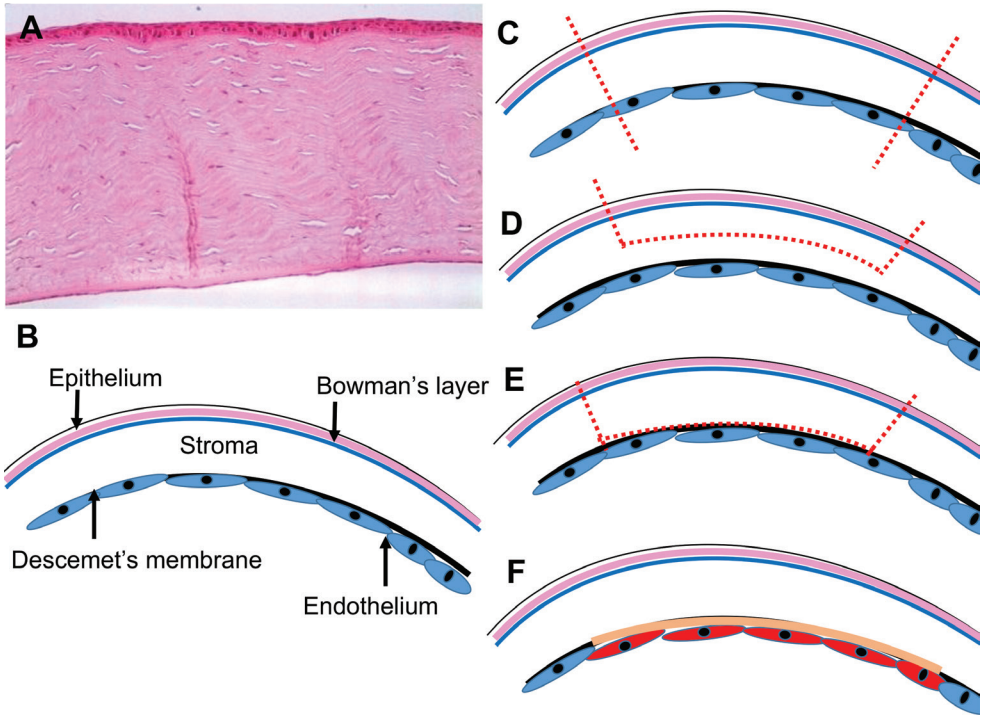


**Figure 1.** Normal anatomy of the cornea and schematic figures of the different types of the keratoplasties. (A) Normal histology of a rabbit cornea in hematoxylin and eosin staining. (B) Schematic figure of a normal cornea which consists of three different cellular layers and two interfaces. (C) Penetrating keratoplasty (PK); a procedure of full thickness replacement of the cornea. (D) Anterior lamellar keratoplasty (ALK); a procedure of partial thickness replacement of the anterior cornea. (E) Deep anterior lamellar keratoplasty (DALK); a procedure of almost the full thickness of stromal layers except Descemet's membrane. (F) Endothelial keratoplasty (EK); a procedure of replacement of the corneal endothelium including Descemet's membrane or posterior stroma.

The cornea is one of the few tissues in the body that enjoy immune-privileged status by passively ignoring or actively modulating immunological reactions [28, 29]. Normal and healthy cornea is devoid of vessels and lymphatic channels, thereby shielding it from immune-mediated attacks by preventing transport of antigens and antigen-presenting cells and thus attenuating the access of immune cells to the graft [28, 29]. Weak or absence of expression of major histocompatibility complex (MHC) class I and II antigens on the corneal cells is also related to the immune privilege of the cornea [29]. In addition, the cornea expresses various cell membrane-bound or soluble immunomodulatory molecules such as Fas ligand (FasL, CD95L), complement regulatory proteins (CRPs), tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), programmed death-ligand 1 (PD-L1), and MHC-Ib that are capable of suppressing immune cells [29]. Interestingly, eye has a unique immune suppression mechanism called anterior chamber-associated immune deviation (ACAID) [29]. In corneal transplantation, the donor allografts are directly contacted with the AC to induce ACAID, a distinctive systemic immune response to alloantigen [28]. ACAID is an active process that induces antigen-specific $CD4^+$ and $CD8^+$ T regulatory cells (Tregs) capable of suppressing cellular immune

responses and protecting a graft from immune rejection in transplantation [29]. However, any infectious or inflammatory events may break down the immunological privilege of the cornea.

The history of corneal transplantation using allografts and xenografts dates back to more than two centuries [3]. Penetrating keratoplasty (PK), a procedure of full thickness replacement of the cornea, has been used as the dominant procedure worldwide [3]. It is a successful method for most causes of corneal blindness. Lamellar transplantation surgery, that selectively replaces only diseased layers of the cornea, consists of anterior lamellar keratoplasty (ALK) and deep anterior lamellar keratoplasty (DALK) [3]. ALK usually replaces partial thickness of the anterior stromal layers and it may induce interface haze between the graft and the recipient corneal stroma. DALK replaces almost the full thickness of stromal layers except Descemet's membrane and endothelial cell layer without inducing interface haze. Both procedures can be applied to patients who have a corneal opacity with an intact endothelial cell layer, and they can eliminate the risk of endothelial rejection [3]. Endothelial keratoplasty (EK) can selectively replace the corneal endothelium in patients with endothelial disease. Rejection risk in PK is higher rather than that in ALK/DLAK or EK [3]. Different types of keratoplasties are schematically shown in **Figure 1**.

## 3. Immunological barriers of corneal xenotransplantation

Although an eye is an immune-privileged site, the innate, humoral, and cellular immune responses are involved in corneal allograft rejection. These immune reactions also happen in corneal xenograft rejection associated with pig antigens. Galactose-alpha-1,3-galactose (e.g. $\alpha$Gal) to which human natural Ig M antibodies are reactive is constantly expressed on porcine cells. This is a critical obstacle to overcome hyperacute xenogeneic rejection in most organ transplantation [30]. Therefore, the distribution of porcine antigens (e.g., $\alpha$Gal, non-Gal) in the cornea has been investigated. It has been found that wild type (WT) porcine cornea expresses $\alpha$Gal mostly in the anterior stromal keratocytes in immunohistochemical or immunofluorescent staining [31, 32]. *In vitro* culture, $\alpha$Gal expression appears on both WT porcine endothelial cells and keratocytes [32]. Based on mass spectrometry, sialylated N-glycans have been identified from both WT porcine corneal endothelial cells and keratocytes [33]. As non-Gal antigens, N-glycolylneuraminic acid (NeuGc) as well as N-acetyl sialic acid (NeuAc) are also identified in both WT corneal endothelial cells and keratocytes [33]. Since $\alpha$1,3-galactosyltransferase gene-knockout (GTKO) pigs that do not express the Gal epitopes have been made [15, 34], the feasibility of GTKO pigs is investigated for the corneal xenograft. In immunofluorescent staining, strong expression of NeuGc has been found in all layers of both WT and GTKO pig corneas [35]. That is to say, both $\alpha$Gal and non-Gal epitopes are widely expressed in WT cornea, whereas antigenic epitopes such as non-Gal are still expressed in GTKO cornea.

*In vitro* study has shown that IgG antibody binding affinities to the cornea or the T cell responses of GTKO pigs are weaker than those of WT pig corneas [35, 36]. NeuGc is a major target of human antibodies, but not a target of nonhuman primate (NHP) antibodies [37, 38]. The absence of $\alpha$Gal or NeuGc on porcine peripheral blood mononuclear cells or corneal cells can significantly

decrease human antibody binding significantly *in vitro* [39, 40]. However, when immune reactions are compared between GTKO/hCD46 and GTKO/hCD46/NeuGc KO pigs, the strength of the human T-cell proliferative response to GTKO/hCD46/NeuGc KO pig cells is similar to that to GTKO/hCD46 pig cells. The absence of NeuGc expression on GTKO/hCD46 pig cells does not diminish human platelet aggregation or decreases the instant blood-mediated inflammatory reaction (IBMIR) to pig cells [41]. In an NHP study, GT KO/CD46 pig corneas are not associated with prolongation of the graft survival or a reduced antibody response compared with WT pig corneas [42]. Taken together, it remains doubtful whether the absence of $\alpha$Gal or NeuGc expression on cornea of the GE pigs might have an advantage over WT cornea in *in vivo* xenotransplantation.

Major histocompatibility complex (MHC) antigens play important roles in corneal allotransplantation [43–45]. Therefore, MHC antigens might have roles in corneal xenotransplantation as in other organ xenotransplantations [46, 47]. In fact, human antiporcine T cell response and binding property of IgG HLA-specific antibodies to pig lymphocytes are similar to an allogeneic responses with both direct and indirect pathways of recognition in the human antiporcine MHC class II responses being functionally intact [48–50]. In DNA microarray, MHC-A has been expressed in both WT porcine corneal keratocytes and endothelial cells [51]. Genetically-engineered Class I MHC knockout pigs have reduced levels of $CD4^{-}CD8^{+}$ T cells in peripheral blood [52]. Modulation of swine MHC by transferring human HLA DPw0401 can reduce human-to-pig cellular response, *in vitro* [53]. Human dominant-negative class II transactivator (CIITA-DN) transgenic pigs that can suppress swine leukocyte antigen (SLA) class II expression have been found to have reduced human T cell response, *in vitro* [54]. Although MHC-related immune response is evidently important in xenotransplantation, *in vitro and in vivo* immune responses against porcine MHCs in corneal xenotransplantation have not been published yet.

An unmodified cellular porcine cornea is defined as a xenotransplant medicinal product, while a decellularized porcine cornea is defined as a medical device [25]. As a medical device, porcine decellularized cornea can be produced in various ways to reduce immunogenicity [55–58]. Decellularized porcine cornea has an advantage on the survival of the graft by reducing immune responses in different animal models as well as in human clinical study [23, 26, 56, 57, 59, 60].

## 4. Rejection mechanism in corneal xenotransplantation through various *in vivo* animal models

In corneal allotransplantation, a $CD4^{+}$ T cell-mediated reaction is primarily involved in graft rejection [8, 61–63], while $CD8^{+}$ T cell- and complement-mediated reactions are partially involved in allograft rejection [64–67].

Rejection mechanisms of corneal xenotransplantation have been investigated using various animal models (**Table 1**) [8, 23, 24, 42, 68–76]. The main rejection mechanism seems to be different depending on the animal model used. Unlike xenotransplantation of the vascular organs, hyperacute rejection (minutes to hours) is not presented in all corneal xenotransplantation models [4, 8].

| Models | Median survival (days) | Proposed rejection mechanism |
|---|---|---|
| Lewis rat-to-guinea pig [68] | 8 | IgM and IgG xenoantibody |
| Guinea pig-to-rat [69] | 7 | T cell, neutrophil, macrophage, Ig G |
| Guinea pig-to-mouse [70, 71] | 9–16 | $CD4^+$ T cell, complement, MHC class II |
| Lewis rat-to-mouse [72] | 9.4 | $CD4^+$ T cell, antibody |
| Pig-to-mouse [73, 74] | 9.0–9.4 | $CD4^+$ T cell, macrophage, complement |
| Pig-to-GTKO mouse [75] | 9.0 | IgG $\alpha$Gal antibody, $CD4^+$ and $CD8^+$ T cell, macrophage |
| WT pig-to-NHP [23, 24] | 26*–189.8** | $CD4^+$ or $CD8^+$ T cell, macrophage, B cell, IgG/IgM antibody, complement |
| GTKO/CD46 pig-to- NHP [42] | 104 | $CD3^+$ T cell, non-Gal pig antibody |
| hCTLA4-Ig pig-to-NHP [76] | 70.3 | Macrophage, $CD3^+CD4^+$ T cell, $CD79^+$ B cell |

GTKO, $\alpha$1,3-galactosyltransferase gene-knockout; WT, wild type; NHP, nonhuman primate; CD46, membrane cofactor protein (MCP).

*Survival of full-thickness keratoplasty (PKP).

**Survival of anterior lamellar keratoplasty (ALK).

**Table 1.** Rejection mechanisms of the corneal xenotransplantation in various animal models.

In Lewis rat-to-guinea pig corneal transplantation, the mean survival time of corneal xenografts has been reported to be 8 days with IgM and IgG xenoantibody production after pre-sensitization [68]. In Guinea pig-to-rat model, the mean survival time of corneal xenografts is reported to be 7 days with a IgG deposition and infiltration of T cells, neutrophils, and macrophages in the graft [69]. In guinea pig-to-mouse corneal xenotransplantation, the median survival time is 9–16 days in wild types, whereas the survival time is extended in mice deficient in the CD4, C3, or MHC class II gene, suggesting that $CD4^+$ T cells, complement, and host antigen-presenting cells might contribute to graft rejection [70, 71]. In Lewis rat-to-mice corneal xenotransplantation, survival time (mean survival time of 44.1 days) of xenograft is found to be longer after treatment with antiCD4 antibody compared to that of the control (mean survival of 9.4 days). However, xenografts treated with antiCD4 antibody are rapidly rejected by antibody-containing serum (mean survival of 21.5 days) [72]. In pig-to-mouse corneal xenotransplantation, median survival time is 9.0 days with macrophages and $CD4^+$ T cells being found in rejected grafts in WT mice, and the survival time is extended in severe combined immunodeficiency (SCID) mice [73]. Natural killer (NK) cells are not involved in the xenogeneic rejection in this model [73]. In pig-to-mouse corneal xenotransplantation, complement depletion has prolonged the survival of xenograft, showing deposition of IgG and IgM in rejected grafts [74]. In pig-to-GTKO mouse corneal xenotransplantation, gradual increase of IgG $\alpha$Gal antibody is evident suggesting that $\alpha$Gal might affect the long-term survival of pig corneal xenografts through antibody-mediated reactions [75].

In pig-to-nonhuman primate (NHP) corneal xenotransplantation, grafts are not hyperacutely rejected, regardless of pig genotypes [7]. In WT pig-to-NHP corneal xenotransplantation,

infiltrations of $CD4^+$ or $CD8^+$ T cells, macrophages, and B cells and deposits IgG/IgM and C3c have been observed in rejected grafts [23, 24]. It indicates that both the cellular and humoral responses are involved in WT corneal xenograft rejection of NHP models as in allograft rejection. In GT KO/CD46 (human complementary regulatory protein) pig-to-NHP corneal xenotransplantation, $CD3^+$ T lymphocytes still infiltrate in the graft accompanied by increased non-Gal pig antibodies in the blood [42]. Cell infiltration in rejected hCTLA4Ig transgenic grafts is mainly composed of macrophages with $CD3^+$, $CD4^+$ T, and $CD79^+$ B cells to a lesser extent than those in WT types of grafts [76]. It indicates that T cell- and antibody-mediated reactions cannot be exempted even in GE pig grafts.

## 5. Anatomical barriers in corneal xenotransplantation

To restore a vision in corneal xenotransplantation as a functional success, anatomical (e.g., diameter, thickness, and tensile strength), physiological (e.g., cellular behaviors), and optical (e.g., refractive power for light to focus on the retina) properties of the substitute cornea should be similar to those of a human cornea. In this regard, WT or GTKO pig cornea is considered as a potential alternative to human cornea (**Table 2**) [4, 7, 77–86].

A major anatomical barrier in corneal xenotransplantation is the difference in corneal thickness between the human recipient and the pig donor. Pig corneal thickness and endothelial cell density are dependent on the age and the breed as shown in **Table 2** [7, 77–79, 81–83]. Pig central corneas are thicker (659–995 μm) than human central corneas (average; 536 μm). The donor thickness should be in the range so that peripheral edges of the cornea between donor and recipient can be appropriately approximated. Unlike human cornea with center to peripheral thickness difference by 150–250 μm, there is no significant difference in the thickness between central (666 μm) and peripheral locations (657–714 μm) of pig cornea [81]. Consequently, a pig cornea whose central thickness is thicker than in human is considered applicable in human in surgical aspect. However, no paper has documented that pig corneal graft with a central thickness of more than 950–1000 μm is capable of being transplanted up to date. Tensile strength of the pig cornea is similar to that of the human cornea which is operable for corneal transplantation, although stress-relaxation of the pig cornea is significantly lower than that of the human cornea [4, 84]. Differences in stress-relaxation do not affect the long-term mechanical maintenance of the graft in NHP studies. Optical power of the pig cornea has been found to be comparable to that of the human cornea [82, 83, 85].

The cornea can maintain transparency by functionally intact corneal endothelial cells. Therefore, endothelial density and proliferative potential in the endothelial cells of the pig cornea should be similar to those of human cornea. The proliferative potentials of pig and human endothelial cells are similar to each other [77, 79]. Endothelial cell density of the pig cornea is decreased depending on age, as similar to that of aged human [77–79, 86]. However, the age-dependent decrease of endothelial cell density in GE pigs ($1714.0 \pm 19.2$ $mm^{-2}$ in 20–25 months old) is higher than that in WT pigs ($2130.2 \pm 193.7$ $mm^{-2}$ in 42 months old) [78]. Considering that more than 2200 $mm^{-2}$ ofthe endothelial cell density is preferred for

| Parameters and breed of the pig | Pig | Human | Mean pig age (months) |
|---|---|---|---|
| **Central corneal thickness (μm)** | | | |
| GE pig (Revivicor, Blacksburg, VA) | 659 [78] | 536 [80] | 1.5 |
| WT Danish Landrace pig (Lars Jonsson Lynge, Denmark) | 666 [81] | | 3.5 |
| WT pig (Wally Whippo, Enon Valley, PA) | 775 [78] | | 5–10 |
| WT SNU miniature pig (Seoul, Korea) | 833 [77] | | 42 |
| Yorkshire pig (Seoul, Korea) | 867 [82] | | 4 |
| GE pig (Revivicor, Blacksburg, VA) | 868 [78] | | 15 |
| Sus scrofa domestica | 877 [83] | | 6–8 |
| GE pig (Revivicor, Blacksburg, VA) | 914 [78] | | 20–25 |
| WT pig (Wally Whippo, Enon Valley, PA) | 995 [78] | | 42 |
| Tensile strength (MPa) [84] | 3.70 | 3.81 | NA |
| Stress-relaxation pattern*; *P* (×100) [84] | 64.6[a] | 85.6 | NA |
| Stress-relaxation pattern*; *K* (−) [84] | 0.0553[a] | 0.0165 | NA |
| Corneal power (Diopter) | 40.2 [82, 83] | 43.7 [85] | 4–8 |
| **Endothelial cell density (/mm$^2$)** | | | |
| WT pig (Wally Whippo, Enon Valley, PA) | 3094 [78] | 2720 [86] | 5–10 |
| GE pig (Revivicor, Blacksburg, VA) | 3022 [78] | | 15 |
| WT SNU miniature pig (Seoul, Korea) | 2625 [77] | | 42 |
| WT pig (Wally Whippo, Enon Valley, PA) | 2130 [78] | | 42 |
| GE pig (Revivicor, Blacksburg, VA) | 1714 [78] | | 20–25 |

The data present average of the parameters.

WT, Wild-type; GE; genetically engineered; NA, not available data.

*$P$ is the value of G (t) at the end of the stress-relaxation test; $K$ is the slope of fitted G (t)-ln t line.

[a]$p < 0.01$ compared with Stress-relaxation pattern in human.

**Table 2.** Anatomical, physiological, and optical properties of the pig cornea compared to those of adult human cornea.

a donation, the age of the pig as a donor should be limited in accordance with endothelial cell density. The age limitation of GE pigs might be different from that of WT pigs. Unlike type-dependent differences of endothelial cell density (WT versus GE), the preservation time-dependent decrease of endothelial cell density in WT pig cornea is not different from that in human cornea [77]. The preservation time-dependent decrease of endothelial cell density in GE pig cornea is not reported.

## 6. Efficacy of corneal xenotransplantation and current progress in *in vivo* animal studies

Survival of a corneal allograft or xenograft is affected by immunologic reaction, graft size, the presence of corneal endothelial cells, and the hierarchical discordancy between the donor and the recipient [87–92]. Therefore, we should compare the survival time of xenografts depending on the various animal models in consideration with the aforementioned risk factors.

Reported results on the survival time of different types of the pig grafts in various animal models are shown in **Tables 3** and **4**. Outcome for small and medium sized animal models is shown in **Table 3**. Decellularized graft survives longer than fresh grafts, and anterior lamellar partial thickness graft without including the endothelial cell layer survives longer than posterior lamellar or full thickness graft that includes the endothelial cell layer (**Table 3**) [56, 57, 60, 73, 93–95].

| Type of pig donor | Recipient | Graft size (mm) | Graft thickness | Median survival (days) |
|---|---|---|---|---|
| Fresh | C57BL/6 mice | 3.0 | Posterior lamellae[+] | 9.0 [73] |
| Fresh | BALB/C mice | 3.0 | Posterior lamellae[+] | 9.0 [73] |
| Fresh | Sprague-Dawley rats | 6.0 | Posterior lamellae[+] | 9.3 [93] |
| Fresh | Sprague Dawley rats | 2.0 | Anterior lamellae | 14.0 [94] |
| Decellularized[ς] | Sprague Dawley rats | 2.0 | Anterior lamellae | 28.0 [94] |
| Fresh | Rabbits | 7.0 | Anterior lamellae | 29.1 [95] |
| Fresh | Rabbits | 7.0 | Full thickness | 16.8 [95] |
| Decellularized[*] | Rabbits | 8.0 | Anterior lamellae | >180 [57] |
| Decellularized[ξξ] | Rabbits | 6.3 | Anterior lamellae | 84 [60] |
| Decellularized[**] | Rabbits | 10.0 | Anterior lamellae | 365 [56] |

[+]Posterior lamellae that includes endothelial cell layer (Anterior lamellae does not include endothelial cell layer).
[ς]Lyophilized graft.
[*]Treated with hypertonic saline.
[ξξ]Treated with 200 U/ml phospholipase A2 and 0.5% sodium deoxycholate.
[**]Treated with sodium dodecyl sulfate.

**Table 3.** The median survival time of various types of the pig grafts in small- or medium-sized animal models.

| Type | Donor pig | Recipient (number) | Immunosuppression | Survival (days) | Reported year |
|---|---|---|---|---|---|
| ALK | WT | Cynomolgus ($n$ = 6) | None | >30[+], >30[+], >30[+], 75, 165, 180 | 2003 [31] |
| ALK | WT | Rhesus ($n$ = 4) | None | >90, >90, >90, >90 | 2007 [22] |
| ALK | WT | Rhesus ($n$ = 5) | None | 180, 15, 180, 180, 180 | 2011 [97] |
| ALK | WT | Rhesus ($n$ = 4) | Local and systemic steroid | >398, >194, 24.5, 24.5 | 2011 [23] |
| ALK | WT | *Macaca fascicularis* ($n$ = 4) | Local steroid | 9, 70, 21, 21 | 2014 [76] |
| DALK | WT | Rhesus ($n$ = 5) | Steroid+antiCD40 antibody | >389, >382, >236, >201, >61 | 2017 [99] |
| ALK[*] | WT | Rhesus ($n$ = 5) | None | 180, 180, 180, 180, 180 | 2011 [97] |
| ALK[*] | WT | Rhesus ($n$ = 5) | Local steroid | 180, 180, 180, 180, 180 | 2011 [97] |
| ALK[*] | WT | Rhesus ($n$ = 5) | Local and systemic steroid | >391, >265, >208, >195, 28 | 2011 [23] |
| ALK | hCTLA4-Ig transgenic | *Macaca fascicularis* ($n$ = 4) | Local steroid | 21, 50, 90, 120 | 2014 [76] |
| ALK | GTKO/hCD39/ hCD55/hCD59/FT | *Macaca fascicularis* ($n$ = 2) | Local steroid | 9,34 | 2014 [76] |
| PKP | WT | Rhesus ($n$ = 4) | Local steroid | 129, 276, 182, 144 | 2007 [22] |
| PKP | WT | Rhesus ($n$ = 6) | Cyclophosphamide+BMT | 32, 42, 40, 34, 38, 30 | 2013 [98] |
| PKP | WT | Rhesus ($n$ = 6) | Cyclophosphamide | 12, 18, 16, 20, 20, 20 | 2013 [98] |
| PKP | WT | Rhesus ($n$ = 3) | Local and systemic steroid | 21, 28, 29 | 2015 [24] |
| PKP | WT | Rhesus ($n$ = 4) | Local and systemic steroid + antiCD154 antibody | >933, >243, 318, >192 | 2015 [24] |
| PKP | WT | Rhesus ($n$ = 4) | Local steroid | 157, 28, 92, 33 | 2017 [42] |
| PKP | GTKO/CD46 | Rhesus ($n$ = 4) | Local steroid | 128, 57, 47, 171 | 2017 [42] |

ALK, anterior lamellar keratoplasty (partial thickness); BMT, bone marrow transplantation; DALK, deep anterior lamellar keratoplasty; FT, fucosyl transferase; GTKO, $\alpha$1,3-galactosyltransferase gene-knockout; hCTLA4-Ig, human cytoxic T-lymphocyte-associated antigen4-immunoglobulin; hCD39, human ectonucleoside triphosphate diphosphohydrolase-1; CD46, membrane cofactor protein (MCP); hCD55, human complement decay-accelerating factor; hCD59, human MAC-inhibitory protein; PKP, penetrating keratoplasty (full thickness).

[+]Sacrificed at 1 month for histology.

*Decellularized cornea.

**Table 4.** Current progress on clinical efficacies in pig-to-NHP corneal xenotransplantation from 2003 to 2017.

Current progress on clinical efficacies in pig-to-NHP corneal xenotransplantation from 2003 to 2017 is shown in **Table 4** [7, 22–24, 31, 42, 76, 96–99]. Some studies have presented encouraging outcomes in lamellar or full-thickness corneal xenotransplantation with or without immunosuppressants. The survival time varies depending on the breed of the donor and recipients, immunosuppressive protocols, and types of the corneal grafts. Processed acellular corneas can prolong the survival time of ALK. With steroid treatment, partial thickness corneal transplantation that does not include endothelial cell layer (ALK) shows better survival than full thickness corneal transplantation (PKP). GE pigs in ALK or PKP do not show significant increase of the survival time compared to the control. With antiCD154 treatment, PKP using WT Seoul National University (SNU) miniature pig has demonstrated the longest survival time in the NHP model. Taken together, corneal xenotransplantation using fresh pig graft still requires stronger immunosuppressant than steroid alone, regardless of the type of donor pig (WT or GE).

## 7. Updates on regulatory aspects of corneal xenotransplantation

In 2013, the first consensus on guidelines for clinical trials of corneal xenotransplantation has been established in Korea [87]. Thereafter, international consensus statement on conditions for undertaking clinical trials of xenocorneal transplantation has been finally published in International Xenotransplantation Society (IXA) in 2014 [25]. IXA consensus statements on conditions for clinical trials of corneal xenotransplantation include the followings; (1) ethical requirement, (2) quality control of source pigs, (3) quality control of pig corneal products, (4) preclinical efficacy and safety data that are required to justify a clinical trial, (5) strategies to prevent porcine endogenous virus transmission (PERV) transmission, and (6) patient selection and informed consent.

Key ethical requirements for clinical trials of corneal xenotransplantation are essentially identical to those required in other areas of clinical trials. These guidelines adhere to the basic ethical principles for clinical trials of islet xenotransplantation established by the Ethics Committee of the IXA and the Changsha Communique of the World Health Organization [25, 100]. Regulatory guidelines for pig sources and strategies to prevent porcine endogenous virus transmission (PERV) are basically the same as those for clinical trials of islet xenotransplantation [101–103].

Guidelines for corneal-specific issues have been intensively discussed on the procurement of porcine corneal products, preclinical efficacy, and safety data to justify initiation of a clinical trial, and inclusion criteria of the subjects. In order to be enrolled, the subject must meet the following criteria; (1) must be diagnosed with legal blindness as defined by the American Medical Association and the United States Congress as best corrected visual acuity of 20/200 or less in the better eye, (2) must be diagnosed with a corneal blindness that can be only cured with a corneal transplantation, (3) must not have timely access to receive corneal allotransplantation, (4) must be over the legal age, (5) must not be pregnant, must not plan to become pregnant, and must not be breast feeding, and (6) should be highly compliant. Keratoconus should be excluded due to the excellent allograft survival and younger age of the subject. Guideline for visual acuity can be exempted in a subject who requires an emergency

operation for actual or impending corneal perforation. Regarding adequate procurement of the corneal xeno-product, the guidelines of the European Eye Bank Association (EEBA) on the preparation of human corneal tissue should be adopted under provision that laboratory tests have confirmed that biological properties of the preserved pig cornea based on EEBA guidelines are comparable to those of the preserved human cornea. To prove preclinical efficacy, NHP data that the pig cornea xenograft should survive for more than 6 months in five of eight consecutive NHPs are required (ideally for 12 months in one or two successful cases). Compared to the 5-year survival rate (70–80%) of the islet allotransplantation, mean 5-year survival rate of corneal allotransplantation among the various corneal diseases is similar to each other (70–80%) [104–106]. Therefore, the same preclinical efficacy that has been accepted for islet xenotransplantation can be applied to corneal xenotransplantation with provisional condition that patient who is diagnosed as keratoconus must be excluded.

In 2016, the IXA consensus statement on conditions for undertaking clinical trials of porcine islet products has been revised for the first time [107–114]. New or under-appreciated topics have been discussed and updated regarding regulatory framework, genetic modification of the source pig, recipient monitoring for preventing disease transmission, patient selection, porcine islet product manufacturing, and quality control of source pigs. To undertake clinical trials of corneal xenotransplantation, under-appreciated topics as follows should also be addressed and revised [2]. (1) In source pigs, PERV-C negative donor pigs should be considered preferable, and donor pig selection criteria should be primarily based on low PERV expression levels and the lack of infectivity. (2) Clinical trial protocols using GE pig products also need to be assessed on a case-by-case basis. (3) For preclinical efficacy in corneal xenotransplantation, the finding that survival in four of six (or five of eight) consecutive NHP experiments may be sufficient to indicate potential success of a clinical trial that is similar to those in islet xenotransplantation. (4) Clinically relevant microorganisms should be included in pig screening programs. (5) When microorganisms are confirmed to be absent in the donor pig by sensitive microbiological examination, recipients need not to be monitored. (6) Lifelong surveillance for PERV should be adjusted based on the clinical sign and the laboratory test if the subjects do not show any suspicious sign of PERV infection by sensitive laboratory examination for 2 years. In a clinical trial of islet cell xenotransplantation using microencapsulated pig islets, PERV DNA and PERV RNA are not detected in peripheral blood up to 113 weeks by real-time RT-PCR [115]. In this clinical trial, the subjects were followed-up for two years. If the risk of PERV transmission is proved to be negligent, follow-up time should be adjusted accordingly. Given that substantial scientific progress has been made in islet xenotransplantation and cornea field, the international consensus statement on corneal xenotransplantation is expected to be updated regarding these under-appreciated issues.

## 8. Future perspectives

Due to progresses made in immunosuppressive protocols, the availability of GE pigs, and appropriate guidelines for clinical trials, corneal xenotransplantation using pig cornea might be a feasible option to solve the shortage of donor corneas in the future. Decellularized porcine

graft also appears to be efficient in a clinical trial. Results of recent experiments of the corneal xenotransplantation in NHP models using cellularized pig grafts are encouraging, and it helps us decide whether we should keep developing xeno-related products of cornea. With better understanding on the antigenicity of pig cornea and the rejection mechanism involved in corneal xenotransplantation, optimized and standardized immunosuppression should be established before conducting a human clinical trial. As for fresh corneal grafts from GE pigs, the further experiments need to be performed to verify their efficacies as substitutes for human corneas.

## Acknowledgements

This study was supported by a grant from the Korea Healthcare Technology R&D Project, Ministry for Health & Welfare, Republic of Korea (Project No. HI13C0954).

## Author details

Mee Kum Kim

Address all correspondence to: kmk9@snu.ac.kr

1 Department of Ophthalmology, Seoul National University College of Medicine, Seoul, Korea

2 Laboratory of Ocular Regenerative Medicine and Immunology, Seoul Artificial Eye Center, Seoul National University Hospital Biomedical Research Institute, Seoul, Korea

## References

[1] Lamm V, Hara H, Mammen A, Dhaliwal D, Cooper DK. Corneal blindness and xenotransplantation. Xenotransplantation. 2014;**21**(2):99-114. DOI: 10.1111/xen.12082

[2] Kim MK. Commentary: Current status and future of corneal xenotransplantation. Transplantation Technologies & Research. 2016;**6**:3

[3] Tan DT, Dart JK, Holland EJ, Kinoshita S. Corneal transplantation. Lancet. 2012;**379**(9827): 1749-1761. DOI: 10.1016/s0140-6736(12)60437-1

[4] Hara H, Cooper DK. Xenotransplantation—The future of corneal transplantation? Cornea. 2011;**30**(4):371-378. DOI: 10.1097/ICO.0b013e3181f237ef

[5] Treasure T. The Falun Gong, organ transplantation, the holocaust and ourselves. Journal of the Royal Society of Medicine. 2007;**100**(3):119-121. DOI: 10.1258/jrsm.100.3.119

[6] Ekser B, Ezzelarab M, Hara H, van der Windt DJ, Wijkstrom M, Bottino R, et al. Clinical xenotransplantation: The next medical revolution? Lancet. 2012;**379**(9816):672-683. DOI: 10.1016/s0140-6736(11)61091-x

[7] Kim MK, Hara H. Current status of corneal xenotransplantation. International Journal of Surgery. 2015;**23**(Pt B):255-260. DOI: 10.1016/j.ijsu.2015.07.685

[8] Kim MK, Wee WR, Park CG, Kim SJ. Xenocorneal transplantation. Current Opinion in Organ Transplantation. 2011;**16**(2):231-236. DOI: 10.1097/MOT.0b013e328344870c

[9] Hayashi R, Ishikawa Y, Sasamoto Y, Katori R, Nomura N, Ichikawa T, et al. Co-ordinated ocular development from human iPS cells and recovery of corneal function. Nature. 2016;**531**(7594):376-380. DOI: 10.1038/nature17000

[10] Griffith M, Osborne R, Munger R, Xiong X, Doillon CJ, Laycock NL, et al. Functional human corneal equivalents constructed from cell lines. Science. 1999;**286**(5447):2169-2172

[11] Carlsson DJ, Li F, Shimmura S, Griffith M. Bioengineered corneas: How close are we? Current Opinion in Ophthalmology. 2003;**14**(4):192-197

[12] Griffith M, Harkin DG. Recent advances in the design of artificial corneas. Current Opinion in Ophthalmology. 2014;**25**(3):240-247. DOI: 10.1097/icu.0000000000000049

[13] Lee JJ, Kim DH, Jang YE, Choi HJ, Kim MK, Wee WR. The attitude toward xenocorneal transplantation in wait-listed subjects for corneal transplantation in Korea. Xenotransplantation. 2014;**21**(1):25-34. DOI: 10.1111/xen.12069

[14] Kissam RS. Ceratoplastice in man. New York Medical Journal. 1844;**2**:281-282

[15] Lai L, Kolber-Simonds D, Park KW, Cheong HT, Greenstein JL, Im GS, et al. Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science. 2002;**295**(5557):1089-1092. DOI: 10.1126/science.1068228 [doi]1068228 [pii]

[16] Cooper DK. Clinical xenotransplantion—How close are we? Lancet. 2003;**362**(9383):557-559. DOI: S0140-6736(03)14118-9

[17] Mou L, Chen F, Dai Y, Cai Z, Cooper DK. Potential alternative approaches to xenotransplantation. International Journal of Surgery. 2015;**23**(Pt B):322-326. DOI: 10.1016/j.ijsu.2015.06.085

[18] Reardon S. New life for pig-to-human transplants. Nature. 2015;**527**(7577):152-154. DOI: 10.1038/527152a

[19] Michel SG, Madariaga ML, Villani V, Shanmugarajah K. Current progress in xenotransplantation and organ bioengineering. International Journal of Surgery. 2015;**13**:239-244. DOI: 10.1016/j.ijsu.2014.12.011

[20] Perkel JM. Xenotransplantation makes a comeback. Nature Biotechnology. 2016;**34**(1):3-4. DOI: 10.1038/nbt0116-3

[21] Yang L, Guell M, Niu D, George H, Lesha E, Grishin D, et al. Genome-wide inactivation of porcine endogenous retroviruses (PERVs). Science. 2015;**350**(6264):1101-1104. DOI: 10.1126/science.aad1191

[22] Zhiqiang P, Cun S, Ying J, Ningli W, Li W. WZS-pig is a potential donor alternative in corneal xenotransplantation. Xenotransplantation. 2007;**14**(6):603-611. DOI: XEN432 [pii]10.1111/j.1399-3089.2007.00432.x [doi]

[23] Choi HJ, Kim MK, Lee HJ, Ko JH, Jeong SH, Lee JI, et al. Efficacy of pig-to-rhesus lamellar corneal xenotransplantation. Investigative Ophthalmology & Visual Science. 2011;**52**(9):6643-6650. DOI: 10.1167/iovs.11-7273

[24] Choi HJ, Lee JJ, Kim DH, Kim MK, Lee HJ, Ko AY, et al. Blockade of CD40-CD154 costimulatory pathway promotes long-term survival of full-thickness porcine corneal grafts in nonhuman primates: Clinically applicable xenocorneal transplantation. American Journal of Transplantation: Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2015;**15**(3):628-641. DOI: 10.1111/ajt.13057

[25] Kim MK, Choi HJ, Kwon I, Pierson 3rd RN, Cooper DK, Soulillou JP, et al. The International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of xenocorneal transplantation. Xenotransplantation. 2014;**21**(5):420-430. DOI: 10.1111/xen.12129

[26] Zhang MC, Liu X, Jin Y, Jiang DL, Wei XS, Xie HT. Lamellar keratoplasty treatment of fungal corneal ulcers with acellular porcine corneal stroma. American Journal of Transplantation: Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2015;**15**(4):1068-1075. DOI: 10.1111/ajt.13096

[27] Nishida T, Saika S. Cornea and Sclera: Anatomy and Physiology. In Cornea (Krachmer, Mannis, and Holland ). 3rd Ed. MOSBY Elsevier Inc. New York. 2011;3-24

[28] Hori J, Vega JL, Masli S. Review of ocular immune privilege in the year 2010: Modifying the immune privilege of the eye. Ocular Immunology and Inflammation. 2010;**18**(5):325-333. DOI: 10.3109/09273948.2010.512696

[29] Treacy O, Fahy G, Ritter T, O'Flynn L. Corneal immunosuppressive mechanisms, anterior chamber-associated immune deviation (ACAID) and their role in allograft rejection. Methods in Molecular Biology. 2016;**1371**:205-214. DOI: 10.1007/978-1-4939-3139-2_13

[30] Vanhove B, Renard JP, Soulillou JP. Genetic engineering in the pig. Gene knockout and alternative techniques. Annals of the New York Academy of Sciences. 1998;**862**:28-36

[31] Amano S, Shimomura N, Kaji Y, Ishii K, Yamagami S, Araie M. Antigenicity of porcine cornea as xenograft. Current Eye Research Journal. 2003;**26**(6):313-318

[32] Lee HI, Kim MK, Oh JY, Ko JH, Lee HJ, Wee WR, et al. Gal alpha(1-3)Gal expression of the cornea in vitro, in vivo and in xenotransplantation. Xenotransplantation. 2007;**14**(6):612-618. DOI: XEN433 [pii]10.1111/j.1399-3089.2007.00433.x

[33] Kim YG, Oh JY, Gil GC, Kim MK, Ko JH, Lee S, et al. Identification of alpha-Gal and non-Gal epitopes in pig corneal endothelial cells and keratocytes by using mass spectrometry. Current Eye Research Journal. 2009;**34**(10):877-895. DOI: 10.3109/02713680903184243 [pii]10.3109/02713680903184243

[34] Phelps CJ, Koike C, Vaught TD, Boone J, Wells KD, Chen SH, et al. Production of alpha 1,3-galactosyltransferase-deficient pigs. Science. 2003;**299**(5605):411-414

[35] Cohen D, Miyagawa Y, Mehra R, Lee W, Isse K, Long C, et al. Distribution of non-gal antigens in pig cornea: Relevance to corneal xenotransplantation. Cornea. 2014;**33**(4):390-397. DOI: 10.1097/ico.0000000000000069

[36] Hara H, Koike N, Long C, Piluek J, Roh DS, SundarRaj N, et al. Initial in vitro investigation of the human immune response to corneal cells from genetically engineered pigs. Investigative Ophthalmology & Visual Science. 2011;**52**(8):5278-5286. DOI: 10.1167/iovs.10-6947

[37] Ezzelarab M, Ayares D, Cooper DK. Carbohydrates in xenotransplantation. Immunology and Cell Biology. 2005;**83**(4):396-404. DOI: 10.1111/j.1440-1711.2005.01344.x

[38] Yeh P, Ezzelarab M, Bovin N, Hara H, Long C, Tomiyama K, et al. Investigation of potential carbohydrate antigen targets for human and baboon antibodies. Xenotransplantation. 2010;**17**(3):197-206. DOI: 10.1111/j.1399-3089.2010.00579.x

[39] Lutz AJ, Li P, Estrada JL, Sidner RA, Chihara RK, Downey SM, et al. Double knockout pigs deficient in N-glycolylneuraminic acid and galactose alpha-1,3-galactose reduce the humoral barrier to xenotransplantation. Xenotransplantation. 2013;**20**(1):27-35. DOI: 10.1111/xen.12019

[40] Lee W, Miyagawa Y, Long C, Ekser B, Walters E, Ramsoondar J, et al. Expression of NeuGc on pig corneas and its potential significance in pig corneal xenotransplantation. Cornea. 2016;**35**(1):105-113

[41] Lee W, Hara H, Ezzelarab MB, Iwase H, Bottino R, Long C, et al. Initial in vitro studies on tissues and cells from GTKO/CD46/NeuGcKO pigs. Xenotransplantation. 2016;**23**(2):137-150

[42] Dong X, Hara H, Wang Y, Wang L, Zhang Y, Cooper DK, et al. Initial study of alpha1,3-galactosyltransferase gene-knockout/CD46 pig full-thickness corneal xenografts in rhesus monkeys. Xenotransplantation. 2017;**23**(3):202-210. DOI: 10.1111/xen.12282. 10.1111/xen.12234

[43] Khaireddin R, Wachtlin J, Hopfenmuller W, Hoffmann F. HLA-A, HLA-B and HLA-DR matching reduces the rate of corneal allograft rejection. Graefe's Archive for Clinical and Experimental Ophthalmology. 2003;**241**(12):1020-1028. DOI: 10.1007/s00417-003-0759-9

[44] Hargrave SL, Hay C, Mellon J, Mayhew E, Niederkorn JY. Fate of MHC-matched corneal allografts in Th1-deficient hosts. Investigative Ophthalmology & Visual Science. 2004;**45**(4):1188-1193

[45] van Essen TH, Roelen DL, Williams KA, Jager MJ. Matching for human leukocyte antigens (HLA) in corneal transplantation—To do or not to do. Progress in Retinal and Eye Research. 2015;**46**:84-110. DOI: 10.1016/j.preteyeres.2015.01.001

[46] Bothwell AL. Characterization of the human antiporcine immune response: A prerequisite to xenotransplantation. Immunologic Research. 1999;**19**(2-3):233-243. DOI: 10.1007/bf02786491

[47] Plenter RJ, Grazia TJ, Doan AN, Gill RG, Pietra BA. CD4 T cells mediate cardiac xenograft rejection via host MHC Class II. Journal of Heart and Lung Transplantation. 2012;**31**(9):1018-1024

[48] Yamada K, Sachs DH, DerSimonian H. Human anti-porcine xenogeneic T cell response. Evidence for allelic specificity of mixed leukocyte reaction and for both direct and indirect pathways of recognition. Journal of Immunology. 1995;**155**(11):5249-5256

[49] Taylor CJ, Tang KG, Smith SI, White DJ, Davies HF. HLA-specific antibodies in highly sensitized patients can cause a positive crossmatch against pig lymphocytes. Transplantation. 1998;**65**(12):1634-1641

[50] Yamada K, Sachs DH, DerSimonian H. Human anti-porcine xenogeneic T cell response. Evidence for allelic specificity of mixed leukocyte reaction and for both direct and indirect pathways of recognition. Molecular Immunology. 2010;**47**(4):809-815. DOI: S0161-5890(09)00760-3 [pii] 10.1016/j.molimm.2009.10.004

[51] Kim MK, Oh JY, Ko JH, Lee HJ, Jung JH, Wee WR, et al. DNA microarray-based gene expression profiling in porcine keratocytes and corneal endothelial cells and comparative analysis associated with xeno-related rejection. Journal of Korean Medical Science. 2009;**24**(2):189-196. DOI: 10.3346/jkms.2009.24.2.189 [doi]

[52] Reyes LM, Estrada JL, Wang ZY, Blosser RJ, Smith RF, Sidner RA, et al. Creating class I MHC-null pigs using guide RNA and the Cas9 endonuclease. Journal of Immunology. 2014;**193**(11):5751-5757. DOI: 10.4049/jimmunol.1402059

[53] Lee JM, Tu CF, Yang PW, Lee KH, Tsuji K, Tsai MK, et al. Reduction of human-to-pig cellular response by alteration of porcine MHC with human HLA DPW0401 exogenes. Transplantation. 2002;**73**(2):193-197

[54] Hara H, Witt W, Crossley T, Long C, Isse K, Fan L, et al. Human dominant-negative class II transactivator transgenic pigs—Effect on the human anti-pig T-cell immune response and immune status. Immunology. 2013;**140**(1):39-46. DOI: 10.1111/imm.12107

[55] Hashimoto Y, Funamoto S, Sasaki S, Honda T, Hattori S, Nam K, et al. Preparation and characterization of decellularized cornea using high-hydrostatic pressurization for corneal tissue engineering. Biomaterials. 2010;**31**(14):3941-3948. DOI: S0142-9612(10)00170-5 [pii] 10.1016/j.biomaterials.2010.01.122

[56] Zhou Y, Wu Z, Ge J, Wan P, Li N, Xiang P, et al. Development and characterization of acellular porcine corneal matrix using sodium dodecylsulfate. Cornea. 2011;**30**(1):73-82. DOI: 10.1097/ICO.0b013e3181dc8184 10.1016/j.biomaterials.2010.01.122.

[57] Oh JY, Kim MK, Lee HJ, Ko JH, Wee WR, Lee JH. Processing porcine cornea for biomedical applications. Tissue Engineering Part C Methods. 2009. DOI: 10.1089/ten.TEC.2009.0022 [doi]

[58] Lee W, Miyagawa Y, Long C, Cooper DK, Hara H. A comparison of three methods of decellularization of pig corneas to reduce immunogenicity. International Journal of Ophthalmology. 2014;**7**(4):587-593. DOI: 10.3980/j.issn.2222-3959.2014.04.01

[59] Hashimoto Y, Funamoto S, Sasaki S, Negishi J, Honda T, Hattori S, et al. Corneal regeneration by deep anterior lamellar keratoplasty (DALK) using decellularized corneal matrix. PLoS One. 2015;**10**(7):e0131989

[60] Wu Z, Zhou Y, Li N, Huang M, Duan H, Ge J, et al. The use of phospholipase A(2) to prepare acellular porcine corneal stroma as a tissue engineering scaffold. Biomaterials. 2009;**30**(21):3513-3522. DOI: S0142-9612(09)00268-3 [pii] 10.1016/j.biomaterials.2009.03.003

[61] He YG, Ross J, Niederkorn JY. Promotion of murine orthotopic corneal allograft survival by systemic administration of anti-CD4 monoclonal antibody. Investigative ophthalmology & visual science. 1991;**32**(10):2723-2728

[62] Yamada J, Kurimoto I, Streilein JW. Role of CD4+ T cells in immunobiology of orthotopic corneal transplants in mice. Investigative ophthalmology & visual science. 1999;**40**(11):2614-2621

[63] Qian Y, Dana MR. Molecular mechanisms of immunity in corneal allotransplantation and xenotransplantation. Expert Rev Mol Med. 2001;**3**(18):1-21. Doi: 10.1017/S1462399401003246

[64] Niederkorn JY, Stevens C, Mellon J, Mayhew E. CD4+ T-cell-independent rejection of corneal allografts. Transplantation. 2006;**81**(8):1171-1178. DOI: 10.1097/01.tp.0000203140.70742.cb 00007890-200604270-00015 [pii]

[65] Gebhardt BM, Shi W. Experimental corneal allograft rejection. Immunologic Research. 2002;**25**(1):1-26. DOI: IR:25:1:01 [pii] 10.1385/IR:25:1:01

[66] Hargrave SL, Taherzadeh S, Hegde S, Niederkorn J. High-risk corneal allografts are capable of stimulating complement dependent cytolytic antibodies. Cornea. 2000;**19**(4):521-525

[67] Hegde S, Mellon JK, Hargrave SL, Niederkorn JY. Effect of alloantibodies on corneal allograft survival. Investigative Ophthalmology & Visual Science. 2002;**43**(4):1012-1018

[68] Ross JR, Howell DN, Sanfilippo FP. Characteristics of corneal xenograft rejection in a discordant species combination. Investigative Ophthalmology & Visual Science. 1993;**34**(8):2469-2476

[69] Ross JR, Sanfilippo FP, Howell DN. Histopathologic features of rejecting orthotopic corneal xenografts. Current Eye Research. 1994;**13**(10):725-730

[70] Tanaka K, Yamada J, Streilein JW, Sonoda K, Higuchi R. Xenoreactive CD4+ T cells and acute rejection of orthotopic guinea pig corneas in mice. Investigative Ophthalmology & Visual Science. 2000;**41**(7):1827-1832

[71] Tanaka K, Sonoda K, Streilein JW. Acute rejection of orthotopic corneal xenografts in mice depends on CD4(+) T cells and self-antigen-presenting cells. Investigative Ophthalmology & Visual Science. 2001;**42**(12):2878-2884

[72] Holan V, Vitova A, Krulova M, Zajicova A, Neuwirth A, Filipec M, et al. Susceptibility of corneal allografts and xenografts to antibody-mediated rejection. Immunology Letters. 2005;**100**(2):211-213. DOI: 10.1016/j.imlet.2005.03.013

[73] Oh JY, Kim MK, Ko JH, Lee HJ, Kim Y, Park CS, et al. Acute cell-mediated rejection in orthotopic pig-to-mouse corneal xenotransplantation. Xenotransplantation. 2009;**16**(2):74-82. DOI: XEN514 [pii] 10.1111/j.1399-3089.2009.00514.x

[74] Oh JY, Kim MK, Lee HJ, Ko JH, Kim Y, Park CS, et al. Complement depletion with cobra venom factor delays acute cell-mediated rejection in pig-to-mouse corneal xenotransplantation. Xenotransplantation. 2010;**17**(2):140-146. DOI: XEN574 [pii] 10.1111/j.1399-3089.2010.00574.x

[75] Choi HJ, Kim MK, Lee HJ, Jeong SH, Kang HJ, Park CS, et al. Effect of alphaGal on corneal xenotransplantation in a mouse model. Xenotransplantation. 2011;**18**(3):176-182. DOI: 10.1111/j.1399-3089.2011.00641.x

[76] Vabres B, Le Bas-Bernardet S, Riochet D, Cherel Y, Minault D, Hervouet J, et al. hCTLA4-Ig transgene expression in keratocytes modulates rejection of corneal xenografts in a pig to non-human primate anterior lamellar keratoplasty model. Xenotransplantation. 2014;**21**(5):431-443. DOI: 10.1111/xen.12107

[77] Kim DH, Kim J, Jeong HJ, Lee HJ, Kim MK, Wee WR. Biophysico-functional compatibility of Seoul National University (SNU) miniature pig cornea as xenocorneal graft for the use of human clinical trial. Xenotransplantation. 2016;**23**(3):202-210. DOI: 10.1111/xen.12234

[78] Lee SE, Mehra R, Fujita M, Roh DS, Long C, Lee W, et al. Characterization of porcine corneal endothelium for xenotransplantation. Seminars in Ophthalmology. 2014;**29**(3):127-135. DOI: 10.3109/08820538.2013.787104

[79] Fujita M, Mehra R, Lee SE, Roh DS, Long C, Funderburgh JL, et al. Comparison of proliferative capacity of genetically-engineered pig and human corneal endothelial cells. Ophthalmic Research. 2013;**49**(3):127-138. DOI: 10.1159/000342978

[80] Doughty MJ, Zaman ML. Human corneal thickness and its impact on intraocular pressure measures: A review and meta-analysis approach. Survey of Ophthalmology. 2000;**44**(5):367-408

[81] Faber C, Scherfig E, Prause JU, Sorensen KE. Corneal thickness in pigs measured by ultrasound pachymetry in vivo. Scandinavian Journal of Laboratory Animal Science. 2008;**35**:39-43

[82] Lee HI, Kim MK, Ko JH, Lee HJ, Lee JH, Wee WR. The characteristics of porcine cornea as a xenograft. Journal of the Korean Ophthalmological Society. 2006;**47**(12):2020-2029

[83] Sanchez I, Martin R, Ussa F, Femandez-Bueno I. The parameters of the porcine eyeball. Graefe's Archive for Clinical and Experimental Ophthalmology. 2011;**249**:475-482

[84] Zeng Y, Yang J, Huang K, Lee Z, Lee X. A comparison of biomechanical properties between human and porcine cornea. Journal of Biomechanics. 2001;**34**(4):533-537

[85] Shufelt C, Fraser-Bell S, Ying-Lai M, Torres M, Varma R. Refractive error, ocular biometry, and lens opalescence in an adult population: The Los Angeles Latino Eye Study. Investigative Ophthalmology & Visual Science. 2005;**46**(12):4450-4460. DOI: 10.1167/iovs.05-0435

[86] Snellingen T, Rao GN, Shrestha JK, Huq F, Cheng H. Quantitative and morphological characteristics of the human corneal endothelium in relation to age, gender, and ethnicity in cataract populations of South Asia. Cornea. 2001;**20**(1):55-58

[87] Kim MK, Lee JJ, Choi HJ, Kwon I, Lee H, Song JS, et al. Ethical and regulatory guidelines in clinical trials of xenocorneal transplantation in Korea; the Korean xenocorneal transplantation consensus statement. Xenotransplantation. 2013;**20**(4):209-218. DOI: 10.1111/xen.12036

[88] Kelley CG, Yamaguchi T, Santana E, Kaufman HE. A primate model of human corneal transplantation. Investigative Ophthalmology & Visual Science. 1984;**25**(9):1061-1064

[89] Epstein AJ, de Castro TN, Laibson PR, Cohen EJ, Rapuano CJ. Risk factors for the first episode of corneal graft rejection in keratoconus. Cornea. 2006;**25**(9):1005-1011. DOI: 10.1097/01.ico.0000240098.01468.4c 00003226-200610000-00002 [pii]

[90] Boisjoly HM, Bernard PM, Dube I, Laughrea PA, Bazin R, Bernier J. Effect of factors unrelated to tissue matching on corneal transplant endothelial rejection. American Journal of Ophthalmology. 1989;**107**(6):647-654

[91] Panda A, Vanathi M, Kumar A, Dash Y, Priya S. Corneal graft rejection. Survey of Ophthalmology. 2007;**52**(4):375-396. DOI: 10.1016/j.survophthal.2007.04.008. S0039-6257(07)00056-2 [pii]

[92] Rose AG, Cooper DK, Human PA, Reichenspurner H, Reichart B. Histopathology of hyperacute rejection of the heart: Experimental and clinical observations in allografts and xenografts. Journal of Heart and Lung Transplantation. 1991;**10**(2):223-234

[93] Lee HI, Kim MK, Oh JY, Ko JH, Lee HJ, Wee WR, et al. The role of cyclosporine and mycophenolate in an orthotopic porcine-to-rat corneal xenotransplantation. Journal of the Korean Ophthalmological Society. 2008;**23**(3):492-501. DOI: 10.3346/jkms.2008.23.3.492 200806492 [pii]

[94] Lee JK, Ryu YH, Ahn JI, Kim MK, Lee TS, Kim JC. The effect of lyophilization on graft acceptance in experimental xenotransplantation using porcine cornea. Artificial Organs. 2010;**34**(1):37-45. DOI: 10.1111/j.1525-1594.2009.00789.x

[95] Oh JY, Kim MK, Ko JH, Lee HJ, Park CG, Kim SJ, et al. Histological differences in full-thickness vs. lamellar corneal pig-to-rabbit xenotransplantation. Veterinary Ophthalmology. 2009;**12**(2):78-82. DOI: 10.1111/j.1463-5224.2008.00680.x VOP680 [pii]

[96] Cooper DK, Satyananda V, Ekser B, van der Windt DJ, Hara H, Ezzelarab MB, et al. Progress in pig-to-non-human primate transplantation models (1998-2013): A comprehensive review of the literature. Xenotransplantation. 2014;**21**(5):397-419. DOI: 10.1111/xen.12127

[97] Li A, Pan Z, Jie Y, Sun Y, Luo F, Wang L. Comparison of immunogenicity and porcine-to-rhesus lamellar corneal xenografts survival between fresh preserved and dehydrated porcine corneas. Xenotransplantation. 2011;**18**(1):46-55. DOI: 10.1111/j.1399-3089.2011.00626.x

[98] Jie Y, Liu L, Pan Z, Wang L. Survival of pig-to-rhesus corneal xenografts prolonged by prior donor bone marrow transplantation. Molecular Medicine Reports. 2013;**7**(3):869-874. DOI: 10.3892/mmr.2013.1294

[99] Kim J, Kim DH, Choi HJ, Lee HJ, Kang HJ, Park CG, Hwang ES, Kim MK, Wee WR. Anti-CD40 antibody-mediated costimulation blockade promotes long-term survival of deep-lamellar porcine corneal grafts in non-human primates. Xenotransplantation. 2017 Apr 10

[100] Sykes M, d'Apice A, Sandrin M. Position paper of the Ethics Committee of the International Xenotransplantation Association. Transplantation. 2004;**78**(8):1101-1107. DOI: 00007890-200410270-00003 [pii]

[101] Hering BJ, Cooper DK, Cozzi E, Schuurman HJ, Korbutt GS, Denner J, et al. The International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes—Executive summary. Xenotransplantation. 2009;**16**(4):196-202. DOI: 10.1111/j.1399-3089.2009.00547.x XEN547 [pii]

[102] Denner J, Schuurman HJ, Patience C. The International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes—Chapter 5: Strategies to prevent transmission of porcine endogenous retroviruses. Xenotransplantation. 2009;**16**(4):239-248. DOI: 10.1111/j.1399-3089.2009.00544.x XEN544 [pii]

[103] Schuurman HJ. The International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes—Chapter 2: Source pigs. Xenotransplantation. 2009;**16**(4):215-222. DOI: 10.1111/j.1399-3089.2009.00541.x

[104] Williams KA, Coster DJ. The immunobiology of corneal transplantation. Transplantation. 2007;**84**(7):806-813. DOI: 10.1097/01.tp.0000285489.91595.13

[105] Reinhard T, Reis A, Bohringer D, Malinowski M, Voiculescu A, Heering P, et al. Systemic mycophenolate mofetil in comparison with systemic cyclosporin A in high-risk keratoplasty patients: 3 years' results of a randomized prospective clinical trial. Graefe's Archive for Clinical and Experimental Ophthalmology. 2001;**239**(5):367-372

[106] Williams KA, Esterman AJ, Bartlett C, Holland H, Hornsby NB, Coster DJ. How effective is penetrating corneal transplantation? Factors influencing long-term outcome in multivariate analysis. Transplantation. 2006;**81**(6):896-901. DOI: 10.1097/01.tp.0000185197.37824.35

[107] Denner J, Tonjes RR, Takeuchi Y, Fishman J, Scobie L. First update of the International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes-Chapter 5: Recipient monitoring and response plan for preventing disease transmission. Xenotransplantation. 2016;**23**(1):53-59. DOI: 10.1111/xen.12227

[108] Spizzo T, Denner J, Gazda L, Martin M, Nathu D, Scobie L, et al. First update of the International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes-Chapter 2a: Source pigs-preventing xenozoonoses. Xenotransplantation. 2016;**23**(1):25-31. DOI: 10.1111/xen.12223

[109] Rayat GR, Gazda LS, Hawthorne WJ, Hering BJ, Hosking P, Matsumoto S, et al. First update of the International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes—Chapter 3: Porcine islet product manufacturing and release testing criteria. Xenotransplantation. 2016;**23**(1):38-45. DOI: 10.1111/xen.12225

[110] Hering BJ, Cozzi E, Spizzo T, Cowan PJ, Rayat GR, Cooper DK, et al. First update of the International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes—Executive summary. Xenotransplantation. 2016;**23**(1):3-13. DOI: 10.1111/xen.12231

[111] Hering BJ, O'Connell PJ. First update of the International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes—Chapter 6: Patient selection for pilot clinical trials of islet xenotransplantation. Xenotransplantation. 2016;**23**(1):60-76. DOI: 10.1111/xen.12228

[112] Cowan PJ, Ayares D, Wolf E, Cooper DK. First update of the International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes—Chapter 2b: Genetically modified source pigs. Xenotransplantation. 2016;**23**(1):32-37. DOI: 10.1111/xen.12224

[113] Cozzi E, Tonjes RR, Gianello P, Buhler LH, Rayat GR, Matsumoto S, et al. First update of the International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes—Chapter 1: Update on national regulatory frameworks pertinent to clinical islet xenotransplantation. Xenotransplantation. 2016;**23**(1):14-24. DOI: 10.1111/xen.12222

[114] Cooper DK, Bottino R, Gianello P, Graham M, Hawthorne WJ, Kirk AD, et al. First update of the International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes—Chapter 4: Pre-clinical efficacy and complication data required to justify a clinical trial. Xenotransplantation. 2016;**23**(1):46-52. DOI: 10.1111/xen.12226

[115] Morozov VA, Wynyard S, Matsumoto S, Abalovich A, Denner J, Elliott R. No PERV transmission during a clinical trial of pig islet cell transplantation. Virus Research. 2017;**227**:34-40. DOI: 10.1016/j.virusres.2016.08.012

Chapter 4

# Immune Response Associated with Islet Xenotransplantation in Small and Large Animal Models

Jennifer Croden, Wenlong Huang and Gina R. Rayat

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.68999

**Abstract**

This chapter will review studies that examine the immune response to porcine neonatal pancreatic cell clusters (NPCC) in small and large animal models; specifically, the immune mechanisms that lead to the rejection of transplanted islet cells in mice, nonhuman primates, and humans will be discussed. In addition, current research on the *in vitro* and *in vivo* human immune responses to porcine NPCC is also included. Research into the immune responses that lead to islet cell death posttransplant allows for further understanding of how to better protect transplanted porcine NPCC in humans. Furthermore, this chapter will examine immune-related strategies that have shown to extend the life and/or function of porcine NPCC *in vitro* and *in vivo*, including techniques that work to modulate the immune system of the islet cell donor and/or the recipient. Finally, this chapter will identify future areas of research that have yet to be examined extensively in the literature, mostly pertaining to the human immune response to porcine NPCC in the clinical setting.

**Keywords:** complement and innate immunology, cell and tissue xenotransplantation, neonatal pancreatic cell clusters (NPCC)

## 1. Introduction

Clinical islet cell transplantation is currently considered as an alternative option for the treatment of unstable type 1 diabetes. Despite recent progress in the field, transplant recipients continue to experience a progressive loss of insulin independence for reasons that are not well understood [1]. In addition, the shortage of human islet cell donors and the necessity of

chronic immunosuppressant drugs are major barriers to the widespread application of islet cell transplantation in clinical practice.

Xenotransplantation addresses the shortage of available human donors in transplantation medicine. Porcine neonatal pancreatic cell clusters (NPCC) remain a strong option for islet cell xenotransplantation due to their relative ease of acquisition and low cost, as well as similarities in physiology between pig and human islet cells [2]. Similar to islet cell allotransplantation, the efficacy of transplanted porcine NPCC into animal models is limited by posttransplant cell damage likely resulting from acute host-mediated inflammatory and oxidative stress, as well as chronic immune cell-mediated responses.

This chapter will review studies that examine the immune response to porcine NPCC in small and large animal models; specifically, the immune mechanisms that lead to the rejection of transplanted islets in mice, nonhuman primates, and humans will be discussed. Research into the immune responses that lead to islet cell death posttransplant allows for further understanding of how to better protect transplanted porcine NPCC in humans. Furthermore, this chapter will examine immune-related strategies that have shown to extend the life and/or function of porcine NPCC both *in vitro* and *in vivo*, including techniques that work to modulate the immune system of the islet cell donor and/or the recipient. Lastly, this chapter will identify future areas of research that have yet to be examined extensively in the literature, mostly pertaining to the human immune response to porcine NPCC in preparation for the transplantation of porcine NPCC in patients with type 1 diabetes.

## 2. Background and history

Islet transplantation began in the 1970s, when Ballinger et al. demonstrated that diabetic rats could be made normoglycemic through injection of islet isografts into the portal vein [3]. Not long afterwards, the University of Minnesota performed successful autologous islet transplantations in patients that had undergone near-complete pancreatectomies [3]. From these experiments arose the goal of clinical islet transplantation as a viable treatment for type 1 diabetes.

However, the integration of islet transplantation into the clinical setting has seen several setbacks. Firstly, islet transplant recipients invariably return to a hyperglycemic state. Long-term follow-up of the earliest successful transplant recipients found that over 80% of these patients did not remain normoglycemic at the end of 2 years, even with adequate immunosuppression [3]. Further understanding of islet isolation protocols and the immune response to islet transplants has allowed for the 2-year failure rate to fall to 50% [1]; however, this remains a large barrier to the use of islet transplantation in a clinical setting. Secondly, as is true across the field of transplantation, there is a large shortage of donor tissue available. Xenotransplantation attempts to address this issue.

While the idea of xenotransplantation dates back to the sixteenth century, it was not until the 1980s that a better understanding of immunosuppression allowed for clinical islet xenotransplantation to be attempted with any success [4]. From 1990 to 1993, Groth et al.

performed islet xenotransplants with NPCC into 10 type 1 diabetic patients [5]. Though all 10 patients remained insulin dependent, 4 patients secreted small amounts of insulin up to 400 days posttransplant. In 2002, at the XIXth International Congress of the Transplantation Society, Valdes-Gonzalaez et al. reported 12 transplants of NPCC into children with type-1 diabetes [6]. At 1-year posttransplant, five of the patients who received transplants required less insulin, and one patient was entirely insulin-independent. Most recently, Matsumoto et al. demonstrated that transplantation of encapsulated NPCC into the peritoneal cavity of patients with type 1 diabetes was able to maintain normoglycemia in these patients for over 600 days posttransplant without immunosuppression [7]. These experiments demonstrate that transplantation of NPCC could have a place in the clinical treatment of type 1 diabetes.

While xenotransplantation comes with its own set of immune-related complications, scientists still believe that islet xenografts are a good alternative to islet allografts. Because they are much less vascular, islet transplants are less immunogenic than full organ transplants, and so do not present the same challenges that a heart or kidney xenotransplantation would present. In addition, NPCC are relatively low cost and easily acquired, and would therefore solve the problem of islet donor shortage. Unfortunately, the problem of islet transplant recipients' inevitable return to a hyperglycemic state is also a problem in xenotransplantation.

As is seen in islet allotransplantation, immunosuppression techniques increase the lifespan of islet xenografts *in vivo* [8–12], demonstrating that islet xenograft failure has an immune-dependent mechanism. Scientists continue to attempt to elicit this mechanism, as therapies targeted at controlling this immune response will allow for longer islet transplantation survival.

## 3. Ideal age of porcine islet donors

### 3.1. Adult porcine islets

Successful autograft, allograft, and xenograft transplantation has been done using adult porcine islets. There are several advantages to obtaining islets from older pigs. Firstly, larger numbers of islets can be obtained from a single adult pig pancreas. Secondly, these mature islets, when isolated, are individually larger in size (**Figure 1**) and the potential for insulin secretion is greater [13]. This has been demonstrated in several studies, which have shown that the return to normoglycemia is faster post transplantation in experiments with mice and nonhuman primates [12, 14, 15]. Lastly, adult porcine islets express certain immunogenic antigens, such as galactose alpha 1,3-galactose (alpha Gal) to a lesser extent than neonatal [16] or fetal porcine islets [13].

Unfortunately, adult porcine islets are delicate. They are more susceptible to ischemic injury and so are difficult to keep viable in culture [17, 18]. Also, the quality of islets obtained from adult pigs varies greatly depending on the exact age and breed of the donor pig [13, 19]. Lastly, although adult porcine islets express certain antigens to a lesser extent as stated above, it is thought that islets isolated from adult pigs are overall more immunogenic than islets from neonatal or fetal pigs, increasing the need for immunosuppressive drug regimens [2, 16].

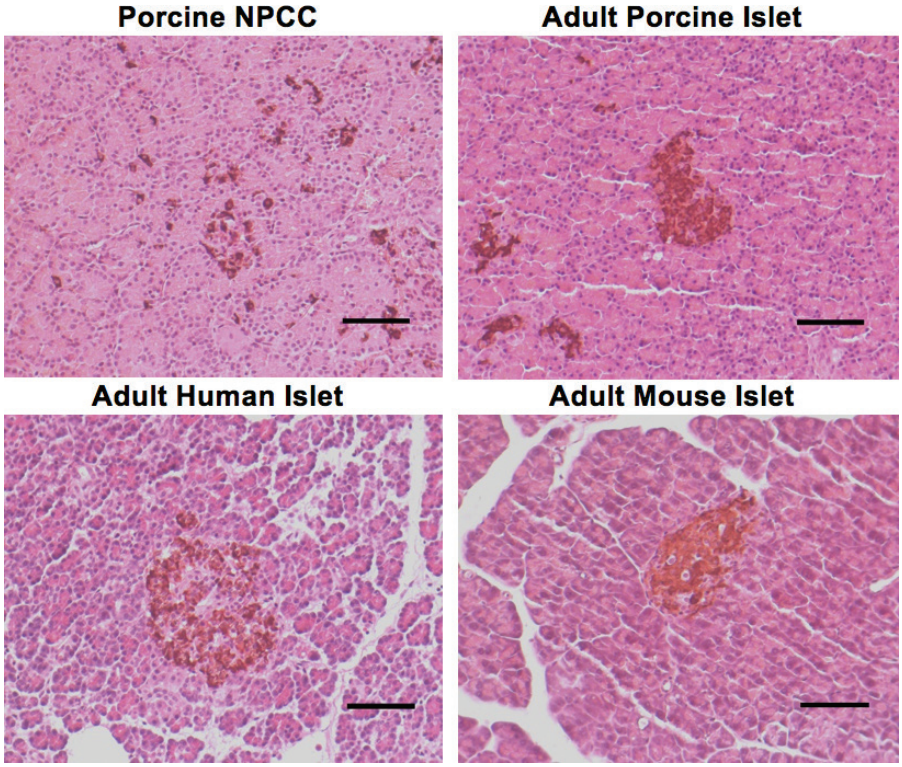


**Figure 1.** Islets in neonatal pig, adult pig, and adult human, adult mouse pancreas. Islets are depicted as brown structures representing insulin-positive beta cells in the islets, which are surrounded with exocrine tissue. Pancreas sections were counterstained with Harris' hematoxylin and eosin. Scale bar represents 100 μm.

### 3.2. Porcine neonatal pancreatic cell clusters

Porcine NPCC have also been used to successfully reverse diabetes in small [8, 10, 20] and large animal models [9, 21]. It is widely believed that neonatal pigs are the best source of islets for xenotransplantation, for several reasons. Firstly, the pancreas of a neonatal pig is less fibrous and the islets are easier to isolate than those of an adult pig. Porcine NPCC also maintain growth capacity after isolation, and may continue to grow even after transplantation [13]. They also appear to be less susceptible to ischemic injury after isolation and keep better in culture [17, 18].

Disadvantages of using porcine NPCC include an increased time to return islet recipients to normoglycemia due to their immature nature compared to adult islets (**Figure 1**). NPCC also have an increased presence of antigens on their surface (i.e., alpha Gal), and require a high number of donor pigs for a single transplantation [2, 13, 18].

### 3.3. Porcine fetal pancreatic cell clusters (FPCC)

Porcine FPCC have many of the same advantages that porcine NPCC have, including the resilience of the cells to ischemic injury and the ability to mature and maintain growth capacity after isolation [13].

However, fetal islets have shown to secrete very small amounts of insulin in response to glucose stimulation and can take up to months to achieve normoglycemia, even in small animal models. Much like porcine NPCC, many donor pigs are required for a single transplantation [13]. Neither large animal islet allotransplantation nor nonhuman primate xenotransplantation has been successfully achieved with porcine FPCC.

## 4. Immune response in mouse

Many studies examining the postporcine NPCC transplantation immune response have been performed in rodent models. Mice are often used as a mammalian model organism, due to relative ease of acquisition, short gestation period, and well-studied and sequenced genome. C57BL/6 mice in particular are the most widely used rodent in laboratory experiments, and have been used extensively in the study of postislet transplantation environment.

### 4.1. Hyperacute rejection

If the serum of the transplant recipient has natural preformed antibodies with specificity for antigens on the transplanted tissue, a process known as hyperacute rejection can occur. This can lead to an antibody-mediated destruction of the transplant that begins immediately after transplantation. The presence of preformed natural antibodies against the transplant can occur from prior exposure to either the antigen present on the transplant or an antigen similar enough that it can be recognized by the same antibody. This becomes especially important in xenotransplantation, as different species express antigens that can be recognized by preformed natural antibodies commonly made by the human immune system.

The main difference in hyperacute rejection of pig-to-rodent transplants is that these species share a similarity that pigs and nonhuman primates and pigs and humans do not. Pigs and rodents, along with most other mammals, synthesize the enzyme alpha 1,3-galactosyltransferase and produce alpha Gal, and so do not produce anti-Gal antibodies [22]. Some nonhuman primates and humans lack this enzyme, and therefore do produce anti-alpha Gal antibodies. Hyperacute rejection occurs in pig-to-nonhuman primates and pig-to-human xenotransplants when preformed natural antibodies in the recipient, largely anti-alpha Gal antibodies, recognize alpha Gal present within the transplanted tissue. Anti-alpha Gal antibody-mediated rejection does not occur in the mouse model. Because of this difference and the resulting lack of clinical application, hyperacute rejection in mouse models is not a main focus of xenotransplantation research.

### 4.2. Instant blood-mediated inflammatory reaction (IBMIR)

Islet transplantation triggers an inflammatory reaction when transplanted intravascularly in mice. This reaction involves activation of the coagulation cascade and complement pathways, which damages the transplanted islets. These events are known to occur in all transplants, including autologous transplants, but it has been shown that IBMIR occurs on a larger scale in xenotransplantation [23].

However, IBMIR in the pig-to-mouse model has been poorly researched. This is because islet transplantation in mice is often not done by injecting the porcine NPCC into the portal vein and is instead done through injecting the porcine NPCC into the peritoneum (for encapsulated porcine NPCC) or under the kidney capsule. This does not allow for an IBMIR response that would be comparable to nonhuman primate or human islet transplantation models.

A study that did attempt transplantation into the portal vein of mice showed that the inflammatory mediators in the posttransplant environment are largely acute-phase cytokines, such as TNF$\alpha$ and IFN-$\gamma$ [24]. It has been shown that blockade of these inflammatory pathways, such as by using an anti-TNF$\alpha$ antibody, improved the survival of the islet transplant and improved glucose tolerance *in vivo* [24].

### 4.3. Adaptive immune response

T cell-mediated rejection is key in the rejection of porcine NPCC xenografts by mouse recipients [25]. There are two pathways of antigen recognition by T cells that are important in transplantation: the direct pathway and the indirect pathway. The direct pathway occurs when T cells recognize an antigen that is presented on the surface of a donor antigen-presenting cell (APC). The indirect pathway occurs when the T cells recognize an antigen that is presented on the surface of a host APC. Activation of either of these pathways can lead to subsequent activation of T cells and destruction of a transplant [18].

The indirect pathway of T cell activation becomes increasingly dominant as the evolutionary disparity between the transplant donor and recipient increases; likewise, the direct pathway of T cell activation is dominant in the rejection of allotransplants. As expected, in the pig-to-mouse xenotransplant, the indirect pathway is responsible for T cell-mediated rejection [25, 26]. It has been demonstrated that CD4+ T cell activation is essential for porcine NPCC xenograft rejection to occur, whereas CD8+ T cells are only minimally involved [25].

## 5. Immune response in nonhuman primate

Nonhuman primates (NHP), specifically old world monkeys, constitute the only research animal in which the occurrence of transplant rejection and the efficacy of immunosuppression can be observed in the presence of a human-like complicated and redundant immune system. As such, numerous studies of pig-to-NHP islet xenotransplantation have been responsible

for the discovery of important immune mechanisms involved in causing posttransplantation graft damage.

### 5.1. Hyperacute rejection

As previously stated, the difference in evolutionary diversity between pigs and monkeys cause a different hyperacute rejection process than in the pig-to-rodent model. The carbohydrate alpha Gal is accepted to be the epitope responsible for immediate xenograft destruction of porcine islets in nonhuman primates [22].

Alpha Gal is expressed by all animal species, including pigs, and many bacterial species. However, in humans and old world monkeys, the evolutionary loss of enzyme alpha 1,3-galactosyltransferase has led to the inability to synthesize alpha Gal. It is hypothesized that exposure to microorganisms shortly after birth cause humans and old world monkeys to synthesize anti-alpha Gal antibodies [27, 28]. These antibodies remain in blood circulation and are thought to be responsible for the destruction of Gal-expressing porcine NPCC within minutes of transplantation [22].

As discussed above, porcine NPCC show the most promise in islet transplantation. Unfortunately, porcine NPCC have a higher expression of alpha Gal when compared to adult porcine islets, which express alpha Gal only minimally [16]. The use of genetically modified pigs that have the enzyme alpha 1,3-galactosyltransferase knocked out (GTKO) remains a large area of research interest for this reason. However, even with the use of GTKO porcine NPCC, acute rejection still occurs (though to a lesser extent) [29]. This suggests that more porcine NPCC antigens are recognized by antibodies in nonhuman primates. Two have been identified: N-glycolylneuraminic acid (NeuGc) and β1,4 N-acetylgalactosaminyltransferase (B4GALNT2) [29]. Interestingly, Stewart et al. have demonstrated that treating nonhuman primates with alpha adrenergic agonist clonidine inhibits the production of these additional "antinon-Gal" antibodies [30].

### 5.2. IBMIR

Porcine NPCC are susceptible to IBMIR when exposed to the blood of nonhuman primates [22]. This reaction involves platelet activation, complement cascade activation, and mononuclear cell infiltration in the first hours to days following transplantation [2]. Alpha Gal is also thought to be implicated in this inflammatory response. When Komoda et al. developed a transgenic pig that overexpresses an enzyme, which prevented the formation of alpha Gal and transplanted NPCC from this pig to diabetic nonhuman primates, the transplant did not undergo hyperacute rejection and showed less activation of the complement cascade [31].

This reaction occurs regardless of immune cell-mediated rejection and is thought to involve tissue factor production, but the specific pathways behind this event are poorly understood. It has been shown that even with the depletion of the components of the complement system in nonhuman primates, IBMIR still occurs, although the destruction of the islet graft is decreased [11].

Innate immune cells have also been implicated in islet xenograft rejection. In nonhuman primate models, neutrophils and macrophages have been temporally associated with the failure of porcine NPCC grafts [2].

### 5.3. Adaptive immune response

Aside from the antibody-mediated rejection of xenografts that was mentioned during the discussion of hyperacute rejection, the response of the adaptive immune system to porcine NPCC in the nonhuman primate model has not been studied to the extent that it has been in the rodent model.

Available research shows through analysis of transcript levels in inadequately immunosuppressed nonhuman primates that a T cell-dependent antibody response occurs posttransplantation, resulting in high levels of antiporcine IgG [22]. The results of other studies support this idea, and have demonstrated that immunosuppressive agents that result in a blockade of T cell costimulation maintain normoglycemia in diabetic monkeys for over a year [22].

## 6. Immune response in human

The immune response to porcine NPCC in human models is poorly understood, due to a lack of a suitable experimental model. Because it is not possible to assess the human immune response *in vivo* in a research setting, scientists rely on *in vitro* experiments and experiments with animals that have been reconstituted with a human immune system.

### 6.1. Hyperacute rejection

Because of the evolutionary similarity between nonhuman primates and humans, the immune response to porcine NPCC transplant may be very similar in both species. As in nonhuman primates, the carbohydrate alpha Gal is accepted to be the epitope responsible for the hyperacute destruction of porcine NPCC when exposed to human blood *in vitro* [32].

As previously stated, evolutionary loss of enzyme alpha 1,3-galactosyltransferase in humans and old world monkeys led to the inability of either species to synthesize alpha Gal. It is hypothesized that exposure to microorganisms shortly after birth causes humans and old world monkeys to synthesize anti-alpha Gal antibodies [27, 28]. This becomes an issue especially with the use of porcine NPCC, as they express alpha Gal on their surface to a significant extent [16].

### 6.2. IBMIR

Much like is seen in the immune response in nonhuman primates, islet grafts undergo IBMIR once exposed to human blood. Studies have shown that this damage affects the integrity and viability of the cell membranes within the islet cell cluster and leads to an initial 25% loss of transplanted islets in *in vitro* models [33]. IBMIR involves platelet activation,

complement cascade activation, and mononuclear cell infiltration in the first hours to days following transplantation.

In studies involving the exposure of porcine NPCC to human blood, activation of the coagulation cascade produced proinflammatory thrombin at high concentrations, which exacerbated the destruction of the transplanted islet cells [34]. In reconstituted animal models, complement activation was demonstrated by the increase in concentration of complement proteins in the serum of transplant recipients [35]. Specifically, complement proteins C4d and C5b-9 have been implicated in IBMIR, implicating the classical complement pathway in xenograft destruction [36]. Complement protein Bb, a marker of the alternative complement pathway, also appears to be involved in IBMIR-related graft destruction, but not when islets from genetically engineered pigs (GTKO/CD46) are used [36].

In addition, activated neutrophils interacting with components of the coagulation cascade appear to be essential in the early loss of xenograft function in human models [34]. It has been demonstrated that the mechanisms by which this loss occurs include phagocytosis and secretion of reactive oxygen species (ROS) and proteinases by neutrophils [34].

### 6.3. Adaptive immune response

The response of the adaptive immune system to porcine NPCC in the human model is poorly studied, aside from what is understood about hyperacute rejection. What is known, however, is that the rejection of porcine NPCC xenografts *in vitro* is predominantly T cell-mediated [37]. This has also been demonstrated by Yi et al., who showed that the reconstitution of mice with regulatory T cells, which suppress effector T cells, prior to reconstitution with a human immune system prevented xenograft rejection [38].

Little research has been done into the specific pathways of T cell rejection of porcine NPCC due to the lack of a suitable experimental model. Murray et al. demonstrated that islet rejection likely occurs via a CD4+ T cell-directed response, with NK cell and CD8+ T cell-mediated injury of the xenograft not contributing to islet loss [37]. Additionally, a study by Lalain et al [39]. examined the adaptive immune response *in vitro* to adult porcine islets in type 1 diabetic and healthy human subjects. It was shown that the immune response to porcine islet cells involves dominantly CD4+ T cells activated through the indirect pathway, as well as CD8+ T cells activated through the direct pathway [39].

Our preliminary results suggest that there are significant differences in the strength and kinetics of *in vitro* proliferation of human peripheral blood mononuclear cells (PBMCs) from individuals with or without type 1 diabetes when stimulated with mitogen, neonatal porcine PBMCs, or porcine NPCC (**Figure 2**). Whether these results could be translated *in vivo* remains to be determined. We initially performed adoptive transfer experiments to identify the human immune cells that are infiltrating the porcine NPCC grafts. Examination of the infiltrating cells showed numerous CD45 positive human leukocytes in porcine NPCC grafts of NOD. SCID gamma mice injected with human PBMCs from donors with or without type 1 diabetes (**Figure 3**). The extent of this infiltration appeared to be similar; however, further quantification is necessary to confirm this observation. In addition, we also found the presence of M2

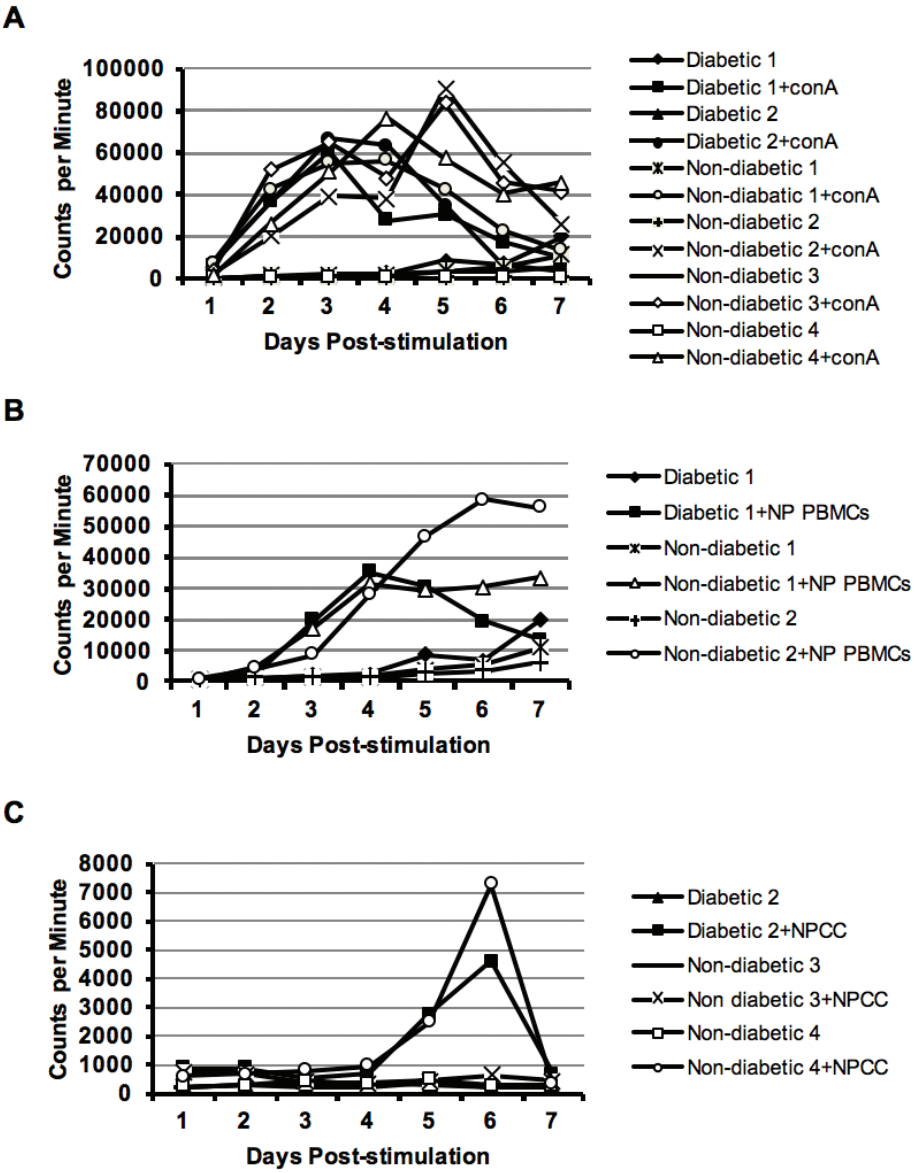


**Figure 2.** *In vitro*proliferation of PBMCs from human individuals with or without type 1 diabetes after stimulation with (A) mitogen (conA), (B) neonatal porcine PBMCs, or (C) porcine NPCC.

(Mac-2) macrophages among the cells infiltrating the porcine NPCC and their role in the rejection of porcine NPCC needs to be elucidated. Further characterization of human immune cells that infiltrated the porcine NPCC are ongoing and continued research in this area is necessary to enhance our understanding on the immune mechanisms involved in the rejection of porcine NPCC in human recipients with type 1 diabetes.

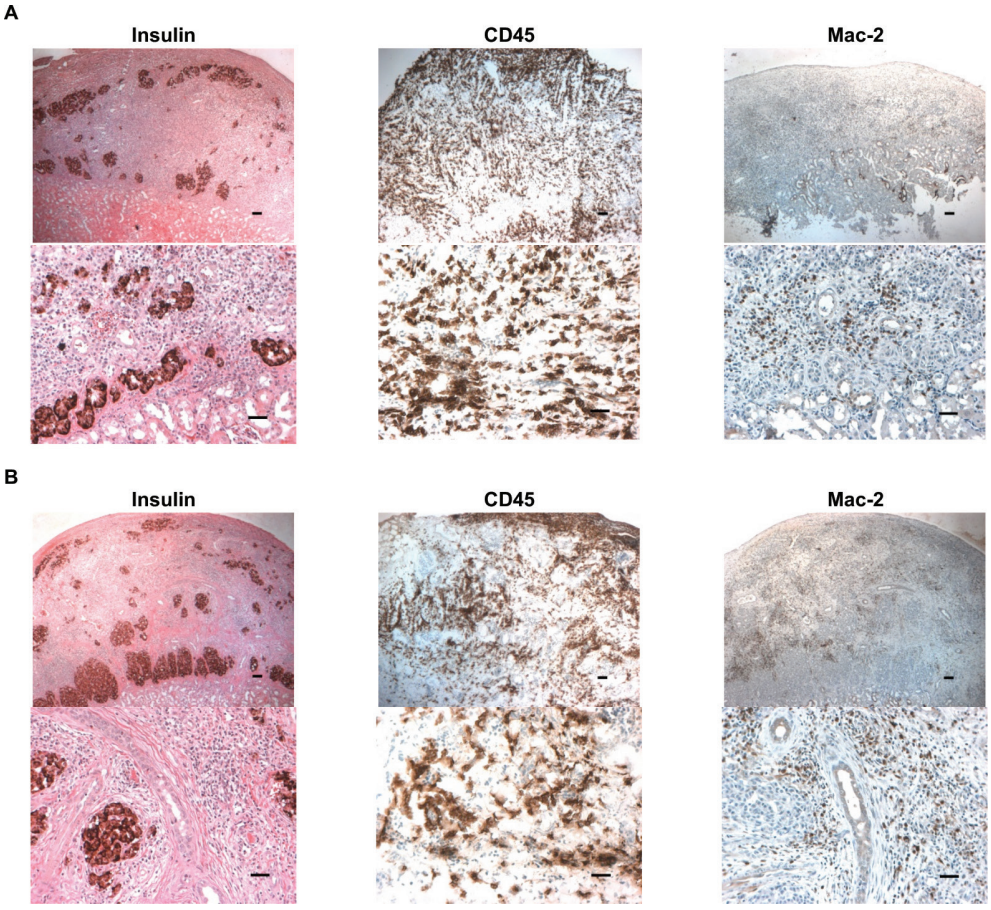


**Figure 3.** Porcine NPCC xenografts in NOD.SCID gamma mice reconstituted with PBMCs from human donors with (A) or without (B) type 1 diabetes. The presence of insulin-positive beta cells, human CD45-, and Mac-2-positive immune cells are shown as brown structures. Islet xenografts were collected 14 days postcell reconstitution. Images were taken at 2.5× and 10× objectives. Scale bar represents 100 μm.

## 7. Strategies and recommendations

There are many potential strategies to improve the viability and the function of islet xenografts. The following are a selection of strategies that do not involve an immunosuppressive regimen, and instead involve making changes to the islets themselves or the posttransplant environment that attempts to minimize damage from the recipient's immune system.

### 7.1. Immune modulation *in vitro*

Modulating the immune response before transplantation of the porcine NPCC into the recipient may allow for increasing the viability and function of the xenograft without the need for

more immunosuppressive drugs. One example of this strategy would be the culturing of porcine NPCC with protective agents, such as antioxidants, prior to transplantation.

It has been shown that oxidative stress is a likely contributor to cellular damage in the post-transplant environment [40]. Luca et al. have demonstrated that treating porcine NPCC with antioxidants vitamin D3 and E results in increased *in vitro* function of the islets for a significantly longer time than untreated controls [40]. In addition, morphologic examination of the porcine NPCC at 16 days after exposure to antioxidants showed increased porcine NPCC size and viability [40].

### 7.2. Immune modulation *in vivo*

Immunoisolation, or attempting to prevent exposure of the transplant to the recipient's immune system, is another method that can be utilized in islet xenotransplantation. Microencapsulation, macroencapsulation, and immunosuppressive scaffolding are all variants of this strategy.

Encapsulation involves placing islets inside a protective barrier, or capsule, prior to transplantation. Microencapsulation and macroencapsulation differ only in the number of islets inside each capsule. Microcapsules contain a single islet or very few islets, whereas macrocapsules contain a greater number of islets. Ideally, these capsules protect porcine NPCC from immune-mediated damage but allow for exchange of oxygen, nutrients, and waste. Advancement in the materials used for encapsulation, which now include cellulose, agarose, alginate, and protamine-heparin complex [19], have resulted in prolonged survival of islet xenografts in mice [20] and nonhuman primates [19]. Similarly, scaffolding involves transplanting islets on a porous, biodegradable material. This offers some of the protection of encapsulation with evidence for longer term function and survival of islet xenografts compared to encapsulation [41].

Other strategies involve administering immunomodulatory (but not immunosuppressive) agents to transplant recipients to prolong the life of the xenograft. As previously mentioned, Stewart et al. have demonstrated that treating nonhuman primates with alpha adrenergic agonist clonidine inhibits the production of anti-pig antibodies by the transplant recipient [30].

### 7.3. Manipulation of islet cell donors

Genetically modifying the pig donors allows for a minimization of hyperacute rejection and the IBMIR-related damage and cell loss that occurs shortly after transplant. An example of this has already been mentioned. Porcine NPCC from GTKO pigs can prevent anti-Gal-mediated damage to the islet graft [29, 32]. Additional genetic engineering of donor pigs can knockout other antigens present on NPCC, such as NeuGc [32]. Donor pigs can also be engineered to knockout tissue factor, a factor needed in coagulation [19], in order to successfully prevent IBMIR.

In addition to knocking out harmful genes, genetic engineering can also be used to add helpful genetic material. Komoda et al. performed a study in which porcine NPCC from *N*-acetylglucosaminyltransferase III (GnT-III) transgenic pigs were transplanted into diabetic cynomolgus

monkeys. GnT-III pigs are bred with an additional residue in the complex *N*-linked sugars that are implicated in "antinon-Gal" antibody formation. The study demonstrated that the engineered porcine NPCC showed a reduced antigenicity and increased survival time compared to the wild-type islet transplants [31].

## 8. Future research directions

There are several directions for future research that have become apparent throughout this chapter. Firstly, a better understanding of the human immune response to porcine NPCC is needed, both *in vitro* and *in vivo*. This must include an examination of the antibodies involved in hyperacute rejection and identification of the epitopes present on porcine NPCC. In addition to simply a greater number of studies needing to be performed, most *in vitro* and reconstituted animal *in vivo* studies of the human immune response to porcine NPCC are performed with blood products from healthy subjects. As a result, little is known specifically about the immune response of patients with type 1 diabetes. Additionally, while there have been *in vivo* studies in nonhuman primates [11, 31] using genetically engineered porcine NPCC, *in vitro* and *in vivo* human studies using transgenic porcine NPCC are needed.

There is also no clear consensus on the best transplant sites for optimizing xenograft function and minimizing immune-mediated damage to the islets. Injection of porcine NPCC into the portal vein, traditionally accepted as the site of islet transplantation, causes a sizable and immediate immune response and results in islet loss [33]. More recently, porcine NPCC have been transplanted into the peritoneal cavity in an attempt to minimize the recipient's immune response; however, it is hypothesized that this transplant site leads to a lag time between islets sensing blood glucose levels and releasing insulin, resulting in poorer glycemic control [7]. Other transplant sites, such as within the omentum or under the skin, have been proposed but not investigated.

Scaffolding and encapsulation allows for protection of the islet grafts from the recipient's immune system without the need for additional immunosuppressive drugs. There are challenges with long-term survival of islet transplants with encapsulation, as encapsulation can prevent revascularization and remodeling posttransplantation. Scaffolding can address these issues, as scaffolds are porous and allow for tissue ingrowth and revascularization. While studies with encapsulated islets have been performed in mice [10, 20, 24, 25], nonhuman primates [19], and humans [7] with success, fewer experiments have been performed using scaffolds and more research is needed into their utility.

Lastly, little research has been done into multiple dose transplantation. Because long-term survival of islet xenotransplantation has not yet been achieved, multiple dose transplantation becomes an important consideration. There are implications for the immunosuppression regime necessary if multiple islet xenotransplants are needed in a single patient, as it is reasonable to assume that the recipient would develop immunological memory to xenoantigens present in the islet graft. This is an important area of future research that has major implications in using islet transplantation as a clinical treatment of type 1 diabetes.

## 9. Conclusion

Islet xenotransplantation addresses the shortage of available human islet donors for clinical islet transplantation and has the potential to become a viable treatment of type 1 diabetes. Porcine islets remain the best option for islet transplantation, due to their ease of acquisition and similar physiology to human islet cells. Neonatal pigs appear to be the best source for transplantable islets because of their resilience to ischemic damage and growth potential postcollection. Like islet allotransplantation, islet xenotransplantation has been limited by posttransplant graft destruction from the recipient's immune response.

The immune response in rodent, nonhuman primate, and human models can be separated into hyperacute rejection, IBMIR, and adaptive immune responses. While each species group has a slightly different immune response to porcine NPCC xenografts, there are also some similarities. Antigens present on the porcine NPCC, such as alpha Gal, are largely responsible for hyperacute rejection in humans and nonhuman primates. However, other xenoantigens that need to be identified also may contribute to this response. In addition, it appears that the indirect pathway of T cell activation is an essential part of xenograft rejection in all species groups, with CD4+ T cells dominating the rejection process.

There are several strategies that can be utilized in islet xenotransplantation to improve the viability and function of the islet grafts that do not involve immunosuppressive drug regimens. These include culturing the islets with immune-modulating agents pretransplantation, transplanting encapsulated or scaffolded islets, or genetically modifying the islet cell donors to dampen the recipient's immune response. Future research directions include eliciting the specific mechanism of islet xenotransplant rejection in the human model, ideally *in vivo*.

## Acknowledgements

We are grateful for the technical assistance of Ping Wu, Mazzen Black, and Eric Boivin as well as funding support by Mr. and Mrs. John Burton, Mr. and Mrs. Ken Cantor, Mrs. Martine Farand, and Colliers International Inc.

## Author details

Jennifer Croden, Wenlong Huang and Gina R. Rayat*

*Address all correspondence to: grayat@ualberta.ca

Department of Surgery, Faculty of Medicine and Dentistry, Alberta Diabetes Institute, Ray Rajotte Surgical-Medical Research Institute, University of Alberta, Edmonton, Canada

## References

[1] Barton FB, Rickels MR, Alejandro R, et al. Improvement in outcomes of clinical islet transplantation: 1999-2010. Diabetes Care. 2012;**35**(7):1436-1445

[2] Hering BJ, Walawalker N. Pig-to-nonhuman primate islet xenotransplantation. Transplant Immunology. 2009 Jun;**21**(2):81-86

[3] Kirk A. Textbook of Organ Transplantation. West Sussex, UK: Wiley Blackwell; 2014

[4] Deschamps JY, Roux FA, Saï P, et al. History of xenotransplantation. Xenotransplantation. 2005;**12**:91-109

[5] Groth CG, Korsgren O, Tibell A, et al. Transplantation of porcine fetal pancreas to diabetic patients. Lancet. 1994 Nov 19;**344**(8934):1402-1404

[6] Valdes-Gonzalaez RA, Elliot RB, Dorantes LM, et al. Porcine islet xenografts can survive and function in type 1 diabetic patients in the presence of both pre-existing and elicited anti-pig antibodies. Transplantation. 2002;**74**(Suppl 4):94

[7] Matsumoto S, Abalovich A, Wechsler C, Wynyard S, Elliott RB. Clinical benefit of islet xenotransplantation for the treatment of type 1 diabetes. EBioMedicine. 2016;**12**:255-262

[8] Appel MC, Banuelos SJ, Greiner DL, et al. Prolonged survival of neonatal porcine islet xenografts in mice treated with a donor-specific transfusion and anti-CD154 antibody. Transplantation. 2004;**77**:1341-1349

[9] Cardona K, Korbutt GS, Milas Z, et al. Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. Nature Medicine. 2006 ;**12**: 304-306

[10] Kobayashi T, Harb G, Rayat GR. Prolonged survival of microencapsulated neonatal porcine islets in mice treated with a combination of anti-CD154 and anti-LFA-1 monoclonal antibodies. Transplantation. 2005;**80**:821-827

[11] Rood PP, Bottino R, Balamurugan AN, et al. Reduction of early graft loss after intraportal porcine islet transplantation in monkeys. Transplantation. 2007;**83**:202-210

[12] Wennberg L, Song Z, Bennet W, et al. Diabetic rats transplanted with adult porcine islets and immunosuppressed with cyclosporine A, mycophenolate mofetil, and leflunomide remain normoglycemic for up to 100 days. Transplantation. 2001;**71**:1024-1033

[13] Nagaraju S, Bottino R, Wijkstrom M, et al. Islet xenotransplantation: What is the optimal age of the islet-source pig? Xenotransplantation. 2015;**22**:7-19

[14] Dufrane D, Goebbels T, Fdilat I, et al. Impact of porcine islet size on cellular structure and engraftment after transplantation: Adult versus young pigs. Pancreas. 2005;**30**:138-147

[15] Kirchof N, Shibata S, Wijkstrom M, et al. Reversal of diabetes in non-immunosuppressed rhesus macaques by intraportal porcine islet xenografts precedes acute cellular rejection. Xenotransplantation. 2004;**11**:396-407

[16] Rayat GR, Rajotte RV, Hering BJ, et al. In vitro and in vivo expression of Galalpha-(1,3)Gal on porcine islet cells is age dependent. The Journal of Endocrinology. 2003 Apr;**177**(1):127-135

[17] Emamaulle JA, Shapiro AM, Rajotte RV, et al. Neonatal porcine islets exhibit natural resistance to hypoxia-induced apoptosis. Transplantation. 2006;**82**:945-952

[18] Rayat GR, Rajotte RV, Korbutt GS. Potential application of neonatal porcine islets as treatment for type 1 diabetes: A review. Annals of The New York Academy of Sciences. 1999;**875**:175-188

[19] Zhu H-T, Wang W-L, Yu L, et al. Pig-islet xenotransplantation: Recent progress and current perspectives. Frontiers in Surgery. 2014;**1**:7

[20] Rayat GR, Rajotte RV, Ao Z, et al. Microencapsulation of neonatal porcine islets: Protection from human antibody/complement-mediated cytolysis in vitro and long-term reversal of diabetes in nude mice. Transplantation. 2000;**69**:1084-1090

[21] Kin T, Korbutt GS, Kobayashi T, et al. Reversal of diabetes in pancreatectomized pigs after transplantation of neonatal porcine islets. Diebetes. 2005;**54**:1032-1039

[22] Cooper DKC, Ezzelarab MB, Hara H, et al. The pathobiology of pig-to-primate xenotransplantation: A historical review. Xenotransplantation. 2016;**23**:83-105

[23] Ekser B, Cooper DK. Overcoming the barriers to xenotransplantation: Prospects for the future. Expert Review of Clinical Immunology. 2010;**6**(2):219-230

[24] Itoh T, Hata Y, Nishinakamura H, et al. Islet-derived damage-associated molecular pattern molecule contributes to immune responses following microencapsulated neonatal porcine islet xenotransplantation in mice. Xenotransplantation. 2016;**23**:393-404

[25] Kobayashi T, Harb G,Rajotte RV, et al. Immune mechanisms associated with the rejection of encapsulated neonatal porcine islet xenografts. Xenotransplantation. 2006;**13**:547-559

[26] Rayat GR, Johnson ZA, Beilke JN, et al. The degree of phylogenetic disparity of islet grafts dictates the reliance on indirect CD4 T-cell antigen recognition for rejection. Diabetes. 2003 Jun;**52** (6):1433-1440

[27] Ezzelarab M, Ayares D, Cooper DK. Carbohydrates in xenotransplantation. Immunology & Cell Biology. 2005;**83**:396-404

[28] Kobayashi T, Cooper DK. Anti-Gal, alpha-Gal epitopes, and xenotransplantation. Subcellular Biochemistry. 1999;**32**:229-257

[29] Bottino R, Trucco M. Use of genetically-engineered pig donors in islet transplantation. World Journal of Transplantation. 2015;**5**(4):243-250

[30] Stewart JM, Tarantal AF, Hawthorne WJ, et al. Clonidine inhibits anti-non-Gal IgM xenoantibody elicited in multiple pig-to-primate models. Xenotransplantation. 2015; **22**(6):413-426

[31] Komoda H, Miyagawa S, Omori T, et al. Survival of adult islet grafts from transgenic pigs with N-acetylglucosaminyltransferase-III (GnT-III) in cynomolgus monkeys. Xenotransplantation. 2005;**12**:209-216

[32] Nagaraju S, Bottino R, et al. Islet xenotransplantation from genetically engineered pigs. Current Opinion in Organ Transplantation. 2013 Dec;**18**(6):695-702

[33] Kanak MA, Takita M, Kunnathodi F, et al. Inflammatory response in islet transplantation. International Journal of Endocrinology. 2014;**2014**:451035

[34] Liuwantara D, Chew YV, Favaloro EJ, et al. Characterizing the mechanistic pathways of the instant blood-mediated inflammatory reaction in xenogeneic neonatal islet cell transplantation. Transplantation Direct. 2016;**2**(6):e77

[35] Ji M, Jin X, Phillips P, et al. A humanized mouse model to study human immune response in xenotransplantation. Hepatobiliary Pancreatic Diseases International. 2012; **11**(5): 494-498

[36] Nagaraju S, Bertera S, Tanaka T, et al. In vitro exposure of pig neonatal islet like cell clusters to human blood. Xenotransplantation. 2015;**22**:317-324

[37] Murray AG, Nelson RC, Rayat GR, et al. Neonatal porcine islet cells induce human CD4+, but not CD8+, lymphocyte proliferation and resist cell-mediated cytolytic injury in vitro. Diabetes. 1999 Sep;**48**(9):1713-1719

[38] Yi S, Ji M, Wu J, et al. Adoptive transfer with in vitro expanded human regulatory T cells protects against porcine islet xenograft rejection via interleukin-10 in humanized mice. Diabetes. 2012;**61**(5):1180-1191

[39] Lalain S, Chaillous L, Gouin E, et al. Intensity and mechanisms of in vitro xenorecognition of adult pig pancreatic islet cells by CD4+ and CD8+ lymphocytes from type I diabetic or healthy subjects. Diabetologia. 1999;**42**:330-335

[40] Luca G, Nastruzzi C, Basta G, et al. Effects of anti-oxidizing vitamins on in vitro cultured porcine neonatal pancreatic islet cells. Diabetes Nutrition & Metabolism. 2000 Dec;**13**(6):301-307

[41] Gibly RF, Zhang X, Lowe WL, et al. Porous scaffolds support extrahepatic human islet transplantation, engraftment and function in mice. Cell Transplantation. 2013;**22**(5): 811-819

Chapter 5

# Xenotransplantation for Islets from Clinical Side

Wei Wang, Qi Liang, Wei Nie, Juan Zhang and

Cheng Chen

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.69233

**Abstract**

Islet transplantation can eliminate severe hypoglycemia symptoms caused by conventional treatment, and has the advantages of less trauma and complications, which is considered as the most promising treatment for type 1 diabetes mellitus (T1DM). Regulatory guidance is needed for a standard pig source. In section 1, the regulation of medical grade designed pathogen free (DPF) donor pig for clinical xenotransplantation consists of five parts: genetic quality control, microbiological surveillance, formula feeds, specification of pathological diagnosis, and requirements of environment and housing facilities. In section 2, we present the current approach and progress in pig donor selecting, pancreatic digestion, isolation and preparation of porcine islet grafts, identification and quality assessment of final islet product in clinical trials. The liver is currently the most preferred site for islet transplantation, even though it is far from ideal. A large number of alternative sites have been used for islet transplantation in experimental animal models to provide improved engraftment and long-term survival. In Section 3, we introduce some commonly used sites in xenotransplantation. The benefits and drawbacks of each parameter above are discussed in an attempt to decide which is the most suitable for clinical use and to direct future research.

**Keywords:** cell and tissue xenotransplantation, clinical study, designated pathogen free status, islet xenotransplantation, regulatory guidance of source pigs, islets preparation, release criteria, sites for islet transplantation

## 1. Regulation of medical grade designed pathogen free (DPF) donor pig for clinical xenotransplantation

Definition of PDF donor pig: the donor pig should be artificially bred and cultivated, genetic background or origin clear. The weight should not be more than 50 kg when 12-month old; WHO designated animal donor as pathogen free; pathogen infection must be prevented by biological safety barrier, antibiotics or vaccines free, for medical xenotransplantation, scientific research, teaching, production, verification, as well as other scientific experiment [1].

**Part 1: Genetic quality control**

In this section, the genetic classification, breeding methods, and the genetic quality criteria of inbred herd and closed herd have been discussed, which are suitable for the genetic quality control of DPF medical donor pigs [2].

1. Genetic classification and nomenclature

1.1. Genetic classification: According to different genetic characteristics, DPF pigs are divided into inbred herd and closed herd.

1.2. Nomenclature

1.2.1. Nomenclature of DPF pigs

Herd, generally named after the capital English letters, can also use capital English letters and Arabic numerals; named symbols should be short as far as possible, such as XENO.

1.2.2. Generation

The generation of herd use F in capital English letters. For example, an inbred herd for 30 generations is written as F30.

2. Breeding, including the principle of breeding, introduction, and methods

2.1. Breeding of inbred herd

2.1.1. Principle of propagation

Keep the inbred herd miniature pigs alleles homozygosity.

2.1.2. Introduction

Breeding pigs of inbred herd medical grade xenotransplantation DPF donor pigs should be from foundation herd or pedigree expansion herd, the basis of genetic background is clear, complete data (including the name of the herd, inbreeding generation, genetic characteristics, and main biological characteristics).

2.1.3. Methods

Inbred herd can be divided into foundation herd, pedigree expansion herd, and production herd. When the production supply of the inbred herd miniature pig is not very large, generally no consanguinity expanding herd, just set foundation herd and production herd.

2.2. Breeding of closed herd

2.2.1. Principle of breeding

Keep the genetic heterogeneity and genetic polymorphism of closed herd miniature pig, avoid generation growth too fast.

2.2.2. Introduction

Breeding pigs of inbred herd medical grade should be defined genetic background or source is clear, complete data (Including the species-group name, origin, genetic characteristics and main biological characteristics, etc.)

According to the way of breeding, ensuring the inbreeding generation growth in each generation under the premise of not greater than 1%, determines the minimal introduction scale. If using cycle copulation, introduction amount shall not be less than 13 pairs of unrelated pigs (within three generations, no common ancestor); if using random mating, introduction amount shall not be less than 25 pairs of unrelated pigs.

2.2.3. Methods

Keep a closed herd of medical grade xenotransplantation DPF donor pigs genetic stability, and try to avoid inbreeding.

3. The genetic quality monitoring of DPF medical donor pig

3.1. Inbred herd

3.1.1. Detection method

Microsatellite DNA markers detection method is generally used.

3.1.2. Sampling

In foundation herd, all animal breeding parents should be tested. In production herd, a random sample from each inbred herd, half male and half female, should be tested.

3.1.3. Result determination

All the alleles of microsatellite DNA markers in sample should conform to the characteristic of the herd, no new alleles appear as qualified medical grade xenotransplantation DPF donor pigs inbred herd, otherwise the sentence to unqualified.

3.1.4. Detection frequency

Genetic quality of medical grade xenotransplantation DPF donor pigs production herd should be tested at least once a year.

3.2. Closed herd

3.2.1. Detection method

Detection method is same as that of inbred herd.

3.2.2. Sampling

A random sample from each inbred herd, half males and half females, should be tested.

3.2.3. Result determination

The evaluation method of the genetic variation of the group is an average heterozygosity index or group balance state.

When the average heterozygosity lies between 0.5 and 0.7, and no obvious difference was found between observed heterozygosity and expected heterozygosity by chi-square test, the medical grade xenotransplantation DPF donor pigs can be qualified for closed herd. Or determine by whether the group is in a balance state, if there is no balanced, indicate that the gene frequency and genotype frequency of population changed, the closed herd of medical grade xenotransplantation DPF donor pigs group is not qualified.

3.2.4. Detection frequency

Genetic quality of closed herd medical grade xenotransplantation DPF donor pigs production herd should be tested at least once a year.

**Part 2: Microbiological surveillance**

In this section, microbiology classification of pathogens, surveillance standard, procedures, methods, rules, results determination, conclusions, sample preservation for xenotransplantation medical grade DPF donor pigs microbiology surveillance [1, 3–5] have been discussed.

| Type | Number | Testing items |
|---|---|---|
| Bacterium | 1 | *Brucella* spp. |
| | 2 | *Leptospira* spp. |
| | 3 | *Serpulina hyodysenteriae* |
| | 4 | *Mycobacterium bovis* |
| | 5 | *Mycobacterium tuberculosis* |
| | 6 | *Mycobacterium avium-intracellulare complex* |
| | 7 | *Mycoplasma hyopneumoniae* |
| | 8 | *Salmonella typhi* |
| | 9 | *Shigella* |
| | 10 | *Bordetella bronchiseptica* |
| | 11 | *Pasteurella multocida* |
| | 12 | *Actinobacillus pleuropneumoniae* |
| | 13 | *Streptococcus suis* type 2 |
| Fungi | 14 | *Pathogenic dermal fungi* |
| | 15 | *Cryptococcus neoformans* |
| | 16 | *Histoplasma capsulatum* |
| Parasites | 17 | *Ectozoa* |
| | 18 | *Ascaris suum* |
| | 19 | *Echinococcus* sp. |
| | 20 | *Isospora* sp. |
| | 21 | *Strongyloides ransomi* |
| | 22 | *Toxoplasma gondii* |
| | 23 | *Trichinella spiralis* |
| | 24 | *Neospora* |
| | 25 | *Fasciolopsis buski* |

| Type | Number | Testing items |
|---|---|---|
| Viruses | 26 | Adenovirus (porcine) |
| | 27 | Encephalomyocarditis virus |
| | 28 | Porcine influenza virus |
| | 29 | Human influenza viruses |
| | 30 | Porcine cytomegalovirus |
| | 31 | Porcine gammaherpesvirus |
| | 32 | Porcine reproductive and respiratory syndrome virus |
| | 33 | Porcine parvovirus |
| | 34 | Rotavirus |
| | 35 | Pseudorabies virus |
| | 36 | Rabies virus |
| | 37 | Foot and mouth disease virus |
| | 38 | Classical swine fever virus |
| | 39 | Japanese encephalitis virus |
| | 40 | Porcine circovirus type 2 |
| | 41 | Porcine transmissible gastroenteritis virus |
| | 42 | Swine vesicular disease virus |

**Table 1.** Microbiological testing items [3].

1. Microbiology classification: conventional (CV) minipig, clean (CL) minipig, specific pathogen free (SPF) minipig
2. Microbiological surveillance standard

2.1. Clinical observation
A visual inspection without abnormality has been done.

2.2. Microbiological testing items
Details see **Table 1**.

2.3. Microbiological testing procedures
Numbering—visual inspection—Blood sampling—Testing—Result determination.

2.4. Sample testing frequency
Testing is done at least once in every 6 months.

2.5. Sampling standard
Choose the pig which is more than 6-month-old for detection. random sampling.

3. Results determination
Results determination is according to the various microbial detection items. For antibody testing item, serum antibody negative is qualified. For antigen and nucleic acid testing item, no positive is qualified.

4. Preservation standard for microbiology testing sample
   Sample data, sample source, animal numbering, sample type and number, save by medical pathology data file management standard. Save time for 1 year.

**Part 3: Formula feeds**

Raw material for feed, nutrition, feed production, sanitary standard for medical grade xenotransplantation DPF donor pigs quality control of feed [6].

1. Raw material for feed

1.1. Principle of selection
Raw material should be certified as green food, or from the product of standard green food production base. Or from self-production green food which is qualified by government. According to the mode of green food production standard.

1.2. Nutrition
Details see **Table 2**.

1.3. Product requirements

1.3.1. Sense
The color should be homogeneous, without mildew, caking and odor.

1.3.2. Water
Compound feed 13.5% or less.

1.3.3. Mixing homogeneity
Compound feed, concentrated feed mixing uniformity coefficient of variation (CV) of 7% or less; Additive premixed feed coefficient of variation (CV) of 5% or less.

1.4. Sanitary requirement
Shall conform to the World Health Organization (World Health Organization, WHO) for medical donor animal food hygiene requirements, within the sterilization period of validity.

| Item | Standard sustaining feed | Growth and reproduction feed |
|---|---|---|
| Water, % ≤ | 11.0 | 11.0 |
| Crude protein, % ≥ | 12.0 | 14.0 |
| Crude fat, % ≥ | 2.5 | 3.0 |
| Crude fiber, % ≤ | 7.5 | 7.0 |
| Crude ash, % ≤ | 8.0 | 7.5 |
| Calcium, % | 0.65–1.0 | 0.75–1.0 |
| Phosphorus, % | 0.55–0.7 | 0.58–0.8 |
| Available phosphate, % | 0.32–0.4 | 0.32–0.4 |

*Modified from Laboratory animal wuzhishan pig, Feed nutrients requirements. (http://down.foodmate.net/standard/sort/15/42411.html. In Chinese).

**Table 2.** Feed nutrition ingredient detection index*.

**Part 4: Specification of pathological diagnosis**

In this section, the contents and methods of pathological examination for medical grade xenotransplantation DPF donor pigs, including examination rules, procedures, clinical pathology, anatomy and results determination and conclusion have been discussed [2, 7, 8].

1. The frequency of examination, sampling requirements, sampling methods, and the number of samples

1.1. Examination frequency
Clinical pathology inspection check should be done at least once in every 6 months; anatomical pathology testing check should be done at least once in every 2 years.

1.2. Sampling requirements

1.2.1. Method
Select 6 months above medical grade xenotransplantation DPF donor pigs for detecting random sampling.

2. Clinical and pathological examination

2.1. Visual examination
Mental state, coat, skin, natural orifice, nutritional status, motions, eating, breathing, etc.

2.2. Hematology detection
RBC: Red Blood Cell, HCT: Hematocrit, MCV: Mean Corpuscular Volume, HGB: Hemoglobin, MCH: Mean Corpuscular Hemoglobin, MCHC: Mean Corpuscular Hemoglobin Concentration, RDW: Red Cell Distribution Width, PLT: Platelets, MPV: Mean Platelet Volume, WBC: White Blood Cell.

2.3. Biochemistry detection
ALT: Alanine Aminotransferase, AST: Aspartate Amino Transferase, Scr:Serum Creatinine, BUN: Blood Urea Nitrogen, TP: Total Protein, ALB: Albumin, GLU: Glucose, T-BIL: Total Bilrubin, TG: Triglyceride, T-CHO: Total cholesterol.

2.4. Autopsy and pathological examination

2.4.1. Surface: the developmental status, nutritional status, mental state, sense organs, respiratory system, coat, skin, and testicles.

2.4.2. Subcutaneous: fat, mammary gland, lymph nodes (lymph nodes under the jaw, neck shallow lymph node, axillary lymph nodes, popliteal lymph nodes), and epididymis.

2.4.3. Head and neck: oral, nasal, brain, cerebellum, brainstem, pituitary gland, tonsil, thyroid, parathyroid gland.

2.4.4. Chest: pleural fluid, the thymus, the lungs and the lung, pericardium, pericardial fluid lymph node and the heart, the aorta, trachea, and bronchi.

2.4.5. Abdominal cavity: peritoneal fluid, spleen and lymph nodes, liver, gallbladder, liver door parts of the blood vessels, bile duct and lymph nodes, pancreas, kidney, adrenal, stomach, intestines, duodenum, jejunum, ileum, caecum, colon, rectum) and mesenteric lymph nodes.

2.4.6. Pelvic: bladder, ureter, prostate, seminal vesicle, ovaries, fallopian tubes, uterus, vagina.

2.5. Histopathological examination
Heart, lung, liver, spleen, kidney, stomach, jejunum, mesenteric lymph nodes, ovary/testis, and gross anatomy examination revealed abnormal organs and tissues.

3. Results determination
   Clinical pathology examination result is divided into four levels: did not see abnormality, minor abnormality, mild abnormality, and obvious abnormality. The pathological diseases are divided into congenital and infectious diseases, no matter what level, noninfectious disease excluded disease individuals, the whole group is ruled out when infectious disease occurs.

3.1. No abnormality: animal appearance without abnormality, at the same time, the blood test indices in the normal reference value range.

3.2. Minor abnormality: animals have no obvious abnormal appearance, but blood tests index one or two higher or lower, the biggest variation is not more than 20% of the normal reference value range threshold.

3.3. Mild abnormality

3.3.1. No obvious abnormal animal appearance, but more than two blood tests index higher or lower, the biggest variation is 20–50% of the normal reference value range threshold.

3.3.2. Mildly abnormal appearance, at the same time more than two blood tests index higher or lower, the biggest variation is 20–50% of the normal reference value range threshold.

3.4. Obvious anomaly

3.4.1. No obvious abnormal animal appearance, but more than two blood tests index higher or lower, the biggest variation is more than 50% of the normal reference value range threshold.

3.4.2. Animal mildly abnormal appearance, at the same time more than two blood tests index higher or lower, the biggest variation is more than 50% of the normal reference value range threshold.

3.4.3. Animal appearance is apparently abnormal

**Part 5: Requirements of environment and housing facilities**

In this section, the requirements of facilities, environmental conditions and drinking water, cushion, cage and transport standard for medical grade xenotransplantation DPF donor pigs [2, 8–11] have been discussed.

1. Construction

1.1. Building site

1.1.1. Chooses a good air quality and natural environment

1.1.2. Should be far away from the urban residential area and places have serious air pollution, vibration or noise of railway, docks, airports, roads, factories, storage, storage area, slaughtered live herd and poultry farms, factories, etc.

1.1.3. Facilities should be reliable to avoid other animal feeding cross infection

1.2. Sanitary requirements

1.2.1. External environment should be clean and tidy, easy to clean, and disinfectant. Drainage should be unblocked, without waste and sewage accumulation.

1.2.2. Set entrance for people, animals, objects, vehicles is dedicated, special disinfection facilities and equipment.

1.2.3. There should be measures to prevent exotic animals in and the experimental animals out.
1.2.4. Structure of the barrier should be solid, nontoxic, and without any radioactive material.
1.3. Facilities requirements
1.3.1. Doors and windows of the building should have good sealing; the observation window should be set up in the feeding door, set appropriate buffer room door interlock device.
1.3.2. Air-tight door of barrier environment facilities should be open in the direction of the higher air pressure, and can be automatically shut down.
1.3.3. The stairs width should not be less than 1.2 m; the corridor width generally should not be less than 1.5 m. The slot width should not be less than 1.0 m. The door width should meet the requirements for equipment to be in and out, width of which should not be less than 0.8 m.
1.3.4. Barrier environment facilities should be according to the need to maintain the correct pressure direction.
1.3.5. Breeding should be a reasonable organization between the location of the outlet and the inlet air flow, avoid blind angle and short circuit.
1.3.6. The pipe orifice of clean areas toward nonclean areas should be sealed. Drains, tank, pipe slope should guarantee the smooth drainage with no dirt accumulation. Drainage pipe diameter should not less than DN150.
1.3.7. There should be an established environmental monitoring system and the level of other facilities according to the need to set up the environment monitoring system.
1.4. Construction requirements
1.4.1. Passageway of goods should set up a ramp or unloading platform. The ramp slope should not be more than 1/10.
1.4.2. Rooms with the drain, drainage slope should not be less than 1%; the ground should be tested for waterproof processing.
1.4.3. Animal feeding room and lab should be set separately.
1.4.4. The autoclave sterilization equipment should be set between cleaning and disinfection room and clean storage room.
1.4.5. Production area (lab area); the height should not be less than 4.2 m.
1.4.6. The surface of Windows and doors, walls, ceiling, floor (ground) surface in clean area should be smooth, the structure and construction cracks reliably airtight measures should be adopted, metope and ground intersect position should have a radius of not less than 30 mm arc processing.
1.5. Water supply and drainage requirements
1.5.1. Water supply
1.5.1.1. The area of water purification should meet the requirements of sterilization.
1.5.1.2. Water supply system in production area (lab area) should be well equipped with the technology layer.
1.5.1.3. Purification pipeline crossing the wall, reliably sealing measures should be taken.
1.5.1.4. Water supply pipe and pipe fittings of purification area should be of corrosion resistance and provided with convenient and reliable connections.

1.5.2. Drainage
1.5.2.1. The septic tank must be individually set up for the drainage of production and lab area.
1.5.2.2. Drainage in barrier environment facilities should be set apart from subsistence drainage.
1.5.2.3. Purification area should not be through the drainage riser.
1.5.2.4. Pipes should be rust and corrosion free.
1.5.2.5. The drain floor of purification area should be airtight.
2. Layout
2.1. Overall layout
2.1.1. According to DPF medical donor pig's physiological needs and behavior characteristics, design and built their living facilities, and to strictly control the in and out of the personnel, goods, animals and the air.
2.1.2. Production area includes quarantine inspection room, the buffer room, shower rooms, corridors, clean storeroom, post disinfection room and board, mating, pregnancy, childbirth, breastfeeding, piglets, breeding pig house, etc.
2.1.3. Lab area includes buffer room, animals bath room, clean storeroom, post disinfection room, corridors, quarantine inspection room, preparation room, operating room, postoperative observation room, breeding room, etc.
2.2. Requirements for the main locale
2.2.1. Breeding room setup requirements
2.2.1.1. Water system should prevent reflux and alien species.
2.2.1.2. Breeding room should be equipped with appropriate feeding equipment and capture tools. Equipment and tools shall ensure to be firm and will not harm the animal.
2.2.2. Operating room setup requirements
2.2.2.1. Should set up comprehensive laboratory, equipped with necessary equipment and according to the demand.
2.2.2.2. Should set up isolation room to independently observe injured and suspected diseased animals.
2.2.2.3. Should set up quarantine room for new animals.
2.2.2.4. According to the need to set up postoperative observation room.
2.2.3. The auxiliary area setup requirements
2.2.3.1. Environmental controls should be strict in the feed storeroom, preventing pathogenic microorganism pollution, parasites pollution, and alien species.
2.2.3.2. Should set up storeroom with storage cages and instruments.
2.2.3.3. Disinfection room space should be accessible for cleaning process. Before and after cleaning equipment should be placed separately. The walls and floor waterproof treatment should be done.
2.2.3.4. Specialized room and (or) equipment should be set up for animal bodies and waste storage
2.2.3.5. Should set up the observation corridor, or observation area, or set up a video surveillance system, used for observing animals.
3. Feeding conditions
3.1. Fence

| Item | | Index |
|---|---|---|
| Temperature, °C | | 20–26 |
| Daily temperature difference, °C, ≤ | | 4 |
| Relative humidity, % | | 40–80 |
| Pressure gradient in the same area, Pa, ≥ | | 10 |
| Air velocity, m/second, ≤ | | 0.2 |
| ventilation rate, /hour, ≥ | | 15 |
| Air cleanliness, level | | 7 |
| Mean concentration of settled bacteria/0.5 hours/Φ 90 mm plating, ≤ | | 3 |
| Detection rate of designed pathogen, %, ≤ | | 0 |
| Ammonia concentration, mg/m$^3$, ≤ | | 14 |
| Noise, dB(A), ≤ | | 60 |
| Illuminance,lx | Working, ≥ | 200 |
| | Animal | 100–200 |
| Photoperiod, hour | | 12–14/12–10 |

*Modified from Laboratory animal wuzhishan pig, Feed nutrients requirements. (http://down.foodmate.net/standard/sort/15/42411.html. In Chinese.)

**Table 3.** Environment factor index*.

3.1.1. Choose a material that is nontoxic, washable, high temperature resistant, and easy to sterilize.

3.1.2. Bar size should meet the miniature pig lying down, feed intake and defecation, or use the fence to establish different function areas. Fences should be strong.

3.2. Manger

3.2.1. Choose a material that is nontoxic, washable, high temperature resistant, and easy to sterilize.

3.2.2. The size of the manger should allow all the animals to eat at the same time.

4. Environment

Details see **Table 3**.

5. Waste disposal

5.1. Sewage

Primary sewage treatment equipment or anaerobic tank should be equipped.

5.2. General waste

Waste packing should be gathered for disposal. Disposable coverall, masks, hats, gloves, and experiment of waste should be treated harmless. Injection needles, razor blades sharp items should be collected in toolbox processed by corresponding qualifications organization.

5.3. Infectious waste

Infectious waste must be high pressure sterilized before processing.

5.4. Animal waste
Animal bodies and tissues should be loaded in special bags stored in the refrigerator or freezer, gathered for harmless disposal.
6. Transport
6.1. Transport cages
6.1.1. Should be strong and can prevent animal damage and escape, have feces and urine collection device, and in accordance with the requirements of the animal health and welfare. Suitable for carrying, is conducive to protect animals, and handling personnel safety.
6.1.2. In accordance with the corresponding microbial control environment, easy to clean, and disinfectant.
6.1.3. With peripheral filter membrane, internal solid cages of biological security isolation function.
6.2. Transportation
6.2.1. The transportation shall be equipped with air conditioning and other equipment and able to keep the environment temperature stable.
6.2.2. It should be able to ensure that there is enough fresh air and the shipping space of the cages, meets the needs of the animals' health, safety, and comfort.
6.2.3. The transportation can be disinfected.
6.2.4. Long-distance transportation (more than 6 hours) should provide drinking water, feed whenever necessary.

**Conclusion**

In conclusion, after strict genetic quality control, close monitoring of pig breeding condition and process, and extensive microbiological screening, we have selected a DPF herd as donors for cell xenotransplantation. This herd is free from all tested conventional and xenotransplantation related pathogens. It can not only minimize microbial negative impact, but also be likely to reduce swine pathogen infection risk, which will promote the development of clinical xenotransplantation from pig donor sources.

## Isolation, purification, and quality control of islet in clinical porcine islet xenotransplantation

Islet cells are mainly divided into three types according to different hormone secretion, namely glucagon secretion $\alpha$-cells, insulin secretion $\beta$-cells, and somatostatin secretion $\delta$-cells. $\beta$-cells can regulate insulin release by sensing the change of glucose level in order to maintain euglycemia. Islet transplantation can eliminate severe hypoglycemia symptoms caused by conventional treatment and has the advantages of less trauma and complications, which is considered as the most promising treatment for type 1 diabetes mellitus (T1DM). In recent years, with the maturing of islet cell transplantation technology and the development of the clinical application, pancreatic islet transplantation has gradually showed satisfactory and

prospective approach in the treatment of T1DM. Nonetheless, the donors' shortage still precluded the development and progression of clinical islet transplantation. Xenotransplantation offers an effective and feasible solution for this limitation. Among many potential candidates, pig is considered as the most ideal donor for future clinical applications. Although a number of encouraging findings have been reported in preclinical trails, the clinically pervasive application of pig islet xenotransplantation still faces the challenges such as inadequate supply of islet cells with high-quality. This section will discuss the current approach and progress in pig donor selecting, isolation, and preparation of porcine islet grafts, quality control, and release criteria of final product in clinical trials.

1. Selection of islet from different sources of donor pig

1.1. Comparison of characteristics of newborn pigs, young pigs, and adult pigs

Islets may be harvested from newborn (neonatal), young, or adult pigs for transplantation into human in clinical trials. For human recipients, pig donor sources not only comply with the regulation of medical grade DPF donor pig for clinical xenotransplantation, but also must conform to human islets in terms of structure and function to regulate the high blood glucose level found in T1DM patients. Despite several years of investigation, no real consensus has been established with regard to the best pig strain to provide enough viable and functional isolated porcine islets for xenotransplantation [12].

Newborn or neonatal (aged 1–5 days) islet-like cell clusters, (NICCs) have several advantages as the preferred source of β-cells for xenotransplantation. Compared to young or adult pig islets, they can be procured and are easy to isolate by enzymatic digestion [13–15] and there is less batch-to-batch variation between isolations. The main problem with NICCs is the need to culture for maturation to achieve functionality, which is as a potential source of cells because they have an inherent ability to proliferate and differentiate *in vivo* [16]. The NICCs are composed of fully differentiated endocrine cells (35%) and endocrine precursor cells (57%) [17].

Reducing warm ischemia time in the surgical procedure is indispensable for processing of pancreases from young or juvenile (12–15 weeks) pigs. However, culturing between weeks 5 and 24 is required for further maturing of the islets acquired from young pigs, which is unpractical for clinical transplantation owing to isolation difficulties and immature capability [17, 18].

Isolation and purification of islets acquired from adult pigs (>2 years) is still demanding to conduct and repeat, although there were many experiences in this field previously. The mature islets from adult pigs are physiologically similar to humans, which can secrete insulin in response to a glucose challenge. However, adult pig islets require mature pigs of more than 2 years of age [19], furthermore, the logistical considerations of keeping and handling large numbers of adult donor pigs in specific pathogen free housing for this period is impractical, which are not present with neonatal or juvenile pigs.

Dufrane D's group reported a protocol providing a greater than 90% chance to obtain a sufficient islet yield for adult pig islet xenotransplantation into no human primates [20].

Furthermore, they demonstrated that the β-cell percentage within islets is significantly affected by pig age (87.0 ± 3.3% versus 82.1 ± 3.6% for young and adult pig donors, respectively) [21]. This was directly correlated with a significant difference in non–β-cell composition between islets from young and adult pig donors (11.8 ± 3.3% versus 16.2 ± 3.4%, respectively).

1.2. Comparison of different methods for isolation and purification of islets of newborn pigs, young pigs, and adult pigs

1.2.1. NICC isolation

Once removed, the pancreata of neonatal pigs were finely chopped and digested with collagenase Type V, 1 mg/ml (Sigma-Aldrich) at 37°C. The digested tissue was washed in Hank's balanced salt solution (HBSS) (Gibco) and filtered through a 500-μm sieve. Tissue was plated into dishes (No tissue culture treated) with Hams F-10 medium (Gibco) containing 10 mM glucose, 50 mM isobutylmethylxanthine, 10% porcine serum, 2 mM L-glutamine, 10 mM nicotinamide, 100 U/ml penicillin, and 100 μg/ml streptomycin, CaCl2 0.236 g/l, hepes 80 mM, NaHCO3 21.3%(Sigma-Aldrich), with full media changes every other day. The cells were cultured at 37°C and 5% $CO_2$.

NICCs were isolated and cultured for up to 27 days postisolation. Number of islet equivalents (IEQ), viability, and function were analyzed each week to determine whether time in culture was important for NICC function *in vivo*. It is reported that culture of NICC for 12 days provided the best outcome of viability and function *in vivo* posttransplantation, which was revealed by better reversal of diabetes, and lower levels of TF expression and higher expression of insulin, glucagon, and Bcl-2 with acceptable cell loss in terms of time and expense [22].

1.2.2. Islet isolation from young pigs

For young pigs, briefly, the pancreas was harvested using rapid surgical procurement (<5 min) and placed in organ preservation solution. Cold ischemia time was limited to less than 30 min. The pancreas was then washed in cold (4°C) HBSS supplemented with hepes and trimmed of surrounding adipose and lymphatic tissue in a sterile environment. The pancreatic tissue was then minced into 2–3 $mm^3$ pieces and digested at 37°C using sigma type V collagenase (2.5 mg/mL in HBSS). The mean digestion time was around 16 min. The islet tissue clusters (50–500 μm) isolated using the method above were allowed to mature into complete islets during culture *in vitro* at 37°C, 5% $CO_2$ at first in recovery maturation media (Optatio LLC) supplemented with 10% porcine serum, 417 mM dornase α, 215 mM aprotinin, 0.5 mM pefabloc, and then in a novel maturation media (Optatio LLC) 48 hours later, supplemented with 10% porcine serum [23, 24].

1.2.3. Islet isolation from adult pigs

The factors as follows influence the islet yield of pancreas from adult pigs: (1) pancreas acquisition (exsanguination and warm ischemia time), (2) the ingredient of cold storage solutions, (3) the various methods of pancreas digestion and purification, and (4) the endotoxin content and enzymatic activity. Researchers concluded some variables through a variety of investigation, which could enhance the yield of islets, for example, the application of blood exsanguination before pancreas procurement, a warm ischemia time within 10 min, the concentration of <30 EU of endotoxin in Liberase batches, etc. To the contrary, the isolation technique (dynamic versus static) and the storage

method (short-term versus long-term) and solution (UW versus modified UW) did not obviously affect the islet yield. Additionally, there was a positive relationship between isolated islet number and the number of islets/cm$^2$ or with the percentage of large islets shown by the pancreas biopsies. Pig pancreases containing more than 82 islets/cm$^2$ and more than 42% of large islets (>100 μm); thus, enabled more than 120,000 islet equivalents to be harvested in 90% of the cases [20].

2. Methods for islet quality control

To ensure the safety and efficiency of islet transplantation, all kinds of measurements have been processed. The quality parameters of islet products involved sterility, purity, viability and activity, cell population, and functionality. It has been suggested that transplantation of poor quality islet product would cause the inconsistencies of the ability of islet transplants to reverse diabetes [25, 26], so islet quality control is critical to both determining the suitability of islets for transplantation as well as to maintain a long-term functional graft in recipients posttransplantation.

2.1. Biological safety

As the main aim of clinical islet isolation is transplantation into a recipient, biological safety of the final product is an essential criterion for product release. This is particularly significant as recipients are immunosuppressed and thus are at an increased risk that infectious pathogens enter the recipient as part of the transplant product and cause infectious disease [27].

To test for the microbial sterility, sample aliquots are taken from the culture medium post-purification and post-culture respectively. Two aliquots from each time point are inoculated aseptically into bactec™ culture vials (Becton Dickinson) specific for aerobic (tryptic soy broth) and anaerobic (soybean-casein digest broth) bacteria culture for 72 hours [28, 29]. Furthermore, samples are also cultured for fungi, mycoplasma, and mycobacteria. At last, the final product is tested for a large series of viruses, consisting of more than 28 viruses.

Endotoxin contaminants are known to lead to islet cell damage and early graft loss. Additionally, microbial contamination is likely to occur at various stages throughout the islet isolation, purification, and culture procedure. The reagents and supplies are possible sources of endotoxins in islet preparations [30, 31], but the most likely source of contaminations is the donor duodenum during pancreas surgical retrieval, as observed from testing of the solution in which the sample of retrieved pancreas is preserved [32].

It is very crucial to determine the sterility of islet preparations for transplantation, and several measures are in place to reduce risk of contamination after isolation and culture. Antibiotics are usually supplied to culture media, and aliquots are taken for Gram staining, endotoxin content measurement, and microbiological culture both after isolation and pretransplant after culturing. In terms of islets release, a negative Gram stain is required, as well as endotoxin content <5 endotoxin units (EU)/kg recipient weight [33]. Several studies have demonstrated that no clinical infection was observed in recipients and long-term graft survival remained unaffected by using these criteria [32].

2.2. Biological characteristics

2.2.1. Quantity (islet equivalent determination, islet count standard)

Islet count was determined by the number of islet equivalents (IEQ = conversion of actual number of islets into number of islets with a diameter of 150 μm) [34]. The final purity of islet products after purification was calculated as the ratio between islets (stained in red by dithizone) and exocrine tissue (unstained by dithizone) on an inverted phase contrast microscope with a calibrated grid in the eyepiece.

2.2.2. Activity

Dithizone staining was also used to determine percentage of purity over the maturation period. Islet viability was analyzed using Newport Green (NPG) (Invitrogen) and propidium iodide, imaged using fluorescence microscopy, and quantified with a microplate reader.

2.2.3. β cell purity

2.2.3.1. Flow cytometry

Newport Green (NPG PDX acetoxymethyl ether) binds to zinc present in β-cells. Apoptosis was measured by tetramethylrhodamine ethyl ester perchlorate (TMRE) selectively binding to mitochondrial membranes. 7-aminoactinomycin D (7-AAD) binds to DNA in dead cells as their membrane permeability is altered. NICCs were dispersed by accutase (Sigma-Aldrich) and then stained, respectively, with NPG, TMRE, and 7-AAD to determine the proportion of β-cells, viable cells, and dead cells. To obtain a numeric product, the β-cell viability index was calculated according to the following formula ((%βcells × %βcells viability)/10,000) [35].

2.2.3.2. DNA content

Two samples of 200 IEQs of islets were obtained and stored at 20°C after washing with citrate buffer. Samples were sonicated and used to measure the DNA content using the Quant-iT Pico Green dsDNA Assay Kit (Invitrogen). Fluorescent excitations ~480 nm, emission ~520 nm was used to read samples by staining with Pico Green fluorescent reagent.

2.2.3.3. Insulin/DNA ratio

Cell suspensions of each sample were homogenized by ultrasonication on ice prior to detection of DNA content using a Quanti-iT Pico Green dsDNA Assay Kit (Invitrogen) and insulin content with an porcine insulin RIA kit (Biosource), respectively. Insulin capacity was obtained by the ratio of the insulin content to the DNA content in microgram in 1000 NICC IEQ [25].

2.2.3.4. Islet function identification

*In vitro* insulin capacity of the islet products was determined using glucose-stimulated insulin release (GSIS). Eight hundred IEQ porcine islets were incubated in low glucose solution (2.8 mM) for 1 hour and then incubating half the cells in low glucose and half in high glucose (25 mmol/l) solution. The stimulation index was obtained as the ratio by dividing the average high glucose by the average low glucose value. Insulin levels were analyzed using a standard porcine insulin enzyme-linked immunosorbent assay (Porcine Insulin ELISA; Mercodia), and absorbance was measured using a microplate reader [36].

Alternative cell functional assays to determine the metabolic activity such as ATP content and oxygen consumption rate can also be frequently used as release

criteria. These assays utilize small number of islets from the pooled product, and can be performed quickly prior to transplantation. Furthermore, they offer a good indication of the metabolic activity and potential functional capacity of the product. Finally, it should also include the reversal of diabetes in immune-deficient mice relative to dose.

The FDA regulations place islets isolated for transplant therapy under the biological products, requiring the released preparation to demonstrate product stability and consistency between lots in addition to complying with standards of product identity, safety, purity, and potency [37, 38]. The release criteria, formally, are based on porcine islet count per recipient weight (10,000–20,000 IEQ/kg for single transplant), with purity greater than 30% (assessed using dithizone staining), viability greater than 70% (assessed using Newport Green/TMRE/7-AAD staining), endotoxin concentration <5 (EU) /kg recipient weight, and no detectable organisms in a Gram stain prior to transplantation, as well as to a glucose stimulation index (ratio of stimulated insulin secretion/basal insulin secretion) ≥1 [39, 40]. Criteria based on these are currently, in formal, applied at the institution for cell transplantation and gene therapy at the 3rd Xiangya Hospital of Central South University, China, where we require each islet preparation from neonatal pigs to reach the determined thresholds of islet number/mass, viability, purity, and sterility before the product is released for transplantation [41].

**Conclusion**

Based on the remarkable progress of islet cell transplantation technology in the experimental and clinical studies, the islet xenotransplantation from porcine donors expected to become one of the potential and fundamental treatments for type 1 diabetes mellitus. The effective separation and purification of functional pig islet for transplantation, has always been a hot research topic in the field of heterogeneous islet transplantation. With the continuous development of suitable sources of pig donors, modification of isolation and purification technology, the improvement of quality control system of islet products, how to establish simple, economic and standardized graft preparation, and evaluation standard as soon as possible will promote islet xenotransplantation technology make greater progress and enter the next step of clinical studies, which will benefit the patients with diabetes by the tangible therapy in the very near future. However, remaining questions and detailed problems need to be adequately addressed.

## Current alternative sites for islet transplantation

A suitable transplantation site could accommodate a large volume of islets for transplantation in an ample space, which is very close to vasoganglion providing enough oxygen and nutrients in the course of revascularization. Furthermore, it should avoid the reduction of early islet from host immune and inflammatory responses, while if necessary, the site is accessible to

transplantation processes [42]. It is a priority of research to define extrahepatic engraftment sites. The purpose of ongoing studies is to find a microenvironment that could offer prompt transplantation and make the inflammation and islet cell death to a minimum at early stage. At the same time, it could realize continuous function, which is of particular interest for researchers. It has been demonstrated in experimental animal models that islet grafts transplantation with or without the strategy of bioengineering in a number of extrahepatic sites, even though translation in clinic for some is unclear [43–45]. Numerous sites have been proposed and tested, both experimentally and in some cases clinically, including the liver, kidney subcapsule, bone marrow, immune privileged sites, and peritoneum spaces. While some alternative sites may be advantageous in experimental models, their feasibility and translation into clinical settings is limited up to date. While it has been proved in clinic that the infusion of intraportal islet could abolish T1DM, there has been long a need for finding a selectable engraftment site to optimize clinical results in the long term. Experimental research has offered potential alternatives to repair normoglycemia, even though a number of methods have implied limitations in terms of technology and/or physiology.

**Liver**

Intrahepatic islet infusion via the portal vein accounts for all clinical islet transplants conducted worldwide. Percutaneous portal vein infusion under ultrasound and fluoroscopic guidance offers a minimally invasive procedure with the ability to regulate glycemic levels through portal insulin delivery [46]. Alternatively, in patients at risk for bleeding, the transplant is performed by cannulation of a tributary of the portal vein using open surgery (minilaparotomy) or laparoscopic approach. It is worth noting the potential procedural risks such as portal thrombosis and bleeding [47]. A significant amount of intraportal islets are lost immediately after transplantation due to instant blood-mediated inflammatory reaction (IBMIR), which negatively influences islet grafts through expression of tissue factor, resulting in platelet adherence, activation, clot formation, and lymphocyte recruitment. In addition, xenotransplantation has more severe obstructions than those of clinical islet allotransplantation because IBMIR is still a major obstacle for islet transplantation. In pig-to-NHP islet xenotransplantation, although the precise mechanisms are yet to be illuminated, simultaneous activation of complement, coagulation, and platelets occurred immediately after monocyte and neutrophil infiltration, which play a pivotal role in this very early islet destruction. In order to solve this problem, a variety of genetically engineered pigs have been developed. The generalized strategies to introduce porcine genetic modification are knocking down or knocking out certain genes for polysaccharide antigens, e.g., $\alpha$-Gal, knocking in human complement regulatory proteins, coagulation and cellular immune response regulatory proteins, respectively, or combinations of the above genetic modifications. Technical speed development for genetically engineering modified pigs provides another approach to realize clinical islet xenotransplantation [48].

**Peritoneum**

The peritoneum has the advantages of accepting both unpurified and microencapsulated islets as tested in many experimental studies. It is unfortunate that research in the

murine mode indicated that a great number of islets are made requests for hyperglycemia reversion, while the insufficiency of parasympathetic re-innervation of the transplant is related to abnormal glucose tolerance tests [49] and morphologic alteration in islet architecture [50]. In the nonobese diabetic mouse mode, intraperitoneal syngeneic islets microencapsulated in 5% agarose hydrogel resisted to the autoimmune attack [51]. In terms of technique, a latest research suggested a minimally invasive laparoscopic process for microencapsulated islet autotransplantation in nonhuman primates [52]. As a result, the transplantation site was promptly applied in a successful clinical experiment [53] after the process of intraperitoneal alginate capsulated islets from neonatal pig was proved safe in the NHP mode [54].

**Bone marrow**

Bone marrow (BM) may represent an ideal microenvironment for islets, attribute to its protected and extravascular (but well vascularized) microenvironment, its broad distribution, and its easy access. Because of its broad distribution and easy access, BM has the potential to overcome not only the physiologic loss of islets, but also the technical limitations and complications encountered with the intraportal infusion [55]. A previous research reported that BM could provide an immunoprotected microenvironment that allogeneic, syngeneic, and xenogeneic islet could survive in nondiabetic rat models without immunosuppression [56]. Based on this, an ongoing pilot clinical trial at San Raffaele Scientific Institute in Milan aims to evaluate the safety and feasibility of BM as a potential site for islet auto/allotransplantation. Some research results showed that the BM is a more suitable site than the liver for the implantation of islets in murine model [57]. However, further research is required to determine whether the results can be reproduced in large animals and eventually in humans.

**Kidney capsule**

The kidney capsule has been applied as a potential site for experimental islet transplants in murine models [58], despite its poor blood supply in a relative manner [59] and the fact that it does not supply an oxygen-rich microenvironment. Correspondingly, the surgical process in murine modes is easy, which lead to hyperglycemia reversion in several days. In addition, it has the advantages on transplant restoration post-nephrectomy by both histologic research and function test on glucose metabolism. Compared with the number of liver, islets that could reverse chemically-induced diabetes were less when transplanted under kidney capsule in a syngeneic murine model of islet transplantation, mainly because the microenvironment for intraliver engraftment is less ideal [60]. In addition, a smaller islet mass is demanded to reverse hyperglycemia in the renal subcapsular space [61, 62]. Furthermore, islet transplants under the kidney capsule allow the cotransplantion with endothelial cells [63], MSCs [64], and BM stem cells [65]. In humans, the invasive surgical procedure is used to release the islets under the kidney capsule, which is the really limited space for a high transplant mass. Additionally, the diabetic nephropathy of recipients signifies the kidney capsule is not an ideal site for islet grafts [66].

**Conclusion**

Although many implantation sites have been proposed, few have found their way into the clinical setting. The experts suggest that islets infusion into the liver through the portal vein has been the chief approach of option. Well characterized sites, e.g., the kidney capsule and other immunoprivileged sites, are significant experimental models but with some limitations in applicability in the clinical setting. Nevertheless, there is clinical potential for possible utilization of both the peritoneum and bone marrow sites; however, further research is required before therapeutic advances can be made here.

## Acknowledgments

The authors would like to thank Bin Ye, Pengfei Rong, Xiaoqian Ma, Cejun Yang, Qiong Dong, Shengwang Zhang for their assistance with this chapter.

## Author details

Wei Wang*, Qi Liang, Wei Nie, Juan Zhang and Cheng Chen

*Address all correspondence to: cjr.wangwei@vip.163.com

Institute for Cell Transplantation and Gene Therapy of the Third Xiangya Hospital, Central-South University, Changsha, China

## References

[1] Schuurman HJ. The International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes Chapter 2: Source pigs. Xenotransplantation. 2009;**16**(4):215-222

[2] Korbutt GS. The International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes–Chapter 3: Pig islet product manufacturing and release testing. Xenotransplantation. 2009;**16**(4):223-227

[3] Fishman JA. Infection in xenotransplantation. Journal of Cardiac Surgery. 2001;**16**(5):363-373

[4] Fishman JA, Patience C. Xenotransplantation: Infectious risk revisited. American Journal of Transplantation. 2004;**4**(9):1383-1390

[5] Fishman JA, Scobie L, Takeuchi Y. Xenotransplantation-associated infectious risk: A WHO consultation. Xenotransplantation. 2012;**19**(2):72-81

[6] Vanderpool HY. The International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes–Chapter 7: Informed consent and xenotransplantation clinical trials. Xenotransplantation. 2009;**16**(4):255-262

[7] Denner J, Schuurman HJ, Patience C. The International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes—Chapter 5: Strategies to prevent transmission of porcine endogenous retroviruses. Xenotransplantation. 2009;**16**(4):239-248

[8] Cooper DK, Casu A. The International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes–Chapter 4: Pre-clinical efficacy and complication data required to justify a clinical trial. Xenotransplantation. 2009;**16**(4):229-238

[9] Vajta G, Zhang Y, Machaty Z. Somatic cell nuclear transfer in pigs: Recent achievements and future possibilities. Reproduction Fertility and Development. 2007;**19**(2):403-423

[10] Cozzi E, et al. First update of the International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes–Chapter 1: Update on national regulatory frameworks pertinent to clinical islet xenotransplantation. Xenotransplantation. 2016;**23**(1):14-24

[11] Rayat GR, et al. First update of the International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes–Chapter 3: Porcine islet product manufacturing and release testing criteria. Xenotransplantation. 2016;**23**(1):38-45

[12] Prabhakaran S, Hering BJ. What strain of pig should be used? Xenotransplantation. 2008;**15**:83-88

[13] Cardona K, Korbutt GS, Milas Z, et al. Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. Nature Medicine. 2006;**12**:304-309

[14] Rajotte RV. Isolation and assessment of islet quality. Xenotransplantation. 2008;**15**:93-97

[15] Korbutt GS, Elliott JF, Ao Z, et al. Large scale isolation, growth, and function of porcine neonatal islet cells. Journal of Clinical Investigation. 1996;**97**:2119-2147

[16] Dufrane D, Gianello P. Pig islet for xenotransplantation in human: Structural and physiological. Transplantation Reviews. 2012;**26**:183-188

[17] Jay TR, Heald KA, Carless NJ, et al. The distribution of porcine pancreatic beta-cells at ages 5, 12 and 24 weeks. Xenotransplantation. 1999;**6**:131-170

[18] Dufrane D, Goebbels RM, Fdilat I, et al. Impact of porcine islet size on cellular structure and engraftment after transplantation: Adult versus young pigs. Pancreas. 2005;**30**:138-184

[19] Mackenzie Dah D, Sollinger HW. Xenogeneic transplantation of porcine islets: An overview. Transplantation. 2003;**76**:887-891

[20] Dufrane D, D'hoore W, Goebbels RM, et al. Parameters favouring successful adult pig islet isolations for xenotransplantation in pig-to primate models. Xenotransplantation. 2006;**13**:204-217

[21] Cabrera O, Berman DM, Kenyon NS, et al. The unique cytoarchitecture of human pancreatic islets has implications for islet cell function. Proceedings of the National Academy of Sciences of the United States of America. 2006;**103**:23342342

[22] Jimenez-Vera E, Davies S, Phillips P,et al. Long-term cultured neonatal islet cell clusters demonstrate better outcomes for reversal of diabetes: In vivo and molecular profiles. Xenotransplantation. 2015;**22**:114-123

[23] Krishnan R, Buder B, Alexander M, et al. Juvenile porcine islets can restore euglycemia in diabetic athymic nude mice after xenotransplantation. Transplantation. 2015;**99**:710-716

[24] Lamb M, Laugenour K, Liang O, Alexander M, Foster CE, Lakey JR. In vitro maturation of viable islets from partially digested young pig pancreas. Cell Transplantation. 2014;**23**(3):263-272

[25] Ma XQ, Ye B, Gao F, et al. Tissue factor knockdown in porcine islets: An effective approach to suppressing the instant blood-mediated inflammatory reaction. Cell Transplantation. 2012;**21**:61-71.

[26] Shapiro AM, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson RP, et al. International trial of the Edmonton protocol for islet transplantation. The New England Journal of Medicine. 2006;**355**(13):1318-1348

[27] Taylor GD, Kirkland T, Lakey J, Rajotte R, Warnock GL. Bacteremia due to transplantation of contaminated cryopreserved pancreatic islets. Cell Transplantation. 1994;**3**(1):103-108

[28] O'Connell PJ, Hawthorne WJ, Holmes-Walker DJ, et al. Clinical islet transplantation in type 1 diabetes mellitus: Results of Australia's first trial. Medical Journal of Australia. 2006;**184**(5):221-225

[29] Kin T, Rosichuk S, Shapiro AM, et al. Detection of microbial contamination during human islet isolation. Cell Transplantation. 2007;**16**(1):9-13

[30] Berney T, Molano RD, Cattan P, et al. Endotoxin-mediated delayed islet graft function is associated with increased intra-islet cytokine production and islet cell apoptosis. Transplantation. 2001;**71**(1):125-156

[31] Vargas F, Vives-Pi M, Somoza N, et al. Endotoxin contamination may be responsible for the unexplained failure of human pancreatic islet transplantation. Transplantation. 1998;**65**(5):722-728

[32] Bucher P, Mathe Z, Bosco D, et al. Microbial surveillance during human pancreatic islet isolation. Transplantation Proceedings. 2004;**36**(4):1147-1154

[33] Gala-Lopez B, Kin T, O'Gorman D, et al. Microbial contamination of clinical islet transplant preparations is associated with very low risk of infection. Diabetes Technology & Therapeutics. 2013;**15**(4):323-329

[34] Hawthorne WJ, Simond DM, Stokes R, et al. Pre-clinical model of composite foetal pig pancreas fragment/renal xenotransplantation to treat renal failure and diabetes. Xenotransplantation. 2011;**18**:390-399

[35] Ichii H, Inverardi L, Pileggi A, et al. A novel method for the assessment of cellular composition and beta-cell viability in human islet preparations. American Journal of Transplantation. 2005;**5**:1635-1645

[36] Kobayashi T, Arefanian H, Harb G, et al. Prolonged survival of microencapsulated Cell Transplant. Transplantation. 2008;**17**:1243-1256

[37] Linetsky E, Ricordi C. Regulatory challenges in manufacturing of pancreatic islets. Transplantation Proceedings. 2008;**40**(2):424-429

[38] U.S. Department of Health and Human Services FDA. Guidance for industry: Considerations for allogeneic pancreatic Islet cell products 2009 [updated 15/09/14.]

[39] Balamurugan AN, Naziruddin B, Lockridge A, et al. Islet product characteristics and factors related to successful human islet transplantation from the Collaborative Islet Transplant Registry (CITR) 1999-2010. American Journal of Transplantation. 2014;**14**(11): 2595-2606

[40] Papas KK, Suszynski TM, Colton CK. Islet assessment for transplantation. Current Opinion in Organ Transplantation. 2009;**14**(6):674-682

[41] Wang W, Mo ZH, Ye B, et al. A clinical trial of xenotransplantation of neonatal pig islets for diabetic patients. Journal of Central South University (Medical Sciences). 2011;**36**(12): 1134-1140

[42] Veriter S, Gianello P, Dufrane D. Bioengineered sites for islet cell transplantation. Current Diabetes Reports. 2013;**13**(5):745-755

[43] Cantarelli E, Piemonti L. Alternative transplantation sites for pancreatic islet grafts. Current Diabetes Reports. 2011;**11**:364-374

[44] Smink AM, Faas MM, de Vos P. Toward engineering a novel transplantation site for human pancreatic islets. Diabetes. 2013;**62**:1357-1364

[45] Brady AC, Martino MM, Pedraza E, et al. Pro-angiogenic hydrogels within macroporous scaffolds enhances islet engraftment in an extrahepatic site. Tissue Engineering Part A. 2013;**19**(23-24):2544

[46] Rajab A. Islet transplantation: Alternative sites. Current Diabetes Reports. 2010;**10**(5): 332-337

[47] Barshes NR, Lee TC, Goodpastor SE, et al. Transaminitis after pancreatic islet transplantation. Journal of the American College of Surgeons. 2005;**200**(3):353-361

[48] Park CG, Bottino R, Hawthorne WJ. Current status of islet xenotransplantation. International Journal of Surgery. 2015;**23**(Pt B):261-266

[49] Fritschy WM. The efficacy of intraperitoneal pancreatic islet isografts in the reversal of diabetes in rats. Transplantation. 1991;**52**(5):777-783

[50] Lorenz D, et al. Transplantation of isologous islets of Langerhans in diabetic rats. Acta diabetologica Latina. 1975;**12**(1):30-40

[51] Kobayashi T, et al. Indefinite islet protection from autoimmune destruction in non-obese diabetic mice by agarose microencapsulation without immunosuppression. Transplantation. 2003;**75**(5):619-625

[52] Qi M, et al. A recommended laparoscopic procedure for implantation of microcapsules in the peritoneal cavity of nonhuman primates. Journal of Surgical Research. 2011;**168**(1):e117-123

[53] Calafiore R, et al. Microencapsulated pancreatic islet allografts into nonimmunosuppressed patients with type 1 diabetes: First two cases. Diabetes Care. 2006;**29**(1):137-145

[54] Elliott RB, et al. Intraperitoneal alginate-encapsulated neonatal porcine islets in a placebo-controlled study with 16 diabetic cynomolgus primates. Transplantation Proceedings. 2005;**37**(8):3505-3512

[55] Casey JJ, Lakey JR, Ryan EA, et al. Portal venous pressure changes after sequential clinical islet transplantation. Transplantation. 2002;**74**:913-915

[56] Salazar-Banuelos A, et al. Pancreatic islet transplantation into the bone marrow of the rat. The American Journal of Surgery. 2008;**195**(5):674-678

[57] Cantarelli E, Melzi R, Mercalli A, et al. Bone marrow as an alternative site for islet transplantation. Blood. 2009;**114**(20):4566-4574

[58] Szot GL, Koudria P, Bluestone JA. Transplantation of pancreatic islets into the kidney capsule of diabetic mice. Journal of Visualized Experiments. 2007;**9**:404

[59] Carlsson PO, et al. Chronically decreased oxygen tension in rat pancreatic islets transplanted under the kidney capsule. Transplantation. 2000;**69**(5):761-766

[60] Van Suylichem PT, et al. Rat islet isograft function. Effect of graft volume and transplantation site. Transplantation. 1994;**57**(7):1010-1016

[61] Lacy PE, Ricordi C, Finke EH. Effect of transplantation site and alpha L3T4 treatment on survival of rat, hamster, and rabbit islet xenografts in mice. Transplantation. 1989;**47**(5):761-766

[62] Mellgren A, Schnell Landstrom AH, Petersson B, et al. The renal subcapsular site offers better growth conditions for transplanted mouse pancreatic islet cells than the liver or spleen. Diabetologia. 1986;**29**(9):670-672

[63] Song HJ, et al. Prolongation of islet graft survival using concomitant transplantation of islets and vascular endothelial cells in diabetic rats. Transplantation Proceedings. 2010;**42**(7):2662-2666

[64] Rackham CL, et al. Co-transplantation of mesenchymal stem cells maintains islet organisation and morphology in mice. Diabetologia. 2011;**54**(5):1127-1135

[65] Sakata N, et al. Bone marrow cell cotransplantation with islets improves their vascularization and function. Transplantation. 2010;**89**(6):686-693

[66] Jindal RM, Sidner RA, McDaniel HB, et al. Intraportal vs kidney subcapsular site for human pancreatic islet transplantation. Transplantation Proceedings. 1998;**30**(2):398-399

Chapter 6

# Pig-to-Nonhuman Primate (NHP) Naked Islet Xenotransplantation

Jun-Seop Shin, Jong-Min Kim, Byoung-Hoon Min,
Jung-Sik Kim, Il-Hee Yoon, Hyunwoo Chung,
Won-Woo Lee, Hee Jung Kang and Chung-Gyu Park

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.69001

**Abstract**

Islet transplantation is an established therapy for selected type 1 diabetes (T1D) patients with severe hypoglycemic unawareness and glycemic liability despite of insulin treatment. However, the donor organ is limited. Porcine islets are the best alternative source to overcome this limitation, and pig-to-nonhuman primate (NHP) naked islet xenotransplantation studies are being performed worldwide. Several studies including our own have presented successful proof-of-concept results based on immunosuppression regimen including the anti-CD154 monoclonal antibody. Particularly, long-term control of diabetes by adult porcine islet transplantation has been demonstrated in five consecutive monkeys, and the longest survival was ~1000 days after transplantation. Currently, pig-to-NHP islet xenotransplantation based on clinically applicable immunosuppression regimen is being pursued. In this chapter, we will describe all the procedures of pig-to-NHP naked islet xenotransplantation: (1) the porcine islet isolation from designated pathogen-free (DPF) miniature pigs, (2) diabetes induction in monkeys, (3) transplantation procedure via the portal vein, (4) immune monitoring comprising humoral and cellular immunity after porcine islet transplantation, and finally (5) liver biopsy and subsequent immunohistochemical procedure in detail.

**Keywords:** porcine islet, nonhuman primate, transplantation, immune monitoring, biopsy

## 1. Introduction

Diabetes, a serious disease and a fast-growing worldwide epidemic, has culminated in that nearly 9% of global population is afflicted [1]. Most patients suffer from type 2 diabetes (T2D) in which genetic predisposition and harmful environmental exposure will lead to β-cell dysfunction and peripheral insulin resistance [2]. About 5–10% of the patients are afflicted with T1D where autoimmunity toward pancreatic β-cells induces β-cell death, and thus regular exogenous insulin treatment is absolutely required for the daily life of the patients [3]. In late 1960s, the procedure yielding large numbers of islets from the rodents was established using a commercial collagenase [4] and its ductal injection, and islet transplantation into the portal vein of diabetic animals could lead to recovery from experimental diabetes [5]. As islet isolation from large animals including the pigs and humans became possible [6, 7], several research groups including the Giessen group attempted clinical islet transplantation in the patients in 1980s–1990s [8]. Although early clinical experience had been unsuccessful with only some of the recipients being insulin-independent for a short while, other procedures such as organ preservation, islet isolation, and immunosuppressive regimen had been steadily improved [9, 10]. In 2000, Shapiro et al. reported a seminal paper in the *New England Journal of Medicine* and demonstrated that islet transplantation could lead to insulin-independence at least for a year in all seven type 1 diabetes (T1D) patients [11]. Since then, an international collaborative team reproduced this result from the clinical trials involving more patients from various ethnicities and continents [12]. Hering et al. very recently published an important result from a Phase 3 clinical trial, which had intended to test the efficacy and safety of the standardized human pancreatic islet product in the patients with impaired awareness of hypoglycemia (IAH) and severe hypoglycemic events (SHEs). The results showed that islet transplantation was effective for preventing hypoglycemic unawareness and providing sustained glycemic control (<5.6% of HbA1c) and thus should be considered for patients with T1D and IAH in whom other less-invasive treatments have been ineffective in preventing SHEs [13]. Although human allogeneic islet transplantation is now considered to be widely applicable to more T1D patients possibly under the coverage of health insurance reimbursement, the supply of donor organ is significantly limited, leading to only 0.1% of the patients gaining access to this promising therapy.

Porcine islets have long been considered to be the best alternative source for the human counterpart [14]. Pigs are easy to breed and have large litters at delivery and, most importantly, have high degree of physiological similarity to humans and a long history of use of porcine insulin for treating T1D patients. In addition, they can be bred in specific pathogen-free (SPF) and/or designated pathogen-free (DPF) status and thus healthier donor pancreata can be supplied unlimitedly, though relatively high cost is required for maintaining them in a clean facility which is regularly monitored for microbial pathogens. To achieve clinical xenotransplantation, several groups started pig-to-NHP islet xenotransplantation from the late 1990s [15]. However, in these early studies, porcine islets that had been transplanted into the portal vein or kidney subcapsule of the monkeys survived only

for several days to weeks despite of using strong immunosuppression regimen. Loss of the significant number of islets immediately after transplantation has been the most difficult obstacle to overcome. Goto et al. had focused on this phenomenon and tried to delineate the cellular and molecular mechanism underlying this early islet loss [16]. Indeed, they coined the term instant blood-mediated inflammatory reaction (IBMIR), and this problem still remains a significant barrier for long-term survival of porcine islet in the monkeys as well as in allogeneic and even in autologous islet transplantation settings [17, 18]. In 2006, two independent groups first succeeded in prolonging the survival of porcine islets (adult porcine islets and neonatal porcine islets) for longer than 3 months, using anti-CD154 monoclonal antibody-based immunosuppression [19, 20]. Since then, the survival duration of the porcine islet graft in the monkeys has been lingering around one year at most in a very limited number of the recipients, even though multiple genetically modified pigs were used [21]. In 2009, International Xenotransplantation Association (IXA) released a consensus statement on conditions for undertaking clinical trials of porcine islet products in T1D [22] and recently updated this document [23]. To justify clinical xenotransplantation, prerequisite results that should be obtained in preclinical NHP studies were to show the maintenance of fasting blood glucose levels (BGLs) of <150 mg/dL and nonfasting levels of <200 mg/dL in the absence of exogenous insulin or in the presence of greatly reduced insulin requirements in at least five out of eight consecutive NHPs (now four out of six consecutive NHPs in an updated version). Follow-up should be for a period of at least 6 months in all cases and ideally for 12 months in one or two successful cases [24, 25]. Recently, our group reported successful results where five consecutive diabetic monkeys achieved normoglycemia for at least 6 months after transplantation of adult porcine islets, with the longest survival day reaching to >603 days [26]. During a follow-up study, one monkey showed normoglycemia up to ~1000 days using a CD40-CD154 blockade such as anti-CD154 or anti-CD40 monoclonal antibody [27]. These two studies pose an important implication that porcine islet graft can survive in the liver, which is rather a harsh environment, for a significantly long duration. The caveat of these studies would be transplanting relatively high numbers of islets (100,000 IEQ/kg) to maintain normoglycemia for a long duration, which would reflect the species incompatibility between NHP and pig. The normal fasting blood glucose level (BGL) of a monkey has been known to be around 60 mg/dL, and the monkey requires higher amount of insulin for glycemic control than pigs and probably humans [28]. As rhesus monkeys have about 25-year lifespan, 1000 days could approximate to 3000 days (>8 years) in humans, suggesting that transplanted porcine islets can treat diabetic patients for a long time, given that immunosuppression should be well maintained with suitable drugs. Currently, our group is actively seeking to develop clinically applicable immunosuppressive regimen in the same pig-to-NHP islet xenotransplantation model.

In this chapter, we will describe the detailed procedures of islet isolation from adult SNU miniature pigs, diabetes induction and islet transplantation, immune monitoring after transplantation, and finally biopsy and subsequent immunohistochemical analyses. Because other related topics including encapsulated pig islets, islet sources, immunosuppression regimen,

and overall results from several pig-to-NHP islet xenotransplantation have been elegantly described elsewhere [15, 29–32], here we will focus on the above themes while briefly reviewing past and current status of each area.

## 2. Porcine islet isolation DPF SNU miniature pigs

### 2.1. History and characteristics of SNU miniature pigs

Islets for transplantation can be obtained from adult, neonatal, fetal, and embryonic pigs. There is still a debate on the best source of the porcine islets, but at least adult, neonatal, and embryonic pancreata have been shown to be efficient for controlling hyperglycemia in higher mammals including NHPs [19, 20, 33]. Because advantages and disadvantages of using each islet source have been repeatedly discussed elsewhere [30, 34], here we focus on adult porcine islets from DPF miniature pigs. Our group had obtained an SPF miniature pig strain from the University of Chicago in 2004 and have been breeding and maintaining a closed herd in a SPF barrier facility. About 41 viral, 35 bacterial, 2 fungal pathogens, and 25 parasites were screened and confirmed negative in microbial examinations that have been performed on a regular basis (at least once two years), implying that this closed herd is in DPF status [35]. Also, all SNU miniature pigs have been tested for the presence of porcine endogenous retrovirus (PERV) via reverse transcription-polymerase chain reaction (RT-PCR). The results showed that PERV A, B, and C genotypes were present in the genome. However, reverse transcription activity of PERV assessed by *in vitro* reverse transcriptase assay in >60 of monkeys that underwent porcine islet transplantation was not observed. This observation is supported by the gene sequence data of PERV in SNU miniature pig, showing that most of the PERV genes were integrated into the chromosome as defective forms such as deletion, insertion, or inversion (data not shown).

### 2.2. Islet yield from SNU miniature pigs

In 2007, we have published the first results of islet isolation from SNU miniature pigs (at that time, this pig was named Chicago Medical School [CMS]). In that report, we compared the islet yield from 9 adult SNU pigs (>12 months old), 6 young SNU pigs (6–7 months old), 4 other adult miniature Prestige World Genetics (PWG) pigs (>12 months old), and 13 adult market pigs, and found significantly higher yield of islets from adult SNU pigs than the other three groups: The yield was ~9600 islet equivalent per 1 g pancreas (IEQ/g), which marked the highest value that had ever been reported worldwide [36]. Moreover, we published the results based on 68 successful cases of isolation attempts and found several parameters that predicted for higher yield of islet isolation in 2009: old age of >2 years, male preference, pregnancy experience in female, and good distension of pancreas by collagenase injection [37]. Since then, we have preferred to use adult SNU pigs older than 2 years and standardized all isolation procedures from pancreas procurement to islet purification (**Figure 1**). The results of islet isolation remained stable during the past 5 years, and the yield was ~6000 IEQ/g pancreas and total ~300,000 IEQ/isolation attempt.

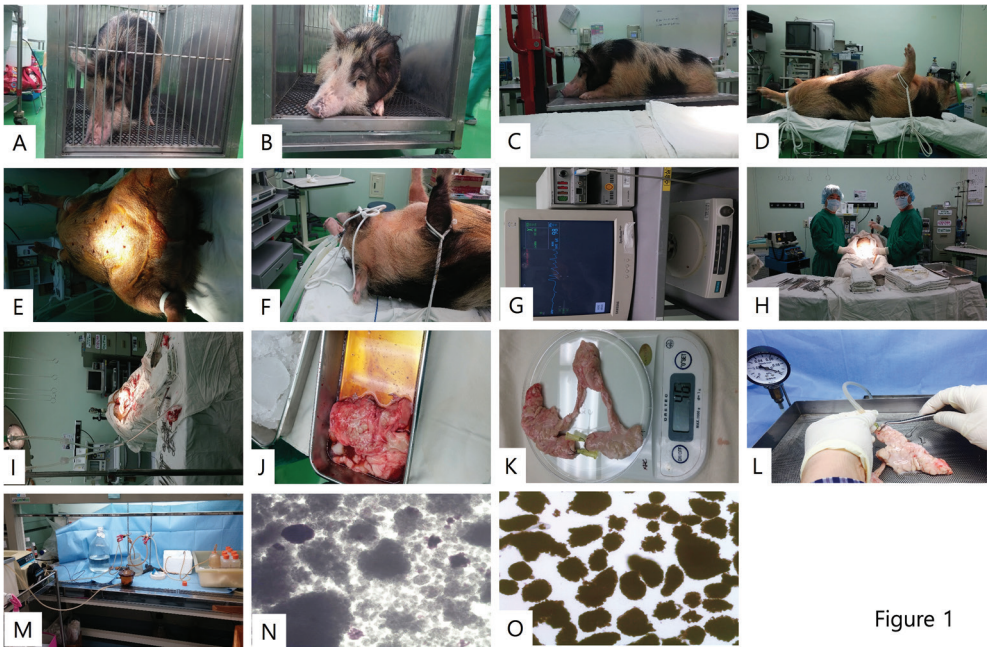


**Figure 1.** Whole procedure of islet isolation from a DPF SNU miniature pig. (A) A pig transferred to an operating room in a cage. (B) The pig was anesthetized with ketamine and xylazine. (C–F) The pig was moved to an operating table using a lift, intubated, and kept under anesthesia under isoflurane gas. G; Oxygen saturation was monitored. (H–I) Surgery was started under aseptic condition. (J–K) The pancreas was removed, trimmed, and weighed. (L) Collagenase was infused into the pancreatic duct in a cold infusion chamber while monitoring the pressure. (M) The pancreas was incubated in a Ricordi chamber for 20–25 min. (N) The tissue digest was examined under a microscope before the purification step using a COBE2991 processor. (O) Final islet preparation that has been stained with dithizone after purification.

### 2.3. Quality control of isolated islets from SNU miniature pigs

In order to gain consistent glycemic control after transplantation of porcine islets in NHPs, quality control of isolated islets is important. For our pig-to-NHP islet xenotransplantation experiments, we performed three independent assays that included (1) islet cell viability test using β-cell specific fluorescent dye Fluozin-3, mitochondrial activity indicator Tetramethylrhodamine, Ethyl Ester (TMRE), and a fluorescence-activated cell sorting (FACS) machine, (2) glucose-stimulated insulin secretion (GSIS), and (3) nondiabetic obese severe combined immunodeficiency (NOD/SCID) mouse bioassay where four streptozotocin (STZ)-induced diabetic mice were transplanted with 2500 IEQ of porcine islets under the kidney subcapsule, and their BGL were monitored 2–3 times per week for at least 2 months (**Figure 2**). Our recent study showed that the isolated porcine islets were >90% pure, contained >80% healthy β-cells, and had >60% diabetes correction capacity, each demonstrated by dithizone staining, FACS analysis, and NOD/SCID bioassay, respectively [26]. Although the fold increase of insulin upon glucose stimulation of porcine islets overall reached >1, the results from GSIS assay were highly variable and did not reflect the potency of the isolated pig islets, unlike those from other species (data not shown).

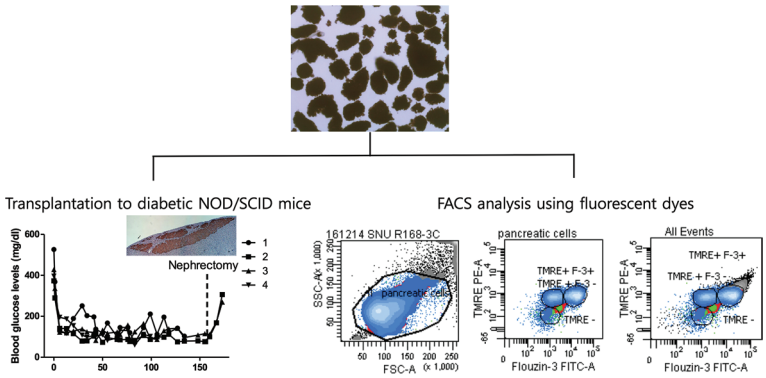


**Figure 2.** Quality control of the isolated porcine islets. A portion of the isolated porcine islets was tested for their potency using two independent assays as follows: *NOD/SCID bioassay; four mice were rendered diabetic by injecting STZ (200 mg/kg) and BGLs were followed after porcine islet transplantation (marginal mass; 2500 IEQ) into subcapsular region of the kidney. After nephrectomy, hyperglycemia was confirmed, and the harvested kidney was immunostained with anti-insulin antibody. FACS index; porcine islets were dissociated into single cells and were stained with Fluozin-3 and TMRE for measuring β-cells and mitochondrial potential, respectively. Then, the stained cells were analyzed by FACS. FACS index was calculated by Fluozin-3 positive (β-cells) × TMRE positive cell fraction.

## 3. Diabetes induction in monkeys and transplantation procedure via the portal vein

### 3.1. Diabetes induction in monkeys

There are several methods to induce diabetes mellitus (DM) in the monkeys such as total pancreatectomy [38], partial pancreatectomy (75% resection of the pancreas) followed by low-dose STZ (15 mg/kg) injection [39], and high-dose STZ (80–150 mg/kg) injection [40]. Pros and cons of each DM induction method are summarized in **Table 1**. STZ is selectively uptaken by the glucose transporter 2 (GLUT2), and induce cell death by massive DNA alkylation [41]. Because GLUT2 is mainly expressed in the pancreatic β-cell, hepatocytes, and basolateral membrane of small intestine and renal tubular cells [42], those organs can be damaged by STZ injection. To prevent systemic side effect of STZ, one group suggested that STZ should be injected into celiac artery and branches supplying blood to the pancreas after temporary embolization of the hepatic and gastric arteries [43]. However, the equipment such as C-arm or fluoroscopy and higher degree of technical skill for arterial catheterization is required to use this method. Zhu et al. reported an in-depth review article for DM induction in NHPs for islet transplantation [44]. Recently, our group published the procedures of STZ-induced DM induction and subsequent DM management before and after islet transplantation in rhesus monkeys [45].

### 3.2. Induction of DM using high dose of STZ injection

A central venous catheter (5Fr. Dual-Lumen PICC; Bard Access Systems, Salt Lake City, UT, USA) was inserted into the right internal jugular vein in monkeys under general anesthesia. Monkeys

| | Total pancreatectomy (TP) | Partial pancreatectomy (PP) + Low dose STZ injection | High-dose STZ injection |
|---|---|---|---|
| Pros | Usage for auto- or alloislet source [81]<br>Unfailing method of DM induction | Usage for alloislet source<br>Less invasive compared to TP<br>Less systemic toxicity compared to high-dose STZ injection | Less invasive<br>More convenient<br>Low possibility of regeneration of β-cell [82] |
| Cons | Invasive<br>Requires a precise surgical technique<br>Loss of exocrine function (sometimes requirement of oral administration of pancreatin and other islet hormones)<br>High surgical mortality | Increased possibility of regeneration of β-cell<br>More invasive compared to high-dose STZ injection<br>More systemic toxicity compared to TP | Instability of STZ in solution<br>Liver and kidney toxicity<br>Nausea and vomiting |

**Table 1.** Pros and cons of each diabetes mellitus induction method.

were fasted overnight and were prehydrated with normal saline (NS; 0.9% NaCl, 5 mL/kg/hr intravenously [i.v.]) via a tether system for 12 h before STZ (Sigma–Aldrich, St Louis, MO, USA) administration to reduce adverse nephrotoxic effects. A high dose of STZ (110 mg/kg) was diluted with 10 mL of normal saline and given i.v. within 10 min at 4 pm to prevent hypoglycemia at 9 am the next day. Because maximum nadir of hypoglycemia usually occurs about 17 h after STZ injection, 5% dextrose solution was infused at 1 h after STZ injection to prevent hypoglycemia and nephrotoxicity. Intravenous glucose tolerance test (IVGTT) and arginine stimulation test (AST) were conducted within 1–2 weeks after STZ injection. For IVGTT, a bolus of glucose solution (0.5 g/kg) was administered into the right saphenous vein. Two mL of blood was collected from the left saphenous vein at baseline, immediately before injection of glucose, 2, 5, 15, 30, 60, 90, and 120 min after injection of glucose to measure blood glucose and C-peptide. BGL were measured using a small electrode-type blood glucose meter (Accu-Chek™, Roche Diagnostics, Seoul, Korea). For AST, 70 mg/kg of arginine (Sigma–Aldrich, St Louis, MO, USA) was administered into the right saphenous vein. Two mL of blood was collected from the left saphenous vein at baseline, 0, 1, 2, 3, 4, 5, and 10 min after administration of arginine to measure C-peptide. Complete DM was confirmed by persistent hyperglycemia and <1 ng/mL of fasting C-peptide levels and absence of C-peptide responses in IVGTT and AST.

### 3.3. Exogenous insulin treatment procedure in diabetic monkeys

Because the monkeys require high doses of insulin to sustain normoglycemia and are easily succumbed to metabolic deteriorations such as ketone body formation if they are not adequately treated, insulin treatment is very important to keep animals healthy after STZ injection. Animals were fed on commercially available certified primate biscuit diet (2050C, Harlan, Indianapolis, IN, USA). Calorie intake was maintained within 70–130 Kcal/kg/day which were divided equally at 9 am and 4 pm. After confirming complete DM induction, BGL was checked at least two times per day. Desired target value of the fasting BGL was approximately 80–150 mg/dL in the diabetic monkeys. To do so, each meal was not fed until the fasting BGL was measured at 9 am and 4 pm. Intermediate-acting form of insulin (NPH; Novolin N, Green Cross Corp., Yongin, Korea) and long-acting form of insulin (glargine; Lantus, Sanofi-Aventis Korea, Seoul, Korea) were injected subcutaneously after feeding at 9 am and 4 pm, respectively. Because insulin glargine and NPH that had been injected at different time points can influence BGL at the time

of subsequent measurement, the algorithm of insulin dose adjustments that have been modified from the method used in human clinic [46] was used to maintain target fasting BGL (**Table 2**).

| Fasting plasma glucose (mg/dL) | Insulin dosing |
|---|---|
| <40 | No insulin injection |
| <60 | Reduce by 1–2 U |
| 60–80 | Reduce by 0.5 U |
| 80–150 | No change |
| 151–250 | Increase by 0.5 U |
| 251–350 | Increase by 1 U |
| >350 | Increase by 1.5 U |

**Table 2.** Insulin dose adjustments according to the fasting blood glucose levels [45].

### 3.4. Transplantation procedure via the portal vein

There are two popular methods to transplant the islets via the portal vein: one is to infuse the islets through a jejunal vein after laparotomy [26]; and the other is to use percutaneous transhepatic portal catheterization guided by ultrasound technology [47]. The latter is less invasive than the former, but special equipment—such as ultrasound and C-arm—and the technique for ultrasound-guided percutaneous transhepatic portal vein catheterization are indispensable. Because of this limitation, most research groups prefer to use the former method in NHP study. In our study, the monkeys were fasted for 12 h and a laparotomy was performed. The jejunal arch was exposed, and a 22-gauge catheter was inserted through the jejunal vein and advanced near the portal vein. The porcine islets were infused under gravity pressure for 8–12 min (**Figure 3**). The vessel was ligated with a 5–0 prolene suture, and the tether system was applied for continuous fluid therapy and infusion of low-dose glucose, if necessary.

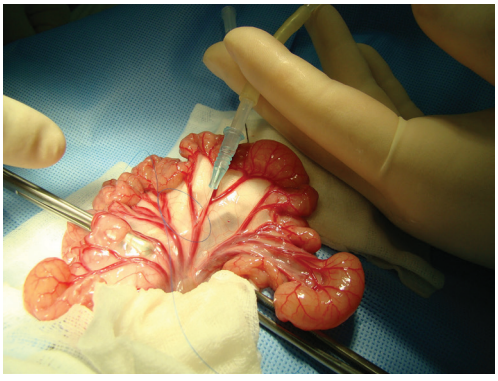

**Figure 3.** Islet transplantation into the portal vein through a jejunal vein. Porcine islets were resuspended in tissue culture media and infused via a jejunal vein of the diabetic rhesus monkey under anesthesia.

## 4. Immune monitoring after porcine islet transplantation

### 4.1. Monitoring of humoral immune responses

Porcine islet transplantation in NHP has been known to elicit humoral responses against porcine antigens, including Gal$\alpha$1,3Gal (Gal), and non-Gal antigens [48]. Gal is a carbohydrate antigen, which is expressed universally in most species including bacteria and fungi, but not in humans and old world NHPs. Anti-Gal is the most abundant form of natural antibody in humans (mostly found as IgM, IgG, and IgA isotypes to a lesser extent). CD40 signaling on B cells, which is acquired through the interaction with CD154 expressed on T cells, is critical for the survival and proliferation of B cells, antibody production, isotype switching, germinal center formation, memory generation, and production of numerous cytokines [49]. Antibodies targeting CD40-CD154 costimulation pathway have been shown to efficiently suppress humoral responses including anti-Gal and anti–non-Gal antibodies in the recipients [19, 20]. Indeed, immunosuppression regimen including anti-CD154 antibody suppressed the induction of anti–non-Gal and anti-Gal antibodies and prolonged islet graft survival for up to >603 days in NHP recipients [26]. In contrast, similar immunosuppression regimen including anti-CD40 antibody, instead of anti-CD154, suppressed xenoreactive IgG responses after islet transplantation as well, but could not sustain the graft function for a prolonged period [50]. Therefore, suppression of humoral responses against xenoantigens seems to be essential, but not quite enough to sustain graft survival in porcine islet transplantation. In our pig-to-NHP islet xenotransplantation model, monitoring of humoral responses after islet transplantation in the recipients is performed as follows: (1) weekly measurement of anti-Gal IgG and IgM using an in-house enzyme-linked immunosorbent assay (ELISA), (2) measurement of anti-donor pig peripheral blood mononuclear cells (PBMC) IgG and IgM using an in-house flow cytometry assay at every 2–3 months interval with the serum samples that had been collected weekly and stored in aliquots, and (3) measurement of anti-porcine endothelial cells (PEC) IgG every 2–3 months by flow cytometry using weekly collected and stored serum aliquots. As a control, the levels of IgG binding to galactosyl transferase knockout (GTKO) PECs (kindly provided by Dr. Shuji Miyagawa in Osaka University, Japan) were measured in parallel.

The levels of anti-Gal IgG and IgM antibodies are measured by ELISA as follows [50]: each well was coated with 100 μL of Gal$\alpha$1,3Gal $\beta$1-4GlcNAc-human albumin (5 μg/mL; GlycoTech, Gaithersburg, MD, USA) and then blocked with 1% human albumin (Green Cross Corp., Yongin, Korea) diluted in phosphate-buffered saline (PBS). Monkey plasma (100 μL) diluted 1:50 (for anti-Gal IgG) or 1:100 (for anti-Gal IgM) in 0.1% human albumin-supplemented PBS was added into each well in duplicate and incubated at 37°C for 30 min. Then, the signal was detected with the peroxidase-conjugated anti-human IgG or anti-human IgM (Sigma-Aldrich, St. Louis, MO, USA) and subsequent color development. Serial dilutions of the selected lot of monkey plasma (as a calibrator) were tested in parallel. A mean absorbance of the sample was compared with those of the calibrator and each antibody level of the sample was calculated from the calibration curve. High-level and low-level control plasma samples were simultaneously tested in each run to validate the performance of assays.

The detection of xenoreactive antibodies binding to porcine cells was performed by flow cytometry [51]. Single-cell suspensions ($10^5$/tube) of cultured PEC cells or PBMC obtained from the donor pigs were mixed with 50 μL of the plasma diluted 1:10 in PBS containing 1% human albumin and 30 mM EDTA, and incubated at 4°C for 30 min. The plasma was then incubated with FITC-conjugated $F(ab)_2$ fragments of a rabbit immunoglobulin specific for human IgG or human IgM (DAKO, Glostrup, Denmark) and measured using a FACSCalibur (BD Biosciences, San Jose, CA, USA) flow cytometer. The extent of antibody binding was expressed as the mean fluorescence intensity (MFI): MFI of the sample subtracted by the MFI of the negative control (porcine plasma). To reduce inter-variation, cohort samples obtained every week were assayed in duplicate in a single run, and the number of samples in each assay did not exceed 20. High-level and low-level control plasma samples were simultaneously tested in each run to validate the performance of assays.

### 4.2. Monitoring of cell-mediated immune responses

Since the current clinical islet transplantation procedure has been performed in the liver through the portal vein, IBMIR mediated by diverse nonimmunological and immunological factors is known to contribute to early islet loss [52]. Although the exact mechanisms underlying IBMIR need to be elucidated further in pig-to-NHP islet transplantation, activation of coagulation cascades together with platelet activation, tissue factor release, and thrombin release is observed during IBMIR, and the extent may be stronger than that observed in allogeneic islet transplantation [53]. Strong complement activation has been observed during IBMIR [54]. In particular, activation of alternative complement pathway was profound in pig-to-NHP islet transplantation [55]. Following immediate responses by soluble inflammatory mediators, infiltration of islet grafts by large numbers of activated $CD11b^+$ neutrophils and macrophage was observed [56]. In turn, the degranulation of cytotoxic granules and release of inflammatory cytokines, such as TNF-$\alpha$ and IL-6 from neutrophils and macrophages, induce the apoptosis of islets. In this sense, infiltrating innate immune cells may strengthen the subsequent adaptive immune responses from T and B cells. Among the diverse immune responses against porcine islets, T cell response has been the most critical barrier against long-term graft survival [57, 58]. Indeed, many T cell-targeting immunosuppressants have been developed to control T cell–mediated immune responses against porcine islets. Particularly, costimulation blockade such as CD40-CD154 and B7-CD28 interactions have been proven to be highly effective for prolonged graft survival [19, 20, 59].

To establish an optimal immunosuppressive regimen and to individualize the immunosuppressive therapy, the existence of reliable and predictable immunological tools for monitoring immunological status after clinical porcine islet transplantation is necessary. Yet, there are only a few reports on predictive immune parameters that can estimate the fate of the graft in pig-to-NHP islet transplantation model. Therefore, we will describe our own experience for finding the appropriate monitoring methods to oversee the immunological events happening during graft rejection. In addition, the role of *de novo* induced-immunosuppressive $CD8^+$ T cells will be discussed for the potential markers for predicting graft rejection.

Enzyme-linked immunosorbent spot (ELISPOT) assay is based on the detection of a cytokine (e.g., IFN-$\gamma$, IL-4, and IL-2) produced by single cells after stimulation with cognate antigens

[60, 61]. The secreted cytokine is detected by specific monoclonal antibodies and revealed by the generation of discrete spots, reflecting the number of cytokine-secreting cells. It has been widely used in measuring antigen-specific responses in the context of vaccine development for infectious diseases [62], cancer [63], and autoimmunity [64]. In the transplantation field, it has been also used to examine the presence of donor-specific T cells in the patients. For example, following human kidney transplantation, it has been proved useful to screen the patients at high risk for acute or chronic graft rejection [65]. Also, an increased number of IFN-γ-secreting donor-specific cells were detected by ELISPOT in the patients who experienced an acute rejection [66]. Standardization and cross-validation of alloreactive IFN-γ ELISPOT assay were reported in clinical allotransplantation [67, 68]. However, ELISPOT assay is yet to be determined for pig-to-NHP islet xenotransplantation model. Recently, our group reported the results of the retrospective IFN-γ ELISPOT assay using a time-series of PBMC samples from the monkeys with long-term surviving islet grafts (**Figure 4**) [69].

Accumulating evidence indicates that immunosuppression after T cell depletion affects $CD8^+$ T cell homeostasis in the periphery, resulting in the loss of CD28 expression on some subset of T cells. Interestingly, repopulated $CD8^+CD28^-$ T cells have been shown to have immunosuppressive activity and be closely related to the graft survival in some allogeneic transplantation [70, 71]. In our pig-to-NHP islet xenotransplantation model, absolute number of $CD8^+CD28^-$ T cell population significantly increased during homeostatic reconstitution of T cell subpopulation [26] in which the monkeys were treated with ATG and immunosuppressive agents such as

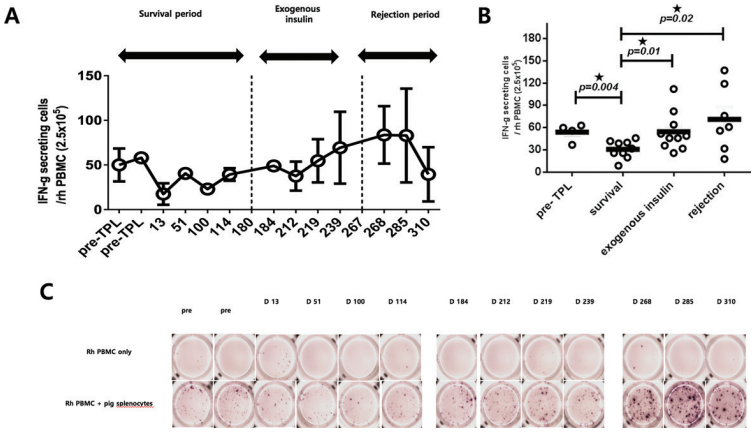


**Figure 4.** One representative IFN-γ ELISPOT result. (A) STZ-induced diabetic monkey (R015) was transplanted with porcine islets (100,000 IEQ/kg) through a portal vein under immunosuppressive regimen comprised of anti-thymoglobulin, sirolimus, anti-CD40 antibody (2C10R4), and tacrolimus. Fasting BGL and porcine and monkey C-peptide were measured. Grey line: fasting BGL, red bar (bar during normoglycemic period): porcine C-peptide, filled inverted triangle (▼): monkey C-peptide, pink bar (bar during hyperglyemic period): exogenous insulin. The values above red bar indicate porcine C-peptide, (B) After porcine islet transplantation, PBMCs from the recipient monkey were sampled at different time-points and stored. Stored PBMCs ($2.5 \times 10^5$) were cocultured with $5.0 \times 10^5$ splenocytes for 40 h and the number of IFN-g producing spots was measured. The number of IFN-γ secreting spots was enumerated and compared according to the status of graft functioning. (C) Raw data showing the images of IFN-γ secreting cells as visualized by chromogen development.

rapamycin or methyl-prednisone. These resurged $CD8^+CD28^-$ T cells were immunosuppressive to $CD4^+$ T cell activation and proliferation *in vitro*, suggesting that these cells are regulatory subsets. Importantly, blood glucose levels indicative of function of the transplanted islet were closely associated with the ratio of $CD8^+CD28^-$ T to $CD4^+$ T cells, and the transient hyperglycemia or terminal graft loss was observed after $CD8^+CD28^-$ T/$CD4^+$ T cell ratio dropped below 2.0 approximately. In this regard, monitoring immunosuppressive $CD8^+CD28^-$ T cell population together with $CD4^+$ T cells will be helpful for predicting graft function in some allogeneic or xenogeneic transplantation. However, it is highly likely that reconstituted $CD8^+CD28^-$ T cells are heterogeneous in nature and are mixed together with regulatory $CD8^+$ T cells and cytotoxic $CD8^+$ T cells. This fact may hinder broad application of CD28 as a regulatory $CD8^+$ T cell marker. Further study for the identification of surface or lineage markers which could differentiate regulatory CD8+ T cell subset among $CD8^+CD28^-$ T cells is warranted.

## 5. Liver biopsy and subsequent immunohistochemical procedure

In organ transplantation, tissue biopsy is the standard way to evaluate graft dysfunction or rejection. Recent advances in the development and expansion of antibody application have generated more sophisticated and powerful diagnostic methods based on tissue biopsy. Since the 1990s, diagnosis and determination of graft rejection through a biopsy have been a routine clinical practice in human kidney, heart, and lung transplantation. In kidney transplantation, standardization of criteria for renal allograft rejection has been published [72]. There were many reports on biopsy-based diagnostic methods for other allograft [73, 74] as well as xenograft rejection [75]. The infiltration of immune cells including T cells, B cells, macrophages, and the deposition of antibody/complement to the graft causing graft dysfunction and rejection can be revealed by histological analysis through biopsies. Thus, the histology-based results were usually semiquantitatively analyzed and correlated to the degree of cell infiltration and antibody/complement deposition [74, 76]. For example, C4d deposition was correlated with the presence of donor-specific anti-human leukocyte antigen (HLA) antibodies [77–79] and was deemed a specific marker for acute humoral rejection [76]. Also, $CD68^+$ and $CD3^+$ cell infiltration in the grafts was highly correlated with the extent of cellular rejection in heart transplantation [74]. However, in islet transplantation, posttransplantation monitoring tools to examine the graft site are limited, because the porcine islets are engrafted throughout the liver in a scattered pattern. Immune-monitoring with peripheral blood after transplantation such as measuring absolute counts of T cells, B cells, neutrophils, and NK cells is simple and less invasive, but it is difficult to predict whether the islets in situ are attacked by immune cells and antibodies in the liver via observing these immune parameters. Particularly, after ATG injection, immune cells such as T cells, B cells, and NK cells are depleted and detected in very low numbers in the peripheral blood. In contrast, a large number of immune cells were observed in the biopsied liver samples, and in some cases, overt graft rejection ensued. Therefore, the histological examination of biopsied liver sample would have higher predictive value in determining the status of immune response against islet graft.

In our pig-to-NHP islet xenotransplantation study, scheduled or event biopsy was performed as needed (**Figure 5**). Ordinarily, biopsies of the distal portion of the liver were performed. Under general anesthesia, the monkey was placed in the supine position. The abdominal wall was incised from the xyphoid process to the umbilicus. The margin of the central lobe of the liver was gently grasped and excised about 10 mm distal to the margin (wedge biopsy; 1 × 1 cm). Hemorrhage from the biopsy site was controlled with electrocautery and absorbable hemostat (SURGICEL®, Ethicon Inc.). Routine abdominal wall closure was then performed [26]. Finally, biopsied sample was washed three times with PBS and transferred to 4% paraformaldehyde in PBS for fixation.

Troxell and Lanciault [73] reported practical applications of immunohistochemistry for human organ transplantation, and elegantly described all the details of antibody selection for immune cells and blood coagulation factors in human tissues. However, when these antibodies are used in NHP experiments, antigen-antibody reaction did not work as expected in many cases. Thus, many researchers have tried to find the antibodies that are suitable for NHP tissues. In 2009, Kap et al. reported an important paper entitled "A monoclonal antibody selection for immunohistochemical examination of lymphoid tissues from non-human primates [80]." In this study, they have tested over 100 antibodies against 69 antigens expressed in tissues from the great apes, old world monkeys, and new world monkeys. This report was of great help in selecting antibodies for use in pig-to-NHP islet xenotransplantation. Antibodies used to determine the distribution of B cell, T cell, and macrophages and deposition of complement, antibody, and $\alpha$-Gal are listed in **Table 3**.

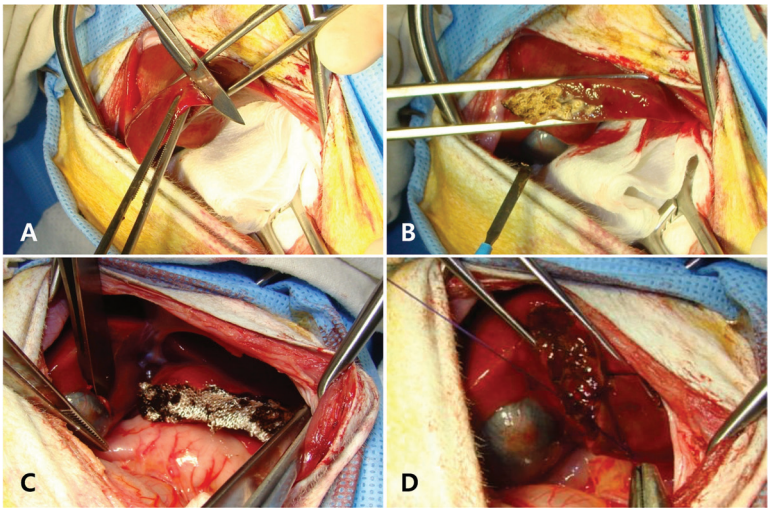


**Figure 5.** Procedure of liver biopsy in pig-to-NHP islet xenotransplantation. (A) Marginal liver of left central lobe (approximately 1 × 2 cm) is resected. (B) After the resection, the resected surface of liver is electro-cauterized for bleeding control. (C) After the electro-cauterization, Surgicel® is applied on it. (D) When anti-coagulant or anti-platelet agents are administered in the recipients, overlapping guillotine suture method is used to control bleeding because those procedures are not enough for bleeding control.

| Antibody | Description | Company | Host | Clonality | Clone |
|---|---|---|---|---|---|
| Anti-αGal | Galactose-α-1,3-galactose | Vector Laboratories | | | Fluorescein labeled *Griffonia Simplicifolia* Lectin I (GSL I) isolectin B4 |
| Insulin | *Pancreatic β-cell marker* | DAKO | Guinea Pig | Polyclonal | |
| Insulin | *Pancreatic β-cell marker* | Abcam | Guinea Pig | Polyclonal | |
| Glucagon | *Pancreatic alpha cell marker* | Santacruz | Rabbit | Polyclonal | |
| CD3 | *T cell coreceptor* | DAKO | Rabbit | Polyclonal | UCHT1 |
| CD3 | *T cell coreceptor* | Santacruz | Mouse | Monoclonal | A1 |
| CD4 | *Glycoprotein found on the surface of immune cells* | Santacruz | Mouse | Monoclonal | 1F6 |
| CD4 | *Glycoprotein found on the surface of immune cells* | Abcam | Mouse | Monoclonal | 1F6 |
| CD8 | *Transmembrane glycoprotein that serves as a coreceptor for the T cell receptor* | Abcam | Rabbit | Polyclonal | |
| CD20 | *Activated-glycosylated phosphoprotein expressed on the surface of all B cells* | Thermo | Mouse | Monoclonal | L26 |
| CD20cy | *Activated-glycosylated phosphoprotein expressed on the surface of all B cells* | DAKO | Mouse | Monoclonal | L26 |
| CD68 | *Glycoprotein which binds to low density lipoprotein. expressed on monocytes/macrophages* | Thermo | Mouse | Monoclonal | KP1 |
| FOXP3 | *Specific marker of natural T regulatory cells* | Abcam | Mouse | Monoclonal | 236A/E7 |
| CD31 | *Platelet endothelial cell adhesion molecule (PECAM-1)* | Santacruz | Rabbit | Polyclonal | H300 |
| CD31 | *Platelet endothelial cell adhesion molecule (PECAM-1)* | DAKO | Mouse | Monoclonal | JC70A |
| C4d | *Complement system activation marker* | LSBio | Mouse | Monoclonal | |
| Fibrinogen | *Glycoprotein that helps in the formation of blood clots.* | Abcam | Rabbit | Polyclonal | |

**Table 3.** Antibody specification of immunohistochemistry for pig-to-NHP islet xenotransplantation.

In our study, triple immunohistochemical staining was routinely performed because it has an advantage: capability of examining multiple cell types in the same tissue section. For example, T cells and B cells or T cells and macrophages can be observed simultaneously in the islet graft site. As it is not easy to identify the islet if the islet has been destroyed or only a few β-cells remain, insulin staining should always be performed at the same time (**Figure 6**). Biopsy samples from

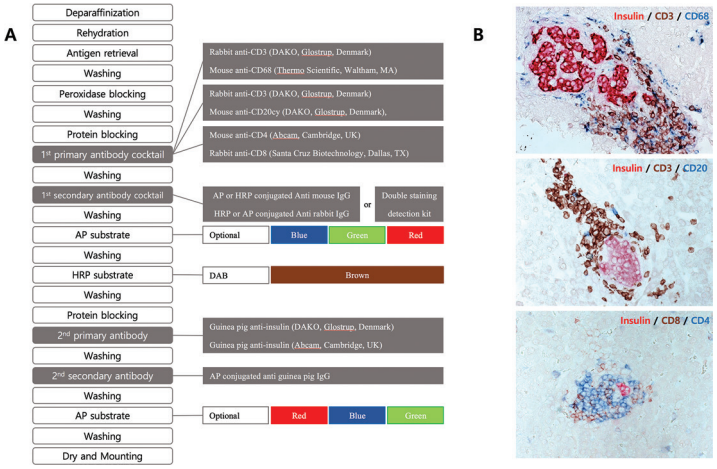


**Figure 6.** Flow chart for anti-insulin antibody-based triple immunohistochemical staining. (A) Whole procedure of triple staining is depicted. (B) One representative result of anti-insulin antibody-based triple staining. These micrographs show insulin positive b-cells (red) and other immune cells that are either infiltrated or peripherally located (blue or brown).

the liver were fixed in 4% paraformaldehyde in PBS and embedded according to the conventional paraffin-embedding protocol. Paraffin-embedded tissues were sectioned at 4-μm thickness using a microtome. The sections were incubated with primary antibody cocktail designed for each combined targets. The sections were subsequently incubated with secondary antibody cocktail of anti-rabbit/HRP + anti-mouse/AP polymers. For color development, the slides were incubated with blue chromogen (Thermo Scientific, Waltham, MA, USA) for AP and DAB chromogen (DAKO, Glostrup, Denmark) for HRP. After the slides were treated with protein block solution (Thermo Scientific, Waltham, MA, USA), they were incubated with guinea pig anti-insulin antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), AP-conjugated goat anti-guinea pig secondary antibody (Abcam, Cambridge, UK) and then incubated with red chromogen substrate (Zytomed Systems, Berlin, Germany). After the staining procedure, all slides were dried at 60°C and mounted with aqueous mounting medium (Thermo Scientific, Waltham, MA, USA). The stained sample was examined by Carl Zeiss Axio Imager A1 microscope, and the images were taken with a micrograph with AxioVision software (Carl Zeiss AG, Oberkochen, Germany).

## 6. Conclusion

Recent advances in preclinical studies in pig-to-NHP naked islet xenotransplantation have granted a huge momentum in the endeavor for clinical adoption of porcine islets to overcome donor organ shortage in islet transplantation as a cure for T1D. Safety concerns related to porcine islet transplantation have also been significantly lessened by the fact that no infection had been detected in several clinical studies using encapsulated neonatal porcine islets and porcine islets cotransplanted with Sertoli cells. Importantly, clinically applicable

immunosuppressive regimen without anti-CD154 antibody is now being developed. Our detailed procedures of porcine islet isolation from DPF miniature pigs, islet transplantation, immune monitoring with peripheral blood after transplantation, and biopsy and subsequent immunohistochemistry described in this chapter will help other scientists to expedite clinical realization of naked porcine islet transplantation using clinically acceptable immunosuppression in the near future. Also, a quantum leap in advances on gene editing technique that will generate multiple genetically modified pigs or possibly PERV-free pigs within a few months, and on generation of interspecies chimera that will provide human organs in the pigs will heighten the potential of xenotransplantation.

## Acknowledgements

This work was supported by a grant from the Korea Healthcare Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry for Health and Welfare, Republic of Korea (Grant No. HI13C0954). Anti-CD40 antibody used in these studies was provided by the Nonhuman Primate Reagent Resource supported by US National Institutes of Health NIAID contract HHSN 272201300031C and grant OD010976. The GTKO porcine endothelial cell line (PEC69) was kindly provided by Dr. Shuji Miyagawa in Osaka University. The authors would like to thank S. K. Park, J. W. Choi, and M. S. Lee for isolating pig islets; H. Y. Nam for conducting FACS and ELISPOT analyses; and W. Y. Jung, M. S. Kim, G. E. Lee, J. E. Kim, and S. E. Kwon for caring for all NHPs.

## Author details

Jun-Seop Shin[1,2,3,4†], Jong-Min Kim[1,2,3,4†], Byoung-Hoon Min[1,2,3,4†], Jung-Sik Kim[1,2,3,4], Il-Hee Yoon[1,2,4], Hyunwoo Chung[1,2,5], Won-Woo Lee[1,2,3,4,5], Hee Jung Kang[6] and Chung-Gyu Park[1,2,3,4,5*]

*Address all correspondence to: chgpark@snu.ac.kr

1 Xenotransplantation Research Center, Seoul National University College of Medicine, Seoul, Korea

2 Department of Microbiology and Immunology, Seoul National University College of Medicine, Seoul, Korea

3 Institute of Endemic Diseases, Seoul National University College of Medicine, Seoul, Korea

4 Cancer Research Institute, Seoul National University College of Medicine, Seoul, Korea

5 Department of Biomedical Sciences, Seoul National University College of Medicine, Seoul, Korea

6 Department of Laboratory Medicine, Hallym University College of Medicine, Anyang, Korea

† These authors equally contributed to this work.

## References

[1] The 7th Edition of the Diabetes Atlas. International Diabetes Foundation; 2015 International Diabetes Federation (IDF) idf@idf.org/www.idf.org

[2] Kahn SE, Cooper ME, Del Prato S. Pathophysiology and treatment of type 2 diabetes: Perspectives on the past, present, and future. Lancet. 2014;**383**:1068-1083

[3] van Belle TL, Coppieters KT, von Herrath MG. Type 1 diabetes: Etiology, immunology, and therapeutic strategies. Physiological Reviews. 2011;**91**:79-118

[4] Lacy PE, Kostianovsky M. Method for the isolation of intact islets of Langerhans from the rat pancreas. Diabetes. 1967;**16**:35-39

[5] Ballinger WF, Lacy PE. Transplantation of intact pancreatic islets in rats. Surgery. 1972;**72**: 175-186

[6] Ricordi C, Socci C, Davalli AM, Staudacher C, Baro P, Vertova A, et al. Isolation of the elusive pig islet. Surgery. 1990;**107**:688-694

[7] Ricordi C, Lacy PE, Finke EH, Olack BJ, Scharp DW. Automated method for isolation of human pancreatic islets. Diabetes. 1988;**37**:413-420

[8] Scharp D, Lacy P, Ricordi C, Boyle P, Santiago J, Cryer P, et al. Human islet transplantation in patients with type I diabetes. Transplantation Proceedings. 1989;**21**:2744-2745

[9] Kin T. Islet isolation for clinical transplantation. Advances in Experimental Medicine and Biology. 2010;**654**:683-710

[10] Iwanaga Y, Sutherland DE, Harmon JV, Papas KK. Pancreas preservation for pancreas and islet transplantation. Current Opinion in Organ Transplantation. 2008;**13**:445-451

[11] Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. The New England Journal of Medicine. 2000;**343**:230-238

[12] Shapiro AM, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson RP, et al. International trial of the Edmonton protocol for islet transplantation. The New England Journal of Medicine. 2006;**355**:1318-1330

[13] Hering BJ, Clarke WR, Bridges ND, Eggerman TL, Alejandro R, Bellin MD, et al. Phase 3 trial of transplantation of human islets in type 1 diabetes complicated by severe hypoglycemia. Diabetes Care. 2016;**39**:1230-1240

[14] Dufrane D, Gianello P. Pig islet for xenotransplantation in human: Structural and physiological compatibility for human clinical application. Transplantation Reviews. 2012;**26**: 183-188

[15] Marigliano M, Bertera S, Grupillo M, Trucco M, Bottino R. Pig-to-nonhuman primates pancreatic islet xenotransplantation: An overview. Current Diabetes Reports. 2011;**11**: 402-412

[16] Goto M, Tjernberg J, Dufrane D, Elgue G, Brandhorst D, Ekdahl KN, et al. Dissecting the instant blood-mediated inflammatory reaction in islet xenotransplantation. Xenotransplantation. 2008;**15**:225-234

[17] Johansson H, Goto M, Dufrane D, Siegbahn A, Elgue G, Gianello P, et al. Low molecular weight dextran sulfate: A strong candidate drug to block IBMIR in clinical islet transplantation. American Journal of Transplantation. 2006;**6**:305-312

[18] Naziruddin B, Iwahashi S, Kanak MA, Takita M, Itoh T, Levy MF. Evidence for instant blood-mediated inflammatory reaction in clinical autologous islet transplantation. American Journal of Transplantation. 2014;**14**:428-437

[19] Cardona K, Korbutt GS, Milas Z, Lyon J, Cano J, Jiang W, et al. Long-term survival of neonatal porcine islets in nonhuman primates by targeting co-stimulation pathways. Nature Medicine. 2006;**12**:304-306

[20] Hering BJ, Wijkstrom M, Graham ML, Hardstedt M, Aasheim TC, Jie T, et al. Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. Nature Medicine. 2006;**12**:301-303

[21] van der Windt DJ, Bottino R, Casu A, Campanile N, Smetanka C, He J, et al. Long-term controlled normoglycemia in diabetic non-human primates after transplantation with hCD46 transgenic porcine islets. American Journal of Transplantation. 2009;**9**:2716-2726

[22] Hering BJ, Cooper DK, Cozzi E, Schuurman HJ, Korbutt GS, Denner J, et al. The International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes—executive summary. Xenotransplantation. 2009;**16**:196-202

[23] Hering BJ, Cozzi E, Spizzo T, Cowan PJ, Rayat GR, Cooper DK, et al. First update of the International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes—executive summary. Xenotransplantation. 2016;**23**:3-13

[24] Cooper DK, Casu A. The International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes—Chapter 4: Pre-clinical efficacy and complication data required to justify a clinical trial. Xenotransplantation. 2009;**16**:229-238

[25] Cooper DK, Bottino R, Gianello P, Graham M, Hawthorne WJ, Kirk AD, et al. First update of the International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes—Chapter 4: Pre-clinical efficacy and complication data required to justify a clinical trial. Xenotransplantation. 2016;**23**:46-52

[26] Shin JS, Kim JM, Kim JS, Min BH, Kim YH, Kim HJ, et al. Long-term control of diabetes in immunosuppressed nonhuman primates (NHP) by the transplantation of adult porcine islets. American Journal of Transplantation. 2015;**15**:2837-2850

[27] Shin JS, Min BH, Kim JM, Kim JS, Yoon IH, Kim HJ, et al. Failure of transplantation tolerance induction by autologous regulatory T cells in the pig-to-non-human primate islet xenotransplantation model. Xenotransplantation. 2016;**23**:300-309

[28] Graham ML, Bellin MD, Papas KK, Hering BJ, Schuurman HJ. Species incompatibilities in the pig-to-macaque islet xenotransplant model affect transplant outcome: A comparison with allotransplantation. Xenotransplantation. 2011;**18**:328-342

[29] Hu Q, Liu Z, Zhu H. Pig islets for islet xenotransplantation: Current status and future perspectives. Chinese Medical Journal. 2014;**127**:370-377

[30] Park CG, Bottino R, Hawthorne WJ. Current status of islet xenotransplantation. International Journal of Surgery. 2015;**23**:261-266

[31] Desai T, Shea LD. Advances in islet encapsulation technologies. Nature Reviews Drug Discovery. 2017;**16**:338-350

[32] van der Windt DJ, Bottino R, Kumar G, Wijkstrom M, Hara H, Ezzelarab M, et al. Clinical islet xenotransplantation: How close are we? Diabetes. 2012;**61**:3046-3055

[33] Hecht G, Eventov-Friedman S, Rosen C, Shezen E, Tchorsh D, Aronovich A, et al. Embryonic pig pancreatic tissue for the treatment of diabetes in a nonhuman primate model. Proceedings of the National Academy of Sciences of the United States of America. 2009;**106**:8659-8664

[34] Nagaraju S, Bottino R, Wijkstrom M, Trucco M, Cooper DK. Islet xenotransplantation: What is the optimal age of the islet-source pig? Xenotransplantation. 2015;**22**:7-19

[35] Jin SM, Shin JS, Kim KS, Gong CH, Park SK, Kim JS, et al. Islet isolation from adult designated pathogen-free pigs: Use of the newer bovine nervous tissue-free enzymes and a revised donor selection strategy would improve the islet graft function. Xenotransplantation. 2011; **18**:369-379

[36] Kim JH, Kim HI, Lee KW, Yu JE, Kim SH, Park HS, et al. Influence of strain and age differences on the yields of porcine islet isolation: Extremely high islet yields from SPF CMS miniature pigs. Xenotransplantation. 2007;**14**:60-66

[37] Kim HI, Lee SY, Jin SM, Kim KS, Yu JE, Yeom SC, et al. Parameters for successful pig islet isolation as determined using 68 specific-pathogen-free miniature pigs. Xenotransplantation. 2009;**16**:11-18

[38] Buss J, Essig E, Osei K, Brodsky S, Hadley G, Rajab A. Steroid-free maintenance of islet allografts using mycophenolate mofetil and cyclosporine in the non-human primate. Annals of Transplantation. 2011;**16**:88-97

[39] Qiao CF, Tian BL, Mai G, Wei LL, Jin X, Ren Y, et al. Induction of diabetes in rhesus monkeys and establishment of insulin administration strategy. Transplantation Proceedings. 2009;**41**:413-417

[40] Thompson P, Badell IR, Lowe M, Turner A, Cano J, Avila J, et al. Alternative immunomodulatory strategies for xenotransplantation: CD40/154 pathway-sparing regimens promote xenograft survival. American Journal of Transplantation. 2012;**12**:1765-1775

[41] Lenzen S. The mechanisms of alloxan- and streptozotocin-induced diabetes. Diabetologia. 2008;**51**:216-226

[42] Karim S, Adams DH, Lalor PF. Hepatic expression and cellular distribution of the glucose transporter family. World Journal of Gastroenterology. 2012;**18**:6771-6781

[43] Tal MG, Hirshberg B, Neeman Z, Bunnell D, Soleimanpour S, Bacher J, et al. Induction of diabetes in nonhuman primates by means of temporary arterial embolization and selective arterial injection of streptozotocin. Radiology. 2004;**230**:163-168

[44] Zhu H, Yu L, He Y, Wang B. Nonhuman primate models of type 1 diabetes mellitus for islet transplantation. Journal of Diabetes Research. 2014;**2014**:785948

[45] Kim JM, Shin JS, Min BH, Kim HJ, Kim JS, Yoon IH, et al. Induction, management, and complications of streptozotocin-induced diabetes mellitus in rhesus monkeys. Xenotransplantation. 2016;**23**:472-478

[46] LaSalle JR. Empowering patients during insulin initiation: A real-world approach. Journal of the American Osteopathic Association. 2010;**110**:69-78

[47] Gao F, Ai SD, Liu S, Zeng WB, Wang W. Percutaneous transhepatic portal catheterization guided by ultrasound technology for islet transplantation in rhesus monkey. Hepatobiliary & Pancreatic Diseases International. 2012;**11**:154-159

[48] Galili U. The alpha-gal epitope and the anti-Gal antibody in xenotransplantation and in cancer immunotherapy. Immunology and Cell Biology. 2005;**83**:674-686

[49] De Silva NS, Klein U. Dynamics of B cells in germinal centres. Nature Reviews Immunology. 2015;**15**:137-148

[50] Kang HJ, Lee H, Park EM, Kim JM, Shin JS, Kim JS, et al. Increase in anti-Gal IgM level is associated with early graft failure in intraportal porcine islet xenotransplantation. Annals of Laboratory Medicine. 2015;**35**:611-617

[51] Kang HJ, Lee H, Park EM, Kim JM, Shin JS, Kim JS, et al. Dissociation between anti-porcine albumin and anti-Gal antibody responses in non-human primate recipients of intraportal porcine islet transplantation. Xenotransplantation. 2015;**22**:124-134

[52] van der Windt DJ, Bottino R, Casu A, Campanile N, Cooper DK. Rapid loss of intraportally transplanted islets: An overview of pathophysiology and preventive strategies. Xenotransplantation. 2007;**14**:288-297

[53] Robson SC, Cooper DK, d'Apice AJ. Disordered regulation of coagulation and platelet activation in xenotransplantation. Xenotransplantation. 2000;**7**:166-176

[54] van der Windt DJ, Marigliano M, He J, Votyakova TV, Echeverri GJ, Ekser B, et al. Early islet damage after direct exposure of pig islets to blood: Has humoral immunity been underestimated? Cell Transplantation. 2012;**21**:1791-1802

[55] Kang HJ, Lee H, Ha JM, Lee JI, Shin JS, Kim KY, et al. The role of the alternative complement pathway in early graft loss after intraportal porcine islet xenotransplantation. Transplantation. 2014;**97**:999-1008

[56] Kirchhof N, Shibata S, Wijkstrom M, Kulick DM, Salerno CT, Clemmings SM, et al. Reversal of diabetes in non-immunosuppressed rhesus macaques by intraportal porcine islet xenografts precedes acute cellular rejection. Xenotransplantation. 2004;**11**: 396-407

[57] Scalea J, Hanecamp I, Robson SC, Yamada K. T-cell-mediated immunological barriers to xenotransplantation. Xenotransplantation. 2012;**19**:23-30

[58] Shin JS, Kim JS, Kim JM, Jang JY, Kim YH, Kim HJ, et al. Minimizing immunosuppression in islet xenotransplantation. Immunotherapy. 2014;**6**:419-430

[59] Bennet W, Groth CG, Larsson R, Nilsson B, Korsgren O. Isolated human islets trigger an instant blood mediated inflammatory reaction: Implications for intraportal islet transplantation as a treatment for patients with type 1 diabetes. Upsala Journal of Medical Sciences. 2000;**105**:125-133

[60] Taguchi T, McGhee JR, Coffman RL, Beagley KW, Eldridge JH, Takatsu K, et al. Detection of individual mouse splenic T cells producing IFN-gamma and IL-5 using the enzyme-linked immunospot (ELISPOT) assay. Journal of Immunological Methods. 1990;**128**: 65-73

[61] Miyahira Y, Murata K, Rodriguez D, Rodriguez JR, Esteban M, Rodrigues MM, et al. Quantification of antigen specific CD8+ T cells using an ELISPOT assay. Journal of Immunological Methods. 1995;**181**:45-54

[62] Slota M, Lim JB, Dang Y, Disis ML. ELISpot for measuring human immune responses to vaccines. Expert Review of Vaccines. 2011;**10**:299-306

[63] Asai T, Storkus WJ, Whiteside TL. Evaluation of the modified ELISPOT assay for gamma interferon production in cancer patients receiving antitumor vaccines. Clinical and Diagnostic Laboratory Immunology. 2000;**7**:145-154

[64] Schloot NC, Meierhoff G, Karlsson Faresjo M, Ott P, Putnam A, Lehmann P, et al. Comparison of cytokine ELISpot assay formats for the detection of islet antigen autoreactive T cells. Report of the third immunology of diabetes society T-cell workshop. Journal of Autoimmunity. 2003;**21**:365-376

[65] Heeger PS, Greenspan NS, Kuhlenschmidt S, Dejelo C, Hricik DE, Schulak JA, et al. Pretransplant frequency of donor-specific, IFN-gamma-producing lymphocytes is a manifestation of immunologic memory and correlates with the risk of posttransplant rejection episodes. The Journal of Immunology. 1999;**163**:2267-2275

[66] Hricik DE, Rodriguez V, Riley J, Bryan K, Tary-Lehmann M, Greenspan N, et al. Enzyme linked immunosorbent spot (ELISPOT) assay for interferon-gamma independently predicts renal function in kidney transplant recipients. American Journal of Transplantation. 2003;**3**:878-884

[67] Bestard O, Crespo E, Stein M, Lucia M, Roelen DL, de Vaal YJ, et al. Cross-validation of IFN-gamma Elispot assay for measuring alloreactive memory/effector T cell responses in renal transplant recipients. American Journal of Transplantation. 2013;**13**:1880-1890

[68] Ashoor I, Najafian N, Korin Y, Reed EF, Mohanakumar T, Ikle D, et al. Standardization and cross validation of alloreactive IFN-gamma ELISPOT assays within the clinical trials in organ transplantation consortium. American Journal of Transplantation. 2013;**13**:1871-1879

[69] Kim HJ, Yoon IH, Min BH, Kim YH, Shin JS, Kim JM, et al. Porcine antigen-specific IFN-gamma ELISpot as a potentially valuable tool for monitoring cellular immune responses in pig-to-non-human primate islet xenotransplantation. Xenotransplantation. 2016;**23**: 310-319

[70] Lin YX, Yan LN, Li B, Wang LL, Wen TF, Zeng Y, et al. A significant expansion of CD8+ CD28–T-suppressor cells in adult-to-adult living donor liver transplant recipients. Transplantation Proceedings. 2009;**41**:4229-4231

[71] Lin YX, Wang LL, Yan LN, Cai P, Li B, Wen TF, et al. Analysis of CD8+CD28- T-suppressor cells in living donor liver transplant recipients. Hepatobiliary & Pancreatic Diseases International. 2009;**8**:241-246

[72] Solez K, Axelsen RA, Benediktsson H, Burdick JF, Cohen AH, Colvin RB, et al. International standardization of criteria for the histologic diagnosis of renal allograft rejection: The Banff working classification of kidney transplant pathology. Kidney International. 1993;**44**:411-422

[73] Troxell ML, Lanciault C. Practical applications in immunohistochemistry: Evaluation of rejection and infection in organ transplantation. Archives of Pathology and Laboratory Medicine. 2016;**140**:910-925

[74] Bocchi EA, Tanigawa RY, Brandao SM, Cruz F, Issa V, Ayub-Ferreira S, et al. Immunohistochemical quantification of inflammatory cells in endomyocardial biopsy fragments after heart transplantation: A new potential method to improve the diagnosis of rejection after heart transplantation. Transplantation Proceedings. 2014;**46**:1489-1496

[75] Ekser B, Klein E, He J, Stolz DB, Echeverri GJ, Long C, et al. Genetically-engineered pig-to-baboon liver xenotransplantation: Histopathology of xenografts and native organs. PLoS One. 2012;**7**:e29720

[76] Mauiyyedi S, Crespo M, Collins AB, Schneeberger EE, Pascual MA, Saidman SL, et al. Acute humoral rejection in kidney transplantation: II. Morphology, immunopathology, and pathologic classification. Journal of the American Society of Nephrology. 2002;**13**:779-787

[77] Collins AB, Schneeberger EE, Pascual MA, Saidman SL, Williams WW, Tolkoff-Rubin N, et al. Complement activation in acute humoral renal allograft rejection: Diagnostic significance of C4d deposits in peritubular capillaries. Journal of the American Society of Nephrology. 1999;**10**:2208-2214

[78] Lederer SR, Kluth-Pepper B, Schneeberger H, Albert E, Land W, Feucht HE. Impact of humoral alloreactivity early after transplantation on the long-term survival of renal allografts. Kidney International. 2001;**59**:334-341

[79] Bohmig GA, Exner M, Habicht A, Schillinger M, Lang U, Kletzmayr J, et al. Capillary C4d deposition in kidney allografts: A specific marker of alloantibody-dependent graft injury. Journal of the American Society of Nephrology. 2002;**13**:1091-1099

[80] Kap YS, van Meurs M, van Driel N, Koopman G, Melief MJ, Brok HP, et al. A monoclonal antibody selection for immunohistochemical examination of lymphoid tissues from non-human primates. The Journal of Histochemistry and Cytochemistry: Official Journal of the Histochemistry Society. 2009;**57**:1159-1167

[81] Watanabe M, Yamashita K, Suzuki T, Kamachi H, Kuraya D, Koshizuka Y, et al. ASKP1240, a fully human anti-CD40 monoclonal antibody, prolongs pancreatic islet allograft survival in nonhuman primates. American Journal of Transplantation. 2013;**13**:1976-1988

[82] Koulmanda M, Qipo A, Chebrolu S, O'Neil J, Auchincloss H, Smith RN. The effect of low versus high dose of streptozotocin in cynomolgus monkeys (*Macaca fascicularis*). American Journal of Transplantation. 2003;**3**:267-272

Chapter 7

# Moving Islet Cell Xenotransplantation to the Clinic

Wayne Hawthorne

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.69379

**Abstract**

The ultimate goal to provide a cure for all patients suffering from type-1-diabetes has remained out of the patients reach despite major advances in technologies. There has however, for a number of decades been a concerted effort to use various forms of porcine β-cells as a replacement transplant alternative to cadaveric human donors. This has seen major advances in the last decade with significant development of multi-transgenic donor pigs that now can potentially be used for xenotransplantation. This has been achieved with cellular transplants leading the way using porcine islet cell transplants as a form of β cell replacement in pre-clinical studies to treat diabetic non-human primates in various guises. These uniquely modified islet cells have the potential to offer an unlimited source of insulin-producing cells once we have solved all of the issues required to prevent loss of the xenotransplant. This chapter provides an in depth overview as to how the most recent advances have been achieved in regards to the genetic modification of donor pigs to provide protection from hyperacute rejection, instant blood mediated inflammatory reaction, xenoantibody and cellular responses to provide long-term functional islet cell xenotransplants to be able to move islet cell xenotransplantation to the clinic.

**Keywords:** clustered regularly interspaced short palindromic repeats, diabetes mellitus, graft rejection, immunosuppression, islets of langerhans, primates, swine, transplantation, hyperacute rejection, instant blood-mediated inflammatory reaction, islet cell transplantation, neonatal islet cell clusters, thrombosis, Type-1-diabetes, transcription activator-like effector nucleases, xenotransplantation, zinc finger nucleases

## 1. Introduction

The major landmark in the development of a treatment for type-1-diabetes (T1D) occurred almost a century ago, with conventional treatment still utilising exogenous insulin therapy.

Clearly for the majority of this time the main stay for its use was that of porcine insulin until it was usurped by recombinant human insulin in the early 1980s [1]. Despite the development of these newer insulin's and their modified treatment regimens still used to this day, insulin's role only remains in the prevention of elevations of blood sugar levels which ultimately give rise to ketoacidosis and ultimately death, where it is not adequately controlled [2]. Even with the advent of the insulin pump (IP) and integrated control with a Glucose Management System (GMS) the fact remains that the life-saving benefits of exogenous insulin therapy are still inadequate to prevent the serious long-term secondary complications of T1D. Sadly, patients with T1D still suffer significantly from cardiac disease, nephropathy, retinopathy and micro vascular disease. Even with the use of insulin pumps, it still has not reduced the severe hypoglycemic episodes resulting in coma and even death of these patients due to the significant lack of biofeedback and blood sugar re-balancing by glucagon secretion as produced by islet cells [3, 4].

The current transplant treatments being offered to patients suffering from T1D, both whole organ pancreas and pancreatic islet cell allotransplantation remain the Gold standard in treatment but are only available to treat small numbers, more or less just subsets of patients with T1D. In the case of simultaneous pancreas and kidney (SPK) transplantation where the whole pancreas is transplanted in combination with the kidney to treat patients with T1D and renal failure these patients do incredibly well, but this transplant is a major surgical procedure that is not an option for all patients suffering from T1D [5, 6]. Likewise islet cell transplantation remains an option only for the small subset of patients suffering severe hypoglycemic unawareness [7, 8]. These patients have seen great benefits from their transplants such as protection from hypoglycemia and from the progression of the severe secondary complications. Despite the benefits pancreas and islet allotransplantation provide, we remain unable to offer these treatments universally to all patients with T1D due to the significant shortage of donor organs and the need for continuous immunosuppression to prevent graft rejection [9, 10].

The major reason we may never be able to offer widespread application of such transplants is the sad and unfulfilled ability to provide what is an extremely valuable resource in that of human organ donor supply, where a gap remains unlikely ever to be filled with a disproportionate gap in the supply as compared to the demand for transplants. To be able to offer an alternative source of tissue for clinical transplantation we must be able to produce cells that are demonstrated to be safe and effective from a reproducible alternative source of β-cell replacement tissues [11]. Decades of concerted effort in the xenotransplantation field has seen the emergence of porcine islets as the most plausible source of tissues to provide a safe, effective, reliable and renewable source of islet cell tissues [12]. However, in order for xeno-islet-transplantation to move to clinical therapy we must clearly provide a safe and stable porcine donor source that avoids the many existing barriers of xenotransplantation, including the necessity for a suitable and effective immunosuppressive regimen [13]. To be able to do this, those of us in the xenotransplantation field have concentrated on utilizing the pig as the donor source due to it being an easily housed and bred animal that has been farmed for centuries. It is also of a large

enough size that organs from pigs can quite readily size match humans for transplantation. Additionally, for such a large animal they are also rather unique, having a relatively short gestational cycle being less than four months (3 months 3 weeks 3 days) and they produce large litters (generally 10 but can have up to 20 in a single litter). This places them in a rather fortuitous position for us to be able to utilize them as a reproducible tissue source for generation of donors for tissues and cells, following appropriate genetic manipulation. Genetic manipulation has been necessary in order to be able to transplant pig tissues across the xenogeneic barriers into humans and this has taken multiple transgenes to achieve [11, 14].

Xenotransplantation leads all other technologies in the race to provide a viable source of transplantable tissues to treat T1D due to the incredible advances in technologies providing the ability to manipulate the donor tissues or cells prior to transplantation. Over the last two decades we have seen incredible advances and changes in the technologies available for such, even since the first genetically manipulated transgenic pigs were produced expressing the human complement regulator CD59 [15], we have seen more dramatic advances due to the rapid adoption of extremely cutting edge technologies which are further discussed below. This is even reflected in the more traditional cloning technologies where zinc finger nucleases (ZFNs) [16, 17] have been used to develop a number of new pig lines. But by far the most effective and potentially further productive multiple transgenic combinations has been the adoption of targeted gene knockouts using transcription activator-like effector nucleases (TALENs) where there have been amazing advances in the production of pigs with multiple genetic knockouts [18]. The more recent advances have slingshot the production of transgenic pigs forward many years by the use of type II clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system [19–21]. These various strategies of genetic modification have 'humanized' the transgenic pig to be more readily usable in the clinical setting. Most notably, these approaches have aimed at either antigen reduction or human transgene expression [22].

This chapter provides an in depth overview as to how the most recent advances have been made in regards to the genetic modification of donor pigs to provide protection from hyperacute rejection, IBMIR, Xeno antibody and cellular responses to provide long-term functional islet cell xenotransplants to be able to move islet cell xenotransplantation to the clinic.

## 2. Overcoming the Islet-specific barriers inhibiting islet xenotransplantation

Islet cell xenotransplantation has been plagued by not only xenotransplant barriers but also islet specific barriers, which have inhibited its success until they have recently been overcome. The most significant barrier in xenotransplantation has been seen at the time of first point of contact with the recipient's blood stream and thus the cellular and antibody targeting cofactors. Primary graft loss can occur almost immediately following the first direct recipient blood contact with the graft. This is due, principally, to hyperacute rejection

(HAR). HAR occurs when the xenoreactive natural pre-formed antibodies to the oligosaccharide galactose $\alpha$1-3 galactose ($\alpha$Gal) of the recipient recognize the xenoantigen on the graft endothelium, and the resulting complement-mediated immune response leads to the triggering of the cascade of humoral and cellular events. These preexisting antigens on the cell surface of the donor tissue are rapidly recognized by the recipient's antibodies after revascularization which initiates the complement coagulation cascade, leading to thrombosis and edema and also the triggering of cellular inflammation that destroys the graft as quickly as within minutes to hours.

More readily observed in whole vascularized organs where HAR is characterized by immediate vascular engorgement and discoloration of the organ as can be seen in **Figure 1** of a kidney transplant one-hour post-transplantation whilst undergoing HAR.

Islets do not have large blood vessels or a significant vascular endothelium for this to occur in the same way. However, islets are infused into the vasculature of the liver usually via the portal vein. Following their transplantation into the liver's sinusoids, they trigger an almost immediate and profound micro and macro-vascular thrombotic change within the liver. This ultimately leads to larger vessel thrombosis and potentially thrombosis of the liver that they have been transplanted into [23].

Also at the time of transplantation islet cells are exposed to other barriers such as islet specific inflammation (instant blood mediated inflammatory reaction) referred to as IBMIR [24]. IBMIR was first demonstrated by William Bennet a PhD student in the Swedish group of Korsgren and Groth where he exposed human islets to freshly collected human ABO-compatible non-anticoagulated blood which he placed together into surface coated-heparinized polyvinyl chloride tubing loops to observe the resultant reactions and then subsequently also performed intraportal transplantation of porcine islets into pigs to observe and report on the advent of IBMIR [24].

Others have subsequently explored this process in depth using both human and xeno-islets. What they discovered was that allo-islets coalesced and ended up embedded in clots, where

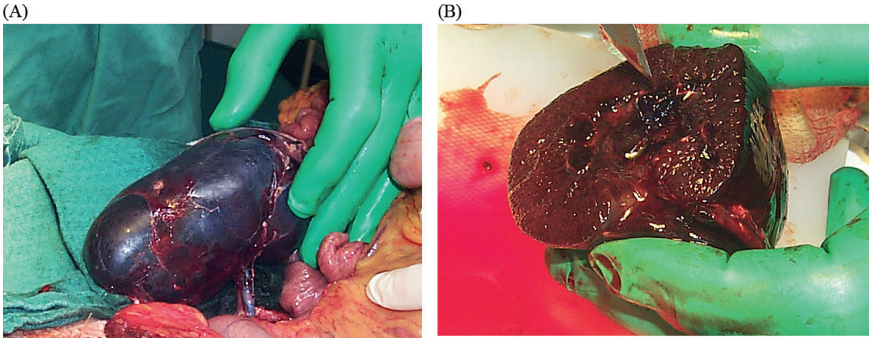


**Figure 1.** (A) Hyperacute rejection of a kidney graft 1 h post revascularisation. (B) Note the extremely engorged appearance of the graft with vascular thrombosis, engorgement and edema of the pulp of the graft.

they were infiltrated specifically with leukocytes and their cellular morphology was disrupted. Xeno-islets tend to also be infiltrated with neutrophils and the process occurring in a more aggressive and destructive manner [25, 26]. The macro-loop system setup provides a useful tool to explore the mechanisms that occur in IBMIR in both allo and xeno islet transplantation. The setup of the loop system can be seen in **Figure 2** where porcine islets are mixed with 7 ml of freshly collected human ABO compatible blood and incubated for an hour.

Fortuitously this process can be abrogated with the addition of anticoagulants such as heparin, which are added to the islet milieu in combination with a soluble complement receptor, completely preventing or delaying this process from occurring. More specifically it can prevent the initial triggering of the inflammation and as such reduce both initial islet loss and subsequent specific immune responses [25, 26]. However, in the Xenotransplantation setting it appears that the same protection may not be offered unless islets are specifically developed to avoid the IBMIR process.

In addition to these immune activated processes, pancreata and islets are also extremely affected by hypoxia that occurs with the organ donation process and removal of the organ from its blood supply. More significantly the loss of islet cell vascularization and the depravation of oxygen to the islets during the islet isolation process cause significant damage and loss of the islets [27].

We therefore have a number of unique possibilities, as we are able to develop transgenic pigs as donors where we can address these various problems and prevent them from occurring. With the genetic engineering of porcine donors we can modify them in such a way that they are more compatible with the human recipients they will be transplanted into. Multiple genetic manipulations have already proven useful specifically in relation to hyperacute rejection, IBMIR, hypoxia, innate immune responses and even T cells with the potential of even further advancement in the near future [28].

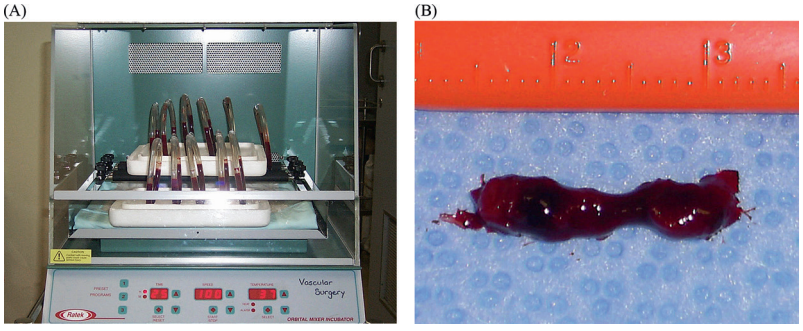


**Figure 2.** (A) An orbital mixer incubator with the PVC tubing loops containing porcine islets mixed with freshly collected non-anticoagulated human ABO-compatible blood. (B) The resultant clot after one hour of incubation of wild type porcine islets mixed with freshly collected non-anticoagulated human ABO-compatible blood.

### 2.1. Overcoming hyperacute rejection by genetic modification

The most impacting of the barriers, HAR, caused by the existence of the pre-formed antibodies to αGal prevent direct xenotransplantation as they are expressed at high levels on pig cells and in particular on islet cells, but the rate differs depending on the age of the pigs, seen at their highest levels on neonatal islet cells [29, 30]. In an eloquent study Rayat et al. examined αGal expression on various porcine islet cell preparations and correlated this with the proportion of cytokeratin 7 (CK7)-positive ductal cells. In vitro and in vivo expression of αGal and CK7 was significantly ($p < 0.05$) higher in less mature neonatal islet cells compared with matured neonatal islet and adult porcine islet cells, while the reverse was observed in the proportion of beta cells [31]. These pre-formed antibodies to the oligosaccharide galactose α1-3 galactose are not on human tissue and thus HAR does not occur in the allo setting [32].

Attempts to prevent HAR, for example by depletion of anti-pig Abs in recipients, were not very successful and it was not until genetic modification of donor pigs that any form of protection was provided. Numerous studies have subsequently utilized an array of genetic modifications in the genetically modified donor pigs for protection. But by far the most dramatic of impacts was that seen when the first genetic modification occurred with the deletion of the xenoantigen αGal known as the Gal Transferase Knockout (GTKO) [33–35], which still remains the pig of choice as the genetic background for application of further genetic modifications. There has been quite profound data showing that following the genetic manipulation of pigs to have transplantation of neonatal porcine GTKO islets into diabetic rhesus macaques, the neonatal porcine GTKO islets had significantly decreased susceptibility of the xenografts to innate immunity mediated by complement and preformed xenoantibody, and increased survival and function when compared to wild type islets [36]. We also saw the same abrogative effects in baboons transplanted with porcine neonatal islet cell clusters (NICC) from pigs with GTKO background when compared to wild type NICC [37]. The profound effects of transplanting wild type NICC into the baboon liver produced an almost immediate production of micro-thrombi surrounding the αGal-positive wild type NICC. This clot contained fibrin, RBC, and leukocytic infiltrate from as early as 1-hour post-transplant. Even using a combination of anticoagulation consisting of heparin and recombinant human antithrombin NICC's underwent HAR. NICC were surrounded by large numbers of platelets, monocytes and neutrophils and areas staining positively for complement C3c in surrounding clot. Neutrophils were seen infiltrating NICC that stained positive for IgG deposition, leading to early destruction [37]. As can be seen in **Figure 3A** the Wild type NICC were trapped in clots, whereas can be seen in **Figure 3B** the GTKO background NICC were not thrombosed at all at any stage post transplant being able to survive long-term for more than a year post transplant.

In a very short space of time technology has leapt forward and we have established a new era in molecular biology with the advent of novel and extremely cutting edge technologies, which make great changes to the way we genetically modify pigs. The most amazing leap forward came with a new tool based on a bacterial CRISPR and CRISPR-associated (Cas), in

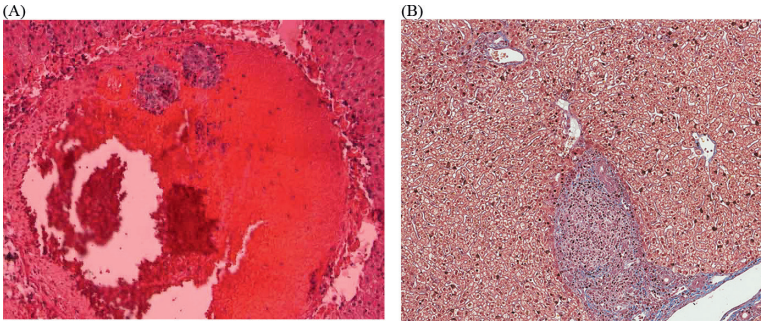


**Figure 3.** (A) A photomicrograph from a histology slide of a biopsy of a baboon liver transplanted with wild type NICC, note the two NICCs contained in a large clot consisting of RCC, platelets, fibrin and infiltrating cells that almost obstruct the entire venule it is sitting in. Section is at 100× magnification and stain is H&E. (B) A photomicrograph from a histology slide of a biopsy from a baboon liver transplanted with NICC from GTKO background neonatal pig, the islet is intact and well revascularized with minimal infiltration at 1 week post transplant, note the open portal venule with no sign of micro or macrovascular thrombosis. Section is at 200× magnification and stain is MSB.

particular the associated protein-9 nuclease (Cas9) from *Streptococcus pyogenes* which has led to a revolution in technology, causing considerable excitement in the entire science world let alone in xenotransplantation. In particular the genes have great potential to be exploited in the system for RNA-programmable genome editing [38]. Now a number of groups have been able to use the CRISPR/Cas9 system to efficiently perform biallelic knockout of the $\alpha$-1,3-galactosyltransferase gene in porcine blastocysts derived from the somatic cell nuclear transfer of $\alpha$Gal epitope-negative cells which also lacked the $\alpha$Gal epitope on their surface [39].

There have also been a number of other quite recent publications in which it has been shown that researchers have targeted various combinations to delete $\alpha$(1,3)galactosyl transferase (GGTA1), the gene for the enzyme cytidine monophospho-N-acetylneuraminic acid hydroxylase (CMAH) resulting in the generation of pigs that do not express NeuGc [20]. We have also seen the use of the slightly older but still cutting edge technology that is still very effective, TALENs which has been designed to target exon 6 of porcine GGTA1 gene resulting in the production of GGTA1-null miniature pigs [40, 41]. Miyagawa and colleagues were the first to generate the $\alpha$1,3 galactosyltransferase and cytidine monophospho-N-acetylneuraminic acid hydroxylase gene double-knockout pigs using this system [40]. ZFNs and somatic cell nuclear transfer have also been used for the generation of GGTA1 biallelic (double) knockout pigs [17, 42].

Quite clearly the multitude of very new and extremely effective technologies have provided an array of avenues to establish a clear platform from which we can progress additional genetic manipulations required to move xeno islets to the clinic.

### 2.2. Overcoming IBMIR by genetic modification

IBMIR is also a well-known mechanism effecting islets and islet like cells, which can occur almost immediately or even up to hours after the islet infusion in the allo, auto and xenograft setting [43, 44]. Islets specifically undergo targeted innate thrombotic and inflammatory

responses, which, like HAR results in coagulation, complement activation, infiltration of immune cells and platelet adhesion [45]. Certainly one of the major players appears to be complement activation, especially via the alternative pathway, which has been shown to greatly contribute to the triggering of IBMIR in the non-human primate (NHP) islet setting [13]. Specifically the triggering of IBMIR revolves around the release of inflammatory mediators including IL-8, MCP-1 and macrophage migration inhibitory factor which is very similar to what occurs in HAR with the triggering of thrombin then promoting the activation of monocytes, neutrophils and platelets that aggregate at the point of contact with the graft. It appears the major trigger for IBMIR therefore is tissue factor, which is expressed on the surface of islets and also the fragments of acinar tissue, which surround or are sometimes attached to the islet [43, 46, 47].

More complex than originally thought, it also appears that there is a synergistic influence between the coagulation cascade and platelets which are thought to exacerbate IBMIR and as such continues the destructive cycle without direct triggering. Experimental complement activation can be controlled by cobra venom factor, coagulation by heparin or low molecular weight dextran, and platelet activation by anti-platelet agents such as Plavix [13]. This is useful as proof of principal in the experimental setting where these drugs and agents have been used, however, it is best that they are not used clinically, so alternative drugs and agents specifically targeting complement such as Compstatin, human factor H (HFH) and intravenous immunoglobulin (IVIG) are under investigation [13]. Quite clearly, as pointed out more than a decade ago, if we are to make inroads into preventing IBMIR of islets or islet like tissues we must block tissue factor or inhibit its expression to prevent the thrombotic response *in vitro* [44, 45].

Fortunately, like the problems of HAR, this barrier has been overcome by the clever genetic modification of the donor pig to avoid $\alpha$Gal but also express human complement regulators such as CD46/55/59 [37]. Promising results have been achieved with porcine islets from hCD46-expressing pigs, which were transplanted into diabetic cynomolgus monkeys, normoglycemia was achieved in four of five monkeys with up to 3 months follow-up [48]. By far the most impressive results to date were achieved when porcine islets from $\alpha$Gal-deficient pigs, protecting against the pre-existing xeno antibodies, and also transgenic for the human complement regulators CD55 and CD59 were transplanted into the immunologically taxing baboon model. The baboons received NICC and were treated with a clinically relevant immunosuppressive protocol. The islets were less susceptible to humoral injury, induced significantly less complement activation and thrombin generation limiting antibody-mediated rejection compared to wild type thus proving that xeno islets can be protected from both HAR and IBMIR in the xenotransplant setting [23].

Following the rapid uptake of the most cutting-edge technologies by the xenotransplant community we have seen dramatic advances in xenotransplantation with the CRISPR/Cas9-directed mutation and human transgene delivery system that have been used to genetically modify and develop specific pigs to prevent HAR. CRISPR/Cas9 was used to delete the GGTA1, cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) and Beta-1,4-N-Acetyl-Galactosaminyl Transferase2 (B4GALNT2) genes. As a very targeted use

of the CRISPR/Cas9 system this approach provided a significant xenoantigen reduction with the abolition of the porcine carbohydrate profiles and as such effectively prevented antibody-mediated complement dependent cytotoxicity from occurring [22].

These genetic modifications to protect against HAR and IBMIR have been mitigated by genetically disrupting the $\alpha$1,3-galactosyltransferase gene and other targeted approaches to reduce xenogenicity as shown above by targeting the many various transgenes such as; GLA, HT, hGnT-III, GT-KO, CMAH, (Neu5Gc)-KO, class I MHC-KO, iGb3s-KO, β4GalNT2-KO [11, 13, 17, 22, 23, 37, 40–42]. Clearly remarkable these have made massive inroads in the direction that we need to travel to be able to move islet xenotransplantation to the clinic.

### 2.3. Overcoming hypoxia and inflammation by genetic modification

Ultimately the overt processes of organ donation and the islet cell isolation strip and denude the islet cells of their vasculature and deprive the islets of the oxygen and nutrients they require to sustain them. Ultimately this causes irreversible damage to the islets resulting in graft loss. Obviously the main cause of this is from hypoxia and as such is contributed to by hypoxia inducible factor (HIF)-1$\alpha$ and activation of its target genes that end up causing impaired islet function, apoptosis and eventually cell death [49–51]. One of the fundamental ways to potentially improve outcomes is to increase the oxygen delivery to the cells during the organ retrieval process. This has been undertaken by the use of various experimental devices such as the use of the two layer method [52, 53] and also by the experimental use of persuflation [11]. The two-layer method (TLM) utilizes a perfluorochemical (PFC) and UW solution to store the pancreas during shipping. The benefits of the use of the PFC are theoretically because it is a biologically inert liquid that acts as an oxygen-supplying media. A pancreas preserved using the TLM is oxygenated through the PFC and substrates are supplied by the UW solution. Reportedly this allows the pancreas to be better preserved using the TLM to generate adenosine triphosphate during storage, prolonging the preservation time [11].

Numerous attempts have been undertaken to improve the supply of oxygen to islets including *in situ* oxygen generation and improved revascularization of the graft and the islets themselves [54] along with possible systemic treatments of the islets during culture and transplant using already clinically approved agents such as desferrioxamine (DFO) [55]. There have been a few ways developed to improve hypoxia by the modification of the xenotransplant utilizing genetic modification of the donor pig to date.

The most specific targets investigated have been using the transgenic expression of the human A20 gene in cloned pigs. The zinc finger protein A20 is an important negative regulator of inflammation; polymorphisms in the corresponding gene, *TNFAIP3*, have been reported to be associated with numerous inflammatory diseases. The A20 gene is thought to provide protection against apoptotic and inflammatory stimuli but studies to date have been restricted to heart, skeletal muscle and porcine aortic endothelial cells (PAECs) of transgenic animals. Cultivated hA20-transgenic PAECs were protected against TNF-$\alpha$-mediated apoptosis, and partially protected against CD95 (Fas)L-mediated cell death and the pig cardiomyocytes were partially protected in ischemia/reperfusion stud-

ies. This study demonstrated that human A20 expression on pig cells could be a promising molecule for protection against hypoxia in xenotransplantation studies and it may also play a role in protection against the innate immune response [56]. More specifically we can produce pigs with transgenic expression of human heme oxygenase-1 (hHO-1), an inducible protein capable of cytoprotection by scavenging reactive oxygen species and preventing apoptosis caused by cellular stress during inflammatory processes [57]. The Korean group of Curie Ahn developed a hHO-1 expressing pig and analyzed the expression and function of the transgene. Human HO-1 was expressed in tissues, including the heart, kidney, lung, pancreas, spleen and skin. Fibroblasts derived from the hHO-1 transgenic pigs were significantly resistant to both hydrogen peroxide damage and hTNF-$\alpha$ and cycloheximide-mediated apoptosis when compared with wild-type pig fibroblasts. Furthermore, induction of RANTES in response to hTNF-$\alpha$ or LPS was significantly decreased in fibroblasts obtained from the hHO-1 transgenic pigs. These findings suggest that transgenic expression of hHO-1 can protect xenografts when exposed to oxidative stresses, especially from ischemia/reperfusion injury, and/or acute rejection mediated by cytokines [57].

More recently, the CRISPR/Cas9 system was used to yield human cells devoid of manganese superoxide dismutase (MnSOD). SOD2-null cells displayed perturbations in their mitochondrial ultrastructure and preferred glycolysis as opposed to oxidative phosphorylation to generate ATP [58]. We have also seen the development of various pigs that have the addition of a potential anti-inflammatory genes such as human CD39 (hCD39), the major vascular nucleoside triphosphate diphosphohydrolase (NTPDase), converts ATP and ADP to AMP, which is further degraded to the antithrombotic and anti-inflammatory mediator adenosine. Deletion of CD39 renders mice exquisitely sensitive to vascular injury, and CD39-null cardiac xenografts show reduced survival. Conversely, upregulation of hCD39 by somatic gene transfer or administration of soluble NTPDases has major benefits in inflammation. We have previously shown its advantages with hCD39 being expressed on NICC that were transplanted into baboons where it had significant benefits in preventing IBMIR and inflammation [37].

There is now clearly abundant protection against hypoxia by the expression or overexpression of hHO-1, HA-hHO-1, hA20, XIAP, INS-XIAP and genetic manipulation to protect against inflammation targeting by hCD39, INS-CD39, hTM, ASGR1-KO, hEPCR, hTFPI, and INS-TFPI [11, 13, 37, 55–58]. The use of such systems shows great advances and promise for the potential of the CRISPR/Cas9 system in making further genetic modifications in various donor pigs in the pursuit of the ultimate pig for xenotransplantation, not just for protection against hypoxia when undergoing islet cell xenotransplantation [59].

### 2.4. Overcoming the innate immune responses by genetic modification

Underpinning the overt innate inflammatory response to pig grafts is an overwhelming production of cytokines that includes IL-6 [60]. Along with this immediate cytokine response,

direct antigen presentation and complement upregulation also occur. Added together this formative assault targets the donor graft once revascularization occurs. Obviously only a targeted approach to prevent this cascade of events from occurring can be undertaken by direct targeting of antigen reduction and direct complement regulation on the human-anti-porcine complement dependent cytotoxic responses. A number of approaches have been undertaken of late to target this by producing genetically modified animals created using CRISPR/Cas9-directed mutation and human transgene delivery. Pigs doubly deficient in GGTA1 and CMAH genes have been produced and have been compared to pigs of the same background that expressed a human complement regulatory protein (hCRP). A third transgenic pig type has also been made deficient in GGTA1, CMAH and B4GalNT2 gene expression. Cells from these animals were subjected to measures of human antibody binding and antibody-mediated complement-dependent cytotoxicity by flow cytometry. Human IgG and IgM antibody binding was unchanged between the double knockout and the transgenic hCRP double knockout pig. IgG and IgM binding was reduced by 49.1 and 43.2% respectively by silencing the B4GalNT2 gene. Compared to the double knockout, human anti-porcine cytotoxicity was reduced by 8% with the addition of a hCRP ($p = 0.032$); It was reduced by 21% with silencing the B4GalNT2 gene ($p = 0.012$). Quite clearly selecting such genes to target effectively mediates human antibody-mediated complement dependent cytoxicity [22].

Although at first they were not thought to be involved at the time of revascularization, natural killer (NK) cells appear to also play a role in xenograft rejection. As such a number of groups have targeted HLA-G and HLA-E in an attempt to provide inhibitory receptors of human NK cells or macrophages with some effect. Weiss et al. have produced HLA-E/human beta2-microglobulin transgenic pigs that provided a small degree of protection against xenogeneic human anti-pig natural killer cell cytotoxicity [61]. Maeda et al. demonstrated that by transfecting swine endothelial cells to express HLA-E and also HLA-G they significantly suppressed the production of pro-inflammatory cytokines from the inflammatory macrophages, which is seen to be an important target to help advance islet xenotransplantation [62]. With genetically modified pigs that have one or more of the cellular immune response inhibitors already produced for xenotransplantation as described above including; hTRAIL, HLA-e/β2 m, pCTLA4-Ig, INS-pCTLA4-Ig, LEA29Y, hFasL, shTNFRI-Fc and CIITA-DN [11, 13, 60–62]. There would appear to be few other variable minor receptors to target, but with the major ones already targeted we are now more than readily able to move toward the clinic in regards to suppression of such responses.

### 2.5. Overcoming T cell responses by genetic modification

Ultimately overcoming the initial immediate barriers is of greatest need but following this we still have to provide a defense against the human T cells that recognize the pig MHC molecules (SLA). It appears that their response to pig tissue is greater than an allo-immune response as seen by the rapid infiltration of islet grafts in the loop model systems even within an hour following contact with human blood [7, 63, 64]. We have used standard clinically used immunosuppressive therapy in a pig to baboon islet transplant model and seen that the

grafts are more rapidly targeted and are eventually rejected by immune mechanisms within a month post transplantation [65]. Thus we require much heavier and directed immunosuppressive modalities to be able to prolong xenograft survival without causing untoward toxicity to the recipient [65].

As outlined previously, xenografts can become infiltrated with NK cells which are recruited by the innate inflammatory response but they can also be stimulated and recruited by upregulation of $CD4^+$ and $CD8^+$ T cells and macrophages. $CD4^+$ T cells are the predominant cell type involved in xenograft rejection with activated $CD4^+$ T cells infiltrating the rejecting pig xenograft resulting in IFN$\gamma$-mediated activation and infiltration of macrophages and NK cells [66–68]. Macrophages participate in recruitment of effector T cells as well as antigen presentation and cytokine production. Proinflammatory cytokines produced by macrophages, including TNF$\alpha$ and IL-6, upregulate tissue factor and can promote both inflammation and activation of coagulation [60].

We have seen in a number of studies that conventional doses of common immunosuppressive drugs such as cyclosporine, tacrolimus and glucocorticoids are not effective. However, some studies using extremely toxic and non-clinically applicable immunosuppression have shown extended graft preservation but lead to an unacceptably high susceptibility to serious infections in baboons [69]. We have seen some inroads to the success of long-term graft survival using the more novel and targeted immunosuppressive agents such as anti-CD154 Ab treatment in pig islet transplants into monkeys [70]. Using a modified Anti-CD154 based immunosuppressive regimen and islets from genetically engineered pigs on an $\alpha$1, 3-galactosyltransferase gene-knockout background with ubiquitous expression of human CD46 (GTKO/CD46 pigs), and additional islet beta cell-specific expression of human tissue factor pathway inhibitor (hTFPI) and/or human CD39 and/or porcine CTLA4-lg, islets were intraportally transplanted into diabetic cynomolgus monkeys demonstrating reduced islet destruction in the first hours after transplantation. Despite encouraging effects on early islet loss, these multi-transgenic islet grafts did not demonstrate consistency in regard to long-term success, with only two of five demonstrating function beyond five months [71].

Despite several issues being raised with the potential thrombogenic side effects of anti-CD154, Cooper's group have recently published its safe and efficacious use in pig islet transplants in monkeys showing no apparent side effects in an extended series of fourteen animals over many months in which they describe the extended treatment of their monkeys with the anti-CD154 and then undertook a critical and extensive analysis of the animals tissues by microscopy looking for any microthrombotic or thromboembolic complications [70]. There are also further studies utilizing a blocking antibody against CD40 (the receptor for CD154), which is showing even more promise for pig heart, and kidney transplants in baboons with no thromboembolic complications [72, 73].

Specific targeting of an immunosuppressive factor to be genetically engineered into islets could be in place of systemic delivery or if the agent was not suitable for repeated systemic treatment. A perfect candidate would appear to be costimulation blockade using anti-CD154 whose questioned thromboembolic effects would be negated by its local production at the

site of the islet. We have also shown suitability of another novel and very effective agent anti-CD2 whose systemic T cell depletion might be undesirable long term, but if produced locally would not affect the majority of the body's T cells. We have clearly shown it to be efficacious in a humanized severe combined immunodeficiency (huSCID) model using transduced NICC secreting anti-CD2 to prevent graft rejection [74]. Novel targeting by agents such as anti-CD2 would appear a very definitive route to take, as CD2 it is expressed on all T cells and subsets of NK cells and unlike most other T cell specific targets; it is expressed more highly on memory T cells [75].

If we are to provide comprehensive coverage of all avenues of protection we can also theoretically target the genetic reduction of the expression of MHC. In fact this has been undertaken in a pig-to-baboon artery patch model. Pig arteries expressing a dominant-negative MHC II transactivator gene to reduce levels of MHC II (including on endothelial cells) had a modest effect. Targeted disruption of MHC I genes in pigs by utilizing CRISPR/Cas9 has also been achieved [19] providing an avenue forward for incorporation with the other already demonstrated to be effective transgenic manipulations to move porcine islet xenotransplantation to the clinic.

### 2.6. Final remarks

There appear very few remaining barriers to be overcome before xenotransplantation can move to the clinic. The recent advent of the cutting-edge molecular tools such as ZFNs, TALENs, and the CRISPR/Cas9 system have all significantly increased efficiency and precision of the production of genetically modified pigs for xenotransplantation [59]. There are a number of proof of concept studies already demonstrating long-term islet xenograft survival due to various [23, 48, 76], genetic modifications to protect against HAR and IBMIR being mitigated by genetically disrupting the $\alpha$1,3-galactosyltransferase gene and other targeted approaches to reduce xenogenicity by targeting GLA, HT, hGnT-III, GT-KO, CMAH, (Neu5Gc)-KO, class I MHC-KO, iGb3s-KO, $\beta$4GalNT2-KO [11, 13, 17, 22, 23, 37, 40–42]. These have also been combined with the transgenic expression of complement regulators hCD59, hCD55/hDAF, and hCD46 [11]. There is also abundant protection against hypoxia by the expression or overexpression of hHO-1, HA-hHO-1, hA20, XIAP, INS-XIAP [11, 13] and genetic manipulation to protect against inflammation targeting by hCD39, INS-CD39, hTM, ASGR1-KO, hEPCR, hTFPI, and INS-TFPI [11, 13, 37, 55–58].

It would appear the way is clearly open for moving to the clinic since the only remaining barrier, the adaptive immune response, can also be surpassed by the use of new systemic immunosuppressive therapies including a combination of local suppression by genetically modifying islets to be resistant to cellular rejection. We also have genetically modified pigs that have one or more of the cellular immune response inhibitors already produced for xenotransplantation including hTRAIL, HLA-e/$\beta$2 m, pCTLA4-Ig, INS-pCTLA4-Ig, LEA29Y, hFasL, shTNFRI-Fc and CIITA-DN [11, 13, 60–62, 71–75]. All that it would appear to be able to move to the clinic is to make the correct selection of the most appropriate combination of genetic manipulations to be able to provide the ideal multi-transgenic xenotransplant donor pig.

## Acknowledgements

My sincere gratitude to my sons Kyle and Liam Hawthorne for proofreading this chapter, they are always interested, supportive and happy to help me despite my many absences due to my work.

I also would like to thank everyone in the Department of Surgery, Westmead Hospital, Sydney Medical School, University of Sydney and the Westmead Institute for Medical Research for providing an academic environment that supports me in undertaking such valuable research work that will hopefully see the advent of islet xenotransplantation a clinical reality.

## Abbreviations

| | |
|---|---|
| GGTA1 | $\alpha$(1,3)galactosyl transferase |
| ASGPR | Asialoglycoprotein receptor |
| B4GALNT2 | Beta-1,4-N-acetyl-galactosaminyl transferase2 |
| CRISPR | Clustered regularly interspaced short palindromic repeats |
| CRISPR/Cas9 | CRISPR-associated (Cas) |
| CMAH | Cytidine monophospho-N-acetylneuraminic acid hydroxylase |
| CK7 | Cytokeratin 7 |
| $\alpha$Gal | Galactose $\alpha$1-3 galactose |
| GTKO | Gal Transferase Knockout |
| GMS | Glucose Management System |
| hCRP | Human complement regulatory protein |
| HFH | Human factor H |
| hHO-1 | Human heme oxygenase-1 |
| hCD39 | Human CD39 |
| EPCR | Human endothelial protein C receptor |
| HFH | Human factor H |
| huSCID | Humanized severe combined immunodeficiency |
| HAR | Hyperacute rejection |
| IBMIR | Instant blood mediated inflammatory reaction |
| IP | Insulin pump |
| IVIG | Intravenous immunoglobulin |
| INS- | Insulin producing |
| LMWD | Low molecular weight dextran |

| | |
|---|---|
| MnSOD | Manganese superoxide dismutase |
| NK | Natural killer cells |
| NICC | Neonatal islet cell clusters |
| NHP | Non-human primate |
| NTPDase | Nucleoside triphosphate diphosphohydrolase |
| PFC | Perfluorochemical |
| PAECs | Porcine aortic endothelial cells |
| SPK | Simultaneous pancreas and kidney |
| T1D | Type-1-diabetes |
| TFPI | Tissue factor pathway inhibitor |
| TALENs | Transcription activator-like effector nucleases |
| TLM | Two-layer method |
| GalT KO or GTKO | Xenoantigen $\alpha$Gal |
| XIAP | X-linked inhibitor of apoptosis |
| ZFNs | Zinc finger nucleases |

## Author details

Wayne Hawthorne

Address all correspondence to: wayne.hawthorne@sydney.edu.au

1 Sydney Medical School, University of Sydney, Westmead, NSW, Australia

2 Department of Surgery, Westmead Hospital, Westmead, NSW, Australia

3 Centre for Transplant and Renal Research, Westmead Institute for Medical Research, Westmead, NSW, Australia

## References

[1] Quianzon CC, Cheikh I. History of insulin. Journal of Community Hospital Internal Medicine Perspectives. 2012;**2**(2). http://dx.doi.org/10.3402/jchimp.v2i2.18701

[2] Azevedo LC, et al. Incidence and long-term outcomes of critically ill adult patients with moderate-to-severe diabetic ketoacidosis: Retrospective matched cohort study. Journal of Critical Care. 2014;**29**(6):971-977

[3] Bergenstal RM, et al. Effectiveness of sensor-augmented insulin-pump therapy in type 1 diabetes. The New England Journal of Medicine. 2010;**363**(4):311-320

[4] Miller KM, et al. Current state of type 1 diabetes treatment in the U.S. Updated data from the T1D exchange clinic registry. Diabetes Care. 2015;**38**(6):971-978

[5] Allen RD, et al. Pancreas and islet transplantation: An unfinished journey. Transplantation Proceedings. 2001;**33**(7-8):3485-3488

[6] Lam VW, et al. Evolution of pancreas transplant surgery. ANZ Journal of Surgery. 2010;**80**(6):411-418

[7] O'Connell PJ, et al. Transplantation of xenogeneic islets: Are we there yet? Current Diabetes Reports. 2013;**13**(5):687-694

[8] O'Connell PJ, et al. Clinical islet transplantation in type 1 diabetes mellitus: Results of Australia's first trial. Medical Journal of Australia. 2006;**184**(5):221-225

[9] Gerber PA, et al. Glycemia, hypoglycemia, and costs of simultaneous islet-kidney or islet after kidney transplantation versus intensive insulin therapy and waiting list for islet transplantation. Transplantation. 2015;**99**(10):2174-2180

[10] O'Connell PJ, et al. Multicenter Australian trial of islet transplantation: Improving accessibility and outcomes. American Journal of Transplantation. 2013;**13**(7):1850-1858

[11] Hawthorne WJ, Lew AM, Thomas HE. Genetic strategies to bring islet xenotransplantation to the clinic. Current Opinion in Organ Transplantation. 2016;**21**(5):476-483

[12] Graham ML, et al. Species incompatibilities in the pig-to-macaque islet xenotransplant model affect transplant outcome: A comparison with allotransplantation. Xenotransplantation. 2011;**18**(6):328-342

[13] Park CG, Bottino R, Hawthorne WJ. Current status of islet xenotransplantation. International Journal of Surgery. 2015;**23**(Pt B):261-266

[14] Fischer K, et al. Efficient production of multi-modified pigs for xenotransplantation by 'combineering', gene stacking and gene editing. Scientific Reports. 2016;**6**:29081

[15] Fodor WL, et al. Expression of a functional human complement inhibitor in a transgenic pig as a model for the prevention of xenogeneic hyperacute organ rejection. Proceedings of the National Academy of Sciences of the United States of America. 1994;**91**(23):11153-11157

[16] Lutz AJ, et al. Double knockout pigs deficient in N-glycolylneuraminic acid and galactose alpha-1,3-galactose reduce the humoral barrier to xenotransplantation. Xenotransplantation. 2013;**20**(1):27-35

[17] Li P, et al. Biallelic knockout of the alpha-1,3 galactosyltransferase gene in porcine liver-derived cells using zinc finger nucleases. Journal of Surgical Research. 2013;**181**(1):e39-e45

[18] Carlson DF, et al. Efficient TALEN-mediated gene knockout in livestock. Proceedings of the National Academy of Sciences of the United States of America. 2012;**109**(43):17382-17387

[19] Reyes LM, et al. Creating class I MHC-null pigs using guide RNA and the Cas9 endonuclease. The Journal of Immunology. 2014;**193**(11):5751-5757

[20] Estrada JL, et al. Evaluation of human and non-human primate antibody binding to pig cells lacking GGTA1/CMAH/beta4GalNT2 genes. Xenotransplantation. 2015;**22**(3): 194-202

[21] Butler JR, et al. Recent advances in genome editing and creation of genetically modified pigs. International Journal of Surgery. 2015;**23**(Pt B):217-222

[22] Butler JR, et al. Silencing porcine genes significantly reduces human-anti-pig cytotoxicity profiles: An alternative to direct complement regulation. Transgenic Research. 2016;**25**(5):751-759

[23] Hawthorne WJ, et al. Control of IBMIR in neonatal porcine islet xenotransplantation in baboons. American Journal of Transplantation. 2014;**14**(6):1300-1309

[24] Bennet W, et al. Incompatibility between human blood and isolated islets of Langerhans: A finding with implications for clinical intraportal islet transplantation? Diabetes. 1999;**48**(10):1907-1914

[25] Akima S, et al. Tirofiban and activated protein C synergistically inhibit the Instant Blood Mediated Inflammatory Reaction (IBMIR) from allogeneic islet cells exposure to human blood. American Journal of Transplantation. 2009;**9**(7):1533-1540

[26] Akima S, et al. Differences in the responses to recombinant human activated protein C against allogeneic and xenogeneic IBMIR. Transplantation. 2006;**82**(1):992

[27] Hameed AM, Hawthorne WJ, Pleass HC. Advances in organ preservation for transplantation. ANZ Journal of Surgery. 2016 Aug 4. doi: 10.1111/ans.13713. [Epub ahead of print] Review. PMID: 27490874

[28] Bottino R, Trucco M. Use of genetically-engineered pig donors in islet transplantation. World Journal of Transplantation. 2015;**5**(4):243-250

[29] Vizzardelli C, et al. Neonatal porcine pancreatic cell clusters as a potential source for transplantation in humans: Characterization of proliferation, apoptosis, xenoantigen expression and gene delivery with recombinant AAV. Xenotransplantation. 2002;**9**(1):14-24

[30] Jimenez-Vera E, et al. Long-term cultured neonatal islet cell clusters demonstrate better outcomes for reversal of diabetes: In-vivo and molecular profiles. Xenotransplantation. 2015 Mar-Apr;**22**(2):114-23. doi: 10.1111/xen.12151. Epub 2015 Feb 10.

[31] Rayat GR, et al. In vitro and in vivo expression of Galalpha-(1, 3)Gal on porcine islet cells is age dependent. Journal of Endocrinology. 2003;**177**(1):127-135

[32] Basnet NB, et al. Deficiency of N-glycolylneuraminic acid and Galalpha1-3Galbeta1-4GlcNAc epitopes in xenogeneic cells attenuates cytotoxicity of human natural antibodies. Xenotransplantation. 2010;**17**(6):440-448

[33] Kolber-Simonds D, et al. Production of alpha-1, 3-galactosyltransferase null pigs by means of nuclear transfer with fibroblasts bearing loss of heterozygosity mutations. Proceedings of the National Academy of Sciences of the United States of America. 2004;**101**(19):7335-73340

[34] Dai Y, et al. Targeted disruption of the alpha1, 3-galactosyltransferase gene in cloned pigs. Nature Biotechnology. 2002;**20**(3):251-255

[35] Phelps CJ, et al. Production of alpha 1,3-galactosyltransferase-deficient pigs. Science. 2003;**299**(5605):411-414

[36] Thompson P, et al. Alternative immunomodulatory strategies for xenotransplantation: CD40/154 pathway-sparing regimens promote xenograft survival. American Journal of Transplantation. 2012;**12**(7):1765-1775

[37] Hawthorne W, et al. Survival of genetically modified porcine neonatal Islet xenografts in baboons. Transplantation. 2014;**98**:414-414

[38] Jinek M, et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 2012;**337**(6096):816-821

[39] Sato M, et al. The combinational use of CRISPR/Cas9-based gene editing and targeted toxin technology enables efficient biallelic knockout of the alpha-1, 3-galactosyltransferase gene in porcine embryonic fibroblasts. Xenotransplantation. 2014;**21**(3):91-300

[40] Miyagawa S, Matsunari H, Watanabe M, et al. Generation of $\alpha$1, 3 galactosyltransferase and cytidine monophospho-N-acetylneuraminic acid hydroxylase gene double-knockout pigs. Journal of Reproduction and Development. 2015;**61**(5):449-457

[41] Xin J, et al. Highly efficient generation of GGTA1 biallelic knockout inbred mini-pigs with TALENs. PLoS One. 2013;**8**(12):e84250

[42] Bao L, et al. Generation of GGTA1 biallelic knockout pigs via zinc-finger nucleases and somatic cell nuclear transfer. Science China Life Sciences. 2014;**57**(2):263-268

[43] Moberg L, et al. Production of tissue factor by pancreatic islet cells as a trigger of detrimental thrombotic reactions in clinical islet transplantation. Lancet. 2002;**360**(9350):2039-2045

[44] Ozmen L, et al. Inhibition of thrombin abrogates the instant blood-mediated inflammatory reaction triggered by isolated human islets: Possible application of the thrombin inhibitor melagatran in clinical islet transplantation. Diabetes. 2002;**51**(6):1779-1784

[45] Moberg L, Korsgren O, Nilsson B. Neutrophilic granulocytes are the predominant cell type infiltrating pancreatic islets in contact with ABO-compatible blood. Clinical & Experimental Immunology. 2005;**142**(1):125-131

[46] Ji M, et al. The importance of tissue factor expression by porcine NICC in triggering IBMIR in the xenograft setting. Transplantation. 2011;**91**(8):841-846

[47] Johansson H, et al. Tissue factor produced by the endocrine cells of the islets of Langerhans is associated with a negative outcome of clinical islet transplantation. Diabetes. 2005;**54**(6):1755-1762

[48] van der Windt DJ, et al. Long-term controlled normoglycemia in diabetic non-human primates after transplantation with hCD46 transgenic porcine islets. American Journal of Transplantation. 2009;**9**(12):2716-2726

[49] Cantley J, et al. The hypoxia response pathway and beta-cell function. Diabetes, Obesity and Metabolism. 2010;**12**(Suppl 2):159-167

[50] Moritz W, et al. Apoptosis in hypoxic human pancreatic islets correlates with HIF-1alpha expression. The FASEB Journal. 2002;**16**(7):745-747

[51] Cheng K, et al. Hypoxia-inducible factor-1alpha regulates beta cell function in mouse and human islets. Journal of Clinical Investigation. 2010;**120**(6):2171-2183

[52] Fujino Y. Two-layer cold storage method for pancreas and islet cell transplantation. World Journal of Gastroenterology. 2010;**16**(26):3235-3238

[53] Brandhorst H, et al. A new oxygen carrier for improved long-term storage of human pancreata before islet isolation. Transplantation. 2010;**89**(2):155-160

[54] Shimoda M, et al. In vivo non-viral gene delivery of human vascular endothelial growth factor improves revascularisation and restoration of euglycaemia after human islet transplantation into mouse liver. Diabetologia. 2010;**53**(8):1669-1679

[55] Stokes RA, et al. Hypoxia-inducible factor-1alpha (HIF-1alpha) potentiates beta-cell survival after islet transplantation of human and mouse islets. Cell Transplant. 2013;**22**(2):253-266

[56] Oropeza M, et al. Transgenic expression of the human A20 gene in cloned pigs provides protection against apoptotic and inflammatory stimuli. Xenotransplantation. 2009;**16**(6):522-534

[57] Yeom HJ, et al. Generation and characterization of human heme oxygenase-1 transgenic pigs. PLoS One. 2012;**7**(10):e46646

[58] Cramer-Morales K, et al. SOD2 targeted gene editing by CRISPR/Cas9 yields Human cells devoid of MnSOD. Free Radical Biology & Medicine. 2015;**89**:379-386

[59] Niemann H, Petersen B. The production of multi-transgenic pigs: Update and perspectives for xenotransplantation. Transgenic Research. 2016;**25**(3):361-374

[60] Ezzelarab MB, et al. Systemic inflammation in xenograft recipients precedes activation of coagulation. Xenotransplantation. 2015;**22**(1):32-47

[61] Weiss EH, et al. HLA-E/human beta2-microglobulin transgenic pigs: Protection against xenogeneic human anti-pig natural killer cell cytotoxicity. Transplantation. 2009;**87**(1): 35-43

[62] Maeda A, et al. The suppression of inflammatory macrophage-mediated cytotoxicity and proinflammatory cytokine production by transgenic expression of HLA-E. Transplant Immunology. 2013;**29**(1-4):76-81

[63] Dorling A, Lombardi G, Binns R, Lechler RI. Detection of primary direct and indirect human anti-porcine T cell responses using a porcine dendritic cell population. European Journal of Immunology. 1996;**26**:1378-1387

[64] Yamada K, Sachs DH, DerSimonian H. Human anti-porcine xenogeneic T cell response. The Journal of Immunology. 1995;**155**:5249-5256

[65] Hering BJ, et al. Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. Nature Medicine. 2006;**12**(3):301-303

[66] Fox A, Mountford J, Braakhuis A, Harrison LC. Innate and adaptive immune responses to nonvascular xenografts: Evidence that macrophages are direct effectors of xenograft rejection. The Journal of Immunology. 2001;**166**:2133-2140

[67] Yi S, et al. T cell-activated macrophages are capable of both recognition and rejection of pancreatic islet xenografts. The Journal of Immunology. 2003;**170**(5):2750-2758

[68] Yi S, et al. CD4+ T cells initiate pancreatic islet xenograft rejection via an interferon-gamma-dependent recruitment of macrophages and natural killer cells. Transplantation. 2002;**73**(3):437-446

[69] Teotia SS, et al. Prevention, detection, and management of early bacterial and fungal infections in a preclinical cardiac xenotransplantation model that achieves prolonged survival. Xenotransplantation. 2005;**12**(2):127-133

[70] Bottino R, et al. Safe use of anti-CD154 monoclonal antibody in pig islet xenotransplantation in monkeys. Xenotransplantation. 2017 Jan;**24**(1). doi: 10.1111/xen.12283. Epub 2017 Jan 6. PMID: 28058735

[71] Bottino R, et al. Pig-to-monkey islet xenotransplantation using multi-transgenic pigs. American Journal of Transplantation. 2014;**14**(10):2275-2287

[72] Iwase H, et al. Pig kidney graft survival in a baboon for 136 days: Longest life-supporting organ graft survival to date. Xenotransplantation. 2015;**22**(4):302-309

[73] Mohiuddin MM, et al. Genetically engineered pigs and target-specific immunomodulation provide significant graft survival and hope for clinical cardiac xenotransplantation. The Journal of Thoracic and Cardiovascular Surgery. 2014;**148**(3):1106-1113; discussion 1113-1114

[74] Brady JL, et al. Anti-CD2 producing pig xenografts effect localized depletion of human T cells in a huSCID model. Xenotransplantation. 2013;**20**(2):100-109

[75] Sutherland RM, et al. Protective effect of CTLA4Ig secreted by transgenic fetal pancreas allografts. Transplantation. 2000;**69**(9):1806-1812

[76] Shin JS, et al. Long-term control of diabetes in immunosuppressed nonhuman primates (NHP) by the transplantation of adult porcine islets. American Journal of Transplantation. 2015;**15**(11):2837-2850

Chapter 8

# Whole-Liver Decellularization: Advances and Insights into Current Understanding

Ibrahim Fathi and Ahmed Eltawila

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.69487

**Abstract**

Whole-liver decellularization comprises the removal of the antigenic cellular content, while maintaining intact 3D extracellular matrix architecture and the complex native vascular cues. As a result, it challenges the classical hurdles of xenotransplantation and hypothetically allows the production of bioengineered human-size liver constructs. The associated technique and understanding of the determinants of successful application evolved rapidly during the last decade. In this chapter, the authors offer a comprehensive walk-through, starting from the simplicity of the concept to the complexity of clinical application. Avoiding repetition, the chapter covers the fundamentals and advances of decellularization, recellularization, ex vivo perfusion culture, and sterilization techniques. The interplay between the main pivots of whole-liver decellularization, namely intrinsic matrix potentials, immune response, and vasculature is described. An effort was made to dissect the hurdles facing the whole-liver decellularization approach and to highlight the gaps in current literature. The authors also offer insights about some critical concepts including intra-scaffold flow dynamics, gradient zonation, critical cell mass/density, mechano-sensitivity, substrate modifications, nondestructive analysis, and the surgeon's perspective, together with the discussion of published in vivo trials and large-scale production parameters.

**Keywords:** liver, decellularization, recellularization, xenograft, mechano-sensitivity, flow dynamics, sterilization, thrombosis, nondestructive analysis, substrate modification

## 1. Introduction

The whole-liver decellularization (WLD) approach, one of the organ-engineering and xenotransplantation approaches, comprises the removal of hepatic cellular content through perfusion decellularization while maintaining an intact 3D extracellular matrix (ECM) scaffold and the native hepatic vascular network. Intact vasculature can then be used to

repopulate the scaffold (a process termed "recellularization"), and thereafter allow the delivery of nutrients/oxygen to the newly seeded cells in a bioreactor setting, and in vivo after transplantation. Thus, a functional human-scale bioengineered liver can be fabricated, which is devoid of the original cellular antigenic content, repopulated with allogeneic or patient-specific cells, and relies on the ECM potentials for supporting cell proliferation and differentiation.

The main target of WLD approach is to provide physiologically matching liver grafts of xenogeneic origin for clinical transplantation and thus significantly expanding the organ harvest pool, which is the main focus herein. However, several other possible applications exist including drug testing, production of hydrogels and flask-coating materials, incorporation into ex vivo liver-support devices, or the use in repair of other tissues in the form of membranes (**Figure 1**). In this chapter, the authors offer a comprehensive walk-through of the different aspects of WLD, with the aim of highlighting the inadequacies and advances, clarifying the gaps in the approach hierarchy, and offering possible explanations and few theoretical insights. The main focus is to discuss and link the findings of the previous relevant studies, rather than inclusively listing them.

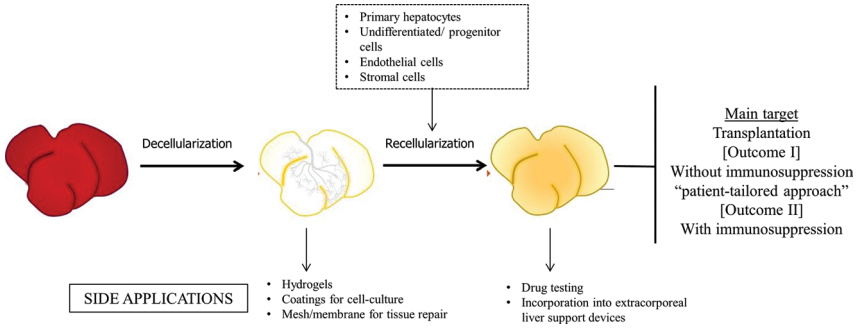


**Figure 1.** Schematic presentation of the whole-liver decellularization applications.

## 2. Concept

The concept of tissue decellularization was designed to evade the classical barriers to xenotransplantation and, thus, offers enormous enlargement of the tissue and organ pool for tissue repair and transplantation, as provoked by donor/patient mismatch crisis. The concept strongly relies on the intrinsic potentials of ECM as its fundamental justification. The whole-organ decellularization strategy was a step forward after clinical and experimental success of decellularized biological membranes by applying the same principles to whole organs in order to supply physiological-size-matched scaffolds for organ engineering. The cornerstone of this advance is the reliance on the native organ vascular system as a cue for perfusion decellularization, recellularization, and

nutrients/oxygen delivery after in vivo transplantation. As a result, an expansion past the limitations of diffusion distance for non-vascularized grafts (about 200 μm) can be achieved. The decellularized whole-liver matrix (wDLM) therefore can hypothetically evade the xenograft immune rejection cascade by the removal of cellular antigens, supply physiological-size-matched constructs through reliance on the native vascular system, and allow for acellular scaffold sterilization, thus diminishing the risk of xenozoonosis.

In fact, the attractive potentials of ECM provoked the design of different approaches to produce ECM scaffolds that can be used for cell seeding or tissue repair, and offered as an on-shelf product. These approaches can be categorized into synthetic and natural ECM scaffold production techniques. Although great advancements were achieved in the ECM synthetic techniques, the complexity of native organ ECM and vasculature hinders the progress into organ constructs through this pathway. Natural ECM scaffolds, on the other hand, can be produced by either in vitro cell culture and further decellularization or borrowing the native tissue/organ ECM through tissue decellularization techniques. The complex liver architecture and vasculature renders the WLD the most attractive approach for liver ECM scaffold production on a logical scale.

Among other xenotransplantation approaches, the simplicity of the WLD approach also acquires it a relative advantage. As opposed to genetic manipulation approaches for instance, WLD rather represents a simple combination of natural ECM potentials and human cell populations, and therefore involves borrowing of functional elements rather than modifying them to fit the human physiology. It simply aims to shift xenotransplants to the allotransplants or auto-transplants zone (if patient-specific cells are used). Genetic modification on the other hand requires more complex techniques and detailed appreciation of all the significant differences in antigenic expression and cell function effectors. However, despite the clear hypothetical simplicity, the application trials repeatedly demanded for deeper understanding and robust refinement of the decellularization/recellularization techniques provoked by suboptimal functions and ultimately the short-term in vivo survival after transplantation.

In order to simplify the hierarchy of WLD, three main parameters that largely govern its success are identified, namely sufficient DLM intrinsic potentials, avoidance of adverse immune/host responses, and maintenance of patent hepatic vasculature. Other parameters including cell sources and sterilization for example can then be considered as cofactors and not integral in the proof of concept paradigm. The impact of insufficiencies related to these three basic elements can, however, overlap in various study designs. Therefore, the exact delineation of error becomes a difficult task. For example, both insufficiencies related to the vascular system and scaffold immunogenicity can result in vascular thrombosis. Also, poor intrinsic ECM properties can result in endothelialization defects and therefore vascular incompetency. Therefore, in order to accurately locate and troubleshoot the problems along the WLD process, isolated testing for each of the three parameters is necessary as distinct main categories, followed by their combination. **Figure 2** shows a simplified theoretical validation flow chart.

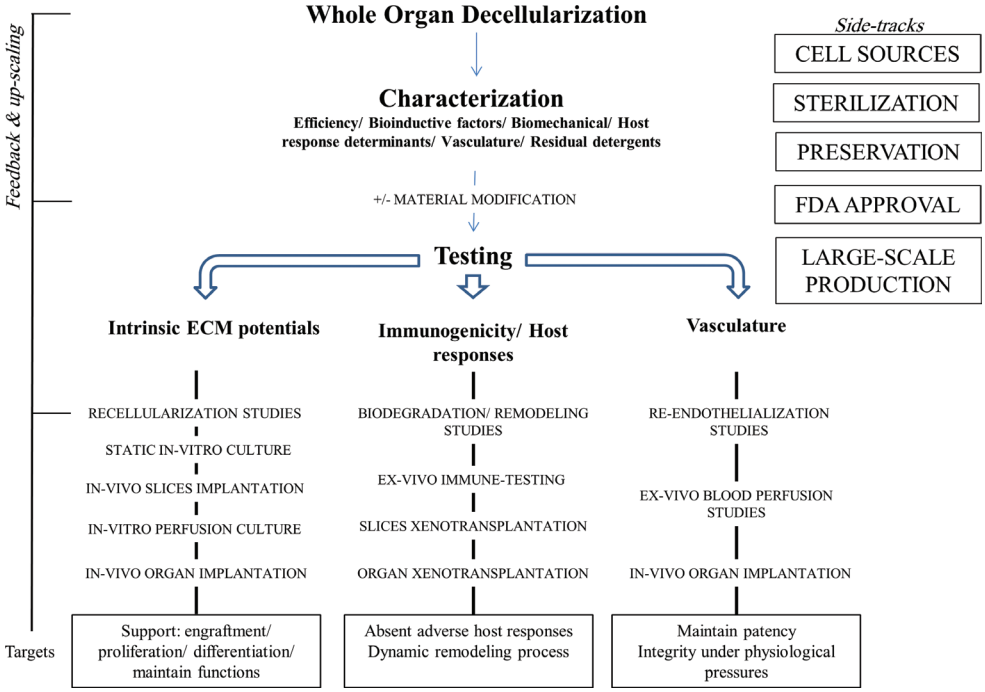


**Figure 2.** Schematic presentation of the whole-liver decellularization validation process.

## 3. Technique

To the authors' knowledge, a patent by Anthony Atala in 2002 offered the earliest discussion of the whole-organ decellularization concept [1]. However, Matthiesen et al. [2] presented the first published abstract reporting a perfusion WLD study. Since then, WLD progressed rapidly rendering it a fast growing field. During this progress, a wide range of decellularization techniques have been reported. In this section, the authors focus on the basic principles and significant advances in decellularization techniques in recent years. For a detailed review of the decellularization agents and techniques, the reviews by Gilbert et al. [3], Crapo et al. [4], and He et al. [5] are excellent references.

The decellularization methods can be categorized into chemical, physical, and enzymatic techniques, or a combination of them [3]. Among the chemical agents, detergents are subdivided into non-ionic (e.g., Triton X-100), ionic (e.g., sodium dodecyl sulfate (SDS), sodium deoxycholate (SDC), and Triton X-200), and zwitterionic detergents (e.g., CHAPS) [3]. The most frequently used detergents for WLD are SDS and Triton X-100, which largely replaced the use of peracetic acid (PAA) used widely in decellularized membranes production. Briefly, Triton X-100 causes lipid-lipid and lipid-protein bonds disruption with an adverse effect on glycosaminoglycans (GAGs) content, while SDS acts through the solubilization of cytoplasmic and nuclear membranes, but can result in protein denaturation, and also removes GAGs [3].

As a general scheme, the liver is removed after vascular cannulation, and the decellularization protocol proceeds after animal termination. The perfusion of detergents is preceded by saline/phosphate-buffered saline (PBS)/or deionized water perfusion to wash out the blood and is followed by another step of perfusion (with or without Triton X-100) to wash out the remnant detergents. In between, decellularization agents are perfused utilizing a peristaltic-perfusion pump (with or without pulse dampeners). The perfusion flow rates and durations, the decellularization agents used, their concentrations, and sequences are largely variable [5, 6]. Constant flow pressure rather than constant flow rate was infrequently used [7, 8]. Gravity-based perfusion was also reported [9]. An approach of lower SDS concentration with longer perfusion duration or gradually increasing concentration was advocated [10, 11], aiming at minimizing the damage to ECM and remnant detergent contents in the decellularized scaffold. In a recent study [12], increasing SDS concentration to 1% resulted in adverse cell outcomes after cell seeding. Substitution of SDS with ammonium hydroxide is possible [13, 14]. Trends also include the higher reliance on enzymatic techniques as an adjunct to detergents. Gessner et al. [15] incorporated phospholipase and nucleases in their rat decellularization protocol, in combination with 1% SDC, which yielded efficient decellularization and matrix preservation. Trypsin-EDTA was used in combination in human decellularized liver matrix (hDLM) production [16]. On the other hand, physical methods are only applied in combination with chemical detergents in WLD. Many protocols included freezing/thawing cycles as a step before detergents perfusion to induce cell lysis. Freezing temperatures reach −80°C [13]. Freezing/thawing cycles using liquid nitrogen to room temperature shifts resulted in deterioration of scaffold's collagen content (in an osteogenic ECM) [17].

The decellularization agents are perfused through one of the hepatic vascular systems, most commonly the portal venous (PV) system. To a lesser extent, perfusion through the hepatic veins (HVs) [16], and arterial system (HA) [18], but not the biliary system was investigated. A comparison of portal and arterial perfusion demonstrated lower DNA and better decellularization homogeneity with the arterial route (not statistically significant), while perfusion through the portal vein resulted in higher hepatocyte growth factor (HGF) content [18]. Only one recent study described a technique for in vivo decellularization by surgically isolating one of the rat liver lobes into a separate perfusion circuit [19]. The application of oscillating pressure in a custom-made chamber to mimic the intra-abdominal pressures also resulted in better decellularization homogeneity, lower residual DNA content (not statistically significant), and higher GAGs content (when the arterial system was used for perfusion) [18].

Despite the large number of studies dealing with WLD, the exact quantitative delineation of the effect of any of the decellularization agents remains a difficult task. The use of complex protocols, the characterization at the end of protocol sequence, and the variable detergent concentration, flow rate, flow duration, and characterization panels are some reasons for this difficulty. Designing specific studies for single detergent effects taking into consideration the perfusion parameters is essential; such analysis was employed in kidney decellularization for instance [20]. Thus far, the authors believe that comparative studies are currently the most useful tool in this context. A comparison of Triton X-100 + SDS proved superior to SDS in decellularization of sheep and rat livers in terms of cell removal and ECM preservation, augmented by the results of in vivo implantation [21]. Maghsoudlou et al. [22] demonstrated the impact of pretreatment with EDTA perfusion for 15 min in a rat 4%-SDC-based decellularization protocol. The authors

reported significant decrease of DNA with EDTA pretreatment; the DNA content was, however, 10% of that in fresh liver. EDTA was more efficient in removing cellular proteins as evidenced by quantitative shotgun proteomics. EDTA pretreatment also yielded a significantly higher content of collagen and elastin, while microarchitecture was more densely packed with reduction in hepatocyte pocket size. This packed microarchitecture could have shared in the higher content of matrix components and rendered the scaffold less suitable for recellularization as concluded by the authors [22]. The use of 1% SDS resulted in the collapse of vascular network compared to its preservation using 0.5 or 1% Triton X-100 as demonstrated by corrosion casting [23]. Mattei et al. [24] compared several decellularization protocols for porcine liver slices with or without ionic detergent (0.1% SDS); a combination of agitation and immersion was, however, used for decellularization rather than perfusion.

Studies demonstrated the use of livers from various animal species for decellularization including rats [10, 25–27], mice [13], ferrets [28, 29], rabbits [9, 30, 31], sheep [14, 21], and swine [8, 11, 32–34]. Decellularization of whole porcine liver was first reported by Matthiesen et al. [2]. Several reasons render pigs the most suitable organ source for human-scale liver engineering based on the current understanding; reasons include the physiological size matching, rapid maturation, availability for organ harvest, and the possible use of genetic engineering techniques [35]. Wu et al. [36] compared three porcine liver decellularization protocols namely 1% SDS, 1% Triton X-100 + 1% SDS, 1% SDC + 1% SDS, all followed by 1% Triton X-100 in PBS perfusion to remove residual SDS. The authors reported better cellular removal, and higher collagen and GAGs content (70% of native GAGs) with Triton X-100 + SDS combination. The same protocol also proved to be more biocompatible after perfusion recellularization with primary rat hepatocytes evidenced by significantly higher urea and albumin media content and higher expression of some liver-specific genes. On the other hand, sheep were also advocated for their size matching and anatomy [14]. Kajbafzadeh et al. [14] compared five perfusion protocols for sheep liver decellularization and found that the utilization of ammonium hydroxide + Triton X-100 was the most appropriate in terms of efficiency and intact vasculature. Recently, human liver decellularization in the form of perfusion WLD [16], or immersion/agitation decellularization of liver tissue discs ([37], preprint) was investigated. The source for human livers was either livers rendered unsuitable for transplantation [16] or obtained after hepatic resection for metastatic/benign liver lesions with no underlying chronic disease ([37], preprint). **Table 1** summarizes human and porcine liver decellularization studies.

Apart from the decellularization protocol, the wDLM scaffold modification during or after the perfusion process is a continuously growing field. Examples include chemical cross-linking (e.g., formalin) [8], NaCl matrix stabilization, and heparin immobilization. Cross-linking preserves the matrix structure, interferes with degradation, and masks the antigenic content. NaCl (as a high-salt buffer) was used to achieve better matrix preservation during the decellularization process [15]. Different techniques for heparin immobilization on the other hand were studied and resulted in decreasing intra-scaffold coagulation in vitro with a relative in vivo vascular patency improvement [7, 15, 32]. Many aspects regarding the effect of these techniques on recellularization, cell-ECM interaction, and scaffold remodeling are, however, still to be elucidated. In a recent study [17], immortalized death-inducible human mesenchymal cells (mesenchymal

| Liver source | Technique | Decell. agents | Total duration | Analysis | Recellularization | In vivo transplantation | Reference |
|---|---|---|---|---|---|---|---|
| *Porcine* | Freeze-thaw, immersion, and agitation (200 rpm) | 2% Triton X-100 + 0.1% ammonium hydroxide, ± followed by 5% PAA (six different wash solutions were compared) | 11 days | Removed 93–96% of DNA, better collagen preservation with water wash, higher GAGs content with PBS and salt wash | HepG2 or 1$^{ry}$ human hepatocytes, cultured on matrix discs for 21 days | None | Lang et al. [33] |
| *Porcine* | Perfusion through PV | 0.25 and 0.5% SDS | 2 days + an average of 100 min | ECM preservation, only few nuclei were observed | Human fetal hepatocytes and fetal stellate cells (co-culture), perfusion culture for 3, 7, and 13 days | Auxillary transplantation of acellular posterior segment into pig, perfusion for 2 h | Barakat et al. [8] |
| *Porcine* | Freeze-thaw, perfusion through portal vein | 0.01–1% SDS, 1% Triton X-100, 0.1% PAA | 4 days or more | Removed 98.8% of DNA, preserved ultrastructure, vascular systems, and 13–20% of GFs | Isolated porcine hepatocytes, perfusion culture for 7 days | None | Yagi et al. [11] |
| *Porcine* | Freeze-thaw, immersion, and agitation | Compared non-ionic (1% Triton X-100), and ionic (0.1% SDS) with three time intervals for each. All followed by 0.1% Triton X-100 in PBS | 3–5 days | Removed 97% of DNA, better matrix preservation with non-ionic protocol | Hepatocytes for cytotoxicity evaluation (compared four sterilization protocols) | None | Mattei et al. [24] |
| *Porcine* | Freeze-thaw, perfusion through PV, and HA ± oscillating pressure | 1% Triton X-100; 1% SDS | 7 h | Better DNA removal, ECM preservation, and homogeneity with oscillating pressure | None | None | Struecker et al. [34] |


http://dx.doi.org/10.5772/intechopen.69487

| Liver source | Technique | Decell. agents | Total duration | Analysis | Recellularization | In vivo transplantation | Reference |
|---|---|---|---|---|---|---|---|
| *Porcine* | Freeze-thaw, perfusion through PV | Wash with 0.1% EDTA; 1% Triton X-100, 1% SDS. Followed by heparin immobilization | <1 day | Almost complete DNA removal; ECM preservation; 70% of GAGs content; elimination of xenogenous antigens | Rat 1$^{ry}$ hepatocytes or HUVECs (seeded in wells over the matrix), cultured for 3 days | Acellular median lobe auxillary transplantation into pigs, vessels were patent at 60 min with heparin immobilization | Bao et al. [32] |
| *Human* | Freeze-thaw, retrograde perfusion through venous system (0.2–0.3 mL/min/g) | 0.025% trypsin-EDTA, 0.01-1% SDS, 3% Triton X-100, 0.1% PAA | 14 days | Absent DNA; architecture preservation; preserved distribution of collagen and fibronectin; decreased elastin | Human cell lines: hepatic stellate cells (LX2); hepatocellular carcinoma (SK-Hep-1); hepatoblastoma (HepG2), cultured for 21 days | Into immunocompetant mice (in the form of acellular tissue cubes) in the SC or omentum. No FB reaction or rejection process | Mazza et al. [16] |
| *Human (and porcine)* | Freeze-thaw, immersion, and agitation | 1 and 0.1% Triton X-100 | 3 days | Heterogeneous outcomes in human livers, while reproducible results in porcine livers | None | None | Mattei et al. ([37], preprint) |

*Abbreviations*: Decell, decellularization; ECM, extracellular matrix; FB, foreign body; GAGs, glycosaminoglycans; GFs, growth factors; HA, hepatic artery; HUVECs, human umbilical vein endothelial cells; PAA, peracetic acid; PBS, phosphate-buffered saline; PV, portal vein; SC, subcutaneous; SDS, sodium dodecyl sulfate.

**Table 1.** Human and porcine liver decellularization studies.

sword of Damocles stromal cell line; MSOD) were used to produce ECM in vitro, and then apoptosis was chemically induced to achieve decellularization. Using MSOD cells overexpressing vascular endothelial growth factor (VEGF) resulted in the production of matrix significantly enriched with VEGF. Applying a similar approach to customize or enforce wDLM composition is not yet investigated.

## 4. Characterization

Defining the outcomes of the decellularization technique is necessary for judging the success of the perfusion protocols and interpretation of the subsequent steps. A standard characterization panel also enables comparison between DLM studies considering the widely variable decellularization protocols. Unfortunately, variation is also evident in the analyzed DLM parameters. For the description of the normal hepatic ECM/biologic scaffolds components and functions, Refs. [38–40] are valuable resources.

The most constant characterization checklist includes matrix and basement membrane structural components (collagen, fibronectin, laminin, and elastin to a lesser extent) and markers of successful decellularization (DNA analysis and absence of nuclear material on histology). Quantitative analysis mostly includes collagen and DNA contents. Accordingly, several protocols resulted in complete preservation of collagen with total/subtotal removal of DNA material; examples include [10, 15, 32]. In case of incomplete removal of DNA, many reports met the criteria previously established for decellularized biological membranes [4]: the absence of visible nuclear material on histological examination using 4′,6-diamidino-2-phenylindole (DAPI) or H&E, the presence of <50 ng dsDNA per mg ECM dry weight, and <200 bp DNA fragment length [4, 41, 42]. Structural components, other than collagens, including elastin [16, 32], reticulin [32], fibronectin, and laminin [24], were less commonly quantified. Few studies showed that elastin was significantly lower in human DLM (hDLM, around 20%) [16], and rat DLM (20–40%) [22] compared to controls despite efficient preservation of collagen. Other ECM components that were evidently affected by decellularization protocols are the glycosaminoglycans, with highest preservation around 77% of native liver [18, 22, 32]. Based on their vital biological roles in cell growth regulations and matrix assembly [43], protocols achieving their maximal preservation are desired. A certain extent of deterioration is, however, reasonable due to their plasma membrane and intracellular components. Regarding growth factors (GFs), an essential component of the bio-functional DLM, Soto-Gutierrez et al. [27] showed the preservation of more than 50% of hepatocyte growth factor (HGF), and around 40% of basic fibroblast growth factor (bFGF). Interestingly, Struecker et al. [18] reported a higher level of HGF in rat DLM compared to native liver. Despite this, the exogenous supply of GFs is feasible during the bioreactor-conditioning phase.

The optimal way of comparison between the test and control samples remains a fundamental issue that needs to be addressed and standardized. Normally, cell removal from hepatic tissue results in collapse of the corresponding cellular spaces and consequently a more densely packed ECM on histological and scanning electron microscopy (SEM) analysis. As a result, more intense staining and marker expression can be expected on histological analysis per unit

area. Mattei et al. ([37], preprint) demonstrated a method for ECM collapse compensation during histological assessment using image analysis. The technique is more easily applicable in porcine liver due to the prominent interlobular septa. Meanwhile, the weight of removed cellular material is compensated by ECM components during quantitative biochemical analysis, resulting in frequently reporting DLM collagen content per unit weight to be higher than native liver, which is obviously an exaggeration. Thus, the accuracy of comparison is compromised for all the quantitative parameters. However, these DLM quantitative analysis figures do represent the true DLM biochemical characteristics as absolute values. For comparison purposes, the use of pre- instead of the post-decellularization weight in quantitative measures can be a valid option, but requires the analysis of the whole liver/liver lobe. Although this can be applicable in small animals, it is not practical for human-sized grafts. Using a weight factor based on the pre- and post-decellularization weights neglects the loss of ECM components and assumes uniformity of decellularization. The authors believe that using predefined anatomical territories or pre-decellularization marked volumes, with pre-decellularization volume-to-weight conversion being a feasible option but lacks validation.

Architectural analysis of the collagen fibers is another crucial aspect of structural integrity beyond mere chemical analysis. It is mostly elucidated using SEM. Satisfactory results were frequently obtained (e.g., [15, 16]). Validation of complete architectural integrity depends on the appreciation of the delicate ECM organization, which in turn guides cell homing, provides the functional niche for hepatic cell populations, and maintains the hepatic acinus gradation. Mazza et al. [16] demonstrated the preservation of delicate ECM architecture in hDLM including the portal triad micro-anatomy, lobular arrangement, and framework of empty hepatocyte spaces on high magnification. Maghsoudlou et al. [22] measured the size of hepatocyte pockets, a fine refinement for SEM analysis. The authors reported a pocket size of 20.9 ± 0.5–11.3 ± 0.3 μm depending on the decellularization protocol. The appreciation of the location of ECM components by immunohistochemical techniques relative to normal liver, rather than validating their presence, has become increasingly evident in recent studies and is integral to the concept of hepatic acinus zonation [15, 44].

The authors did not observe the utilization of scoring systems as a conjugate to histological techniques and SEM. This can provide quantitative outcomes of decellularization and architectural preservation. On the other hand, scoring systems were developed in case of kidney decellularization. Caralt et al. [45] developed two semi-quantitative scores to compare different kidney decellularization protocols for basophilia and architectural preservation. The scores were applied in relation to glomeruli, tubules, and vasculature. Each component was assessed in 5–10 high-powered fields per histological section. This type of scores does not only reflect the efficiency uniformity throughout the specimen and the differential effect of decellularization protocol on tissue components but also allow for comparison between different studies. The use of image analysis for histological comparison between DLM and native liver ([37], preprint), and the appreciation of decellularization homogeneity [18] were the only close reflections of this concept in WLD.

Surprisingly, Gal epitope, a main barrier to xenotransplantation, is not a frequent characterization parameter in wDLM. In one study, Gal epitope showed remarkable reduction in

porcine wDLM by immunohistochemistry [32]. The study also demonstrated the absence of swine leukocyte antigen DR-alpha, swine leukocyte antigen-2, Sus scrofa cytochrome B, and porcine beta-actin in DLM compared to native liver. Generally, investigators utilize the DNA content and histological cellular appreciation as markers of efficient decellularization. This does not exclude the presence of antigenic cell components. In fact, Gal epitope was detected in several decellularized matrix products [46], however, with no clear adverse effect on the final remodeling, suggesting a threshold level for adverse outcomes. In view of the short-term graft survival of wDLM in in vivo studies, investigating the contribution of Gal epitope in this cascade is essential. Meanwhile, mass spectroscopy is increasingly being utilized for ECM characterization in recent studies. Recently, White et al. [12] used time-of-flight secondary ion mass spectroscopy (ToF-SIMS) to analyze the surface of decellularized porcine urinary basement membranes; results showed fragment remnants of SDS, Triton X-100, SDC, and cellular material in the form of phosphate and phosphocholine peaks, depending on the decellularization protocol used (with variable dsDNA content). Although these results are protocol dependent and the tissue source is different, the findings propose ToF-SIMS as a valuable tool for the assessment of decellularized materials.

Because the liver is neither a weight-bearing nor a contractile organ, it could be assumed that mechanical properties of the scaffold are not necessary for the physiologic functions of the engineered liver. However, Engler et al. [47] showed that the mechanical properties of the substrate can guide the differentiation lineage of stem cells. Lozoya et al. [48] further investigated the mechano-sensitivity principle on hepatic stem cells; variation in E-cadherin expression in response to alterations in mechanical properties was demonstrated by embedding in hydrogel with different mechanical parameters, suggesting a role of substrate consistency in guiding the cell remodeling and organization. Hepatocytes also showed better viability and function with the use of perfusion bioreactor, or trans-well devices mimicking sinusoidal circulation, when compared to conventional culture. The superior results with dynamic perfusion techniques can, however, be as well explained by better oxygen/nutrients delivery [49–51]. Hsu et al. [52] demonstrated that better hepatocyte viability and functionality are achieved with lower parenchymal pressure in a liver-assist device with a parenchymal chamber design. The biomechanical environment of the liver thus includes the substrate structural properties, as well as the pressures induced by the blood flow and interstitial fluid (although further investigations are necessary to delineate their effects). Another contributor to the hepatic mechanical environment is the intra-abdominal pressures during the respiratory cycle. The increased intra-abdominal pressure during inspiration squeezes the blood through the hepatic veins, and alternatively lower intra-abdominal pressure during expiration results in increased portal venous flow; this alternating cycle of squeeze/aspiration (exemplified as a sponge) was reported to improve the hepatic microcirculation and perfusion [18, 53]. Therefore, achieving an optimal mechanical environment for cellular differentiation, proliferation, and function relies on both the decellularization protocol, which determines the substrate mechanical properties, and the bioreactor conditioning, which supplies the dynamic component of the mechanical environment. Few studies investigated the effect of decellularization on mechanical properties of DLM. Evans et al. [54] compared the mechanical properties of perfused native liver and DLM of ferrets at the tissue and cellular levels. The study demonstrated a

significant reduction in the liver stiffness by the decellularization process with a long-term Young's modulus at tissue level of 10.5 kPa in native liver versus 1.18 kPa in DLM, and 4.4 kPa in native liver versus 0.91 kPa in DLM at the cellular level. Mattei et al. [24] also compared matrix stiffness between native porcine liver and porcine DLM and observed significant reduction in the compressive elastic modulus after decellularization (1.62 ± 0.13 kPa for native liver compared to 1.25 ± 0.07 and 1.31 ± 0.09 kPa for two different decellularization protocols). This protocol independency implicates the cell removal as the cause of stiffness reduction, as concluded by the study's authors [24]. Sabetkish et al. [21] demonstrated comparable tensile-testing parameters of rat and sheep wDLM compared to native liver; the maximal load was more similar to native liver when Triton X-100 + SDS were used compared to SDS. The ability to maintain equivalent mechanics in the absence of cells is logically questionable; a more realistic approach can obtain comparable characteristics after recellularization. Characterization related to vascular integrity and infectious potential of wDLM is discussed later in Sections 8 and 11, respectively.

In parallel to the standardization of DLM protocols and the characterization of scaffolds' fine constituents, progress in the correlation of DLM parameters with the recellularization, cell behavior, and in vivo outcomes is necessary for efficient feedback tuning. Klaas et al. [55] demonstrated alteration in ECM composition in regenerating liver after liver damage and its contribution in tissue remodeling. The current concept is that maximum preservation of ECM is desired; a deeper understanding based on cell/host-ECM interaction can lead to a tailored approach that can be suitable for the developing liver and fit the cell types used for recellularization. Although tissue sampling is feasible, the development of nondestructive techniques for scaffold characterization is highly desired as the progress continues toward in vivo experimentation. The study by Geerts et al. [56] is perhaps the most relevant in this context, in which the authors used CT and perfusate analysis as nondestructive tools to assess decellularization parameters. DNA levels showed good correlation with the liver Hounsfield unit, and perfusate analysis allowed the assessment of the degree of GAGs' depletion. Nondestructive characterization is also available for vascular analysis. Besides magnetic resonance imaging (MRI) and conventional computed tomography (CT) angiography [21], Gessner et al. [15] reported a nondestructive imaging technique to evaluate the DLM's vascular patency, leakage, and flow rates using ultrasound modalities. The application of this technique to human-scale liver, however, was not yet elucidated. Hagen et al. [57] reported the application of X-ray phase contrast computed tomography (PC-CT) as a nondestructive tool for the assessment of anatomical details of the scaffold and reflected some microarchitectural parameters; the technique is also quantitative.

## 5. Intrinsic DLM potentials

The decellularized matrix potentials can be mainly attributed to its bio-inductive and biomechanical properties [5]. The authors find this functional categorization (bio-inductive/biomechanical) most suitable for simplification of the whole-organ ECM concept, although the mechanical properties contribute to the bio-inductive potentials as previously discussed.

The main hypothesis is that the liver ECM—although neither the main nor the viable component of the liver—is able to provide a framework to harness the heterogeneous cell populations of the native liver, maintain their function, and guide their maturation, differentiation, and the graft regeneration process.

Before discussing the evidence and shortcomings regarding this assumption, a major conceptual question should be raised, namely how long should the DLM provide these functions? In vivo studies of ECM-derived membranes for wound healing and tissue reconstruction demonstrated the dynamic interaction between the ECM material and the host environment resulting in initial degradation followed by remodeling. Small intestinal submucosa used to repair canine Achilles tendon was 60% degraded within 1 month, and complete degradation occurred in a 3-month period as host cells took over ending in successful remodeling [58]. In other words, the decellularized scaffold largely acts as a catalyst and guide for the process of cell migration and initial proliferation, thus a remodeling process can be initiated. In this paradigm, the ECM degradation acts as a release mechanism for bioactive molecules and thus is required for optimal host interaction [38]. However, the concept of biodegradation is not clarified in case of whole-organ scaffolds due to the limitations facing the in vivo experiments. As a result, the fate of the recellularized whole-organ scaffold remains rather vague, taking into consideration that the DLM serves as a transplantable functioning cell reservoir rather than simply guiding the healing process as in case of membrane or tissue repair scaffolds.

DLM-functional expectations include the ability to provide the volume and support for efficient cell engraftment, for a heterogeneous group of cell types (discussed in Section 6), and to grant a bio-inductive environment for their initial proliferation and function (in a bioreactor in case of ex vivo prepping, or in the recipient in case of auxiliary liver transplant). Studies have indeed showed that perfusion DLM proved superior or at least equivalent to regular culture conditions or collagen-sandwich technique in terms of hepatocytic functions and gene expression. For example, immortalized human fetal hepatocytes (IHFHs) showed 2.5–3.5 times increase in mRNA expression of albumin (Alb) and alpha-1-antitrypsin (AAT) after 7 days of culture on DLM compared to regular culture conditions [59].

On the other hand, in vivo studies highlighted the transient functionality of the recellularized DLM (r-DLM). Of significance in this regard are the studies in which seeded DLM slices have been implanted in immuno-compromised animal models, as the adverse host immune response and complex vasculature can be largely excluded, and as a result a more clear appreciation of the in-built DLM potential is achieved. Zhou et al. [59] showed that the activity of IHFH-seeded DLM decreased gradually reaching 2.65% at 8 weeks using luciferase/bioluminescence in vivo monitoring in immune-deficient mice, which proved superior to both IHFH-seeded-Matrigel implants and splenic infusions. In the same study, primary hepatocytes remained viable and functioning on DLM slices in vivo for 6 weeks as shown by green fluorescent protein (GFP) labeling and polymerase chain reaction (PCR) analysis of the explanted scaffolds, where functions comparable to freshly isolated hepatocytes were observed. The change of the primary hepatocytes functions along the 6-week duration was not, however, detailed. Microscopic examination showed the migration of GFP-negative cells into the scaffolds, the nature of which was not also demonstrated. Migrating cells were observed after

cross-species implantation of hDLM in another study [16]. This time, negative smooth muscle actin (SMA) staining suggested a fibroblastic nature. The temporary function of r-DLM in vivo raises doubts about the inherent abilities of DLM, the core of the WLD hierarchy. A suboptimal construct oxygenation and neovascularization, single-cell-type seeding, and the absent role of positive immune modulation may provide other explanations. The visualization of DLM as a slow release device for bioactive molecules obviously suggests that the inherent stores are gradually drained and therefore required to be replenished by either the seeded cell populations or host contributions in which a positive immune response can be an effector. In this context, the aforementioned fibroblastic infiltration should be considered. It is worth mentioning that different liver cell populations are implicated in the process of ECM production during fibrosis [60]. A study also demonstrated that the behavior of hepatocytes and stellate cells in this regard varied in isolated versus co-culture conditions [61].

The gradient nature of the hepatic acinus ECM is largely overlooked in decellularization experimental studies. Briefly, the ECM components vary from Zone 1 to Zone 3 of the hepatic acinus and therefore provide variable micro-environments for intrahepatic cell populations. Consequently, hepatocytes from different zones showed variation in size and enzymatic functions [62–64]. Zone 1 (periportal) ECM provides the suitable environment for hepatoblastic nature where proliferation is promoted, while Zone 3 ECM (pericentral) promotes cell differentiation. This gradient was evident in ECM components of the space of Disse, in which the Zone 1 ECM resembles fetal/neonatal composition while Zone 3 ECM resembles adult composition [65]. Thus, this delicate ECM gradient allows for corresponding transition of cells from undifferentiated/progenitor cells to hepatoblast-like cells and ending in differentiated hepatocytes toward Zone 3 region. In vitro, ECM components like laminin, collagen III, and collagen IV (Zone 1 components) stimulated cologenic expansion of human hepatic progenitor cells, while cell arrest and hepatocytic differentiation were induced by collagen I which is a component of Zone 3 [63]. The appreciation of zonal gradient should be more evident in the design and interpretation of liver decellularization-recellularization studies. Hypothetically, a whole-liver model should preserve the ability to finely tune the functions of different cell population and therefore has to maintain the differential zonal composition. Gessner et al. [15] showed that Hep3B cell line seeded into decellularized rat livers demonstrated zonal-related markers expression. Only cells in zone 1 expressed epithelial cell adhesion molecule (EpCAM), which is a marker of undifferentiated/progenitor cells, compared to higher albumin expression in Zone 2 and 3 regions. Characterization of the used rat scaffold also showed the preservation of matrix components in their normal zonal location [44]. Therefore, it can be concluded that delicate decellularization protocols can not only preserve a certain zonal structural gradation but also the respective zonal potentials. The extent of this preservation remains unverified. Klaas et al. [55] also showed that differential changes occur in ECM composition throughout the zonal distribution in response to damage and guide the remodeling process. Establishment of this understanding paves the way for tailored ECM approaches. On the other hand, this gradient of hepatocyte maturation may not only be a function of ECM composition but also has been classically linked to oxygen gradient. In a recent review, Kietzmann proposed interplay between oxygen gradient, beta-catenin signaling, and hedgehog pathway to underlie the classical acinus zonation [66].

As aforementioned, studies highlighted the influence of the mechanical properties of the scaffold on cellular behavior and subsequently contribute to the intrinsic bio-inductive arsenal. With this understanding, should an optimum human-scale liver scaffold resemble adult liver regarding its composition and mechanical aspects or rather the fetal and regenerating liver properties? If biodegradation and remodeling is desired, should the DLM preserve almost 100% of its chemical constituents? Should matrix-stabilizing techniques be used? Answers to these questions remain largely vague. Another issue to be considered is the discrepancy in the ECM composition/architecture between human and animal liver tissue. The porcine liver ECM is most consequential as it is considered the model capable of providing human-scale liver scaffolds. Indeed, the porcine liver is classically demarcated from human liver by its complete interlobular septa [67]; comparative analysis of ECM composition in regard to architecture and zonation is, however, still lacking. Accordingly, differences in interaction of porcine-derived DLM with human-derived cells may exist.

## 6. Recellularization

The ability to repopulate the DLM with human cells is an integral part of the WLD scheme. Animal-derived cells were frequently used to test the WLD hypothesis and improve the techniques. However, the repopulation with human cells is essential for clinical application for immunological and functional reasons. In order to simplify the process, recellularization will be discussed in terms of cell sources, recellularization technique, and optimal cell mass/density.

The liver is composed of a heterogeneous cellular environment, including hepatocytes, hepatoblasts, endothelial cells (ECs), progenitor cells, fibroblasts, Kupffer cells, undifferentiated cells, and cholangiocytes. An ex vivo-engineered liver should contain all of these cell populations whether by seeding them as differentiated cells or as undifferentiated/progenitor cells along their respective cell lineages and allowing them to differentiate in ex vivo bioreactor setting. In vivo differentiation, although a hypothetical alternative, may lead to blood sequestration issues if the scaffold is insufficiently recellularized due to void intra-matrix spaces.

Different cell sources including cell lines and/or freshly isolated cells were used in recellularization studies to demonstrate the efficiency of the technique, cyto-compatibility of the scaffold, and its intrinsic ability to maintain cell functions or guide the differentiation process. Some of these cell sources are, however, not suitable for clinical application, and others face evident limitations regarding in vitro expansion. As an example, fetal hepatic cells were used to demonstrate the feasibility of DLM recellularization and intrinsic potential of DLM to maintain hepatocyte-specific functions including urea and albumin production [8, 21, 29, 59, 68]; they are, however, not suitable for clinical application [69] but can be used for liver-support devices [70]. Primary hepatocytes were frequently investigated in recellularization studies [69] and on the functional level are ideal on the background of hepatocyte transplantation research. The main source for primary human hepatocytes is the harvested livers that were found unsuitable for transplantation, which represent a very limited resource [71]. The difficulty with 1ry

hepatocyte propagation in cell culture is another limitation [72], taking into consideration the minimum number of cells required for clinically valid engineered liver (discussed later). On the other hand, the use of stem cells, whether of embryonic origin (ESCs) or induced pluripotent stem cells (iPSCs), offers several attractive advantages including the capacity to differentiate into various cell lineages and therefore covers the spectrum of parenchymal, vascular and stromal cell components, feasibility of in vitro expansion, and the suitability for patient-specific recellularization approach (in case of iPSCs where the patient's autologous cells can be utilized). However, wDLM recellularization with ESCs and iPSCs, and the potential of DLM to efficiently drive the differentiation into the different cell lineages (with or without differentiation cocktails) remain largely uninvestigated [69]. Despite that mesenchymal stem cells (MSCs) offer a differentiation potential along a more restricted range of cell lineages, they provide a source for stromal components, chemokines, and cytokines. Jiang et al. [13] used bone marrow MSCs to repopulate DLM and showed their hepatic differentiation with hepatocyte-related expression profiles; the recellularized DLMs were able to rescue a model of hepatic failure after their in vivo implantation. The aforementioned cell types can be used alone or in combination to repopulate the liver parenchyma. In case of repopulation with 1$^{ry}$ hepatocytes, the seeding of other cell components is necessary for vascular endothelialization and to reconstitute the hepatic stromal compartment. On the other hand, stem cells may be able to repopulate the three compartments (parenchymal, vascular, and stromal), confirming that these assumptions are, however, still required. Cell sources used for vascular re-endothelialization are discussed in Section 8. Stromal cells including mural cells, stellate cells, and Kupffer cells all share in the hepatic microenvironment, and therefore it is necessary to reestablish them. Baptista et al. [29] showed that the use of fetal hepatic stellate cells with fetal hepatocytes for recellularization is superior to hepatocytes alone. Cells that are not naturally native to human liver may also represent an addition to the recellularization armamentarium. Examples of these cells are the regulatory T-cells (tREGs) that are increasingly investigated for their role in immunomodulation of xenograft rejection and may therefore be a valuable conjugate [73]. A degree of host-cell migration into the wDLM is possible, which can share in replenishing the stromal compartment or the remodeling process. Few studies demonstrated the migration of host cells into the DLM slices, but the nature of these cells was not fully elucidated [32, 74]. Mazza et al. [16] showed the migration of SMA −ve host cells (probably fibroblasts) into human DLM implanted in rats. The fate of migrating host cells and their contribution in DLM remodeling requires further investigation.

Regarding in vivo recellularization, Sabetkish et al. [21] compared in vitro recellularization with in vivo recellularization of rat and sheep perfusion DLM placed in the sub-hepatic region. The authors concluded that in vitro recellularization is superior to the in vivo approach. Histological examination after 8 weeks showed an evidence of angiogenesis, binuclear cells, fibroblasts, and inflammatory cells, with more superior findings with one of the decellularization protocols (Triton X-100 + SDS) and homografts. Liver enzymes were evidently inferior to both in vitro recellularization and native liver [21]. Bao et al. [7] observed a tiny number of PECAM-1 and von Willebrand factor (vWF)-positive cells in non-endothelialized wDLM after in vivo implantation (that lasted 72 h). The study's authors suggested that the cells may have extended from the portal system. However, the in vivo approach may not be suitable

for vascularized grafts unless thrombosis can be efficiently prevented. Vascular thrombosis was reported to occur in 20 min after non-recellularized DLM transplantation. Blood sequestration in the empty matrix spaces is another concern [32]. In fact, the urgent need for organ replacement in acute liver failure and the debilitated general condition of end-stage liver failure patients make the in vivo recellularization clinical scenario unclear.

The two main recellularization techniques are vascular infusion and direct parenchymal injection. Despite that a report by Shirakigawa et al. [23] showed that directly injected hepatocytes/spheroids (in gelatin) were able to reach a peri-vascular location in the DLM, the vascular pathway was the most commonly used for whole-organ recellularization as it allows for uniform distribution of infused cells and can be suitable for recellularization of all hepatic compartments (parenchymal, stromal, and vascular). Soto-Gutierrez et al. [27] showed better engraftment of cells using vascular perfusion compared to direct injection (≥67 and 13%, respectively). Cell seeding through the portal and hepatic venous systems was the most commonly used with variation in resulting spatial cell distribution. Generally, portal seeding results in higher peri-portal engraftment, while hepatic venous seeding results in higher peri-central engraftment [29]. The simultaneous use of both routes can thus enable better distribution [29]. Hassanein et al. [75] demonstrated that neonatal cell slurry seeded through the biliary tree repopulated the parenchymal regions, CK-7 positivity also marked the presence of cholangiocytes. A comparison between biliary and portal seeding showed that more cells entered the parenchyma with the biliary approach (80% vs. 20% only) [76]. Regarding the infusion technique, stepwise infusion with periods of 10–15-min static resting intervals has repeatedly achieved better engraftment outcomes (≥86% vs. 70% engraftment, respectively) [10, 11, 13, 15, 27], implying the importance of stasis for engraftment. Several perfusion cycles can, however, be required to achieve the desired cell mass.

It is rational that the seeding of whole DLM requires the use of a perfusion bioreactor, as this setting will be mandatory for nutrients and oxygen delivery to seeded cells once the recellularization is undertaken apart from the technique of seeding (bioreactor conditioning is discussed in a separate section). The way the infused cells reach the intra-matrix spaces is yet controversial. Baptista et al. [29] suggested that cell migration occurs through the gaps caused by decellularization detergents that render the vascular wall permeable to infused cells or through selective matrix binding. Interestingly, in a study by Gessner et al. [15], the authors showed that recellularization was successful despite the integrity and non-leakage of the vascular network for 1–5-μm micro-bubble contrast material. The authors postulated that cells leave the intact vascular system through a technique similar to hepatocyte transplantation, where migration into the liver parenchyma was shown to occur through sinusoidal endothelial disruption which provides sufficient spaces for cell migration into the parenchyma [77]. Similarly, the authors suggested a sequence of cell attachment and squeeze through the sinusoidal fenestrations [15]. For an overview of decellularized organ recellularization studies, the review by Scarritt et al. [69] is an excellent resource.

Despite the aforementioned advances with recellularization trials, a recellularized liver cell mass/density valid for clinical transplantation is yet difficult to achieve. To understand the recellularization targets, it is necessary to elaborate regarding the minimum requirements for

clinical transplantation. Thus, the authors believe it is necessary to differentiate between three parameters, namely "functional" cell mass, graft size, and cell density. In a thoughtful review by Caralt et al. [62], the authors suggested that a cell mass equivalent to 5–10% of host's liver weight is the minimum requirement of scaffold cellular content based on the results of clinical hepatocyte transplantation in patients with acute liver failure. It should, however, be clarified that in hepatocyte transplantation, an intact liver is preserved and transplanted hepatocytes function partially by stimulating liver regeneration besides sharing in the repopulation process (the latter considered as the main mechanism) [78] and therefore may not exactly mimic the seeded-scaffold situation. The aforementioned cell mass was also advocated for acute liver failure and not standardized for all transplantation indications. On the other hand, it is widely accepted as a standard for clinical liver transplantation to use graft weight/recipient weight (GRWR) ratio of 0.8% as the minimum for transplantation. Meanwhile, the 0.8%-GRWR graft possesses native liver hepatocyte density and functions. Accordingly, a 60-kg individual will require a minimum graft weight of 0.48 kg. Hepato-cellularity assessment showed that human liver contains $139 \pm 25 \times 10^6$ hepatocytes/g [79]. Therefore, successful engraftment of 49,920–78,720 billion hepatocytes may be required to achieve comparable hepato-cellularity (compared to approximately 10 billion hepatocytes if a cell mass equivalent to 5–10% of human liver weight is required), provided that the cell functionality is similar, otherwise a higher number may be necessary. To date, functional parameters of recellularized wDLM equivalent to native liver were not achieved [10, 21]. The authors of this chapter believe that clinical liver transplantation calculations may represent a closer estimation for the whole-organ-engineering requirements and are more generalizable to various transplantation indications in the context of orthotopic liver transplantation. To further augment this, portal implantation of recellularized DLM containing approximately 10% of total liver cell mass could not achieve long-term survival in 90% hepatectomized rats ($n = 40$), despite prolonging survival from 16 to 72 h [7]. The 10% rule may, however, be applicable if auxiliary liver transplantation or bridging is desired. The short-term failure in [7] can also be attributed to suboptimal function as an alternative explanation.

The second parameter is the optimal cell density. Human liver contains $1.23 \times 10^8$ hepatocytes' nuclei per milliliter of liver tissue compared to $1.69 \times 10^8$ in rat liver [80]. A study using porcine hepatocytes in bio-artificial liver demonstrated a cell density of $5 \times 10^6$ cells/mL to be optimal for most of the functional parameters [81]. Interestingly, hepatocytes demonstrated lower functional profiles and viability at low densities, while a density of $5 \times 10^6$ or higher was associated with superior parameters in alginate scaffold [82]. Thus far, equivalent densities to native liver could not be achieved considering the inferior cell numbers per gram of recellularized tissue ([7] for example), which can be logically considered to have at least an equal volume to a gram of native liver. The delivery of high concentration of cells can also face technical issues in the form of vascular blockage [62] and insufficient oxygen/nutrients delivery.

Based on the inferior cell density and intra-scaffold function, the graft size in case of recellularized DLM will be consequently larger than that for clinical transplantation. Finally, many parameters regarding recellularization efficiency and substrate potentials require considerable improvement; in addition, the limited cell sources are considered a major hurdle facing the recellularization concept. Dependency on harvested livers deemed unsuitable

for transplantation as the source for primary hepatocytes is insufficient. Therefore, the development of alternative cell sources including undifferentiated cells/progenitors, the technical refinement of in vitro cell expansion, and bioreactor conditioning is necessary to achieve clinical relevant cell mass.

## 7. Immunologic aspects

The efficient removal of xenoantigens by the decellularization process and thus evading a destructive rejection cascade are imperative to the success of WLD. Repopulation of the scaffold by autologous or allogeneic cells is designed to allow the use of no or routine immunosuppressive therapy, respectively. The oligosaccharide $\alpha$-Gal (Gal$\alpha$1,3-Gal$\beta$1–4GlcNAc-R; Gal epitope), which is mainly found as a cell membrane antigen, and xenogeneic DNA are considered the main antigens stimulating the rejection cascade for xenogeneic biomaterial [46].

Studies showed that commercially available decellularized biologic scaffolds and prosthesis retain a variable amount of Gal epitope. An increase in anti-Gal antibody levels was also noted after transplantation of xenogeneic material [83, 84]. The use of galactosidase resulted in a decrease in T-lymphocytic infiltration in porcine cartilage grafts [84]. Ex vivo exposure of small intestinal submucosa (SIS) of porcine origin to human plasma showed predominant IgG2 fraction conjugation; Gal epitope was shown to stimulate the same fraction [85, 86]. However, complement activation did not occur, probably due to the low density of the epitope [87, 88], suggesting a threshold for adverse outcomes. Raeder et al. [89] showed that the implantation of SIS in Gal knockout mice resulted in the formation of anti-Gal antibodies; furthermore, pre-sensitization with sheep erythrocytes resulted in more intense early inflammatory cellular infiltration. Despite that these findings highlight the retention of a variable amount of Gal epitope after tissue processing, the host response did not affect the final remodeling outcome. On the other hand, analysis of commercially available ECM products also showed the presence of DNA material [90, 91]. Although remnant DNA was shown to drive an inflammatory process, the clinical success of these materials implies that adverse host responses are also unlikely to occur below a certain threshold amount of DNA fragments retained. The ECM biodegradation process should normally include the remnant DNA content as well [46]. Crapo et al. [4] suggested criteria for remnant DNA that are necessary to avoid an adverse inflammatory/immune response and therefore allow graft remodeling; these criteria are the absence of visible nuclear material on histological examination using 4′,6-diamidino-2-phenylindole (DAPI) or H&E, the presence of <50 ng dsDNA per mg ECM dry weight, and <200 bp DNA fragment length [4, 41, 42]. These criteria were frequently met in WLD studies. A recent study demonstrated complete removal of DNA from human DLM [16]. However, Gal epitope analysis is not routinely included in DLM characterization panel as aforementioned. Bao et al. [32] demonstrated remarkable reduction in Gal epitope compared to native liver by immunohistochemical staining. Although both the consistent efficiency of the decellularization outcome ([37], preprint) and the complete removal of all cellular components [4] are debated, the current decellularization parameters of wDLM were enough for the clinical success of the decellularized biologic membrane scaffolds [4].

Transplantation of porcine organs into primates results in rapid innate immune response driven by natural antibodies, which bind to vascular endothelium and result in complement activation and hyper-acute rejection (HAR). An innate cellular response comprising macrophages, neutrophils, monocytes, and natural killer cells coexists. Innate cellular immune response results in the development of a delayed form of rejection commonly named acute vascular rejection (AVR) if HAR was successfully evaded [35, 73]. The extent of adaptive immune response and the contribution of T-cell in acute cellular rejection (ACR), a classical component of allo-response, are, however, controversial [73].

Few in vivo studies have examined the immunologic response to perfusion DLM slices across species. The first [74] examined a pig DLM to rat xenotransplantation model and rat DLM to rat allotransplantation model, where DLMs were implanted in the subcutaneous dorsal adipose tissue. The specimens showed no capsulation, exudation, or a noticeable adverse host response in the adjacent tissue in both models along 28 days. The total WBCs count did not show a significant increase as well over 28 days. Although cellular infiltration was evident after 7 and 28 days, low to no $CD3^+$ T-lymphocyte activation was noted, the infiltrating cells showed positivity for the pan-macrophage marker (CD68), but neither M1 nor M2 phenotypic markers were evident. The second [16] examined a human DLM to rat xenotransplantation model. Cubic DLM fragments were implanted both subcutaneously and in the omentum. Mild inflammatory response was observed in the surrounding tissue after 7 days in the form of polymorphonuclear (PMN) cells and lymphocytes infiltration, while reduced or no inflammation was detected after 21 days. Analysis demonstrated predominance of CD3+ T-cells. SMA −ve cells (probably fibroblasts) were observed at the time of explantation. In both aforementioned studies, scaffolds were well characterized regarding the DNA content and structural components, and no adverse immune response was observed. On the other hand, Sabetkish et al. [21] reported inferior results of in vivo recellularization of xenografts (sheep to rat) compared to homografts (rat to rat) after sub-hepatic implantation of DLMs; xenografts also showed more marked inflammation and fibrosis.

It is important, however, to highlight that the biological membrane and in vivo DLM slices studies may not accurately mimic the immune response to bioengineered whole-organ transplants for four reasons: (1) the techniques of decellularization and processing of biological membranes differ from that for DLM; therefore, host responses can vary. A comparison of host responses to five different ECM products—four of them were xenogeneic—showed a considerable variation in host response, explained by their different processing techniques [46, 92]. (2) Studies demonstrated the difference in immune response between vascularized and non-vascularized grafts represented in the antigen immune dominance and the strength of indirect allo-response [93, 94]. Although these findings are related to HLA antigens, which have less/no significance (apart from the current debate) in clinical liver transplantation and obviously no direct link with decellularized material, a discrepancy in the response may still exist. The presence of Gal epitope on vascular endothelial cells is in fact considered the main drive for the hyper-acute rejection of xenotransplants [46, 95–97], and this cannot be elucidated using DLM slices. The three previous studies used non-vascularized grafts and thus the classical sequence of HAR and AVR (the main pivots of xenotransplantation rejection

cascade) could not be examined. (3) Transient immune/host response considered as benign in the aforementioned studies can be sufficient to induce vascular/sinusoidal thrombosis/blockage in the vascularized model. In vivo studies of xeno-response to vascularized DLMs are still missing. The last two assumptions are yet impossible to investigate due to the short-term graft survival in wDLM transplantation trials, taking into consideration however that an immune/inflammatory response can contribute to this short-term failure. (4) Finally, despite the fact that human-to-rat, pig-to-rat, and sheep-to-rat models are xenotransplantation models by definition, their relevance to pig-to-human xenotransplantation model is largely questionable. The aforementioned xenotransplantation models do not represent Gal-positive to pre-sensitized Gal-negative transplantation models [35]. It would be very interesting to elucidate the immune response in a complement-enriched, pre-sensitized Gal-knockout or non-human primate (old world monkeys) recipient after vascular anastomosis.

Two important approaches should be mentioned in this context because of their capability to largely bypass the host/immune response to xeno-grafts dilemma if all the other parameters were optimized. The first is using human livers for the decellularization process; livers that are found unsuitable for transplantation are good candidates for this approach [16]. However, despite the structural and immunological advantage, this approach does not massively expand the organ pool. The second is using the native liver for an in vivo decellularization process. In a very interesting study, Pan et al. [19] showed the possibility of in situ liver decellularization by constructing a separate perfusion circuit in vivo for one of the rat liver lobes and using it for the decellularization and recellularization sequence. This may represent the optimal approach regarding the immunological aspect and organ conservation; however, it requires healthy ECM and structural integrity (and thus excludes malignancy, cirrhosis, and biliary atresia for example as an indication for transplantation). Also, many parameters should be addressed in the ex vivo setting before transferring them into an in vivo model. Both models will not be further discussed in order to keep the context of xenotransplantation.

## 8. Vasculature

An intact vascular network able to convey oxygen and nutrients to the deeply seated heterogeneous cell populations is perhaps the most attractive feature of wDLM scaffolds due to the difficulty of the artificial imitation of such complexity. Intact vasculature is essential for the transition of ECM applications from membranes/slices to the complex 3D organ format. Meanwhile, thrombus formation and blockage of the vascular network is largely adopted as the explanation for the repetitive failure of wDLM in vivo experiments, even when heparinization is employed.

The liver contains three vascular networks namely the portal venous system, the arterial system, and the hepatic venous drainage, in addition to the biliary system. Although all the routes except for the biliary system have been utilized for perfusion decellularization, the portal venous system remains the most widely used cue for both decellularization and recellularization as discussed previously. Ideal characteristics of the vascular system after

decellularization include the following overlapping parameters: full integrity, sustainable patency, non-thrombogenicity, ability to withstand blood pressures within the physiological ranges without leakage or rupture, complete decellularization, and efficient recellularization. Most decellularization studies include tests for vascular integrity as a part of their characterization panel, including corrosion casting [8, 10, 25, 27], CT angiography [21], fluoroscopy [9, 28, 29], dye injection, MRI [21], ultrasonography modalities, and confocal microscopy [29], or a combination of them [8, 29]. SEM on the other hand is frequently used to demonstrate the vascular architecture and the inter-vascular relations. However, characterization is sometimes restricted to the portal and hepatic venous system as it is the route used for perfusion and presumptively suffers the maximum burden of the decellularization reagents. Nondestructive approaches (e.g., angiography, fluoroscopy, MRI, and ultrasonography) that are applicable in human-sized liver model are more suitable for the clinical approach. The ability to demonstrate leakage, besides structural integrity, is also important [15]. An intact DLM portal venous system has been sufficiently demonstrated in several reports. Gessner et al. [15] demonstrated the patency and integrity of DLM portal/hepatic vasculature using 1–5-μm micro-bubble contrast agent, which did not leak into the scaffold matrix. An advantage of this technique is the validation of sinusoidal compartment integrity. The patency of the arterial and biliary systems has been less frequently demonstrated using the corrosion-casting technique [10, 27]. Their patency/integrity is, however, necessary for clinical application. Although the combination of corrosion casting with SEM can offer some quantitative parameters for the vascular tree [98], the ability of corrosion casts to demonstrate leaks through minute gaps in the vascular wall is questionable.

Blockages by cellular elements or thrombosis can interfere with vascular patency in vivo. The prevention of in vivo occlusion/thrombosis is, however, a complex task and is currently viewed as the bottleneck for the progression of wDLM in vivo experimentation. Herein, the authors try to dissect the confounding factors of this adverse outcome. For the decellularized vessels to be non-thrombogenic, two conditions need to coexist: (1) efficient decellularization/antigen removal, with successful evasion of intravascular inflammation, HAR, and AVR cascades. An inflammatory response with leukocyte recruitment can act as the nidus initiating thrombus formation. Acute rather than chronic inflammation was associated with an increased risk of venous thrombosis [99]; and (2) efficient vascular re-endothelialization. Thus, the exposure of underlying matrix components that can provoke platelet adhesion/aggregation ending in thrombus formation can be prevented. It is thus important herein to refer to the classical Virchow triad of venous thrombosis, including stasis, changes in vessel wall, and blood changes. Incomplete recellularization results in considerable empty matrix spaces and therefore permits pooling of blood and stasis. On the other hand, a suboptimal vascular endothelium falls under the "vessel wall changes" component along with the actual vessel wall damage by the decellularization protocols. Appreciation of the functional parameters of seeded endothelium along with the seeding efficiency, viability, cell attachment, and endothelial distribution is essential. Of special significance is the expression of anticoagulant proteins, which can again be affected by hypoxia and inflammation [99]. Robertson et al. [100] described the application of an ex vivo thrombomodulin assay to assess the anticoagulant functions of seeded endothelium in decellularized heart scaffold. This is achieved through perfusion of

human alpha-thrombin and protein C followed by the assessment of thrombomodulin and thrombin-mediated protein C activity. Seeded scaffolds showed six- to eightfolds significantly higher thrombomodulin and thrombin-mediated protein C activity compared to acellular ones, signifying their capability of coagulation cascade inhibition. Further investigations of the seeded-endothelial-functional profile in wDLM are necessary.

In case of small-caliber decellularized vascular grafts, an animal study demonstrated the patency of most of the small-caliber (1.5-mm inner diameter) decellularized arterial xenografts (without pre-implantation endothelialization) after 4 weeks when used for the repair of carotid arteries [101]. The patency of 4-mm-diameter decellularized vascular grafts was improved by surface heparin treatment resulting in only 8% thrombosis after 6 months in another study; vessels also showed efficient in vivo cellular migration and remodeling [102]. On the contrary, in vivo implantation of non-recellularized porcine DLM resulted in complete vascular occlusion after only 20 min, compared to preserved patency at 60 min when a heparin immobilization technique was used [32]. Several factors including the smaller vascular diameters, the length of the vascular tree, and blood flow dynamics inside the scaffold (e.g., flow rate and turbulence) can result in this discrepancy when the wDLM and decellularized vascular xenograft models are compared. Complete vascular recellularization at the time of implantation is therefore necessary in wDLM [69]. Baptista et al. [29] performed ex vivo blood perfusion in ferret wDLM with or without endothelial cell seeding and reported significantly less platelet adhesion in seeded scaffolds. Human umbilical vein endothelial cells (HUVECs)-seeded scaffolds also demonstrated less leakage compared to non-seeded scaffolds in another study [23].

For parenchymal recellularization, choosing the appropriate cell sources, seeding technique, and maturation process design are paramount to optimal vascular re-endothelialization. Basically, autologous cells should be used for vascular recellularization if a non-immunogenic DLM is the target. However, using allogeneic cells is another option with the use of immune-suppressants, with reference to clinical transplantation. The cells used should possess a considerable proliferative capacity in vitro to allow the repopulation of the entire hepatic vascular surface area. Therefore, other alternative cell sources besides endothelial cells are needed. Bone-marrow MScs, iPScs, and progenitor cells were suggested for endothelialization of decellularized vascular grafts [103]. Another desired characteristic is the ability to differentiate into the different vascular wall components (mainly ECs and smooth muscle cells “SMCs”); otherwise, they should be supplied independently. Interaction with endothelial ECM was demonstrated to guide the endothelial differentiation of MSCs without other stimulants [104]. MSCs from bone marrow and adipose tissue also possess the ability to differentiate into SMCs, rendering MSCs an attractive option for vascular recellularization [105, 106]. The review by Bajpai et al. [103] is an excellent review of stem cell sources in vascular graft engineering.

ECs of different sources were the only cell type investigated for wDLM re-endothelialization. Uygun et al. [10] used microvascular ECs to seed rat DLM previously recellularized by hepatocytes; the ECs were seen lining the vascular elements after 3 days of culture. Baptista et al. [29] seeded ECs into ferret DLM; although evidence of vascular coverage was noted, technical

limitations did not allow the confirmation of complete coverage of the vascular system. In the same study, seeded HUVECs through portal vein showed a distribution of capillary pattern around larger vascular structures. Shirikagawa et al. [23] also demonstrated the seeding of HUVECs into rat DLM. The seeded HUVECs despite not leaking outside the vascular system and attachment to internal surface of vascular element, they were only observed in a limited cavity. ESCs and iPSCs were used for vascular recellularization of other decellularized organs but not DLM [69]. Infusion recellularization is the technique generally adopted for DLM endothelialization. Interestingly, Ko et al. [107] reported a two-step endothelialization technique comprising a static and dynamic phases starting with the static component. The technique resulted in efficient re-endothelialization with the use of antibody conjugation to DLM. The portal vein is the route commonly utilized for this purpose [10, 23, 29]. As previously mentioned, Baptista et al. [29] compared the portal and hepatic venous recellularization approaches for epithelial cells and concluded that using multiple routes can result in better scaffold recellularization. Ko et al. [107] simultaneously used inferior vena cava (IVC), PV, and HA for vascular re-endothelialization of porcine DLM but without comparison to perfusion through a single route.

After the process of vascular recellularization, a period of in vitro maturation in a dynamic bioreactor design is necessary for cell differentiation, expansion, production of ECM, and remodeling before implantation. For decellularized vascular grafts, the maturation can be achieved in 3 weeks [108], in which exposure to physiological cyclic pressures inside the bioreactor setting is a constant core concept.

Bao et al. [32] applied heparin immobilization to a well-characterized porcine whole DLM using three different techniques. End-point attachment technique proved to be the most efficient. Interestingly, thrombosis did not occur in heparin-immobilized DLM after the auxiliary transplantation of the median lobe into pigs, and blood flow continued for 60 min though not endothelialized compared to 20 min in control DLM. The vessels proved to be patent by histological examination at explantation. However, it was not clear why perfusion was not continued after the 60-min period. Also, the arterial system was not anastomosed and the in vitro study showed that heparin is released from the scaffold to reach 3.6% at the seventh day. The authors concluded that heparin immobilization can boost the anti-thrombogenic nature of DLM and showed that it did not interfere with cell seeding. The effect of heparin immobilization on endothelial cell seeding is, however, unknown. The study also highlighted the discrepancy between the outflow and inflow rates, which the authors explained by sequestration of blood inside the DLM. This finding highlights the need for efficient parenchymal recellularization before transplantation, as stagnation of blood in the suboptimally recellularized matrix zones can also promote intra-parenchymal blood clotting and interfere with oxygen and nutrient delivery.

In a trial to maximize endothelialization of wDLM, Ko et al. [107] used anti-endothelial antibody conjugation to porcine wDLM, coupled with endothelial cell seeding. The authors reported efficient re-endothelialization of 80–90% of the extra-capillary vasculature evidenced by green fluorescent protein utilization. The endothelialized scaffolds showed three- to four-folds lower platelet adhesion in vitro and maintained superior vascular patency 24 h after in

vivo transplantation. Lastly, the mechanical strain effected by the blood flow and pressure within the physiological ranges represents part of the mechanical environment of the liver [62]. As aforementioned, mechanical factors can influence the cell behavior and affect the hepatocytic and endothelial cell functions. Therefore, imitation of these physiological pressures in vitro in view of the dual nature of hepatic blood supply can have an impact on vessel wall acclimatization and cell functions.

## 9. Bioreactor conditioning

Once a whole-liver scaffold is recellularized, the use of a perfusion culture is necessary to allow nutrients and oxygen delivery to the depth of the scaffold. The term bioreactor is commonly used to describe the perfusion culture setting that should also include an oxygenation system, bubble trapping, and allow for media replacement and sampling. The bioreactor does not only serve as a temporary viability maintainer till transplantation but is incorporated in many study designs as a station for scaffold preparation and optimization. The bioreactor setting also provides a 3D in vitro culture model to assess the different parameters of cell-ECM interactions.

An optimal scaffold/bioreactor combination should be capable of supplying the most suitable microenvironment for cell proliferation and differentiation of stem/progenitor cells along the hepatic lineages. Defining the parameters of the optimal environment and developing the appropriate techniques to achieve them are thus necessary; neither, however, is a simple endeavor. Mimicking the native human liver perfusion dynamics is generally visualized as most appropriate for mechanical acclimatization and optimal cell functionality. Briefly, the liver receives 75% of its blood supply through the PV with a low flow pressure (4–10 mmHg, non-pulsatile flow), low $pO_2$ (30–40 mmHg), and carries nutrients absorbed from the intestines. The HA supplies the other 25% of blood supply with a flow pressure around 120 mmHg, high $pO_2$ (90–100 mmHg), and a pulsatile flow pattern [62]. Apart from the technical difficulty to achieve such a complex dual perfusion pattern (considering the single outflow and the differential $pO_2$ content at least), the suitability of these parameters for the developing recellularized graft is questionable for several reasons: (1) in the absence of complete endothelial barrier, the shear stress resulting from the high flow pressures can have adverse effects on the hepatocyte viability and function [62]. (2) These defined flow parameters may not be the optimum for the developing liver tissue, in which higher proliferation and maturation activity are expected, or in case of recellularization with stem/progenitor cells. For example, the developing fetal liver receives its blood supply from the umbilical vein, the PV (low oxygen and nutrient content), and the HA, with differential contributions to the right and left hepatic lobes, where portal vein supplies only the right lobe [109]. Therefore, a design based on the cell/target-specific requirements can be more appropriate at the developing stage than mimicking the developed liver parameters. (3) Mimicking natural flow parameters in the perfusion system does not guarantee mimicking natural equivalent values at the cell level due to the discrepancy between the intra-hepatic flow pattern in the decellularized/recellularized graft (e.g., turbulence) and the native liver, besides the use of artificial oxygen delivery modalities. Nishii et al. [110] studied the mechanical

micro-environment in decellularized versus native ferrets liver in an ex vivo setting with portal perfusion (flow rates 3–12 mL/min), and integrated the data into multi-scale computational model. Decellularization resulted in 82% decrease in vascular resistance with mean fluid pressures of 0.6–2.4 mmHg and mean velocities of 250–840 μm/s along four different studied flow rates. The authors also reported a 5.6 times increase in hydraulic conductivity as a measure of tissue permeability in decellularized livers. These findings are of great relevance to the process of perfusion recellularization and bioreactor conditioning. However, further similar studies for recellularized livers are desired as the seeding process can expectedly impact the vascular flow dynamics. In fact, Bao et al. [7] reported portal hypertension and ascites after portal implantation of recellularized grafts rather than decreased vascular resistance, which can result from the seeding process (hepatocyte spheroids in this case). Also, the arterial flow dynamics and the application of similar computational methods in porcine liver need to be explored.

In order to evade high shear stress, most studies adopted a sub-physiological PV flow rate ranging from 0.5 [29] to 15 mL/min [10]. The duration required for conditioning is a function of the cell source/mass and the desired degree of recellularization. More than 1 month can be needed for stem cell differentiation [62]. Decellularized vascular grafts on the other hand require a period of 2–3 weeks for maturation [108]. Despite the development of hepatocyte-specific functions and hepatocyte-related gene expression in the perfusion culture setting comparable to levels in collagen-sandwich culture, they only represented 20 and 30%, respectively, compared to in vivo levels of albumin production and gene expression [10]. Such sub-physiological levels can be explained by either an inferior cell-cell and cell-ECM interaction or an unsuitable bioreactor setting. The failure of a heterotopic rat-transplant model containing around 10% of liver total cell mass to support long-term survival despite sustained native portal perfusion after 90% hepatectomy fortifies the first possibility.

Studies of hepatocyte hypoxia and oxygen tension at cellular level rather than cell viability are needed. Oxygen delivery methodology should also be reevaluated. The development of nondestructive modalities for monitoring cell parameters inside a bioreactor is necessary for continuous troubleshooting. Ren et al. [111] developed a nondestructive technique to assess cell viability in decellularized whole organs based on a resazurin reduction perfusion assay. The application in decellularized rat lung seeded with endothelial cells showed good matching with histology and interestingly showed no significant difference between constant flow rate (2 mL/min) and gradually increasing flow rate (from 2 to 8 mL/min) along 7 days.

## 10. Vascularized DLM in vivo studies

The transplantation of recellularized DLM (vascular/parenchymal) across a physiologically relevant xenotransplantation model through vascular anastomosis is the best tool to collectively test the whole-liver decellularization hypothesis. However, reasons for adverse outcomes in this setting consequently can be multifactorial. A limited number of studies investigated the in vivo vascularized graft survival/function or vascular patency using different combination of the aforementioned parameters. Uygun et al. [10] reported a rat-to-rat heterotopic transplantation

of recellularized DLM (1ry hepatocytes only) using arterio-venous anastomosis for portal flow reconstruction, with harvest after 8 h. Despite retaining the location, morphology, and some functional markers of hepatocytes, the authors observed minimal damage to hepatocytes due to the shear stress caused by arterial flow and minimal ischemic damage. No comments were offered regarding the vascular tree patency and platelet adhesion; anticoagulation was, however, used. Bao et al. [32] reported heterotopic pig-to-pig transplantation of decellularized median lobe (without recellularization) to assess the effect of scaffold heparin immobilization in preventing vascular thrombosis; the portal inflow was established using the left renal vein. Complete vascular thrombosis occurred in controls after 20 min, while patency was sustained for 60 min in case of heparin immobilization. The immunologically relevant status of the donor/recipient match was not elucidated in the aforementioned studies. Bao et al. [7] described a rat-to-rat (inbred) heterotopic recellularized DLM transplantation (hepatocyte only); the DLM was modified by layer-by-layer heparin immobilization before cell seeding. The recipients were 90% hepatectomized before transplantation of the graft, which was implanted in continuity with the portal vein. After 72 h, the hepatocytes maintained morphology, organized into aggregates with vessel-like structures formation, and expressed several liver-related genes and liver-specific functions with minimal apoptosis and detected proliferation marker (BrdU). The authors observed a tiny number of PECAM-1- and vWF-positive cells that may have extended from the portal system. Rats also developed ascites and gastrointestinal congestion due to portal hypertension, which was explained by the authors as small-for-size syndrome. Although the heterotopic liver slowed down the rise of ammonia level and was able to significantly prolong the survival time after hepatectomy from 16 to 72 h, it did not allow for long-term rat survival. The authors did not comment on the extent of vascular thrombosis in this transplantation scenario. However, in the same study, non-recellularized DLM with heparin immobilization was thrombo-resistant for 3 h after heterotopic transplantation. Two very important inferences of this study are (1) if the vascularized DLM can be able to function long enough without thrombosis, it is probable that endothelial cell ingrowth from the nearby vasculature can occur as suggested by the authors [7], a similar fashion to which occurs in decellularized vascular grafts may apply, and (2) the hurdles against in vivo application are not restricted to vascular patency; the function capabilities of recellularized vascular grafts in vivo were also non-optimal (taking into consideration that the graft contained a mean of 10.65% of the whole rat liver cell mass as evidenced by DNA content). Surprisingly, except for the last study, the rationale behind the time frame of the in vivo study was not clearly elucidated. Bruinsma et al. [112] also examined the layer-by-layer heparinization technique. The authors confirmed that recellularization with primary rat hepatocytes was not affected by the process. The heparinized grafts showed no visible clots and better flow during ex vivo perfusion. However, after heterotopic transplantation, the heparin layering did not improve the flow or transplantation outcomes despite lower evidence of thrombosis. A main difference between the last two in vivo studies with heparin immobilization is the 90% hepatectomy performed in the former one, which may have affected the coagulation profile.

Ko et al. [107] on the other hand reported in vivo implantation of efficiently endothelialized porcine DLM making use of antibody conjugation technique. The grafts demonstrated evidently better patency and lower platelet adhesion compared to acellular grafts. **Table 2** summarizes the trials of vascularized DLM transplantation. No immunological studies of vascularized DLM

transplantation yet exist. Further in vivo transplantation studies are highly desired for both isolated and combined parameters. The advances in recellularization and the upgraded understanding of the underlying scaffold and blood-flow mechanical contributions pave the way for in-depth analysis of in vivo experiments.

## 11. Sterilization

A prerequisite for the success of WLD is the efficient elimination of infectious potential of the scaffold to prevent xenosis. The absence of cellular content allows the use of sterilization techniques, since no concerns regarding cellular damage exist. However, validation of the efficiency and cytotoxic effects of individual sterilization techniques is necessary for recellularization and in vivo progress.

Techniques used for sterilization of acellular scaffolds include ethylene oxide (EO), peracetic acid, and ultraviolet and gamma radiations (GRs). Kajbafzadeh et al. [14] compared six different sterilization protocols on sheep DLM including EO, GR, freeze-drying (FD), EO + GR, FD + GR, and PAA + GR. Interestingly, the protocols utilizing a single agent did not achieve efficient sterilization compared to full sterilization by combination protocols. However, combination with FD resulted in inferior mechanical outcome, while EO + GR and PAA + GR had no mechanical influence. Mattei et al. [24] investigated the cytotoxic effects of three sterilization protocols on porcine DLM, including PAA, exposure to chloroform gas, $H_2O_2$ gas plasma, or a combination of the last two agents, all after FD. The study identified PAA and chloroform gas as the best agents in terms of cyto-compatibility.

Bao et al. [32] investigated the effect of decellularization protocol on the infectious potential of porcine DLM. Interestingly, the DLMs were devoid of porcine endogenous retroviruses (PERVs), a major concern in porcine grafts, and PERV polymerase compared to native liver. Sarikaya et al. [113] demonstrated the antibacterial activity of ECM extracts derived from porcine small intestinal submucosa and urinary bladder submucosa against Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli*. Extracts were able to inhibit bacterial growth for at least 13 h. Antibacterial and antifungal medications can also be added to the matrix during the process of perfusion to improve sterilization.

## 12. Large-scale production

It is rational that the success of the clinical trials should be achieved before seeking large-scale production of on-shelf wDLM scaffolds. However, in this section, the authors try to delineate the parameters highlighted by previous studies along the pathway to on-shelf commercialization.

*Animal source*: a consensus regarding the most suitable animal species and age for organ harvesting was not yet reached. However, the most prevalent opinion favors the use of porcine liver. Despite the previously outlined discrepancy in ECM structure between human and porcine

| Study* | Donor | Decell graft modification | Recellularization | Recipient | Technique | Heparinization | Graft survival/ harvest for analysis | Findings | Reasons for study termination |
|---|---|---|---|---|---|---|---|---|---|
| Uygun et al. [10] | Female Lewis rats | None | 1$^{ry}$ rat hepatocytes | Male Lewis rats | After Lt. nephrectomy, LRA to PV, IVC to LRV | 1 mL saline containing: heparin (100 U) + Fab of chimeric monoclonal Ab (0.25 mg/kg) was injected into the penile vein. | 8 h | *Flow:* efflux within 5 min. *Hepatocytes:* minimal damage and ischemia, retained parenchymal position, morphology, hepatic functions (albumin, G6pc, Ugt1a) by IHC. *Thrombosis:* NM | NM |
| Bao et al. [7] | Male Lewis rats | Layer-by -layer self-assembly heparin immobilization (8 BL) | Hepatic cells | Male Lewis rats (90% hepatecto-mized, syngeneic)/ $n = 40$ | Right median lobe only. In continuity with the PV | Systemic heparinization | 72 h | Did not support long-term survival but significantly prolonged survival from 16 to 72 h. PHTN with GIT congestion and ascites. *Hepatocytes:* expressed several liver-related genes and liver-specific functions. Evidence of tiny amount of PECAM-1 or VWF +ve cells. Minimal apoptosis, evidence of proliferation. | Liver failure |


http://dx.doi.org/10.5772/intechopen.69487

| Study* | Donor | Decell graft modification | Recellularization | Recipient | Technique | Heparinization | Graft survival/ harvest for analysis | Findings | Reasons for study termination |
|---|---|---|---|---|---|---|---|---|---|
| Ko et al. [107] | Piglets | Test: conjugation of rat anti-mouse CD31 antibodies and re-endothelialization Control: No modification** | Mouse vascular endothelial cells (MS1) in test group | Female Yorkshire pigs/*n* = 3 per group | After Lt nephrectomy, LRA to PV, IHIVC to LRV | NM | 24 h | *Flow*: after 24 h, test maintained flow at inflow, outflow, and intra-parenchymal. Absent flow into control implants. *ECs* were present within vasculature of test group. *Thrombosis*: maintained patency in test. Complete absence of patency in control except for main portal branches. Significantly lower plt. adhesion in test | Harvest for assessment |
| Bao et al. [32] | Male Bama miniature pigs | Test: Heparin immobilization by EPA Control: No heparin immobilization | None | Pigs/*n* = 12 | Median lobe only, RV to PV, IVC to SV | Before implantation, 100 mL saline with 25 IU/mL heparin was injected through PV into scaffold | Complete vascular occlusion at 20 min in control. Test maintained patency at 60 min | *Flow:* significant difference between inflow and outflow (? Bld sequestration) *Acellular grafts Thrombosis:* complete vascular occlusion after 20 min in control, test patent at 60 min | Control: vascular occlusion Test: NM |

| Study* | Donor | Decell graft modification | Recellularization | Recipient | Technique | Heparinization | Graft survival/ harvest for analysis | Findings | Reasons for study termination |
|---|---|---|---|---|---|---|---|---|---|
| *Bruinsma et al.* 2015 [112] | Female Lewis rats | Test: Layer-by-layer self-assembly heparin immobilization (4 and 8 BL) Control: No heparin immobilization. | $1^{ry}$ rat hepatocytes | Female Lewis rats (syngeneic)/$n = 3$ per group | Median lobe only. After Rt nephrectomy, proximal IHIVC to PV, SHIVC to distal IHIVC | Preconditioned with heparinized dil. whole blood (16.67 U/mL). | 24 h | *Flow:* no significant bleeding, congested proximal IHIVC after 30 min, minimal outflow at harvest in all groups. *Hepatocytes:* NM *Thrombosis:* profuse erythrocytes and thrombosis in control, reduced thrombosis in 8 BL group. | Harvest for assessment |

*Abbreviations*: Ab, antibody; BL, bilayer; Decell, decellularized; Dil, diluted; ECs, endothelial cells; EPA, end-point attachment; IHC, immunohistochemistry; IHIVC, infra-hepatic inferior vena cava; IVC, inferior vena cava; LRA, left renal artery; LRV, left renal vein; Lt, left; NM, not mentioned; plt, platelet; PV, portal vein; Rt, right; RV, renal vein; SHIVC, supra-hepatic inferior vena cava; SV, splenic vein.

*Two other studies [8, 29] demonstrated in vivo transplantation of vascularized grafts, but were not tabulated due to insufficient published post-transplantation data.

**Decellularized matrices contained 20% remnant DNA after decellularization.

**Table 2.** In vivo transplantation of vascularized decellularized liver matrix.


http://dx.doi.org/10.5772/intechopen.69487

liver, porcine liver possesses several significant advantages. These include size/weight matching, animal availability, the ability to define the breeding and environmental exposure, and the availability of established techniques for genetic modification which can be combined with the decellularization protocols.

*Standardization*: standardization is a requirement for both the decellularization and sterilization protocols. Studies of whole-liver decellularization show great technical variability. A trial to define the optimal characterization cutoffs for all ECM constituents based on the in vivo outcomes seems futile at this stage. In a study by Mattei et al. ([37], preprint), the authors showed variation in decellularization efficiency of human immersion/agitation DLM from five donors despite using the same protocol. However, this observation was not highlighted in human perfusion DLM, where standard deviation for DNA content was 5.82 ng/mg [16]. Several studies described a relatively fast decellularization protocols for porcine [18], rabbit [9], and rat livers [25]. The utilization of automated perfusion systems, however, renders the duration of decellularization process a less important parameter, as opposed to the duration required for recellularization and ex vivo prepping.

*Multi-organ decellularization*: Park et al. [114] reported a technique for simultaneous multi-organ decellularization in rat through carotid artery and portal vein catheters. The process resulted in efficient decellularization of heart, liver, kidney, and other organs (e.g., stomach, intestine, etc.). This approach can prove practical for large-scale production of decellularized whole organs. On the other hand, the simultaneous decellularization of multiple whole livers using a multi-channel perfusion system is also possible.

*Storage/preservation*: cryopreservation and lyophilization are the techniques commonly advocated for the preservation of ECM products. Two techniques for cryopreservation include freezing and vitrification; hindering ice-crystal formation is the main challenge in both of them [108]. Washing of cryo-preservative agents is necessary after storage before proceeding with reseeding and implantation for cytotoxic concerns [108]. Poornejad et al. [115] studied the effects of freezing/thawing as a preservation technique for porcine whole-kidney decellularized scaffolds, without using cryoprotectants. Freezing/thawing did not affect the elastic modulus or adversely affect recellularization. However, this resulted in a decreased arterial pressure (as a measure of structural integrity) by a factor of 4 and caused partial damage of collagen and elastic fibers. The preservation of recellularized wDLMs remains unexplored.

*Food and Drug Administration* (*FDA*): eventually, decellularized/recellularized whole livers will have to meet FDA regulations in a similar fashion to commercially available decellularized human dermal grafts (e.g., AlloDerm©, Lifecell©, etc.) for both the pre-market and post-market prerequisites. As a regenerative medicine technology, recellularized scaffolds fall in the "combination product" category which involves products that combine two or more regulated components that are produced as a single entity. Engineered whole livers can obtain market approval as either a "biologic" or "medical device" based on the mechanism of action [69].

*Market potential*: artificial organ market estimates consistently show enormous potentials due to the expanding transplantation demands. In 2014, a report by a US-based market research and consulting company estimated that the global artificial organ and bionics market can

reach USD 38.75 billion by 2020. Artificial kidney took the lead in 2013 by global market of USD 12.21 billion. Interestingly, the artificial liver category was regarded as the most rapidly growing segment from 2014 to 2020 [116].

## 13. Surgeon's perspective

Engineered whole organs are considered a very attractive solution for organ-shortage crisis. It is thus important to elaborate on some fundamental parameters related to clinical application. Liver transplantation is indicated for a wide range of pathologies. Although indication-specific differences exist concerning the surgical technique and perioperative care, the graft-functional requirements are basically similar. Taking into consideration the variable approaches investigated for WLD, it can be noted that not all approaches are suitable for the different indications of liver transplantation. For example, a fully functioning liver graft will be required for fulminant liver failure, ex vivo liver-support device, end-stage liver failure, and malignancies. For these indications, lengthy ex vivo conditioning and in vivo proliferation approaches may not thus be suitable. Long ex vivo conditioning can be, however, acceptable in patients with biliary atresia, congenital absence of portal vein, or cirrhotic patients with lower model for end-stage liver disease (MELD)/pediatric end-stage liver disease (PELD) scores. In vivo proliferation can only be applied where auxiliary transplantation is feasible. Another example is in vivo decellularization; reasonably, this approach will not be suitable when an architectural abnormality is present as in the case of cirrhosis or biliary atresia, or in malignant conditions.

Implications of functional cell mass were discussed in Section 6. Problems related to the size of the graft can be anticipated in pediatric recipients, as a larger graft volume, in comparison to allografts, will be required to attain the required critical cell mass based on the current seeding densities. A smaller cell mass may be required in case of bridging, metabolic liver conditions, and acute liver failure. Feasibility of surgical implantation in different animal models was verified by the previously discussed in vivo experiments. PV anastomosis was feasible even without recellularization [32]. However, these studies did not tackle arterial or biliary anastomosis. In vivo studies that can accurately define the transplantation requirements are still lagging. Finally, the ability to produce an engineered liver that can be transplanted without the need of immunosuppression is highly desired. Still, the engineering of a liver graft from xenogeneic source that requires standard immunosuppression regimens similar to allograft will be a breakthrough, especially with the ever-increasing advances in the field of immune-suppressive medications.

## 14. Conclusions

In this chapter, an effort was made to put all the parameters of the WLD approach on display. This was done in order to link the different steps of the process together, to provide an overall insight of the approach progress and deficiencies, and to highlight the gaps in the published literature. It is undeniable that the understanding of the perfusion

decellularization and the determinant factors of wDLM potentials has evidently evolved since the introduction of the concept in 2002. This understanding greatly challenged the simplicity of the approach viewed originally as crude borrowing of natural ECM platform and demanded for delicate appreciation of the ECM effectors. The simplicity of borrowing the human cell machinery and the patient-specific-tailoring concept on the other hand still lends the approach a very attractive edge among the other approaches of xenotransplantation. A higher appreciation of cell mechano-sensitivity, hepatic zonation, intra-wDLM flow dynamics, spatial cell-seeding organization, critical cell mass, and substrate modification can be easily noted in the recent publications. The WLD experimentation in a parallel, instead of sequential, fashion offered great insights and allowed a degree of feedback-based modifications. An awareness of the advances in the non-hepatic whole-organ decellularization trials, the other approaches for xenotransplantation, and material modification science is encouraged when dealing with WLD, together with accurate outlining of the clinical target. Therefore, a collaborative teamwork is necessary to offer complementary envisions. Finally, the suboptimal recellularization, intra-bioreactor cell function, and failures with in vivo long-term graft survival highlight deficiencies with two of the three main previously suggested players, namely the intrinsic DLM potentials and vascular competency. The third player (immune/host responses) remains largely unexplored for wDLM. The need for robust stepwise optimization is clear.

## Author details

Ibrahim Fathi[1,2]* and Ahmed Eltawila[3]

*Address all correspondence to: I_Fathy201010@alexmed.edu.eg

1 Department of Surgery, Faculty of Medicine, University of Alexandria, Alexandria, Egypt

2 Division of Transplantation and Regenerative Medicine, Tohoku University School of Medicine, Tohoku, Japan

3 Department of Materials Science, Institute of Graduate Studies and Research, University of Alexandria, Alexandria, Egypt

## References

[1] Atala A. Methods and compositions for organ decellularization. US Patent; US 6376244 B1, 2002

[2] Matthiesen TS, Ott HC, Goh S-K, Taylor DA. Large solid organ perfusion decellularization—A start for human-sized tissue scaffolds? Circulation. 2007;**116**(Suppl 16):II_103

[3] Gilbert TW, Sellaro TL, Badylak SF. Decellularization of tissues and organs. Biomaterials. 2006;**27**(19):3675-3683. DOI: 10.1016/j.biomaterials.2006.02.014

[4] Crapo PM, Gilbert TW, Badylak SF. An overview of tissue and whole organ decellularization processes. Biomaterials. 2011;**32**(12):3233-3243. DOI: 10.1016/j.biomaterials.2011.01.057

[5] He M, Callanan A. Comparison of methods for whole-organ decellularization in tissue engineering of bioartificial organs. Tissue Engineering. Part B: Reviews. 2012;**19**(3):194-208. DOI: 10.1089/ten.teb.2012.0340

[6] Wang Y, Nicolas C, Chen H, Ross J, De Lorenzo S, Nyberg S. Recent advances in decellularization and recellularization for tissue-engineered liver grafts. Cells Tissues Organs. 2017;**203**:203-214. DOI: 10.1159/000452761

[7] Bao J, Shi Y, Sun H, Yin X, Yang R, Li L, Chen X, Bu H. Construction of a portal implantable functional tissue-engineered liver using perfusion-decellularized matrix and hepatocytes in rats. Cell Transplantation. 2011;**20**(5):753-766. DOI: 10.3727/096368910X536572

[8] Barakat O, Abbasi S, Rodriguez G, Rios J, Wood RP, Ozaki C, Holley LS, Gauthier PK. Use of decellularized porcine liver for engineering humanized liver organ. Journal of Surgical Research. 2012;**173**(1):e11–e25. DOI: 10.1016/j.jss.2011.09.033

[9] Fathi I, Elhammady H, Sakr M, Nabawi A, Marei M. Rapid hepatic perfusion decellularization: Technique and critique. Xenotransplantation. 2015 Nov-Dec;**22**(6):451-457. PubMed PMID: 26669725. Epub 2015/12/17. eng. DOI: 10.1111/xen.12212

[10] Uygun BE, Soto-Gutierrez A, Yagi H, Izamis M-L, Guzzardi MA, Shulman C, Milwid J, Kobayashi N, Tilles A, Berthiaume F. Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. Nature Medicine. 2010;**16**(7):814-820. DOI: 10.1038/nm.2170

[11] Yagi H, Fukumitsu K, Fukuda K, Kitago M, Shinoda M, Obara H, Itano O, Kawachi S, Tanabe M, Coudriet GM. Human-scale whole-organ bioengineering for liver transplantation: A regenerative medicine approach. Cell Transplantation. 2013;**22**(2):231-242. DOI:10.3727/096368912X654939

[12] White LJ, Taylor AJ, Faulk DM, Keane TJ, Saldin LT, Reing JE, Swinehart IT, Turner NJ, Ratner BD, Badylak SF. The impact of detergents on the tissue decellularization process: A ToF-SIMS study. Acta Biomaterialia. 2017;**50**:207-219. DOI: 10.1016/j.actbio.2016.12.033

[13] Jiang W-C, Cheng Y-H, Yen M-H, Chang Y, Yang VW, Lee OK. Cryo-chemical decellularization of the whole liver for mesenchymal stem cells-based functional hepatic tissue engineering. Biomaterials. 2014;**35**(11):3607-3617. DOI: 10.1016/j.biomaterials.2014.01.024

[14] Kajbafzadeh A-M, Javan-Farazmand N, Monajemzadeh M, Baghayee A. Determining the optimal decellularization and sterilization protocol for preparing a tissue scaffold of a human-sized liver tissue. Tissue Engineering. Part C: Methods. 2013;**19**(8):642-651. DOI: 10.1089/ten.tec.2012.0334

[15] Gessner RC, Hanson AD, Feingold S, Cashion AT, Corcimaru A, Wu BT, Mullins CR, Aylward SR, Reid LM, Dayton PA. Functional ultrasound imaging for assessment

of extracellular matrix scaffolds used for liver organoid formation. Biomaterials. 2013;**34**(37):9341-9351. DOI: 10.1016/j.biomaterials.2013.08.033

[16] Mazza G, Rombouts K, Hall AR, Urbani L, Luong TV, Al-Akkad W, Longato L, Brown D, Maghsoudlou P, Dhillon AP. Decellularized human liver as a natural 3D-scaffold for liver bioengineering and transplantation. Scientific Reports. 2015;**5**:13079. DOI: 10.1038/srep13079

[17] Bourgine PE, Gaudiello E, Pippenger B, Jaquiery C, Klein T, Pigeot S, Todorov A, Feliciano S, Banfi A, Martin I. Engineered extracellular matrices as biomaterials of tunable composition and function. Advanced Functional Materials. 2017;**27**(7). DOI: 10.1002/adfm.201605486

[18] Struecker B, Butter A, Hillebrandt K, Polenz D, Reutzel-Selke A, Tang P, Lippert S, Leder A, Rohn S, Geisel D. Improved rat liver decellularization by arterial perfusion under oscillating pressure conditions. Journal of Tissue Engineering and Regenerative Medicine. 2017;**11**(2):531-541. DOI: 10.1002/term.1948

[19] Pan J, Yan S, Gao J-j, Wang Y-y, Lu Z-j, Cui C-w, Zhang Y-h, Wang Y, Meng X-q, Zhou L. In-vivo organ engineering: Perfusion of hepatocytes in a single liver lobe scaffold of living rats. The International Journal of Biochemistry & Cell Biology. 2016;**80**:124-131. DOI: 10.1016/j.biocel.2016.10.003

[20] He M, Callanan A, Lagaras K, Steele J, Stevens M. Optimization of SDS exposure on preservation of ECM characteristics in whole organ decellularization of rat kidneys. Journal of Biomedical Materials Research Part B: Applied Biomaterials. 2016. DOI: 10.1002/jbm.b.33668

[21] Sabetkish S, Kajbafzadeh AM, Sabetkish N, Khorramirouz R, Akbarzadeh A, Seyedian SL, Pasalar P, Orangian S, Beigi RSH, Aryan Z. Whole-organ tissue engineering: Decellularization and recellularization of three-dimensional matrix liver scaffolds. Journal of Biomedical Materials Research Part A. 2015;**103**(4):1498-1508. DOI: 10.1002/jbm.a.35291

[22] Maghsoudlou P, Georgiades F, Smith H, Milan A, Shangaris P, Urbani L, Loukogeorgakis SP, Lombardi B, Mazza G, Hagen C. Optimization of liver decellularization maintains extracellular matrix micro-architecture and composition predisposing to effective cell seeding. PLoS One. 2016;**11**(5):e0155324. DOI: 10.1371/journal.pone.0155324

[23] Shirakigawa N, Ijima H, Takei T. Decellularized liver as a practical scaffold with a vascular network template for liver tissue engineering. Journal of Bioscience and Bioengineering. 2012;**114**(5):546-551. DOI: 10.1016/j.jbiosc.2012.05.022

[24] Mattei G, Di Patria V, Tirella A, Alaimo A, Elia G, Corti A, Paolicchi A, Ahluwalia A. Mechanostructure and composition of highly reproducible decellularized liver matrices. Acta Biomaterialia. 2014;**10**(2):875-882. DOI: 10.1016/j.actbio.2013.10.023

[25] De Kock J, Ceelen L, De Spiegelaere W, Casteleyn C, Claes P, Vanhaecke T, Rogiers V. Simple and quick method for whole-liver decellularization: A novel in vitro three-dimensional bioengineering tool? Archives of Toxicology. 2011;**85**(6):607-612. DOI: 10.1007/s00204-011-0706-1

[26] Shupe T, Williams M, Brown A, Willenberg B, Petersen BE. Method for the decellularization of intact rat liver. Organogenesis. 2010;**6**(2):134-136. DOI: 10.4161/org.6.2.11546

[27] Soto-Gutierrez A, Zhang L, Medberry C, Fukumitsu K, Faulk D, Jiang H, Reing J, Gramignoli R, Komori J, Ross M. A whole-organ regenerative medicine approach for liver replacement. Tissue Engineering. Part C: Methods. 2011;**17**(6):677-686. DOI: 10.1089/ten.tec.2010.0698

[28] Baptista PM, Orlando G, Mirmalek-Sani S-H, Siddiqui M, Atala A, Soker S, editors. Whole organ decellularization-a tool for bioscaffold fabrication and organ bioengineering. Conference Proceedings—IEEE Engineering in Medicine and Biology Society. 2009;**2009**:6526-6529. DOI: 10.1109/IEMBS.2009.5333145

[29] Baptista PM, Siddiqui MM, Lozier G, Rodriguez SR, Atala A, Soker S. The use of whole organ decellularization for the generation of a vascularized liver organoid. Hepatology. 2011;**53**(2):604-617. DOI: 10.1002/hep.24067

[30] Federico S, Ayelen R, Romina C, Nelly BA, Adrian NG, Alicia SN, Paula CM, editors. Obtaining liver tridimensional scaffold through the decellularization of rabbit whole liver in 24 hours. Journal of Physics: Conference Series. 2016;**705**(1):012011. DOI: 10.1088/1742-6596/705/1/012011

[31] Nari GA, Cid M, Comin R, Reyna L, Juri G, Taborda R, Salvatierra NA. Preparation of a three-dimensional extracellular matrix by decellularization of rabbit livers. Revista Espanola de Enfermedades Digestivas: Organo Oficial de la Sociedad Espanola de Patologia Digestiva. 2013;**105**(3):138-143. DOI: 10.4321/S1130-01082013000300004

[32] Bao J, Wu Q, Sun J, Zhou Y, Wang Y, Jiang X, Li L, Shi Y, Bu H. Hemocompatibility improvement of perfusion-decellularized clinical-scale liver scaffold through heparin immobilization. Scientific Reports. 2015;**5**:10756. DOI: 10.1038/srep10756

[33] Lang R, Stern MM, Smith L, Liu Y, Bharadwaj S, Liu G, Baptista PM, Bergman CR, Soker S, Yoo JJ, Atala A, Zhang Y. Three-dimensional culture of hepatocytes on porcine liver tissue-derived extracellular matrix. Biomaterials. 2011 Oct;**32**(29):7042-7052

[34] Struecker B, Hillebrandt KH, Voitl R, Butter A, Schmuck RB, Reutzel-Selke A, Geisel D, Joehrens K, Pickerodt PA, Raschzok N, Puhl G, Neuhaus P, Pratschke J, Sauer IM. Porcine liver decellularization under oscillating pressure conditions: A technical refinement to improve the homogeneity of the decellularization process. Tissue Engineering Part C Methods. 2015 Mar;**21**(3):303-313

[35] Zhang Z, Bédard E, Luo Y, Wang H, Deng S, Kelvin D, Zhong R. Animal models in xenotransplantation. Expert Opinion on Investigational Drugs. 2000;**9**(9):2051-2068. DOI: 10.1517/13543784.9.9.2051

[36] Wu Q, Bao J, Zhou Y-j, Wang Y-j, Du Z-g, Shi Y-j, Li L, Bu H. Optimizing perfusion-decellularization methods of porcine livers for clinical-scale whole-organ bioengineering. BioMed Research International. 2015;**2015**:785474. DOI: 10.1155/2015/785474

[37] Mattei G, Magliaro C, Pirone A, Ahluwalia A. Decellularised human liver is too heterogeneous for designing a generic ECM-mimic hepatic scaffold. Pre-print. bioRxiv 2016:073270. DOI: 10.1101/073270

[38] Badylak SF, Freytes DO, Gilbert TW. Extracellular matrix as a biological scaffold material: Structure and function. Acta Biomaterialia. 2009;**5**(1):1-13. DOI: 10.1016/j.actbio.2008.09.013

[39] Martinez-Hernandez A, Amenta P. The hepatic extracellular matrix. II. Ontogenesis, regeneration and cirrhosis. Virchows Archiv A, Pathological Anatomy and Histopathology. 1992;**423**(2):77-84. DOI: 10.1007/BF01606580

[40] Martinez-Hernandez A, Amenta PS. The hepatic extracellular matrix. I. Components and distribution in normal liver. Virchows Arch A, Pathological Anatomy and Histopathology. 1993;**423**(1):1-11

[41] Nagata S, Hanayama R, Kawane K. Autoimmunity and the clearance of dead cells. Cell. 2010;**140**(5):619-630. DOI: 10.1016/j.cell.2010.02.014

[42] Zhang Q, Raoof M, Chen Y, Sumi Y, Sursal T, Junger W, Brohi K, Itagaki K, Hauser CJ. Circulating mitochondrial DAMPs cause inflammatory responses to injury. Nature. 2010;**464**(7285):104-107. DOI: 10.1038/nature08780

[43] San Antonio JD, Iozzo RV. Glycosaminoglycans: Structure and biological functions. In: eLS. Chichester: John Wiley & Sons Ltd.; 2001. DOI: 10.1038/npg.els.0000704LS

[44] Wang Y, Cui CB, Yamauchi M, Miguez P, Roach M, Malavarca R, Costello MJ, Cardinale V, Wauthier E, Barbier C. Lineage restriction of human hepatic stem cells to mature fates is made efficient by tissue-specific biomatrix scaffolds. Hepatology. 2011;**53**(1):293-305. DOI: 10.1002/hep.24012

[45] Caralt M, Uzarski JS, Iacob S, Obergfell KP, Berg N, Bijonowski BM, Kiefer KM, Ward HH, Wandinger-Ness A, Miller WM. Optimization and critical evaluation of decellularization strategies to develop renal extracellular matrix scaffolds as biological templates for organ engineering and transplantation. American Journal of Transplantation. 2015;**15**(1):64-75. DOI: 10.1111/ajt.12999

[46] Badylak SF, Gilbert TW, editors. Immune response to biologic scaffold materials. Seminars in Immunology. 2008;**20**(2):109-116. DOI: 10.1016/j.smim.2007.11.003

[47] Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. Cell. 2006;**126**(4):677-689. DOI: 10.1016/j.cell.2006.06.044

[48] Lozoya OA, Wauthier E, Turner RA, Barbier C, Prestwich GD, Guilak F, Superfine R, Lubkin SR, Reid LM. Regulation of hepatic stem/progenitor phenotype by microenvironment

stiffness in hydrogel models of the human liver stem cell niche. Biomaterials. 2011;**32**(30): 7389-7402. DOI: 10.1016/j.biomaterials.2011.06.042

[49] Dash A, Simmers MB, Deering TG, Berry DJ, Feaver RE, Hastings NE, Pruett TL, LeCluyse EL, Blackman BR, Wamhoff BR. Hemodynamic flow improves rat hepatocyte morphology, function, and metabolic activity in vitro. American Journal of Physiology-Cell Physiology. 2013;**304**(11):C1053–C1063. DOI: 10.1152/ajpcell.00331.2012

[50] Miki T, Ring A, Gerlach J. Hepatic differentiation of human embryonic stem cells is promoted by three-dimensional dynamic perfusion culture conditions. Tissue Engineering. Part C: Methods. 2011;**17**(5):557-568. DOI: 10.1089/ten.tec.2010.0437

[51] Schmelzer E, Triolo F, Turner ME, Thompson RL, Zeilinger K, Reid LM, Gridelli B, Gerlach JC. Three-dimensional perfusion bioreactor culture supports differentiation of human fetal liver cells. Tissue Engineering. Part A. 2010;**16**(6):2007-2016. DOI: 10.1089/ten.tea.2009.0569

[52] Hsu W-M, Carraro A, Kulig KM, Miller ML, Kaazempur-Mofrad M, Weinberg E, Entabi F, Albadawi H, Watkins MT, Borenstein JT. Liver-assist device with a microfluidics-based vascular bed in an animal model. Annals of Surgery. 2010;**252**(2):351-357. DOI: 10.1097/SLA.0b013e3181e982ba

[53] Schön MR, Kollmar O, Wolf S, Schrem H, Matthes M, Akkoc N, Schnoy NC, Neuhaus P. Liver transplantation after organ preservation with normothermic extracorporeal perfusion. Annals of Surgery. 2001;**233**(1):114-123. DOI: 10.1097/00000658-200101000-00017

[54] Evans DW, Moran EC, Baptista PM, Soker S, Sparks JL. Scale-dependent mechanical properties of native and decellularized liver tissue. Biomechanics and Modeling in Mechanobiology. 2013 Jun;**12**(3):569-580. DOI: 10.1007/s10237-012-0426-3

[55] Klaas M, Kangur T, Viil J, Mäemets-Allas K, Minajeva A, Vadi K, Antsov M, Lapidus N, Järvekülg M, Jaks V. The alterations in the extracellular matrix composition guide the repair of damaged liver tissue. Scientific Reports. 2016;**6**:27398. DOI: 10.1038/srep27398

[56] Geerts S, Ozer S, Jaramillo M, Yarmush ML, Uygun BE. Nondestructive methods for monitoring cell removal during rat liver decellularization. Tissue Engineering. Part C: Methods. 2016;**22**(7):671-678. DOI: 10.1089/ten.tec.2015.0571

[57] Hagen CK, Maghsoudlou P, Totonelli G, Diemoz PC, Endrizzi M, Rigon L, Menk R-H, Arfelli F, Dreossi D, Brun E. High contrast microstructural visualization of natural acellular matrices by means of phase-based X-ray tomography. Scientific Reports. 2015;**5**:18156. DOI: 10.1038/srep18156

[58] Gilbert TW, Stewart-Akers AM, Simmons-Byrd A, Badylak SF. Degradation and remodeling of small intestinal submucosa in canine Achilles tendon repair. Journal of Bone and Joint Surgery. 2007;**89**(3):621-630. DOI: 10.2106/00004623-200703000-00020

[59] Zhou P, Lessa N, Estrada DC, Severson EB, Lingala S, Zern MA, Nolta JA, Wu J. Decellularized liver matrix as a carrier for the transplantation of human fetal and

primary hepatocytes in mice. Liver Transplantation. 2011;**17**(4):418-427. DOI: 10.1002/lt.22270

[60] Wells RG. Cellular sources of extracellular matrix in hepatic fibrosis. Clinics in Liver Disease. 2008;**12**(4):759-768. DOI: 10.1016/j.cld.2008.07.008

[61] Arnaud A, Fontana L, Angulo AJ, Gil Á, López-Pedrosa JM. Proliferation, functionality, and extracellular matrix production of hepatocytes and a liver stellate cell line: A comparison between single cultures and cocultures. Digestive Diseases and Sciences. 2003;**48**(7):1406-1413. DOI: 10.1023/A:1024192100775

[62] Caralt M, Velasco E, Lanas A, Baptista PM. Liver bioengineering: From the stage of liver decellularized matrix to the multiple cellular actors and bioreactor special effects. Organogenesis. 2014;**10**(2):250-259. DOI: 10.4161/org.29892

[63] McClelland R, Wauthier E, Uronis J, Reid L. Gradients in the liver's extracellular matrix chemistry from periportal to pericentral zones: Influence on human hepatic progenitors. Tissue Engineering. Part A. 2008;**14**(1):59-70. DOI: 10.1089/ten.a.2007.0058

[64] Susick R, Moss N, Kubota H, Lecluyse E, Hamilton G, Luntz T, Ludlow J, Fair J, Gerber D, Bergstrand K. Hepatic progenitors and strategies for liver cell therapies. Annals of the New York Academy of Sciences. 2001;**944**(1):398-419. DOI: 10.1111/j.1749-6632.2001.tb03851.x

[65] Reid LM, Fiorino AS, Sigal SH, Brill S, Holst PA. Extracellular matrix gradients in the space of Disse: Relevance to liver biology. Hepatology. 1992;**15**(6):1198-1203. DOI: 10.1002/hep.1840150635

[66] Kietzmann T. Metabolic zonation of the liver: The oxygen gradient revisited. Redox Biology. 2017. DOI: 10.1016/j.redox.2017.01.012

[67] Beresford W, Henninger J. A tabular comparative histology of the liver. Archivum Histologicum Japonicum. 1986;**49**(3):267-281. DOI: 10.1679/aohc.49.267

[68] Wang X, Cui J, Zhang BQ, Zhang H, Bi Y, Kang Q, Wang N, Bie P, Yang Z, Wang H. Decellularized liver scaffolds effectively support the proliferation and differentiation of mouse fetal hepatic progenitors. Journal of Biomedical Materials Research Part A. 2014;**102**(4):1017-1025. DOI: 10.1002/jbm.a.34764

[69] Scarritt ME, Pashos NC, Bunnell BA. A review of cellularization strategies for tissue engineering of whole organs. Frontiers in Bioengineering and Biotechnology. 2015;**3**:43. DOI: 10.3389/fbioe.2015.00043

[70] Hu X, Yang T, Li C, Zhang L, Li M, Huang W, Zhou P, editors. Human fetal hepatocyte line, L-02, exhibits good liver function in vitro and in an acute liver failure model. Transplantation Proceedings. 2013;**45**(2):695-700. DOI: 10.1016/j.transproceed.2012.09.121

[71] Palakkan AA, Hay DC, TV K, Ross JA. Liver tissue engineering and cell sources: Issues and challenges. Liver International. 2013;**33**(5):666-676. DOI: 10.1111/liv.12134

[72] Walldorf J, Aurich H, Cai H, Runge D, Christ B, Strom S, Fleig W. Expanding hepatocytes in vitro before cell transplantation: Donor age-dependent proliferative capacity of

cultured human hepatocytes. Scandinavian Journal of Gastroenterology. 2004;**39**(6):584-593. DOI: 10.1080/00365520410005586

[73] Satyananda V, Hara H, Ezzelarab MB, Phelps C, Ayares D, Cooper DK. New concepts of immune modulation in xenotransplantation. Transplantation. 2013;**96**(11):937. DOI: 10.1097/TP.0b013e31829bbcb2

[74] Mirmalek-Sani S-H, Sullivan DC, Zimmerman C, Shupe TD, Petersen BE. Immunogenicity of decellularized porcine liver for bioengineered hepatic tissue. American Journal of Pathology. 2013;**183**(2):558-565. DOI: 10.1016/j.ajpath.2013.05.002

[75] Hassanein W, Uluer MC, Langford J, Woodall JD, Cimeno A, Dhru U, Werdesheim A, Harrison J, Rivera-Pratt C, Klepfer S. Recellularization via the bile duct supports functional allogenic and xenogenic cell growth on a decellularized rat liver scaffold. Organogenesis. 2017;**13**(1):16-27. DOI: 10.1080/15476278.2016.1276146

[76] Ogiso S, Yasuchika K, Fukumitsu K, Ishii T, Kojima H, Miyauchi Y, Yamaoka R, Komori J, Katayama H, Kawai T. Efficient recellularisation of decellularised whole-liver grafts using biliary tree and foetal hepatocytes. Scientific Reports. 2016;**6**:35887. DOI: 10.1038/srep35887

[77] Gupta S, Rajvanshi P, Sokhi R, Slehria S, Yam A, Kerr A, Novikoff PM. Entry and integration of transplanted hepatocytes in rat liver plates occur by disruption of hepatic sinusoidal endothelium. Hepatology. 1999;**29**(2):509-519. DOI: 10.1002/hep.510290213

[78] Kholodenko IV, Yarygin KN. Cellular mechanisms of liver regeneration and cell-based therapies of liver diseases. BioMed Research International. 2017;**2017**. DOI: 10.1155/2017/8910821

[79] Sohlenius-Sternbeck A-K. Determination of the hepatocellularity number for human, dog, rabbit, rat and mouse livers from protein concentration measurements. Toxicology in Vitro. 2006;**20**(8):1582-1586. DOI: 10.1016/j.tiv.2006.06.003

[80] Budd G. Liver stereology. In: Motta P, Didio L, editors. Basic and Clinical Hepatology. Vol. 2. Springer Science & Business Media, Netherlands; 2012. p. 111

[81] Xu Q, Sun X, Qiu Y, Zhang H, Ding Y. The optimal hepatocyte density for a hollow-fiber bioartificial liver. Annals of Clinical & Laboratory Science. 2004;**34**(1):87-93

[82] Dvir-Ginzberg M, Gamlieli-Bonshtein I, Agbaria R, Cohen S. Liver tissue engineering within alginate scaffolds: Effects of cell-seeding density on hepatocyte viability, morphology, and function. Tissue Engineering. 2003;**9**(4):757-766. DOI: 10.1089/107632703768247430

[83] McPherson T, Liang H, Record R, Badylak S. Gal$\alpha$ (1, 3) Gal epitope in porcine small intestinal submucosa. Tissue Engineering. 2000;**6**(3):233-239. DOI: 10.1089/10763270050044416

[84] Stone KR, Ayala G, Goldstein J, Hurst R, Walgenbach A, Galili U. Porcine cartilage transplants in the cynomolgus monkey: III. Transplantation of $\alpha$-galactosidase-treated porcine cartilage. Transplantation. 1998;**65**(12):1577-1583. DOI: 10.1097/00007890-199806270-00007

[85] Hamadeh R, Jarvis G, Galili U, Mandrell R, Zhou P, Griffiss J. Human natural anti-Gal IgG regulates alternative complement pathway activation on bacterial surfaces. Journal of Clinical Investigation. 1992;**89**(4):1223. DOI: 10.1172/JCI115706

[86] Yu PB, Holzknecht ZE, Bruno D, Parker W, Platt JL. Modulation of natural IgM binding and complement activation by natural IgG antibodies: A role for IgG anti-Gal alpha1-3Gal antibodies. Journal of Immunology. 1996;**157**(11):5163-5168

[87] Brljggemann M, Williams GT, Bindon CI, Clark MR, Walker MR. Comparison of the effector functions of human immunoglobulins using a matched set of chimeric antibodies. Journal of Experimental Medicine. 1987;**166**(5):1351-1361

[88] Galili U. Interaction of the natural anti-Gal antibody with $\alpha$-galactosyl epitopes: A major obstacle for xenotransplantation in humans. Immunology Today. 1993;**14**(10):480-482. DOI: 10.1016/0167-5699(93)90261-I

[89] Raeder RH, Badylak SF, Sheehan C, Kallakury B, Metzger DW. Natural anti-galactose $\alpha$1, 3 galactose antibodies delay, but do not prevent the acceptance of extracellular matrix xenografts. Transplant Immunology. 2002;**10**(1):15-24. DOI: 10.1016/S0966-3274(01)00044-2

[90] Derwin KA, Baker AR, Spragg RK, Leigh DR, Iannotti JP. Commercial extracellular matrix scaffolds for rotator cuff tendon repair. Journal of Bone and Joint Surgery (American). 2006;**88**(12):2665-2672. DOI: 10.2106/00004623-200612000-00014

[91] Zheng M, Chen J, Kirilak Y, Willers C, Xu J, Wood D. Porcine small intestine submucosa (SIS) is not an acellular collagenous matrix and contains porcine DNA: Possible implications in human implantation. Journal of Biomedical Materials Research Part B: Applied Biomaterials. 2005;**73**(1):61-67. DOI: 10.1002/jbm.b.30170

[92] Valentin JE, Badylak JS, McCabe GP, Badylak SF. Extracellular matrix bioscaffolds for orthopaedic applications. Journal of Bone and Joint Surgery (American). 2006;**88**(12):2673-2686. DOI: 10.2106/00004623-200612000-00015

[93] Kant CD, Akiyama Y, Tanaka K, Shea S, Connolly SE, Germana S, Winn HJ, LeGuern C, Tocco G, Benichou G. Primary vascularization of allografts governs their immunogenicity and susceptibility to tolerogenesis. Journal of Immunology. 2013;**191**(4):1948-1956. DOI: 10.4049/jimmunol.1202092

[94] Kwun J, Malarkannan S, Burlingham WJ, Knechtle SJ. Primary vascularization of the graft determines the immunodominance of murine minor H antigens during organ transplantation. The Journal of Immunology. 2011;**187**(8):3997-4006. DOI: 10.4049/jimmunol.1003918

[95] Galili U, Macher BA, Buehler J, Shohet SB. Human natural anti-alpha-galactosyl IgG. II. The specific recognition of alpha (1----3)-linked galactose residues. Journal of Experimental Medicine. 1985;**162**(2):573-582. DOI: 10.1084/jem.162.2.573

[96] Good A, Cooper D, Malcolm A, Ippolito R, Koren E, Neethling F, Ye Y, Zuhdi N, Lamontagne L. Identification of carbohydrate structures that bind human antiporcine antibodies: Implications for discordant xenografting in humans. Transplantation Proceedings; 1992;**24**:559-562

[97] Platt JL, Fischel RJ, Matas AJ, Reif SA, Bolman RM, Bach FH. Immunopathology of hyperacute xenograft rejection in a swine-to-primate model. Transplantation. 1991;**52**(2):214-220. DOI: 10.1097/00007890-199108000-00006

[98] Hossler FE, Douglas JE. Vascular corrosion casting: Review of advantages and limitations in the application of some simple quantitative methods. Microscopy and Microanalysis. 2001;**7**(03):253-264. DOI: 10.1017/S1431927601010261

[99] Esmon CT. Basic mechanisms and pathogenesis of venous thrombosis. Blood Reviews. 2009;**23**(5):225-229. DOI: 10.1016/j.blre.2009.07.002

[100] Robertson MJ, Dries-Devlin JL, Kren SM, Burchfield JS, Taylor DA. Optimizing recellularization of whole decellularized heart extracellular matrix. PLoS One. 2014;**9**(2):e90406. DOI: 10.1371/journal.pone.0090406

[101] Nagaoka Y, Yamada H, Kimura T, Kishida A, Fujisato T, Takakuda K. Reconstruction of small diameter arteries using decellularized vascular scaffolds. Journal of Medical and Dental Sciences. 2014;**61**(1):33-40. DOI: 10.11480/610105

[102] Wang XN, Chen CZ, Yang M, Gu YJ. Implantation of decellularized small-caliber vascular xenografts with and without surface heparin treatment. Artificial Organs. 2007;**31**(2):99-104. DOI: 10.1111/j.1525-1594.2007.00348.x

[103] Bajpai VK, Andreadis ST. Stem cell sources for vascular tissue engineering and regeneration. Tissue Engineering. Part B: Reviews. 2012;**18**(5):405-425. DOI: 10.1089/ten.teb.2011.0264

[104] Lozito TP, Kuo CK, Taboas JM, Tuan RS. Human mesenchymal stem cells express vascular cell phenotypes upon interaction with endothelial cell matrix. Journal of Cellular Biochemistry. 2009;**107**(4):714-722. DOI: 10.1002/jcb.22167

[105] Liu JY, Swartz DD, Peng HF, Gugino SF, Russell JA, Andreadis ST. Functional tissue-engineered blood vessels from bone marrow progenitor cells. Cardiovascular Research. 2007;**75**(3):618-628. DOI: 10.1016/j.cardiores.2007.04.018

[106] Marra KG, Brayfield CA, Rubin JP. Adipose stem cell differentiation into smooth muscle cells. In: Gimble J, Bunnell B, editors. Adipose-Derived Stem Cells: Methods and Protocols. Humana Press, New York; 2011. pp. 261-268. DOI: 10.1007/978-1-61737-960-4_19

[107] Ko IK, Peng L, Peloso A, Smith CJ, Dhal A, Deegan DB, Zimmerman C, Clouse C, Zhao W, Shupe TD. Bioengineered transplantable porcine livers with re-endothelialized vasculature. Biomaterials. 2015;**40**:72-79. DOI: 10.1016/j.biomaterials.2014.11.027

[108] Seifu DG, Purnama A, Mequanint K, Mantovani D. Small-diameter vascular tissue engineering. Nature Reviews Cardiology. 2013;**10**(7):410-421. DOI: 10.1038/nrcardio.2013.77

[109] Haugen G, Kiserud T, Godfrey K, Crozier S, Hanson M. Portal and umbilical venous blood supply to the liver in the human fetus near term. Ultrasound in Obstetrics & Gynecology. 2004;**24**(6):599-605. DOI: 10.1002/uog.1744

[110] Nishii K, Reese G, Moran EC, Sparks JL. Multiscale computational model of fluid flow and matrix deformation in decellularized liver. Journal of the Mechanical Behavior of Biomedical Materials. 2016;**57**:201-214. DOI: 10.1016/j.jmbbm.2015.11.033

[111] Ren X, Tapias LF, Jank BJ, Mathisen DJ, Lanuti M, Ott HC. Ex vivo non-invasive assessment of cell viability and proliferation in bio-engineered whole organ constructs. Biomaterials. 2015;**52**:103-112. DOI: 10.1016/j.biomaterials.2015.01.061

[112] Bruinsma BG, Kim Y, Berendsen TA, Ozer S, Yarmush ML, Uygun BE. Layer-by-layer heparinization of decellularized liver matrices to reduce thrombogenicity of tissue engineered grafts. Journal of Clinical and Translational Research. 2015;**1**(1):pii: 04. DOI: 10.18053/jctres.201501.004

[113] Sarikaya A, Record R, Wu C-C, Tullius B, Badylak S, Ladisch M. Antimicrobial activity associated with extracellular matrices. Tissue Engineering. 2002;**8**(1):63-71. DOI: 10.1089/107632702753503063

[114] Park K, Woo H, editors. Systemic decellularization for multi-organ scaffolds in rats. Transplantation Proceedings. 2012;**44**(4):1151-1154. DOI: 10.1016/j.transproceed.2012.03.017

[115] Poornejad N, Frost TS, Scott DR, Elton BB, Reynolds PR, Roeder BL, Cook AD. Freezing/thawing without cryoprotectant damages native but not decellularized porcine renal tissue. Organogenesis. 2015;**11**(1):30-45. DOI: 10.1080/15476278.2015.1022009

[116] Global Artificial Organ and Bionics Market. United States: Grand View Research. 2014. Available from: http://www.grandviewresearch.com/press-release/global-artificial-organ.