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Comprehensive Study

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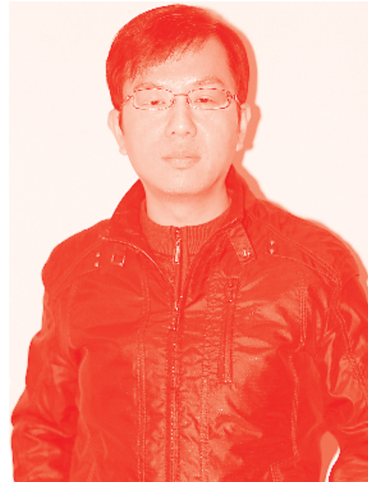

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Xenotransplantation - Comprehensive Study
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Preface

It is a great honor for me to edit the third version of *Xenotransplantation* in IntechOpen's Xenotransplantation – Comprehensive study. Since the second version, almost two years have passed. During this period, the 3rd WHO Global Consultation on Regulatory Requirements for Xenotransplantation Clinical Trials in Changsha-2018, the 14th IXA-2017 in Baltimore, and the 15th IXA-2019 in Munich were held. This field seems to be progressing quickly. Now, in a sense, we are standing at the dawn of the age for xenotransplantation.

As the most imminent issue for clinical xenotransplantation, this version first focused on pig islets transplantation. Several authors wrote about it from their own point of view. The new Regulation by WHO in Changsha is discussed.

In addition, a past clinical case including the review of heart xenotransplantation is introduced.

Concerning the progress of preclinical xenotransplantation studies during these years, the reported maximum survival days were more prolonged in several organs, as indicated below.

*Heart~heterotopic/Orthotropic: 945 days/195 days

*Kidney: 499 days, *Liver: 25 days, *Lung: 31 days

**Islets: 603 days, *Cornea: 903 days

(**: reported in the past, using wild-type pigs)

On the other hand, as the technology progresses, especially the CRISPR/Cas9 system, many new genetic engineered (GE) pigs are produced all over the world, such as the quadripartite knockout (KO) pigs [αGal/CMAH/β4GalNT2/SLAclassI-KO], the tripartite KO plus six genes transgenic (TG) pigs [αGal/CMAH/β4GalNT2-KO + CD46/CD55/CD47/TBM/EPCR/HO1-TG], and [αGal/CMAH/β4GalNT2-KO + CD46/CD55/CD47/TBM/EPCR/Lea29Y-TG]. These pigs were also introduced in the last IXA-2019 in Munich. The data of the preclinical studies using these new GE pigs will be reported in the next meeting and they must process the data indicated above.

In addition, chapters for bio-engineering focus on two reviews related to 3D bio-printing and decellularization. These field have also seen much progress.

Finally, this version contains several chapters related to regeneration studies, such as "Blastocyst complementation" and "Organogenic niche method" that present the research to use pigs as a scaffold for foster human Embryonic stem (ES) cells and

induced pluripotent stem cells (iPS). These topics were also presented as special lectures in the 15th IXA in Munich.

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Section 1

Clinic and Pre-Clinic

Chapter 1

Biosafety Barrier to Xenotransplantation

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

Abstract

Biosafety barrier is most important for xenotransplantation clinical trial. Source animals used in xenotransplantation should be bred in a closed herd and raised in a well-controlled, pathogen-free environment with high standards of animal welfare. To ensure the source animals' freedom from known pathogens under adequate biosecurity and surveillance, extensive tests must be done. Biosafety of DPF source pig should be proved by animal model before clinical trial. In addition, inclusion criteria for transplant recipients and clinical safe transplantation protocol should be established. Comprehensive anti-immune rejection treatment based on immune tolerance program can significantly prolong the xenograft survival and reduce the adverse impact on the immune system, which is suitable for clinical application. According to the clinical follow-up plan of the xenograft recipients, the patients should come back to the hospital for a check at regular intervals after the transplantation. The database of clinical trials for xenotransplantation should be established, including specimens, paper documents, and electronic documents. The information and samples of xeno-transplantation donors and recipients should be preserved for long time.

Keywords: biosafety barrier, donor animal, xenotransplantation, clinical trial

1. Introduction

The demand for a new source of organs and cells for clinical transplantation has been exacerbated for decades. And xenotransplantation (e.g., from pigs to human) could resolve this issue.

In 2008, the WHO and International Xenotransplantation Association (IXA) released a consensus statement on xenotransplantation from pig to human for clinical trials. In this statement, it proposed the criterion for biosafety of source animals in clinical trials. The source animals should be bred in a closed herd for the purpose and kept under a well-controlled and pathogen-free environment with complete animal welfare. Even source animals are housed in appropriate biosecurity and under surveillance, extensive detection must be done to ensure freedom from known pathogens and infectious disease.

Therefore, this chapter will draw attention to the significant biosafety barriers need to be overcome before xenotransplantation from pig to human can become a clinical therapy.

2. DPF source pig

The term "DPF" (Designated Pathogen Free) is used to describe animals, animal herds, or animal facilities that have been rigorously documented to be free of specified infectious agents and that are maintained using well-defined routines of testing for designated pathogens and utilizing rigorous SOPs (Standard operating procedures) and practices of herd husbandry and veterinary care to assure the absence of the designated pathogens [1]. So far, there is no normative document specifying the pathogens specified in DPF pig. DPF standards are dynamic and need to be updated over time according to the geographical environment of the animal population and new pathogens emerging. Generally speaking, there are two types of pathogens that need to be excluded from DPF pig: (1) Pathogens that affect animal health; (2) Pathogens that can cause cross-species transmission.

Experts in this field met to agree on the most comprehensive list of bacteria, fungi, parasites and viruses that should not be present in DPF pig [2]. Endogenous viruses are not listed. PERV (Porcine endogenous retrovirus) is the only one endogenous virus we known in pigs [3]. PERV has three subgroups including PERV-A, PERV-B and PERV-C. In general, PERV-A and PERV-B can infect both pig and human cells, but PERV-C can only infect pig cells. It is noteworthy that PERV-A/C recombine were be found in vitro co-culture system using cells from miniature swine, which means PERV-C can also infect human cells in some condition [4]. To monitor the status of DPF pigs, the pigs' samples including blood, serum, tissues and feces must be tested regularly.

The DPF pigs must be raised in biosecure barrier environment. Biosecure barrier facility includes many aspects.

2.1 Facility environment and building

1. The proposed DPF facility will be sited at a property to be confirmed.

2. The building will be on rural land where there are no other pig farms within a radius of 10 km. The grounds of the facility will be protected and planted with trees, and the grass mowed regularly. There are to be no other animals or livestock within the area boundary.

3. The building is to be fully protected by a secure fence and electric gate entry. The main entrance door is also to be a security door with key access and protected by security alarms 24 hours a day.

4. The facility is designed with two separate areas, outside the barrier (external) and within the barrier (termed "inside the barrier").

5. The external area houses a delivery bay, storerooms for feed and bedding, staff lunchroom facilities, office, laundry area, external change rooms, and rooms to supply goods through the barrier.

6. Inside the barrier, the building is to have a HEPA (High efficiency particulate air) -filtered air supply and it will only contain goods that are sterile, staff who have showered and are wearing sterile clothes, and the pigs themselves which will be free of all specified diseases. There are to be two rooms holding the pig pens, internal gown-up areas, office, treatment room, reception room, and feed and bedding storerooms.

7. There will be two rooms of animal pens, a further unit for the sow farrowing, and a quarantine area, all with an air lock entry. The air pressure in all animal areas will be positive to the corridors (monitored by magnehelic gauges). Rooms will have controlled fluorescent lighting, temperature and humidity, and 15 to 20 air changes per hour of HEPA -filtered air.

8. Animal pens will have gates of the metal farm type, allowing pigs to see out and receive physical contact from other pigs and staff, with aisles between pens and a drain running in front of each row of pens. There will be windows in the walls between each pen to allow pigs to see each other.

9. Each pen will have a valve supplying filtered drinking water and individual stainless steel bowls for feed.

10. Music will be piped into the units by speakers set into the ceiling and serviced from the mezzanine level. Music will be controlled from the main office.

2.2 Facility operation

1. The DPF facility will operate as a full sterile barrier facility. Therefore, all goods entering the facility must be sterile and all staff should go through a full shower procedure and gown-up in sterile suits, boots, hats and gloves. All original breeding stock in the facility will be cesarean-derived, colostrum-deprived, and hand-reared.

2. To enter the facility, staff must shower and don a complete clothing and footwear and wear gloves.

3. All activities that take place will be fully documented in the SOP Manual, including inwards receipt of goods through the facility barrier using such methods as an autoclave, dunk tank, and UV pass-through hatch.

4. There will be SOP-documented regular health screening of pigs and staff.

5. A comprehensive pest control system will be used inside and outside the building and managed by a contracted pest control company. Records of all inspections will be documented.

6. Pig care and welfare are a top priority, as described above for the Invercargill facility.

2.3 Health monitoring of DPF pigs

All pigs are uniquely identified and individual records should be maintained, including animal breeding and genetic records.

1. Regular veterinary should attendance at the pig facilities ensures that the staff is trained in disease recognition and that the veterinarian is called immediately in the event of signs and symptoms of disease in any animal. The veterinarian should report any such incident in writing.

2. The donor herd should continue to test the porcine pathogens and parasites.

3. All donor piglets should be necropsied by a veterinarian within 6 hours of cell harvesting. Any pathological changes must be noted and appropriate specimens taken. The veterinarians' report should be documented.

4. Donor piglet tissue retention samples collected include brain, heart, kidney, liver, lung, pancreas, and spleen. Duplicate samples are stored at 80°C in two separate locations.

5. Duplicate donor piglet serum retention samples are also stored in two separate locations.

6. In addition, duplicate final product retention samples are stored at 80°C in two separate locations.

7. A positive result in any of the infection monitoring tests described in this section, will lead to the donor animal and the batch of isolated islets being discarded.

The pigs are conveyed to the DPF breeding center. They must be disinfected in buffer rooms before entering inspection and quarantine where they are isolated for a month. After isolation the pigs give cesarean birth to the first generation of purified pigs. Compared to vaginal births Cesarean section can eliminate or reduce the risk of infecting with pathogens from sow's vajina. These newborns are fed in isolation under aseptic conditions and grow into adulthood. They are then impregnated and naturally deliver the second generation. After being tested for specified pathogens this second generation enters into a DPF area. The first generation of pigs should not be used as source pigs but the pigs in a second or higher generation can be used as DPF source pigs [1, 5].

3. Other biosafety issues for xenotransplantation clinical trials

Donor pigs are the basis for ensuring the biosafety of xenograft clinical trials. Other biosafety issues are also worthy of attention, including immunosuppression protocols, clinical treatment protocols, sample/data retention programs, and case-tracking programs.

3.1 Immunosuppression and tolerance-inducing strategies for xenotransplantation

The principal challenges that must be faced to make xenotransplantation a clinical reality, which include determining a repeatable strategy for efficient preparation of xenogeneic tissues and organs and tracing the potential transmission of porcine pathogens to human. In addition, it is necessary to overcome the rejection barrier with clinically practicable immunosuppression and tolerance induction strategies. The application of xenotransplantation faces insurmountable immunological barriers, including: (1) hyperacute rejection (complement activation mediated by antibody) which is trigged by natural xenoreactive antibodies against Gal (1,3) and non-Gal antigens, (2) acute rejection of humoral xenograft which is mediated by antibodies that are dependent on T cells, (3) acute cellular xenograft rejection due to T cell mediated cellular responses.

3.1.1 Immunosuppression protocols for xenotransplantation

Continuous administration of multiple immunosuppressive drugs has been required and attempts to minimize immunosuppression. Immunosuppression in

preclinical models of xenotransplantation usually consists of B-cell and plasma cell therapeutics like Rituximab and Bortezomib in addition to the standard triple drug immunosuppression. One or more rounds of immuno-adsorption or plasmapheresis are essential to remove antibodies from the recipient's circulation. These regimens are often associated with serious side effects such as pancytopenia and sepsis.

The xenogeneic T cell response is supposed to be similar to that of typical allogenic responses, even larger. Consider this challenging barrier, most successful immunosuppressive therapy include a T cell depletion method like mono- or polyclonal anti-T cell antibodies, chemotherapeutic agents like cyclophosphamide, or whole body or thymic radiation therapy [6]. And anti-thymocyte globulin (ATG) is still the most commonly utilized option.

The engagement of TCR (T cell receptor) with foreign antigen without co-stimulatory signal will lead to T cells unresponsive to the antigen (known as T-cell anergy), thereby suppressing antigen induced response. The possible mechanism was that the CTLA4Ig fusion protein blocked CD28/B7 co-stimulatory signaling of the primary pathway, which eventually induced differentiation bias of T helper cells (Th cells [7]). Anti-CD154 antibodies, known to be effective in blocking indirect pathway of allorecognition [6], is also a critical component of effective immunosuppressive strategies in preventing cellular rejection in pig-to-NHPs (Non-human primates) xenotransplantation [8] yet its clinical application is restricted due to high risk of thromboembolic complications [9]. However, in pig-to-NHPs models, immune tolerance achievement approached by utilizing co-stimulatory blocking agents and other immunosuppressants in long-term treatments.

The transgenic pigs expressing graft-protecting factors has been shown to require a less toxic immunosuppressive protocol [10] which gives another path to explore. Using advanced gene editing technologies, xenotransplantation from multitransgenic alpha-1,3-galactosyltransferase knockout pigs (GTKO pigs) has demonstrated marked prolongation of xenograft survival. In addition, the incidence of hyperacute rejection was further reduced with organs from the GTKO pigs expressing one or more human complement-regulatory proteins (GTKO/hCRPs pigs), such as CD46, CD55, or CD59.

3.1.2 Tolerance-inducing strategies across xenogeneic immunological barriers

A better but much more complex approach is to try to achieve immunological tolerance to the xenograft. Three successful tolerance induction approaches have been explored in large animal models: the use of mixed hematopoietic chimerism [11, 12], T regulatory cells [13, 14] and thymic transplantation [15] . It has been demonstrated that tolerance is possible in humans by successful clinical application of the mixed chimerism approach to renal transplantation [16] and by the T regulatory cell approach to liver allografts [17]. Despite the greater immunologic differences between species than within species, both mixed chimerism and thymic transplantation approaches have been shown to be capable of tolerizing human T cells to porcine xenografts in humanized mouse models [18]. Moreover, treatment with in vitro expanded regulatory T cells (Treg) prevents porcine xenograft rejection in humanized NOD-SCID IL-2 receptor gamma null (NSG) mice by the suppression of the T cell-mediated graft destruction, which suggesting the feasibility of pig-to-primate xenograft tolerance.

For xenografts, the level of immunosuppressive agents needed to fully suppress immune responses is greater than for allografts, which would likely lead to greater side effects. Thus, adoption of tolerance strategies is inevitable. Even though current immunosuppression seems to be controlling T cell responses in long-term acceptors [19, 20], it appears likely that low levels of T cell-dependent antibodies [21] and

activation of innate responses still develop [22], potentially leading to xenograft loss. Tolerance induction has the potential to avoid such persistent immune reactivity and therefore overcome the antibody-mediated response as well. Although tolerance induction in vivo has not yet been achieved in pig-to-baboon models, recent results are encouraging that this goal will be attainable through genetic engineering of porcine donors. It may be that current and future suppressive regimens that fully suppress the immune system will function sufficiently to benefit rejection of xenograft. Regardless of application, the study of tolerance continues to provide an excellent way to explore the functioning and control the immune system.

3.2 Data archive for xenotransplantation clinical trials

A database of clinical trials for pig islet xenotransplantation should be established, including specimens, paper documents, and electronic documents.

The information of xenotransplantation donors, including the number of animals, test reports, will be preserved for long time. All the samples will be prepared in duplicate and one for long-period preservation in −80° C refrigerator or liquid nitrogen tank. The information of transplant recipients and his/her spouses, such as name, hospital number, clinical data and patient records, will be recorded and maintained for long. When the patient comes to the hospital for review, the sample should be kept, including the following [23]: (1) all serum and plasma of the recipient and his/her spouse will be prepared in duplicate [24]; (2) storage of all samples at −80°C or liquid nitrogen tank for long time; (3) preservation of samples for post-transplant cytokine detection, pathogen detection, etc.; and (4) development of standard operating procedures.

3.3 Postoperative follow-up

The purpose of follow-up after xenotransplantation is to monitor the occurrence of rejection and adverse events. The goal of patient management is to improve their understanding of the disease, actively participate in and achieve partial self-management, improve compliance and achieve long-term survival and higher quality of life.

Postoperative follow-up of biosafety of clinical trials of recipients and spouses include: time-point, biosafety assays and treatment plan. (1) The patient and their spouses was reviewed 1 month before surgery, 1 month, 3 months, 6 months, 12 months, 2 years, 3 years, 4 years, and 5 years after xenotransplantation, and the sample in duplicate was kept. (2) Biosafety assays include fungal, bacterial, parasitic, viral, nucleic acid, cytokine and lymphocyte population detection. (3) If the biosafety assays are negative, the patient continues the symptomatic treatment, but if positive, then quarantine and treatment, personal protection and report to CDC (Centers for Disease Control and Prevention).

The medical record about postoperative follow-up of a xenograft recipient must contain the following information including the recipient's health status, all xenograft-related information, such as: (1) the contact information system of xenograft recipients. (2) If there is an infection related to xenotransplantation, or the pathogen from xenogeneic origin is identified, the health department of local government and the NHFPC(National health and family planning commission) shall be notified promptly. (3) The institution must have a reliable specimen and data preservation system and a complete information reporting system with the competent department. (4) The protocol must clearly address how patients are monitored for efficacy, biosafety, and period, including the draft clinical follow-up plan of xenotransplantation recipients.

4. Conclusions

Source donor pigs fulfilling the Designated Pathogen-Free (DPF) status have been available from a closed colony by GMP(Good Manufacturing Practice) rigorous routines, operational SOPs and rigorous data retention. Above all are very important for the operation of GMP barrier facility for biosafety of DPF source pig. A list of designated pathogens has been excluded from the DPF donor pig by long-term monitoring program of microbiological surveillance and pathological diagnosis. In addition, the consistently known DPF animals should be bred, grown and developed normally in the closed colony.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 2

Pig Islet Transplant

Masayuki Shimoda

Abstract

Islet transplantation is an effective treatment for insulin-dependent diabetes, but the shortage of donors is a problem. To overcome this, porcine islets have been widely studied as an alternative source. This chapter focuses on recent advances in porcine islet transplantation, placing particular emphasis on new transgenic pig models, islet encapsulation, and biological safety. Genetic modifications aimed at reducing the immunogenicity of islet cells to prolong graft survival or improve insulin secretory function have been reported. Microencapsulation and macro-encapsulation of porcine islets may be able to control rejection with little or no immunosuppression. Also, the risk of porcine endogenous retrovirus infection is considered low because several clinical and preclinical studies have found no such evidence. Appropriate pathogen screening, animal selection, and microbiological and quality control measures should improve the safety and efficacy of porcine islet transplantation in future clinical trials.

Keywords: xenotransplantation, islet transplantation, porcine islet

1. Introduction

The islet transplantation protocol used for patients with type 1 diabetes, published by a team of researchers at the University of Alberta in 2000, was called the Edmonton Protocol and became the starting point for clinical islet transplantation [1]. The characteristics of the Edmonton Protocol were that multiple transplants were performed using multiple donors to transplant sufficient amounts of islets, no steroids were used for immunosuppression, and transplants were performed as soon as possible after islet isolation.

Clinical results were reported 5 years after the Edmonton Protocol was announced [2], and several problems were identified. For example, the insulin-free status is not sustained for a long time, the probability of being able to obtain islets of sufficient quality and quantity for transplantation even with islet isolation is about 50%, and there were many side effects, mainly from immunosuppressants.

Islet transplantation has been found to stabilize blood glucose levels and could prevent severe hypoglycemia, defined as hypoglycemia requiring another person's assistance. Because severe hypoglycemia can be life-threatening for patients with type 1 diabetes, islet transplantation will likely be positioned as a measure for preventing severe hypoglycemia. Indeed, allogeneic islet transplantation is an established treatment for severe hypoglycemia in Canada and other European countries. In addition, in 2016, a phase 3 clinical trial of allogeneic islet transplantation for type 1 diabetes patients with a history of severe hypoglycemia found that islet transplantation has a preventive effect for severe hypoglycemia [3]. Therefore, allogeneic

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islet transplantation has also come to be recognized as standard treatment for severe hypoglycemia in the United States. Data on allogeneic islet transplantation are registered in the Collaborative Islet Transplant Registry (CITR). According to CITR data, the C-peptide positivity rate after islet transplantation alone was 80% after 1 year and 61% after 3 years, but the severe hypoglycemia prevention rate was 94 and 88%, respectively. This indicates that even if the concentration of C-peptide is below the lower limit of detection for a positive result (0.3 ng/ml), it would be effective in stabilizing blood glucose levels and preventing hypoglycemia. According to the data from the International Pancreas Transplant Registry, the pancreatic graft survival rate in simultaneous kidney and pancreas transplantation was 89% at 1 year and 82% at 3 years after transplantation. In other words, islet transplantation outperforms simultaneous pancreas and kidney transplantation in terms of rates of preventing severe hypoglycemia. The current status and direction of beta cell replacement therapy were discussed at a consensus meeting of the beta cell replacement therapy opinion leaders held at Oxford University in 2014 [4]. According to the consensus report, there are 15–20 million patients with type 1 diabetes worldwide, they are mostly at >20 years after onset of type 1 diabetes, 1 in 6 patients develop hypoglycemia unawareness, and ~10% of deaths in type 1 diabetes patients are due to hypoglycemia. It was announced that β cell replacement therapy was optimal for hypoglycemia in patients with hard-to-control type 1 diabetes. However, only 0.1% of patients with type 1 diabetes could receive beta cell replacement therapy due to a shortage of donors. In Japan, cardiac arrest donor islet transplantation [5] and living donor islet transplantation [6] have been carried out, but in order to fundamentally solve the donor shortage, β cell replacement therapy not relying on human organ donors is considered essential. Under these circumstances, pig organs are attracting attention as an alternative to organs from human donors.

2. Pancreatic islet transplantation using porcine islets

To realize successful porcine islet transplantation, exploratory clinical research began several decades ago. **Table 1** shows an overview of the history of porcine islet transplantation. In the 1990s in Sweden, Groth et al. transplanted islet cells from fetal pigs into type 1 diabetic patients on immunosuppressants after kidney transplantation [7]. Porcine C-peptide was positive for several months after transplantation, which indicated that porcine islets were successfully engrafted in the human body. Yet, no clinical effect such as a decrease in the amount of insulin injection was observed. In other works, Valdes et al. implanted an angioplasty device with newborn pig islets and Sertoli cells subcutaneously into type 1 diabetes patients [8]. Eleven patients received additional transplantation 6–9 months after the initial transplantation, and four received additional transplantation in the third year. Two patients achieved insulin-free status for several months after transplantation. In New Zealand, Elliott et al. transplanted newborn pig islets encapsulated in hydrogel microcapsules into the peritoneal cavity of type 1 diabetic patients. Because the islets were embedded in the immunoisolation capsule, no immunosuppressant was used. Insulin and glucagon staining of encapsulated pig islets, which were removed after 9.5 years of transplantation, showed that the encapsulated pig islets could be engrafted for a long time [9].

Thus, xenogeneic islet transplantation for type 1 diabetes patients using porcine islets has been performed in several clinical trials overseas. The risk of infection due to xenotransplantation was a concern.

Year	Events	Ref.
1994	Groth et al. reported that fetal pig islet transplantation to diabetic patients	[7]
1997	Patience et al. reported that PERV could infect human cells	[11]
2005	Valdes-Gonzales et al. reported a 4-year course after transplantation of neonatal pig islets and Sertoli cells	[8]
2006	Dufrane et al. showed that encapsulated adult porcine islets survived in the cynomolgus monkey body for more than 6 months	[12]
2006	Hering et al. achieved long-term insulin-free status in diabetic monkeys by transplantation of wild-type adult porcine islets	[13]
2006	Cardona et al. achieved long-term insulin independence in diabetic monkeys by transplantation of neonatal porcine islets	[14]
2007	Elliott et al. reported that about 9.5 years after transplantation, encapsulated porcine islets were recovered and insulin staining was positive	[9]
2013	Wang et al. commenced neonatal porcine islet transplantation with Tregs at Central South University, China	—
2014	Matsumoto et al. reported porcine islet transplantation under New Zealand regulations	[23]
2015	Yang et al. announced that they used CRISPR/Cas9 to inactivate all PERVs	[31]
2016	Matsumoto et al. reported clinical efficacy with encapsulated pig islet transplantation	[24]
2017	Yamaguchi et al. succeeded in creating a mouse pancreas in a rat using blastocyst complementation	[32]

Table 1.
Chronological overview of clinical and preclinical islet xenotransplantation.

3. Designated pathogen-free status and porcine endogenous retrovirus

Pigs for clinical use must have a designated pathogen-free (DPF) status, which means they are free of pathogens that can infect humans and pigs [10]. DPF status is achieved by delivering a piglet by cesarean section from a sow that has been confirmed to be free of transplacental pathogens, and after cleaning and decontamination, the piglet is placed in a biosecure barrier facility.

These facilities are defined at several levels. First, it is necessary that the facility itself be sited away from the pig farming facility. The breeding building must be completely isolated from the outside environment with an air filter, water decontamination system, radiation sterilization, and autoclave for all incoming materials. Piglets are fed with pasteurized milk, not breast milk, and enteric bacteria are provided separately. For waste disposal, especially liquid waste, special consideration is necessary to avoid backflow. Staff must pass through antiseptic showers both when entering and exiting the facility and must change into special sterilized clothes. Routine health checks of personnel are also conducted. In general, all procedures must follow standard operative procedures. It is also important to incorporate current good manufacturing practices in accordance with regulatory guidelines.

Nevertheless, in coculture of PK-15 pig kidney cell line (PK15 cells), and human fetal kidney cells 293 (HEK293 cells), infection of HEK293 cells by porcine endogenous retrovirus (PERV) naturally released from PK15 cells has been reported [11]. The problem of PERV infection via porcine xenotransplantation has emerged, and because PERV-A and PERV-B are integrated into all porcine genes, they are extremely difficult to eliminate. Thus, with regard to PERV, instead of exclusion, denial of infectivity and monitoring of transplanted patients and their close relatives are recommended.

4. Pig islet transplantation experiment using nonhuman primates

Dufrane et al. demonstrated that mature pig islets embedded in alginate capsules and transplanted into cynomolgus monkeys without immunosuppressants survived up to 6 months after transplantation [12]. Hering et al. at the University of Minnesota reported that wild-type (unmodified) adult porcine islets transplanted into the portal vein of rhesus monkeys with streptozotocin-induced diabetes mellitus achieved long-term insulin independence [13]. Also, Cardona et al. from the University of Alberta reported that wild-type newborn porcine islets transplanted into the portal vein of monkeys with pancreatectomy-induced diabetes resulted in long-term insulin-free status [14]. Recently, Park et al. reported more advances with modification of immunosuppressants [15]. These reports have brought great hope for islet transplantation using porcine islets. However, the importance of prevention of infections including PERV has been recognized.

5. Guidelines

While xenotransplantation holds great promise for overcoming donor shortages, the global problem of xenogeneic infection must be considered. Therefore, in 2008 the World Health Organization (WHO) held a conference on xenotransplantation in Changsha, China, and presented the main points as the First WHO Global Consultation on Regulatory Requirements for Xenotransplantation Clinical Trials [16]. This statement, referred to as the Changsha Communique, is the basis for xenotransplantation worldwide. The content summary is shown in **Table 2**.

Based on the Changsha Communique, in 2009 the International Xenotransplantation Association (IXA) announced a consensus statement of conditions for the initiation of clinical trials of porcine islet products for type 1 diabetes [17]. This consensus statement consists of seven chapters and addresses the requirements of the Changsha Communique. Because remarkable progress has been made in research in this field, the statement should be updated.

Since the consensus statement for the initiation of xenogeneic islet transplantation in IXA was announced in 2009, clinical findings of xenotransplantation including clinical xenogeneic islet transplantation in New Zealand have been accumulated, and the consensus statement was updated in 2016 [18]. The contents of the chapters are:

Chapter 1. Key ethical requirements and progress toward the definition of an international regulatory framework: ethical requirements and progress toward establishing an international regulatory framework.

Chapter 2. Source pigs: pig requirements for donor sources.

Chapter 3. Pig islet product manufacturing and release testing: manufacturing, quality control, and release testing.

Chapter 4. Pre-clinical efficacy and complication data required to justify a clinical trial: appropriate pre-clinical trial.

Chapter 5. Strategies to prevent transmission of porcine endogenous retroviruses: concept and prevention strategy for PERV.

Chapter 6. Patient selection for pilot clinical trials of islet xenotransplantation: appropriate patient selection.

Chapter 7. Informed consent and xenotransplantation clinical trials: ideal informed consent procedure.

In particular, because PERV infection and cross-species infection did not occur at all, these infections were regarded as a “theoretical risk” and were considered

1	Xenotransplantation can be used to treat serious diseases such as diabetes, heart disease, and kidney disease. Also, patients who cannot currently receive transplants may be able to receive transplants
2	Medical animals can provide high-quality cells, tissues, and organs. Genetically modified animals may further improve outcomes. Medical animals are limited to closed colonies. Breeding should be done at a well-controlled pathogen-free facility, with high standards for animal welfare. Medical animals are verified by testing for the absence of known pathogens and, moreover, must be kept free of infectious diseases by continuous observation
3	Xenotransplantation is a complex procedure with risk of rejection, poor graft function, and known or unknown infections. There is a risk of developing serious or new infections, and patients, relatives, or other humans and animals may be infected
4	Because of the risk to the community at large, clinical trials of xenotransplantation should be conducted under strict regulation. Xenotransplantation should not be performed in the absence of national regulations. These regulations should have legal basis and be able to prohibit nonregulatory transplants. Furthermore, this regulatory framework should ensure transparency to the general public and should include both science and ethics
5	Given the risk to the community, the benefit to the patient should be high. In particular, preclinical studies should be conducted using animal experiments with predictable effects to demonstrate the safety and efficacy recommended by the international scientific community. Proposed clinical trials should be assessed by the relevant regulatory authorities to minimize risk
6	Personnel responsible for clinical trials should explain the inclusion criteria in order to justify the clinical trial. Patient selection must be done at the patient's own discretion based on informed consent. Patients and relatives must be effectively educated to ensure compliance and minimize risks to themselves and to society
7	Participation in xenotransplantation usually takes a long time. Samples from donor animals, patients before and after surgery, and all records should be kept. Patients who have had transplants need lifetime follow-up, and close relatives may need similar follow-up. The results of clinical trials should be analyzed rigorously. Patients who have undergone xenotransplantation should be registered in an appropriate database, which should also be able to track donor animals. At the same time, the patient's privacy has to be protected. All records, data, and samples must be prepared for submission to regulatory authorities for a designated period
8	The health-care team must have adequate experience and an understanding of the risks to the patient, the health-care team itself, and the community. Because of the risk of transmission to the community, a system of vigilance and surveillance should be established to ensure that any infection associated with the xenotransplant will be identified and addressed immediately
9	There is a need to establish a system for worldwide information exchange, prevention of unregulated xenotransplantation, vigilance and monitoring of xenotransplantation, and response in case of suspected infection
10	Considering the benefits of successful xenotransplantation, from the early stages, the treatment should be considered widely accepted as the treatment is completed, and the public sector is recommended to support

Table 2.
Summary of the contents of the Changsha communique.

unlikely under the adequate control of suitable donors and recipients. In addition, clinical data have been accumulated, infection diagnostic techniques have progressed, clinical protocols have been improved, the risk of PERV-related infection is better understood, DPF facilities and dietary restriction methods have advanced, and the role of sample archives has been clarified. As a result of these efforts, cost-effective generation of donor pigs will be possible, and it is expected that porcine islets will be delivered to many patients who truly need this treatment modality.

Some countries have responded to this consensus statement. In Japan, the Ministry of Health, Labour and Welfare also revised the "guidelines on public health infection problems associated with the implementation of xenotransplantation" in 2016.

Recently, in response to the resumption of clinical xenotransplantation, the Third WHO Global Consultation on Regulatory Requirements for Xenotransplantation Clinical Trials was held in 2018, and the contents were announced as the 2018 Changsha Communique [19]. The points of the revision are:

1. Prohibition of clinical trials in countries without national regulations and subsequent prohibition of medical tourism to such countries

2. Emphasis on reproducible preclinical data

3. Development of quality control measures and standards for genetically modified pigs

4. Deletion of sample retention period requirements

The Communique emphasizes safety while taking into consideration the actual situation of clinical xenotransplantation, social conditions, and technological advances.

6. Encapsulation of islets

Although islet transplantation has proved to be successful for patients with type 1 diabetes, one of the limitations is the requirement for lifelong immunosuppression. An encapsulation strategy that can prevent rejection of xenogeneic islets can potentially overcome this challenge (**Figure 1**). Such capsules have fine holes that allow the passage of oxygen, glucose, and insulin but not immune cells. Blocking immune cells allows islet transplantation without the need for immunosuppressants. The capsules have been studied in various materials and sizes. There are three main sizes: macro, micro, and nano [20, 21]. The macro-capsule is used to seal islets in centimeter-order devices, which are easy to handle and can be removed and replaced. However, the problem is that substance permeability is low, and foreign body reactions are likely to occur, and the survival rate of internal cells is low. The microcapsule is several hundreds of micrometers to millimeters order in size, is

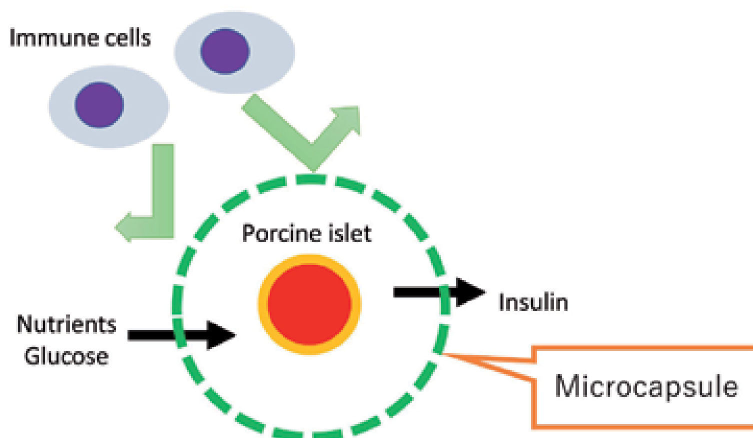

Figure 1.
Schematic representation of an encapsulated porcine islet. Pancreatic islets isolated from DPF pig are encapsulated with an immunoisolation hydrogel. The capsule has fine holes that allow passage of oxygen, glucose, and insulin but not immune cells.

made mainly of hydrogel, and contains one to several islets. It is compatible in terms of substance permeability and immune isolation ability. However, it is too large for endovascular transplantation and recovery after transplantation is difficult. The nano-capsule has a thin-layered surface coating enclosing pancreatic islets comprising a variety of polymers and therapeutic agents. The permeability is high but stability is an issue. In addition, a surface modification with immune-privileged cells is another concept of encapsulation. Each of these encapsulation techniques has advantages and disadvantages, but the technique is very promising.

7. Micro-encapsulated neonatal porcine islet transplantation

In 1980, Lim and Sun applied microcapsules in diabetes treatment, showing prolonged islet graft survival using alginate-poly-L-lysine-polyethyleneimine microcapsules [22]. Since then, this promising technology has been considerably improved.

In 2014, clinical results were reported in which neonatal porcine islets isolated from DPF pigs encapsulated with alginate and poly-(L)-ornithine were transplanted in 14 patients with type 1 diabetes [23]. The patients were divided into four groups according to transplantation dose, and 5000, 10,000, 15,000, and 20,000 IEQ/kg of encapsulated islets were transplanted intraperitoneally, respectively, according to body weight. No immunosuppressant was used. After transplantation, in the low-dose groups of 5000 and 10,000 IEQ/kg, the frequency of occurrence of hypoglycemia unawareness was halved compared to that before transplantation.

In 2016, the same group reported results of a clinical trial in which 5000 and 10,000 IEQ/kg of encapsulated neonatal porcine islets were transplanted twice at intervals of 3 months [24]. After transplantation, HbA1c decreased significantly in all patients, and the frequency of occurrence of hypoglycemia unawareness was significantly reduced in the group that received a transplant of 10,000 IEQ/kg twice. Moreover, the group that received a transplant of 10,000 IEQ/kg maintained an average HbA1c of $\leq$7% over 2 years after transplantation and showed a long-term effect. Clinical effects have been shown in islet xenotransplantation.

8. Porcine islet transplantation combined with regulatory T cell (Treg)

A clinical trial of transplantation of neonatal porcine islets and autologous Tregs in type 1 diabetes patients is underway and is being conducted by Wang et al., Central South University, China (ClinicalTrials.gov Identifier NCT03162237). The transplanted dose is 10,000 IEQ/kg of islets, 2×10^6/kg of Tregs, and the immunosuppressants are tacrolimus, mycophenolate mofetil, and belatacept. The primary end point is stable blood glucose level and prevention of ketoacidosis and hypoglycemia and a 30% reduction in required insulin. The authors reported that the condition of these patients improved substantially (http://en.xy3yy.com/document/show_12/184.html). These results are encouraging and add value to this field of research.

9. Gene editing and blastocyst complementation

One of the advantages of xenotransplantation is the possibility of genetic modification in the donor. Advances in gene editing, such as the CRISPR/Cas9 system, have facilitated editing of specific genes.

Recent advances in genetic engineering and gene editing of donor pigs may overcome the challenge of islet rejection and improve their engraftment and ability to secrete insulin. The required set of genetic modifications will depend on the source of islets (fetal, neonatal, and adult), mode of delivery (encapsulated, free), and the transplantation site. Genetic modification of pigs has been developed mainly via deletion of one or more of the major porcine antigens such as GGTA1, CMAH, and β4GalNT2, and/or insertion of human complement (such as hCD46, hCD55, and hCD59) which suppress the coagulation reaction [25, 26], and/or knockout or insertion of other genes. Simultaneous knockout of two or three major pig antigens has been achieved, and consequently the binding of human antibodies to these cells is significantly reduced. Other genes include the expression of proteins that inhibit co-stimulation of T cells such as hCTLA4Ig [27]. The combinations of multiple gene editing were promising [28, 29]. Currently, the modifications being carried out in pigs span over 24 genes including coagulation regulatory genes, immune cell regulatory genes, and anti-inflammatory genes [30]. Simultaneous modification of more than five genes has been performed in some pigs [30]. These genetically modified pigs will contribute to the improvement of transplantation outcome.

The technology has also been applied to elimination of PERV, and Yang et al. of Harvard University reported inactivation of all PERV genomes using the CRISPR/Cas9 system [31]. They launched a venture company called eGenesis, aiming to create a human friendly medical pig with the added advantage of PERV inactivation. Thus, it is considered that a medical pig suitable for islet transplantation will be created by gene editing technology.

Yamaguchi et al. of the University of Tokyo complemented mouse-induced pluripotent stem cells (iPS), cells with blastocysts of pancreatic-deficient rats, and succeeded in inducing the rats to develop mouse pancreas [32]. The pancreas derived from mouse iPS, which was produced by this blastocyst complementation method, was the size of the rat pancreas and had a sufficient number of pancreatic islets that could be isolated for transplantation to the mouse. These islets were transplanted with small amounts of an immunosuppressant drug to diabetic mice syngeneic with the iPS cells to normalize blood glucose levels. In addition, this research group also succeeded in inducing apancreatic pigs to produce different pig-derived pancreases by blastocyst complementation [33]. In the future, it may be possible to use a human iPS cell line to generate a medical pig for a human pancreas by blastocyst complementation. If the patient's own iPS-derived pancreas can be obtained from a pig, it is essentially an autologous transplantation, and it thus becomes possible to perform islet transplantation without the need for immunosuppressants.

10. Summary

Allogeneic islet transplantation is being established as a standard treatment for hypoglycemia unawareness and severe hypoglycemia, but a shortage of human donors has become a problem. Islet xenotransplantation using DPF pigs is considered as a promising fundamental solution to the donor shortage. However, cross-species infection, especially PERV infection, poses risks to the community, and discussions among key opinion leaders have been implemented by the WHO. As a result, the IXA Consensus Statement was published in 2016, envisioning a future where cost-effective delivery of islet transplants to diabetic patients is facilitated by medical pigs. With the risk of infection always kept in mind, cases of clinical islet xenotransplantation have been accumulated, and steady progress has been made toward a feasible, safe, and effective treatment for diabetic patients. In addition, the development of donor pigs optimal for transplantation using the

recently publicized CRISPR/Cas9 technology and blastocyst complementation that could enable the creation of an individual's pancreas in pigs could provide for safer and more effective islet xenotransplantation. Proper pathogen screening, animal selection, microbiological control, and long-term monitoring of recipients will be required for clinical application of porcine islet transplantation.

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Chapter 3

Porcine Islet Cell Xenotransplantation

Rajeswar Chinnuswami, Abid Hussain, Gopalakrishnan Loganathan, Siddharth Narayanan, Gene D. Porter and Appakalai N. Balamurugan

Abstract

This article reviews the rationale, sources and preparation of pig islets for xenotransplantation. Pancreatic islet cell transplantation is an attractive alternative and an effective treatment option for type 1 diabetes, however, donor pancreas shortages prevent islet transplantation from being a widespread solution as the supply cannot possibly equal the demand. Porcine islet xenotransplantation has the potential to address these shortages, and recent preclinical and clinical trials show promising scientific support. Pig islets provide a readily available source for islet transplantation, with the recent trials in non-human primates (NHPs) demonstrating their potential to reverse diabetes. The risk of zoonosis can be reduced by designated pathogen-free breeding of the donor pigs, but porcine endogenous retroviruses (PERVs) which are integrated into the genome of all pigs, are especially difficult to eliminate. However, clinical trials have demonstrated an absence of PERV transmission with a significant reduction in the number of severe hypoglycemic episodes and up to 30% reduction in exogenous insulin doses. A number of methods are currently being tested to overcome the xenograft immune rejection. Some of these methods include the production of various transgenic pigs to better xenotransplantation efficiency and the encapsulation of islets to isolate them from the host immune system. Furthermore, ongoing research is also shedding light on factors such as the age and breed of the donor pig to determine the optimal islet quantity and function.

Keywords: type 1 diabetes, xenotransplant, porcine islets, encapsulation, transgenic

Keypoints

- Preclinical studies show improvements in pig islet survival after transplantation.
- Clinical pig islet xenotransplantation studies prove no transmission of PERV.
- Pig islets can be successfully transplanted using encapsulation technology.

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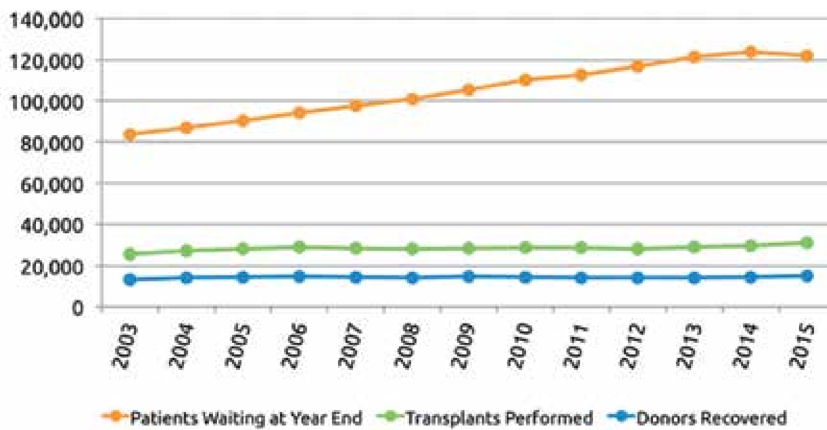
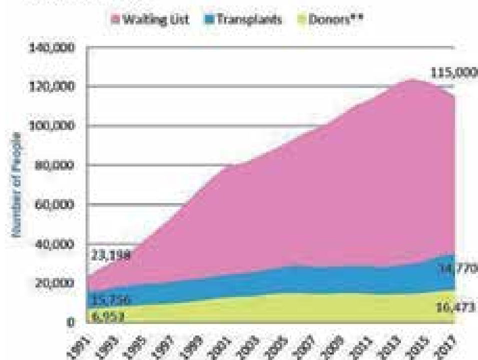
1. Introduction to islet xeno-transplantation

Exogenous insulin is the most common treatment option for type I diabetes (insulin-dependent diabetes mellitus), a chronic metabolic disorder caused by the failure of the beta cells of pancreatic islets most often due to T-cell mediated auto immune reaction which result in hyperglycemia [1]. While the standard insulin therapy treats patients with diabetes, however, it does not cure the disease, nor does it prevent the development of the secondary complications leading to end stage organ failures along with its morbidity and mortality [2]. Technical advancements in the production of exogenous insulin, better glucose monitoring system and optimal insulin therapy can reduce HbA1C but still has not addressed the issues of increasing hypoglycemic episodes in patients. Achievement of normoglycemia and exogenous insulin independence is the goal of diabetes treatment. The International Diabetes Federation (IDF) estimated the number of adults suffering from DM in 2017 to be 425 million: this number is expected to increase to 629 million patients in 2040 [3]. Whole pancreas and pancreatic islet transplantation





As of July 2019, there are more than 113,000 candidates for transplant on the U.S. national waiting list. Two out of every three people on the waiting list are over the age of 50. Almost 2,000 children under 18 are on the waiting list. Over 67,000 people (59 percent) on the list are ethnic minorities.

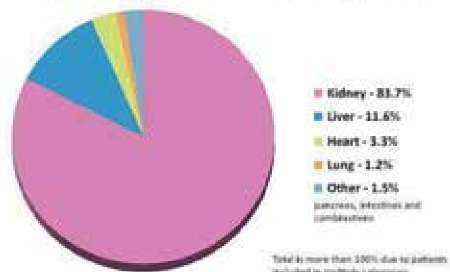


Figure 1.
Trends in the number of organ donors (blue), organ transplants (green), and patients on the waiting list (Orange) in the US, 2003–2015. In 2003, there were 13,285 donors, 25,473 organ transplants, and 83,731 patients on the waiting list. By 2015, there were 15,068 donors, 30,975 organ transplants and 122,071 patients on the waiting list. Source: http://optn.transplant.hrsa.gov.

are effective treatment options for diabetes by which insulin independence in T1D patients can be achieved [4]. Unfortunately, both whole organ and cellular transplantation face challenges due to a wide gap between the ever-increasing transplant waiting list and the supply of donor organs [5]. Data from the Organ Procurement and Transplant Network (OPTN) from 2003 to 2015, indicates a 145% increase in the wait list for all organs, while donor availability increased by only 113% (**Figure 1**) [6]. Similarly, the total number of pancreases available is insufficient to match the need for pancreatic islet allo-transplantation [7–9].

Due to this shortage, xenotransplantation using porcine islets has emerged as a potential alternative source for beta cell replacement. Porcine islets have structural and physiological similarities to human islets. Porcine insulin (differs from human insulin by only one amino acid) is used to treat diabetes in clinical practice [10, 11]. Intact functional islets have been successfully isolated from the pig pancreas [12], and these islets have shown the ability to reverse diabetes when transplanted into NHPs [13]. This review article will present the evolution, current practices, challenges and perspectives for pig islet xenotransplantation.

2. History of islet xeno-transplantation

Xenotransplantation has been attempted for the past 300 years or so and blood xenotransfusion was tried as early as the seventeenth century by Jean Baptiste Denis [14]. This was later followed by corneal transplantations from pigs to humans and kidney transplantations in NHP [15, 16]. The first pancreatic xenotransplantation was performed by Watson et al., implanted three ovine fragments into the subcutaneous plane of a diabetic patient. Though clinically significant blood glucose reduction was not demonstrated, the blood sugar level did decrease [17]. This pioneering work was followed by many experimental xenotransplantations, but results were mostly inconclusive [18–21]. Shumakov et al. reported 53 fetal porcine xenotransplants and 18 fetal bovine xenotranaplants in diabetic patients [22]. A century later, Groth et al. performed clinical xenotransplantation trial using fetal porcine islet cell-like clusters (ICCs) and provided preliminary data regarding the function and survival of grafts. After porcine islets were transplanted into 10 insulin-dependent diabetic kidney-transplant patients, detectable levels of porcine C-peptide were identified in the urine for up to 400 days and in one case, renal graft biopsy showed insulin and glucagon positive cells after staining [23]. Several xenotransplantation studies have also been performed in NHPs [20], and have succeeded in reversing diabetes [24–27] and in reducing daily insulin dosage requirement [28]. Transplanted porcine islet grafts were also shown to survive and function in NHPs for longer than 6 months with immunosuppression [25, 27, 29]. The longest survival rate is now over 603 days according to Shin et al., [30]. Studies have also shown that microencapsulation of the transplanted islets and immune-isolation lead to better survival rate without the need for aggressive immunosuppressive therapy [26].

3. Pig islets as alternative source

The success of porcine insulin and its role in the treatment of T1D has been well established since its discovery in the 1920s [11, 12, 25]. The structural and physiological similarities between human and pig organs, along with its unlimited supply, have made them an excellent translational research model [25]. Insulin extracted from pig islets has been used for the treatment of diabetes for decades [10, 11, 20, 33]. Because porcine islets produce insulin patterns similar to those found in

humans, and because they are readily available [20], studies strongly suggest that islets obtained from pigs could be a promising substitute for human islets in the treatment of T1D. Recent studies on genetically engineered pigs suggest that these pigs are more suitable for xenotransplantation. For example, alpha 1,3-galactosyltransferase gene knockout (GTKO) pigs, have decreased the incidence of immune-rejection and improved compatibility between the donor and recipient [31–36].

The major advantages for using pigs as an islet source for xenotransplantation are as follows:

1. Ethically acceptable source.

2. The pig pancreas has structural and physiological similarities to the human organ.

3. Unlimited availability.

4. Easy to breed and produce large litters.

5. Rapid growth into adult organs (6 months).

6. Significantly low cost of maintenance.

7. Elective and emergent availability of the organs.

8. Low risk of zoonosis.

9. Facilities available to breed pigs under 'clean' conditions.

10. Obviates 'cultural barriers' to human organ transplant (e.g. Japan); illegal organ trafficking; deleterious effects on organs in brain dead patients; living donor organ donation.

11. Advanced and safe immunosuppression protocols.

12. Cloning and genetic modification of cells to reduce immune destruction.

13. Islet encapsulation to combat immune challenge.

Modified from Ekser et al. [5]; Cooper et al. [37, 38]; Cheng et al. [20].

4. Selection of pig and sources of pig islets

Islet quantity and quality varies with the breed of pigs. Readily available market pigs have shown to yield lower when compared to the well-studied breeds of pigs like Landrace pigs, Chicago Medical School (CMS) miniature pigs and Chinese Wuzhishan (WZS) miniature pigs [23, 25, 27]. Two major factors which have been studied in relation to the source of pig islets for xenotransplantation are the breed and age of the donors. Some well-studied breeds are the Landrace pigs, Chicago Medical School (CMS) miniature pigs, and the Chinese Wuzhishan (WZS) miniature pigs. Market weight pigs are easily available, but studies have shown lower yields than for other breeds [39]. Landrace pigs have been shown to yield large sized (>250 μm) islets with a high islet volume density [39, 40]. Adult Chicago Medical School (CMS) miniature pigs

are bred under specific pathogen-free (SPF) conditions, and contain large-sized islets. The yield is greater than market or other miniature pigs (9589 ± 2838 IEQ/g), making CMS pigs one of the best sources for obtaining islets [39, 41–45]. Another miniature pig, the Chinese Wuzhishan (WZS) pig has also shown an islet yield greater than that of market pigs [39, 46]**.** Though no consensus has been arrived at the optimal breed for the preclinical/clinical studies, these breeds has been well documented to yield better islets than others. Higher expression of extracellular matrix (ECM) protein in islet capsules makes isolation easier and German Landrace pigs have higher ECM [24].

Additionally, age [43, 44, 47–49] and size of the donor pigs [36, 50–52] are major factors that affect islet isolation outcomes. Some studies have also suggested that gender may play a role in the final islet yield [39, 53, 54]. Pig islets can be obtained at four distinct life-stages: embryonic, fetal, neonatal and adult [55], and **Table 1** summarizes the significance, advantages, and disadvantages of pig islets from

Islet source	Significance	Advantages	Disadvantages
Embryonic	In the dorsal pancreatic primordial, strands of insulin positive cells are seen as early as week 4 [43]. From week 13, cells exhibiting intense immunoreactivity for insulin are distributed throughout the pancreas [43, 57].	Embryonic pancreatic tissue exhibit predominantly insulin-positive beta cells without evidence of alpha cells [43, 58]. Use of embryonic primordial pancreas is better than pluripotent stem cells as they do not need steering toward pancreatic differentiation and have lower risk of teratoma [59]. Following transplantation, the exocrine tissue does not proliferate. Hence, there is decreased immune response and inflammatory complications. Pancreatic primordia obtained on day 28 successfully reversed diabetes in rhesus monkey when compared to that obtained on day 35, which underwent rejection [43, 60, 61].	Immaturity takes 8–12 weeks (~6 months) for maturation *in vivo* [43]. Poor insulin response post-transplantation due to immaturity [39, 62–64]. Higher expression of alpha-1,3 galactose (Gal) when compared to adult—more susceptible for humoral rejection. Low yield—only a small number of islets can be isolated, requiring large number of pigs which limits large scale clinical application, with ethical issues.
Fetal	Porcine islets are isolated from fetuses of 60–69 days gestational age [36, 65]. Islets lack a definite shape and capsule and are organized in clusters (ICCs) [36]. These cellular clusters are composed of <40% endocrine cells (6–8% beta cells) with the majority being the cytokeratin-positive epithelial cells [65]. Their ability to proliferate makes them a potential source of islet cells [27, 36, 66–68].	Isolation process is very simple, involving digestion of the pancreatic tissue to free the islet clusters [65, 69]. No gradient purification necessary. Easily scalable to provide clinical product. Isolation not dependent on the enzyme collagenase, (activity is variable between enzyme lots). The use of alpha 1,3-galactosyltransferase GTKO strains has demonstrated better transplant outcomes than wild-type strains [43, 70].	Cellular culture is required for 5–9 days to form cellular aggregates. Maturation of islets is delayed Demands higher number of pigs to provide sufficient islets due to lower yield [27, 36, 71]. Because of their clustered appearance, it is difficult to separate islets from the surrounding exocrine and other non-islet cells [36].

Islet source	Significance	Advantages	Disadvantages
Neonatal	The neonatal period is up to 30 days after birth. NPIs are usually obtained from the pancreas within the first week of life [43]. NPIs comprise ~35% of endocrine cells and ~57% of epithelial cells—islet precursor cells [39, 72, 73]. Correct hyperglycaemia in diabetic animal models as the precursor cells also differentiate and proliferate into beta cells [27, 36, 39, 74, 75]. The cellular aggregates are composed of <40% endocrine cells (20–25% beta cells) with majority being cytokeratin-positive epithelial cells [65]. About 10–13 days after birth, the ICCs begin to resemble adult islets [43, 57].	Isolation process is very simple—the process involves digesting pancreatic tissue simply to free islet clusters [65, 72]. No gradient purification. Easily scalable to provide clinical product. Isolation not dependent on the enzyme collagenase (activity is variable between lots). Isolation process is less expensive than for adult islets. Maintenance of neonates is easy and inexpensive as they are maintained only for few days postpartum. Exhibit strong resistance to inflammatory and hypoxia-induced injury. Lower T-cell reactivity than adult pigs [39, 76, 77]. Potential alternative to adult pig islets as xenografts.	Maturation is delayed when compared to adult islets but is faster than for fetal ICCs. Cellular culture is required for 5–9 days to form cellular aggregates. Lower yield—limits clinical usage. Only 50,000 aggregates can be obtained from a single pancreas when compared to adult.
Adult	Adult pig islets (APIs) are the major source of islet cells for xenotransplantation [39, 78–80]. APIs are well differentiated with distinct and intact capsule and vasculature with very few insulin positive cells outside these islets [43, 57]. Antigenicity is from N-linked sugars and not from Gal Ag [39, 43, 81–83]. The expression of Gal Ag decreases and becomes negligible as the pig reaches adulthood [43, 81–86]. >2 yrs. is the optimal age [36, 39, 50, 54, 87]. Adult islets are predominantly islet endocrine cells.	Morphologically distinct—can be extracted and purified as a single unit [36]. Mature cells—response to hyperglycemia is immediate following transplantation without latency [36, 39, 43, 87–90]. Insulin independence in diabetic NHPs is achieved when ≥10,000 IEQs are transplanted. (islets pooled from 2 to 4 adult pigs) [39, 80]. Do not require culturing of the isolated islets [65]. Islet yield is greater than for fetal and neonatal pigs [43, 78, 91].	Isolation is technically challenging, complex and expensive [36, 43, 65, 79, 92–94]. More fragile islets [65]. Difficult to scale-up [65]. Highly dependent on the enzyme lot and activity [65]. Requires gradient purification [65]. Very high cost of maintenance and breeding in a clean isolated environment [36, 43, 47]. Bigger size of the animal is associated with surgical complications during organ procurement [36, 50].

Table 1.
Different sources of pig islets; significance, advantages, and disadvantages.

different donor life-stages. Adult pigs have been preferred for their higher yield of mature islet cells that have the potential to secrete insulin soon after transplantation. However, the higher costs, fragility of the islets and the difficulty in isolation are the disadvantages. Neonatal and fetal islets are easy and inexpensive to isolate but the main disadvantage is the significant delay in functioning after transplantation due to their immaturity and their high expression of Galactose-α-1,3-galactose (αGal), the major antigenic target for primate anti-pig antibodies [56].

5. Pig islet isolation

Adult pig islet preparation is very similar to human islet isolation methods [55] but the digestion process is a lot more gentler as the porcine islets are extremely fragile. Methods of islet preparation may vary depending on the life-stage of the donor pancreas. Fetal pig ICCs and neonatal pig islets (NPIs) are immature cells and can be easily isolated by enzymatic digestion [55] but must subsequently be cultured prior to transplantation to promote re-aggregation of islet clusters and to help eliminate exocrine cells [55]. The digestion procedure for the adult pig pancreas is significantly different over the fetal or neonatal pancreas. Many factors, such as the type of donor pigs, blood exsanguination, warm ischemia time, cold ischemia time, enzyme lot and activity, perfusate, and the isolation-purification process significantly affect the final islet yield, function and viability [39, 54, 55, 79, 95–97].

5.1 *In vitro* and *in vivo* assessment of pig islet function

In vitro studies investigating the insulin response of islets from donor pigs of different ages have shown that the insulin response from adult pig islets is more pronounced and sustained, and that they have a higher stimulation index over young pigs [36]. Islets from different age of donor pigs have also been compared *in vivo*. Two groups of diabetic nude mice populations were implanted with either young and young adult porcine islets or adult islets. One out of 11 recipients of young and young-adult islets achieved normoglycemia, whereas 32 out of 39 transplanted with adult islets became normal, the blood glucose reaching normal range within 4 weeks post-transplantation. Graft function was confirmed as the cause for normoglycemia, as all 32 mice reverted back to hyperglycemia after islet graft removal [36]. Many studies using NHP models have demonstrated the benefits of the pig islets as xenotransplants, with a potential cure for diabetes [25, 39, 98–101]. These studies have shown diabetes reversal with prolonged graft survival in diabetic NHPs.

5.2 Hurdles for xenotransplantation

Prevention of the transmission of porcine endogenous retrovirus (PERV) and immunological reactions have been the major hurdles for xenotransplantation in preclinical and clinical trials. Though the risks of zoonosis have been downplayed significantly with the introduction of genetically modified pigs, immunological responses like instant blood mediated inflammatory response (IBMR) dictate the success of the graft survival. One of the most important risk to overcome during xenotransplantation is the prevention of zoonosis [102]. Porcine endogenous retroviruses (PERVs) are of special concern as they are found integrated with porcine genomes and are difficult to eliminate [102]. The degree of risk of PERV being able to infect the human host is unknown, but evidence has shown that PERV can infect human cells when co-cultured with human EK-293 cells [55, 103]. Cross-species transmission has also been documented in pig to SCID mice xenotransplantation [55, 104]. However, no evidence of transmission has been documented in T1D patients who received porcine islet transplants, even after prolonged follow-up [55, 105].

Apart from PERV, other pathogenic organisms including the herpes virus, lymphotropic herpes virus, and cytomegalovirus can also be transmitted. [55]. Methods of combatting these pathogens include careful assessment and screening protocols, designated pathogen-free (DPF) breeding and housing of PERV gene knockout pigs, all of which can help minimize the risk of zoonotic infections [29]. DPF herds

Immune related islet injury	Genetic modifications	References
Ischemia/reperfusion injury and inflammatory cytokine related injury	Expression of human heme oxygenase-1 GTKO pigs/hCRP pigs	[55, 109] [110–112]
Humoral rejection	GTKO CD46 (membrane cofactor protein) CD59 (MAC-inhibitory protein) CD55 (decay accelerating factor)	[55, 113–115] [116] [117]
IBMIR and coagulation dysfunction	TF knockout and overexpression of human antithrombotic genes (CD39/thrombomodulin) ENTPD1 expression Mesenchymal stem cell (MSC) co-transplantation	[55, 118] [39, 119] [110, 120]
Cellular rejection	CTLA4Ig gene expression GTKO pigs/hCRP pigs MSC co-transplantation—downregulate T-cell response (immunomodulator)	[55, 121] [110–112, 122] [39, 120, 123] [117]

Table 2.
Genetic modifications in pigs to overcome immunological rejection.

must be free from a comprehensive and list of specified microorganisms [29, 106] and meticulous documentation and standard operating procedures (SOPs) must be implemented to maintain this status [29] including feed restrictions [29].

5.3 Immunological response

Pig islet cells express different surface proteins that play a major role in the immunological rejection seen following transplantation [102, 107]. Immunological responses are much more complex than seen in allo-transplantation [102]. Immune mediated inflammatory response have been brought down by significantly by genetic modifications as summarized in **Table 2**. Hyper acute rejection (HAR), Instant blood mediated inflammatory response (IBMIR), and cellular rejection are the types of responses seen in graft rejection of which IBMIR is the most crucial. Portal vein site provides good revascularization and drainage for islet transplantation but due to the severe complications like bleeding, thrombosis, and hepatic steatosis, it is no longer an optimal site [108]. Immunological issues observed during xenotransplantation are similar to those seen in allo-transplantation but are much more complex [102]. Pig islets express different types of surface proteins, and these play a critical role in the immunologic rejection seen following transplantation [107]. Multiple genetic modifications in pigs have been proposed to significantly reduce immune mediated inflammatory response, and these are summarized in **Table 2**.

There are four known major routes for islet cell loss following transplantation and these are summarized in the following sections.

5.3.1 Hyper acute rejection (HAR)

HAR occurs due to the presence of pre-existing host antibodies to surface proteins on the porcine islets. These surface proteins can be broadly categorized into Gal and non-Gal proteins [34, 38, 110]. The Gal epitope is absent in humans, apes and old-world monkeys but many bacteria, NHP and new world monkeys express

the Gal epitope abundantly. In pigs, the expression of Gal antigens decreases as they grow into adults [84, 102, 110, 124, 125].

As the human body is continuously exposed to micro-organisms (including bacteria), it develops immunity to the Gal antigen and has pre-formed, circulating anti-Gal antibodies [107, 126], which make up around 1% of the circulating antibodies [102, 124]. Once the pigs islets are transplanted, these pre-formed antibodies kill the islet cells rapidly by complement mediated destruction [107, 124] resulting in substantial islet loss [102, 107, 127].

Antibodies are also produced for other surface epitopes (non-Gal Ag) such as N-glycolylneuraminic acid (NeuGc) also known as Hanganutzu-Deicher and beta 1,4 N-acetylgalactosaminyltransferase (B4GALNT2) [107, 128–130] which are also involved in complement mediated destruction of xenografts [107].

There are two known strategies for prevention of HAR. Knockout of genes responsible for adding the Gal epitope and other epitopes such as Neu5Gc to the cell surface can prevent their expression [34, 102]. Secondly, expression of complement regulatory proteins such as hCD46, hCD55 and hCD59 can be induced on the surface of the islet cells [102, 131]. Double knockout pigs (deficient in alpha-gal (GTKO) and Neu5Gc) have been produced, which has significantly reduced the incidence of humoral rejection [102, 132]. The Gal antigen is highly expressed in fetal and neonatal pig pancreas, but its expression decreases as the pigs reach adulthood. The use of GTKO pigs is more validated when using fetal or neonatal pancreas [85, 116], but is not as essential when using adult pigs [116]. However, increasing titres of anti-Gal IgG antibody have been noted when immunosuppression is stopped after adult pig islet transplant [30, 116], so GTKO pigs may prove beneficial even for islets isolated from adult pigs.

5.3.2 Instant blood mediated inflammatory reaction (IBMIR)

Following the intra-portal infusion of the pig islets, the elevated expression of tissue factor by the islets initiates IBMIR [39]. The IBMIR contributes to significant islet loss in the early post-transplant phase through a series of events involving simultaneous complement activation (alternative pathway) [81, 86], activation of intrinsic and extrinsic coagulation pathways, and platelet activation (platelet aggregates around the islets P6) followed by neutrophil and monocyte infiltration [110, 116, 133, 134]. IBMIR can result in 60–80% of islet loss in the immediate post-transplant period [39, 55, 110, 118, 135], but studies in NHPs have shown that if a sufficient number of islet cells survive, they can establish normoglycemia for several months [110]. Genetically modified pigs have been produced [110, 136] to combat IBMIR by decreasing the load of xenoantigens but it failed to provide long-term protection against host response [137]. Experimental studies involving control of complement activation by cobra venom factor, and platelet aggregation and coagulation by anti-platelet agents and low molecular weight heparins are not proven clinically safe, [138, 139]. Peritoneal cavity and omentum offer alternative sites for transplantation of encapsulated islets [140].

5.3.3 Cellular rejection

Cellular rejection, a $CD4^+$ T-cell-dependent process [55, 141–143], plays a major role in islet destruction [39, 118, 144, 145]. Acute cellular rejection occurs within 24 h to 20 days post-transplant, and is characterized by a massive infiltration of macrophages and T-cells ($CD4^+$ and $CD8^+$cells). Two signaling pathways required for the full activation of T cells are the T cell receptor signaling, and the co-stimulatory signaling [55, 146]. Since T cell activation requires double signaling

involving TCRs and co-stimulatory molecules [39], blockade of co-stimulatory cell surface molecules such as CD870/86- CD28 and/or CD40L (CD154)- CD40 have significantly improved graft survival, even without immunosuppression [39, 147–149]. The addition of targeted immunosuppression to multi-molecular blockade may further increase effectiveness, and provide an even more promising option to prevent cellular destruction of the transplanted islets [39].

5.3.4 Islet cell revascularization

Islet revascularization is critical for the survival of transplanted pig islets. Islet grafts are cut off from their native vascular supply and after transplantation, are solely dependent on diffusion for nutrient supply, until functional revascularization is established with the host vasculature. This process takes place within 10–14 days post-transplantation [41, 49, 141].

6. Islet encapsulation approaches

Islet encapsulation provides the means for islet cell survival in the absence of immunosuppressive drugs. The principle of encapsulation is that transplanted cells are contained within an artificial compartment separated from the immune system by a semipermeable membrane. The capsule should protect the cells from potential damage caused by antibodies, complement proteins, and immune cells. Therefore, the capsule is often referred to as an "immunoisolation device." As well as the protective mechanism provided by the capsules, islet cells within the capsules can also release insulin to control blood glucose levels, since this membrane enables small molecules to diffuse in (glucose, oxygen, and nutrients) and out (metabolic wastes) [39, 150–152]. Thus, the encapsulation system is also regarded as a "bioartificial pancreas." The immunoisolation device or bioartificial pancreas can be commonly separated into two categories, intravascular and extravascular devices. The latter can further be divided into macroencapsulation and microencapsulation devices. Intravascular and extravascular classifications are based on whether or not it is connected directly to the blood circulation.

The macroencapsulation and microencapsulation classifications depend on whether it contains one or more islets in the device [153, 154]. Alginate is the most commonly used capsule material for microencapsulation, but other materials such polyethylene glycol have also been tested [153].

Although the capsule is selectively permeable, islets can be damaged due to hypoxia or inadequate nutrients, and slow glucose and insulin diffusion can delay insulin response to changing glucose levels [155]. Despite the protection offered from direct immune attack, islets can still be damaged by immune responses. Inflammatory cytokines, produced against the capsules can enter the capsule and damage islets. The encapsulated islets themselves may release such cytokines and cause self-damage [156]. Approaches investigated to overcome these problems include testing different sites of implantation, creating biocompatible capsules, and optimizing the capsule size. The use of genetically engineered pig islets within capsules to promote graft survival and function have also been studied [156]. Several clinical trials of encapsulated pig islets to improve long-term survival outcomes of xenografts are currently being conducted around the world [117, 157]. A phase I/IIa clinical study in Moscow has tested the clinical applicability of a commercially available encapsulated pig islet product called Diabecell [39, 158, 159]. Additional phase I/IIa clinical trials are ongoing in New Zealand and Argentina. These trials have demonstrated an absence of PERV transmission, a significant

reduction in the number of severe hypoglycaemic episodes and up to 30% reduction in exogenous insulin doses [29, 160]. A 10 year follow up of another study involving xenotransplantation of encapsulated porcine islets into the peritoneum of a T1D patient has shown long-term islet survival and function, with no evidence of PERV infection [39, 150].

7. Regulatory aspects

Any new therapeutic substance or procedure, safety and efficacy of the drug substance have been inveterate before starting government approved clinical trials. In line with guidance in consensus statements from the International Xenotransplantation Association and the WHO on xenotransplantation, geographical location will impact choice of the microbiological mitigation strategy. Risk management at the source would include the definition of pathogens circulating in the countries of origin [161], establishment of reliable detection, and screening methods and assessment of risk from animal feed. Given the source animals to be utilized will be from specific pathogen-free/designated pathogen-free or high hygienic herds from a single location, the pathogen risk compared with standard slaughter herd animals is significantly reduced. Further testing during the manufacturing process, that is, islet isolation and encapsulation will provide tissue specific data that should further confirm safety of the final product. Moreover, alginate encapsulation allows keeping the islets in culture for longer periods thus giving enough time to perform viral screening on islet products before transplantation. Other release quality controls related to islet morphology, viability, purity, quantity, and potency should also be established in order to guarantee that only well characterized and functional islet preparations are used in patients. The use of genetically modified donor pigs to reduce islet cells immunogenicity and improve their secretory function stipulates that these genetic modifications should be well characterized. Integration of transgene expression cassettes should be in well-defined genomic locations, preferably in the form of a single-targeted integration that would ensure stable expression of the transgene across herds without affecting other cell functions or rendering them tumorigenic. In this context, it should be noted that encapsulation limits the risk of tumor cells spreading since it confines the cells and eliminates the need for immunosuppression meaning that in case the integrity of the encapsulation device would be compromised, xenogeneic pig cells would most probably be rejected by the host immune system. The use of nonhuman primates in research is subjected to very strict ethical and regulatory considerations but the pig-to-primate model is still considered as a gold standard for pig islet xenotransplantation, so that safety and efficacy data obtained using this model are required before initiating clinical studies [162].

8. Conclusion

Porcine islets represent an excellent alternative source to replace human islets in diabetic patients. Pig islets can be obtained from different life-stages (embryos to adults) and has several other advantages making it an indispensable resource for xenotransplantation. Active research have resulted in standardization of protocols, thereby bettering isolation outcomes. In addition, incorporation of multiple strategies such as generating transgenic pigs together with developing cellular and molecular therapies to sustain long-term xenograft survival have brought porcine islets closer to clinical applications. Despite the risk of zoonosis and other factors which

contribute to islet loss post-transplantation, tremendous progress has been made within the field such as developing encapsulated islets to combat host immunity and utilizing host stem cells to aide islet revascularization. Pig islet xenotransplantation currently acts as a bridge between allo-transplantation and stem-cell therapies. With all the tremendous progress made within the field, ongoing research focuses on a better understanding of various factors such as donor characteristics, isolation procedures, microbiological safety, and immunological tolerance to improve pig islet yield, function and transplantation outcomes. Furthering this understanding will require multiple clinical trials directed toward establishing porcine islets as a safe, effective and robust alternative for treating patients with T1D.

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Conflicts of interest

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Chapter 4

Challenge of Xenotransplantation in Pediatric Heart Transplantation

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Abstract

Although surgical techniques have progressively improved in the field of congenital heart disease (CHD), even such as hypoplastic left heart syndrome, pediatric heart transplantation is the most effective surgical option for complex CHD and cardiomyopathy with severe heart failure. However, even now, donor heart availability has been poor in children. Although technologies for ventricular assist device (VAD) have been progressing even in children, VAD cannot grow as the pediatric recipient grows. Therefore, pediatric cardiac xenotransplantation has a great possibility to save and grow children with end-stage heart failure. In this chapter, I would like to introduce the first pediatric baboon-to-human heart transplantation and its basic animal experiments done by Bailey's group and the following attempts for pediatric cardiac orthotopic xenotransplantation (rhesus monkey-to-baboon and pig-to-primate combination).

Keywords: concordant and discordant xenogeneic orthotopic heart transplantation, pediatric heart transplantation, clinical trial, antibody absorption, primates, pig, goat, lamb

1. Introduction

Clinical heart transplantation (HTx) was the unambiguous goal of the laboratory research at Stanford University in the mid-1960s [1]. They were making tremendous progress in their understanding of the host immune response, and how to control that response with drugs of that era, while at the same time avoiding lethal infection. Then, unexpectedly, on December 3, 1967, Christiaan Barnard et al. performed the first clinical HTx in Cape Town, South Africa [2]. Their recipient survived only 18 days, dying of pneumonia. Nevertheless, it stirred worldwide enthusiasm for HTx, and, more importantly, it opened the door for the Stanford group to develop the procedure in human.

But with regard to infants, Adrian Kantrowitz at New York attempted HTx in a newborn from an anencephalic baby just 3 days after Barnard's first HTx [3]. The recipient died 6 and a half hour after the procedure, and Kantrowitz never pursued clinical HTx. In the 1970s, there have been great progresses in medical and intensive management and surgical technology for neonates and infants with complex congenital heart. However, almost all neonates and infants with too complexed congenital anomaly, especially hypoplastic left heart syndrome (HLHS), could not survive surgery. Theoretically, these neonates, with naïve immune systems and

uniformly lethal heart disease, should be excellent candidates for HTx that included aortic arch reconstruction. But around for a decade since the first HTx, clinical HTx was limited to only a handful of progressive institutions, and none was spearheading research in neonatal HTx except a little Leonard L Bailey's group at Loma Linda University.

His laboratory was using neonatal goats as recipients, and, initially, goats as donors. In 1981, the Sandoz Laboratory, a pharmaceutical house in Basal, Switzerland, agreed to provide them with an investigational agent called cyclosporine-A (CsA). With CsA immunosuppression alone, they observed remarkable survival, maturation, and reproductive capacity among goats that were orthotopically transplanted as newborns with allografts [4]. Even recipients of cross-species grafts from lamb to goat experienced unprecedented survival [5].

2. Lamb to goat orthotopic concordant xenoHTx

Fourteen newborn (less than 7 days old) goats underwent orthotopic HTx with a size-matched lamb's heart [5]. Ten goats survived longer than 24 hours after HTx. Recipient animals received CsA 48 and 24 hours before HTx and daily after HTx on a gradually reducing daily protocol. Recipients were also given pulse doses of methylprednisolone (100 mg/kg) and azathioprine (3 mg/kg) once a week, the dosage schedule being gradually reduced. Azathioprine was discontinued on postoperative day 60. Survival among the 10 recipients was 24, 32, 44, 47, 60, 60, 78, 90, 120, and 165 days. Average survival was 72 days. Serial left ventricular ejection fractions measured by radionuclide left ventriculography from 1 to 4 months postoperatively in four recipients averaged 50, 58, 45, and 45%. There were no significant infections. Most animals showed mild-to-moderate subacute and chronic graft rejection at autopsy. One host showed no gross or microscopic graft rejection at autopsy on postoperative day 47. Tumor was not observed. These data suggest that long-term survival may be feasible for newborn recipients of cardiac xenografts with CsA therapy and limited supplemental immunosuppression.

3. Attempt of a baboon-to-human orthotopic concordant xenoHTx

Neonatal and small infant heart donors were not available in the early 1980s; hence, the Bailey's group focused on the possibility of using immature baboons as donors for neonates with HLHS. They purchased a panel of infant baboons and studied them extensively for infectious diseases. They performed HLA-typing, two-way mixed lymphocyte cultures, and ex vivo perfusion studies to assess their compatibility with human neonates. They thought it might be possible to actually select a "best" baboon donor for any individual baby with HLHS. They began an arduous 14-month process of obtaining Institutional Review Board (IRB) approval for experimental clinical trials of baboon-to-human baby concordant xenoHTx. Sandra Nehlsen-Cannarella, a transplant immunologist and Medawar protégé, was one of external reviewers, helped their works, and finally joined their team after the IRB was approved in October 1984 [6].

Then, in late July of 1984, Dr. Magdi Yacoub and his team at the National Heart Hospital in London transplanted an 11-day-old newborn with HLHS [7], but the recipient had a complex postoperative course and died of respiratory failure on postoperative day 4. Later that same year, in October, the Bailey's group were confronted with the potential to activate our IRB-approved protocol. A neonate

with HLHS named “Baby Fae” was transplanted on October 26, 1984, with the heart of a highly selected infant baboon [8, 9]. She lived for only 20 days, and despite careful observations and analysis, the cause(s) of her death remains somewhat of an enigma. She did heighten awareness, however, and her transplant led directly to the first successful neonatal HTx, again as treatment for HLHS, in November of 1985. That infant is now a 34-year-old man working in Las Vegas. Baby Fae’s legacy is found among the hundreds of neonates and small infants who are living today because of primary or secondary HTx in the world. However, donor shortage had been still severe, and continuous experimental efforts to achieve clinical infant xenoHTx had been performed in the Bailey’s group.

4. The immunological effects of concordant xenograft bridging to cardiac allografting in baboon

Human neonatal xenoHTx evolved around the idea of xenograft bridging to cardiac allografting. The important question relating to this approach was whether the bridged recipient would develop an antibody response to the initial xenograft that would be cross-reactive with the allograft donor. This question was initially explored by Alonso de Begona [10] using a heterotopic HTx model from African green monkey to juvenile baboons treated with CsA (**Table 1**). These 5 grafts are rejected over a period of 5–65 days. Lymphocytotoxic xenoantibody was identified in recipient blood samples. The rejected xenografts were removed, and the recipient circulating xenoantibody titers were observed to peak over 24–48 hours. Using cardiopulmonary bypass primed without blood, the immature baboon recipients then underwent orthotopic allogeneic HTx and were treated with varying degrees using a cyclosporine (CSA) protocol. All survived the secondary allogeneic HTx without any evidence of hyperacute, antibody-mediated rejection. The recipients survived 10, 58, 65, 198, and 164 days. Despite a high titer of circulating xenoantibody in each of the host baboons, orthotopic allogeneic engraftment was possible in all five recipients. Each was immunosuppressed with gradations of CSA-based therapy. Survival to 5 and 6 months of the last two consecutive animals (which were ultimately euthanized) was not unlike that expected for

	Cardiac heterotopic xenograft (African green monkey)			Cardiac orthotopic allograft (common olive baboon)			
	Therapy[a]	Rescue therapy	Survival (days)	Therapy[a]	Rescue therapy	Survival (days)	Allograft rejection
1	A	None	11	A	None	10	Severe
2	A	None	5	A	B	58	Moderated to severe
3	A	None	6	A	B	65	Moderated to severe
4	A + B	B + C	13	A	B + C	198[b]	None
5	A + B + C	None	65	A + B	None	164[b]	None

[a]*Immunosuppression. (A) cyclosporine + azathioprine + solumedrol; (B) goat anti-human T cell IgG; (C) monoclonal antibody.*
[b]*Electively euthanized.*

Table 1.
Survival of xenografts and allografts and host therapy employed in a xenograft bridge to allograft model using an immature baboon recipient.

allotransplanted hosts. Xenoantibody did not appear to alter acute or chronic survival of baboon recipients managed with a clinically applicable regimen of immune regulation. The two chronic survivors had well-functioning allografts that were free of significant rejection injury. These findings have subsequently been confirmed and elaborated on by Michler et al. [11].

5. Rhesus monkey to baboon orthotopic concordant xenoHTx

Orthotopic concordant xenotransplantation in a juvenile primate model was examined [12, 13]. Eighteen donor rhesus monkeys weighing 2.4–3.8 kg (mean 2.9 kg) were matched with juvenile baboons, aged 9–19 months (mean 12.7 months) and weighing 3.2–4.8 kg (mean 3.9 kg), using ABH blood type and mixed lymphocyte culture. In order to examine plasma level of tacrolimus (Tac) in infant baboons and establish immunosuppressive regimen before starting orthotopic xenoHTx experiments [14], seven of these baboons already received two courses of 4-week immunosuppressive therapy prior to HTx. All baboons underwent splenectomy at the time of HTx.

Twelve animals were divided into three groups; five baboons received no immunosuppressive therapy (Group-C). Five baboons were pretreated (Group-P) and the other seven (Group-NP) was not pretreated. Twelve baboons received sheep antilymphocyte globulin (ALG; IV 15 mg/kg) induction for 3 days before the operation and 5 days after xenoHTx and oral tacrolimus (Tac; 18 mg/kg) and intravenous methotrexate (MTX; 0.1–5 mg IV twice weekly) daily after xenoHTx. The baboons in Group-P received two courses of 4-week immunosuppressive therapy prior to xenoHTx; the first course consisted with Tac (18 mg/kg p.o. daily) alone and the second one consisted with Tac (12 mg/kg p.o. daily) and methotrexate (MTX; 25 mg IV weekly). Pretreated baboons had drug-free intervals for 37 days between two courses and for 83–110 days between the second course and xenoHTx. Intravenous methotrexate, methylprednisolone, ALG, and their combination were used as rescue therapy (**Table 2**).

Baboons in group-C had a mean survival of 8 days; all died as a result of classic severe cellular rejection. Baboons in Group-NP had a mean survival of 51.3 days (25–75 days), and those in Group-P had a mean survival of 198 days (35–502 days). Two in Group-NP died during rescue therapy for rejection, and three in Group-NP and two in Group-P died of cytomegalovirus (CMV) infection. One in Group-NP died of massive micro-pulmonary embolism. The remaining two in Group-P died of *Klebsiella pneumoniae* and renal failure aggravated by ganciclovir, respectively.

The longest surviving baboon, named Max, had been a healthy, active, growing baboon with normal cardiac function assessed by echocardiography and left ventriculography and coronary arteries normal in size and distribution assessed by coronary arteriograms at 1 year after xenoHTx. After these examinations, we tried to convert him to oral medications, and his level of immunosuppression fluctuated widely, which led to a late, powerful rejection response. This xenograft rejection was reversed successfully using corticosteroids and ALG. The additional bolus immunosuppression, however, permitted the development of generalized CMV disease and eventually bacterial sepsis from which Max (**Figure 1**) ultimately died. The animal's autopsied xenograft was almost free of cellular rejection but with mild coronary graft atherosclerosis [15].

Management of CMV infection in this splenectomized series of baboon recipients proved to be at least as difficult as controlling the immune response toward their cardiac xenografts. However, Tac coupled with low-dose maintenance

methotrexate and splenectomy has produced prolonged host survival in this xenotransplantation model. Results suggest that concordant xenotransplantation would be a suitable biologic bridge to allotransplantation.

	Survival (days)	Histological findings of autopsied xenograft	Rescue therapy (onset day after transplant)	Cause of death
Group-C[a]				
1	6	Moderate cellular rejection	None	Rejection
2	7	Moderate cellular rejection	None	Rejection
3	8	Severe cellular rejection	None	Rejection
4	8	Severe cellular rejection	None	Rejection
5	9	Severe cellular rejection	None	Rejection
6	10	Severe cellular rejection	None	Rejection
Group-NP[a]				
1	25	CMV infection, no rejection	ALG (21)	Systemic CMV infection
2	32	CMV infection, no rejection	None	CMV infection (graft)
3	43	Cellular infiltration to coronary arteries	None	CMV infection (lung, kidney)
4	53	Mild cellular rejection	ALG (68)	CMV infection (lung)
5	57	Mild cellular rejection	ALG + MP (13), Up[b] Tac + ALG + MP (38)	During rejection treatment
6	74	Mild cellular rejection Mild graft atherosclerosis	Up[b] MTX (25)	Pulmonary embolism
7	75	Mild cellular rejection	ALG + MP (29, 62)	During rejection treatment
Group P[a]				
1	35	Mild cellular rejection	None	*Klebsiella* pneumonia
2	96	No rejection	ALG + MP (71)	Renal failure
3	123	Patchy fibrosis in septum and inferior wall	None	CMV infection (lung, kidney)
4	234	Toxoplasmosis	ALG (94d)	Toxoplasmosis
5	502	Mild cellular rejection Mild graft atherosclerosis	Up[b] Tac and MTX (68, 238), ATGAM+MP (392), MP (482)	Liver failure and CMV infection

ALG, sheep antilymphocyte globulin; CMV, cytomegalovirus; MP, methyl prednisolone; Tac, tacrolimus; MTX, methotrexate; ATGAM, equine anti-thymocyte globulin.
[a]Group-C: controls. Group-NP: intravenous sheep antilymphocyte globulin (ALG) induction at −3 and + 5 days perioperatively, daily oral tacrolimus (Tac), and twice weekly intravenous methotrexate (MTX) after transplantation. Group-P: two courses of 4-week immunosuppressive therapy (1st course, oral Tac alone; 2nd course, oral Tac and intravenous MTX) prior to transplantation and the same immunosuppressive therapy after transplantation as for Group-NP.
Groups NP and P subjects had splenectomy at the time of heart transplantation.
[b]increase dose.

Table 2.
Results of orthotopic cardiac xenotransplantation between immature baboon recipients and rhesus monkey donors.

Figure 1.
Max, an immature baboon recipient of an orthotopic cardiac xenotransplant acquired from a donor rhesus monkey.

6. Toward discordant xenoHTx

Although the high degree of evolutionary relatedness between human beings and primates both suggests that xenotransplantation of primate organs and tissue might be successful, particular concerns are raised by the use of primates, such as baboons. The characteristics, for example, of intelligence and complex social interactions of these closely related higher primates appear to be so like those of human beings that use members of those species as sources for xenotransplantation which might well be seen as ethically unacceptable [16]. The potential risk of extinction, even to a species like the baboon that is not currently endangered, must be taken seriously. The possible transmission of disease from higher primates to human beings and the welfare of the animals should be concerned. From these concerns, it is currently agreed that the use of primates would be ethically unacceptable.

Given the ethical concerns raised by the use of primates for xenotransplantation, attention has turned to developing the pig as an alternative source of organs and tissue, because the use of pigs for xenotransplantation raises fewer ethical concerns. Attention has focused in particular on pigs, since their organs are comparable in size to human ones, and they breed rapidly and could thus be used to supply transplant material on a large scale. The use of pigs as a domestic animal that is farmed and eaten is long established, and many would have fewer concerns about their use for xenotransplantation than the use of primates. If pigs are used for xenotransplantation, they are likely to have been genetically modified so the human immune response to the pig organs and tissue is reduced [16].

When a pig organ is transplanted into a human or nonhuman primate, an immediate immune response occurs with hyperacute rejection (HAR). This has been defined as destruction of the graft in less than 24 hours; however, it usually occurs

within the first hour. This is due to the binding of the preformed anti-pig antibodies (Ab) to the endothelial cells of the graft. Ab deposits initiate a complement-mediated response with endothelial injury, resulting in thrombosis, interstitial hemorrhage, and edema, with subsequent graft dysfunction [17]. Later, it was determined that Ab bind to the carbohydrate epitope, galactose–a1,3-galactose (Gal), expressed in the pig vascular endothelium. This oligosaccharide is present in other mammals, except humans and primates. These Ab are produced in response to viruses and microorganisms that express Gal and colonize the gastrointestinal tract of primates [18].

7. Pig-to-baboon orthotopic discordant xenoHTx

The feasibility of transplanting across discordant xenogeneic barriers in an orthotopic newborn pig-to-juvenile baboon model was first explored in the Bailey's

	Treatment		Survival (hours)	Pathology of autopsied xenograft	Cause of death
	Lung perfusion	Exsanguination			
Group-C[a]					
1[b]	None	None	4.5	HAR	Rejection
2	None	None	18	HAR	Rejection
Group-D[a]					
1[c]	Donor lung	None	6.5	HAR	Rejection
2	Donor lung	None	10	HAR	Rejection
3[d]	Donor lung	None	375	Mild DXR and GCAS	CMV infection
Group-LD[a]					
1	Large pig lung	None	99	Pneumonia	Pneumonia
2	Large pig lung	None	111	DXR	Rejection
Group-D + E[a]					
1	Donor lung	Blood replacement	117.5	DXR	Brain death
2[c]	Donor lung	RBC/serum replacement	100	DXR	Rejection
3	Donor lung	RBC/serum replacement	111	DXR	Rejection
4	Donor lung	RBC/serum replacement	123	DXR	Rejection
5	Donor lung	RBC/serum replacement	174.5	DXR, CR	Rejection

[a]*Group-C, controls; Group-D, donor lung perfusion; Group-LD, perfusion with another large pig lungs; Group-D + E, donor lung perfusion, exsanguination, and replacement with whole blood pretreated or packed red blood cell (RBC) and serum pretreated.*
[b]*No immunosuppression therapy.*
[c]*Kidney perfusion in case of suspected antibody-mediated rejection.*
[d]*Thymic injection with donor myocardium (left atrium).*
All subjects had pretransplant splenectomy. CMV, cytomegalovirus; RBC, red blood cell; HAR, hyperacute rejection; DXR, delayed xenograft rejection; CR, cellular rejection.

Table 3.
Results of orthotopic cardiac xenotransplantation between juvenile baboon recipients and piglet donors.

laboratories during the early 1990s. Because HAR was at that time the single most important factor in limiting discordant xenoHTx, early strategies were directed toward eliminating or reducing baboon preformed xeno Ab to pig sugar antigens [19, 20].

All recipient baboon underwent splenectomy 2 weeks before HTx. Donor hearts were obtained from 12 newborn piglets of either sex age 2–7 days and weighing 1.8–3.1 kg (mean 2.3 ± 0.1 kg) and transplanted orthotopically with deep hypothermia and circulatory arrest in recipient juvenile baboon age 252–459 days (mean 362 ± 19 days) and weighing 2.4–3.5 kg (mean 2.9 ± 0.1 kg). All animals received an infusion of nafamostat mesylate (FUT-175) at a dose of 2 mg/kg/h for 2 h at the time of reperfusion. The recipient baboon received 15 mg/kg CsA orally or 5 mg/kg intravenously and 5 mg/kg 15-deoxyspergualin (DSG) intramuscularly, from the day before HTx until death.

In two baboons, no antibody adsorption (AbA) using pig lungs was performed for control (Group-C). In 10 baboons, the blood in the bypass circuit was perfused into a pig lung to absorb baboon anti-pig antibody during circulatory arrest at the time of HTx. In three baboons (Group-D), the donor lung was perfused, and in two baboons (Group-LD), a lung larger than the donor pig (weighing 5–7 kg) was perfused. In five baboons (Group-D + E), the donor lung was perfused, and exsanguination was also performed at the beginning of cardiopulmonary bypass (CPB), and the baboon blood was replaced with pretreated whole blood (N = 1) or packed red blood cell (RBC) and 50 ml of pretreated plasma (N = 4). The pretreated blood (N = 1) and serum (N = 4) were made by perfusing with other large pig lung (weighing 15 and 20 kg) before xenoHTx. Two baboons underwent pig kidney perfusion using an extracorporeal shunt from the right femoral artery to vein, 5 and 6 days after xenoHTx, because antibody-mediated rejection was suspected.

Figure 2.
An immature baboon recipient of an orthotopic cardiac xenotransplant acquired from a donor pig, which survived 6 days after xenotransplant.

The two control animals survived 4.5 and 18 hours, and the pathological changes of the grafts were compatible with HAR. The other animals survived 125 ± 33 h (10–375 hors). The longest surviving baboon who survived 375 hours was in Group-D, but other two in Group-D died of HAR. All baboons in Group-LD and Group-D + E survived more than 4 days after XenoHTx. One in Group-D died of CMV infection and one in Group-LD died of pneumonia. One in Group-LD and four in Group-D + E died of acute cellular rejection. In summary, examination and echocardiography revealed no evidence of hyperacute rejection in baboons surviving more than 1 day. The longest survivor (375 hours) died of CMV infection with microscopic evidence of mild delayed HAR and graft coronary atherosclerosis. A variable amount of delayed xenograft rejection (DXR) was observed histologically, among the other recipient baboons (**Table 3** and **Figure 2**) [20].

Another baboon which underwent large pig lung perfusion and is given Tac + MTX without splenectomy survived 16 days, and the autopsied graft showed mild DXR and moderate GCAS [20].

8. The role of anti-pig antibody in pig-to-baboon xenoHTx rejection

To investigate the role of anti-pig Ab in discordant xenograft rejection, these 12 baboons were divided into 2 groups: Group-S (n = 4) died within 24 hr. of HTx and Group-L (n = 8) survived more than 24 hr. [19]. Mean survival period was 9.8 ± 3.0 h in Group-S and 151 ± 33 h in Group-L. Baboon anti-pig Ab was measured before CPB, before circulatory arrest, during AbA, at the end of CPB, and daily after HTx. Anti-RBC Ab was measured by the titration method at temperatures of 4 degrees C and 37 degrees C (RAb-4 and RAb-37). Anti-endothelial cell Ab (EAb) and anti-white blood cell Ab (WAb) titers were measured with enzyme-linked immunosorbent assay (ELISA). RAb titration > or = 1/4 and EAb and WAb > or = 1/256 were determined to be seropositive. Seropositive rate of RAb-37 at the end of CPB (endCPB) in Group-L was significantly higher than that in Group-S (8/8 vs. 1/4; $P < 0.05$). The seronegative rates of RBC-4 and EAb (endCPB) in Group-L were higher than those in Group-S (7/8 vs. 1/4 and 6/8 vs. 1/4, respectively), but not significantly. There was no difference in seronegative rate of WAb (endCPB) between both groups. More than fourfold decrease in RAb-4 and RAb-37 by AbA with a pig lung was observed in 5 and 7 of 8 baboons, while EAb and WAb did not change by AbA. In all of Group-L, RAb-4 reverted to seropositive within 3 days after HTx. In four of Group-L, RAb-37 became S(+), 1 or 2 days before death by rejection. EAb became seropositive in all of Group-S, but five of them survived more than 5 days after seroconversion. It was concluded that a pig lung absorbed RAb-4 and RAb-37 may play a role in DXR.

After I came back to Japan, the role of RAb-37 on pig-to-baboon xenoHTx was investigated using sequential heterotopic HTx [21]. Fifteen pig hearts were obtained from pigs weighing 6.4–91 kg. Eleven hearts from pigs larger than the recipient were used for perfusion, and four hearts from a pig of the same size as the recipient for heterotopic transplant donor heart. Four female baboons weighing 5.9–8.1 kg received Tac (12 mg.kg) and CAM (50 mg/kg) p.o. daily 2 weeks before and after xenoHTx. After perfusion with two or three large pig hearts, a pig heart was heterotopically transplanted in the right neck of recipient baboon. As the second and third recipient baboons died of hypotension during the third pig heart perfusion and could not undergo heterotopic xenoHTx, the last baboon underwent two pig heart perfusion and subsequent heterotopic xenoHTx. All first perfused hearts and two second perfused hearts were hyperacutely rejected within 30 minutes of perfusion, but the other two second and all third

	RAb-4	RAb-37	EAb-IgM	EAb-IgG
Adult	582 ± 579	296 ± 291	288 ± 189	853 ± 264
Cord blood	144 ± 181*	69/96*	21 ± 8.3**	683 ± 264
Infant <38 days old	80 ± 58*	30 ± 19*		
Infant > = 38 days old	689 ± 678***	239 ± 149***		

RAb-4 and RAb-37: human anti-pig red blood cell antibody titer at temperature of 4°C and 37°C, respectively.
EAb-IgM and EAb-IgG: human anti-pig endothelial cell antibody (immunoglobulin M and G) titers, respectively.
$^{*}p < 0.01$ vs. adult.
$^{**}p < 0.05$ vs. adult.
$^{***}p < 0.01$ vs. cord blood or infant younger than 38 days old.

Table 4.
Human anti-pig antibody against red blood cell and endothelial cell.

Treatment			Survival after releasing AXC (minutes)	Off CPB and chest closure	Extubation	Pathology of autopsied xenograft
	Donor pig	DAF pig organ perfusion				
Group-C[a]						
1[b]	F1 pig	None	21	No	No	Severe HAR
2	F1 pig	None	132	Yes	No	Severe HAR
Group-DAF[a]						
1 [c]	DAF[b] transgenic pig	None	104	Yes	No	Mild HAR
2	DAF[b] transgenic pig	None	135	Yes	Yes	Mild HAR
3	DAF[c] transgenic pig	None	126	Yes	Yes	Mild HAR
Group-DAF+P[a]						
1	DAF[b] transgenic pig	Heart	211	Yes	No	Moderate HAR
2	DAF[b] transgenic pig	Heart	310	Yes	No	Moderate HAR
3 [d]	DAF[b] transgenic pig	Lung	305	Yes	Yes	Mild HAR
Group-GnT-III[a]						
1	GnT-III transgenic pig	None	73	Yes	No	Mild to moderate HAR
2 [c]	GnT-III transgenic pig	None	257	Yes	Yes	Mild to moderate HAR
3	GnT-III transgenic pig	None	493	Yes	Yes	Mild to moderate HAR

DAF, decay-accelerating factor; GnT-III, beta-D mannoside beta-1,4-N-acetylglucosaminyltransferase III; AXC, aortic cross-clamping; CPB, cardiopulmonary bypass; HAR, hyperacute rejection; DXR, delayed xenograft rejection.
[a]Group-C, controls; Group-DAF, transplanted DAF transgenic pig heart; Group-DAF + P, transplanted DAF transgenic pig heart and perfused with another pig heart; Group-GnT-III, transplanted GnT-III transgenic pig heart.
[b]Hetero DAF transgenic pig.
[c]Homo DAF transgenic pig.

Table 5.
Results of orthotopic cardiac xenotransplantation between rhesus monkey recipients and transgenic pig donors.

perfused hearts were not rejected within 2 hours after perfusion. The first and last transplanted pig hearts stopped beating 6 days and 18 hours after xenoHTx. Histological examination showed no rejection findings in the myocardium of the graft taken at 1 hour after xenoHTx, but the explanted grafts after cardiac arrest showed massive necrosis with ischemic change which suggested some kinds of DXR. RAb-37 prior to perfusion in all baboons was 1: 256 or 1:512, but that at 1 hour after XenoHTx was less than 1:4 which was considered to be negative. These findings suggested that RAb-37 may play an important role in DXR in pig-to-baboon combination.

We also investigated the differences between newborn and adult natural heterophile anti-pig red blood cell IgM xenoantibodies as correlates of xenograft survival [22] (**Table 4**). Newborns and younger infants have significantly lower titers of anti-pig RAb-4 and RAb-37 and anti-pig EAb-IgM than adult.

After coming back to Japan, Kawauchi M also investigated ontogeny of RAb-37 and HAR in 15 macaque monkeys [23]. Ten hearts from newborn Gottingen miniature swine (6–12 days old) were heterotopically transplanted into 10 infant macaque monkeys (52, 59, 75, 101, 108, 114, 129, 151, 181, and 192 days old) without immunosuppressive therapy. RAb-37 prior to xenoHTx were gradually increased according to the age of the monkeys. All six donor hearts in the recipients younger than 4 months survived 6 hours, and then the animals were killed while the donor hearts were beating. Donor hearts in four infant recipients ages 129, 151, 181, and 192 days were hyperacutely rejected at 19, 22, 29, and 9 minutes. The pig hearts in the recipients younger than 4 months showed no findings of HAR.

These two findings may suggest that newborn and younger infants may be more suitable recipient of discordant xenoHTx.

9. Transgenic pig-to-rhesus monkey orthotopic discordant xenoHTx

As Miyagawa et al. demonstrated the effect of the human beta-D mannoside beta-1,4-N-acetylglucosaminyltransferase III (GnT-III) gene in downregulating the xenoantigen of pig heart grafts, using a pig to cynomolgus monkey transplantation model suggests that this approach may be useful in clinical xenotransplantation in the future [24]. Moreover, they showed the possibility that both the decay-accelerating factor (DAF) and GnT-III double transgenic pig skin xenografts could be used in place of human skin allografts in the cases of severe burns [25].

Then, after coming back to Japan, the author and Japanese colleagues underwent orthotopic discordant xenoHTx using DAF and GnT-III transgenic pig heart xenografts (unpublished data). Donor hearts were obtained from two F1 pigs, six DAF transgenic pigs (five hetero DAF and one homo DAF), and three GNT-III transgenic pigs and transplanted orthotopically in adult rhesus monkey with deep hypothermia and circulatory arrest. All animals received no immunosuppressive drugs.

In two baboons, a F1 pig heart was transplanted for control (Group-C). In three baboons, the blood in the bypass circuit was perfused into a hetero DAF pig heart or lung to absorb baboon anti-pig antibody during circulatory arrest at the time of xenoHTx (Group-DAF + P).

In the one control animal, the graft stopped beating 21 minutes after aortic unclamping before weaning from cardiopulmonary bypass (CPB). All other 10 rhesus monkeys could wean from CPB and undergo chest closure, but only one in Group-DAF, one in Group-DAF + P, and two in Group-GNT-III could be removed from a ventilator. Two grafts in Group-C and two perfused pig hearts showed severe HAR. Other grafts showed various degree of HAR. These data suggested that

transgene of DAF or GNT-III might not be enough to suppress HAR in adult rhesus monkey which had high titers of anti-pig xenoantibodies.

10. Recent concerns about xenotransplantation in children

Xenotransplantation has been proposed as a method of reducing the especially acute shortage of organs for babies and children. Early clinical trials of xenotransplantation will be a form of therapeutic research. Therapeutic research must offer some prospect of genuine benefit for the patient, but it involves greater uncertainties than treatment, and therefore greater caution must be exercised. Many working parties concerning xenotransplantation, such as the British Pediatric Association and the Medical Research Council, have advised that therapeutic research should not involve children if it could equally well be performed with adults. It would be difficult to justify the involvement of children in major and risky xenotransplantation trials before some of the uncertainties have been eliminated in trials involving adults. Therefore, the FDA and WHO also recommend that the first xenotransplantation trials involve adults rather than children.

Then, although the authors tried to continue animal experiment to start clinical pediatric xenoHTx in the mid-2000s, we resigned.

11. Current status of pediatric heart transplantation in the world and Japan

After the Bailey's first xenoHTx, hundreds of neonates and small infants with end-stage heart failure are living today because of primary or secondary HTx in the world. The number of pediatric HTx has been increasing (**Figure 3**), and their survival has been acceptable in every recipient age (**Figure 4**).

When the author came back to Japan in 1994, there was no Transplant Act in Japan. In 1988, the Japan Medical Association professed that it would accept brain death as human death. In 1990, the Provisional Commission for the Study on

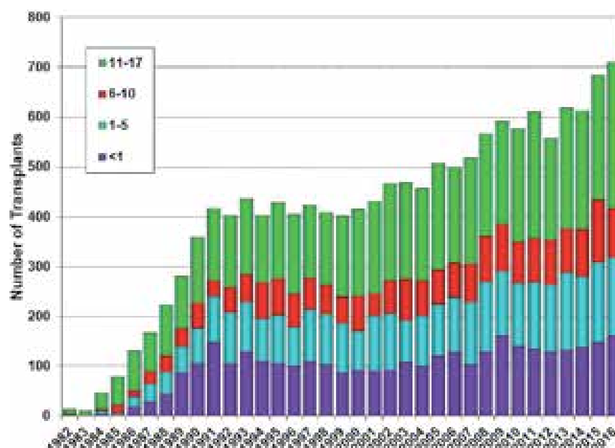

Figure 3.
Pediatric heart transplants. Recipient age (in years) distribution by year of transplant.

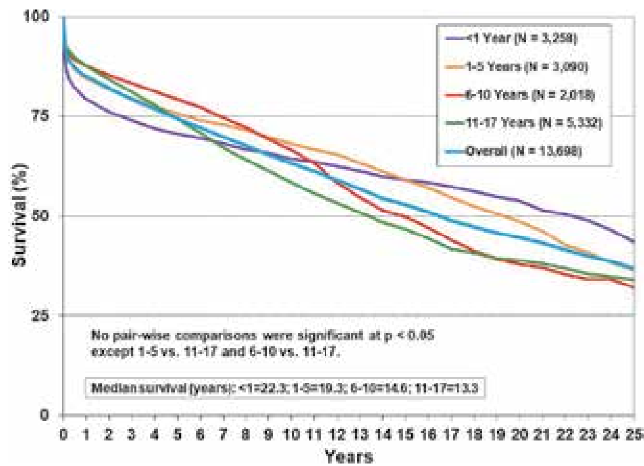

Figure 4.
Pediatric heart transplants. Kaplan–Meier survival (transplants: January 1982–June 2016).

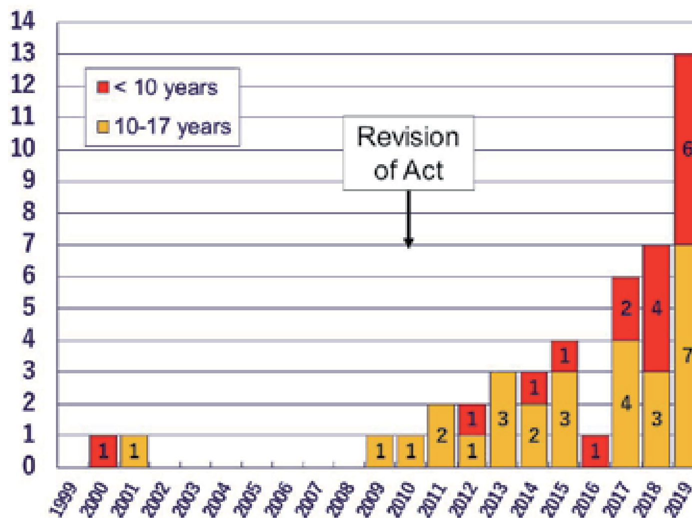

Figure 5.
Pediatric heart transplant in Japan.

Brain Death and Organ Transplantation was set up in 1990. The draft of the Organ Transplantation Law was proposed in 1994. Finally, on October 16, 1997, the Organ Transplant Act took effect, which enabled brain dead organ donation only if the person expressed in writing prior to death his/her intent to agree to donate his/her organs. In addition, the Act states that “only persons 15 years and above can express to donate.” Then, heart transplants to small children become impossible.

So, we started to send children with end-stage heart failure to Dr. Bailey as other pediatricians did (**Figure 5**) and continued to perform xenoHTx experiments. But as mentioned above, we finished experiments due to the FDA and WHO recommendation against pediatric xenotransplantation. Since 2003, the author and members of Japanese Associations of Transplant patients made many efforts to revise the Act, and finally the Act was revised in 2010. After then, the

number of pediatric HTx has increased and finally exceeded that of HTx abroad (**Figures** 5 and **6**).

Unexpectedly, Dr. Bailey (**Figure** 7) died of cancer in May 2019.

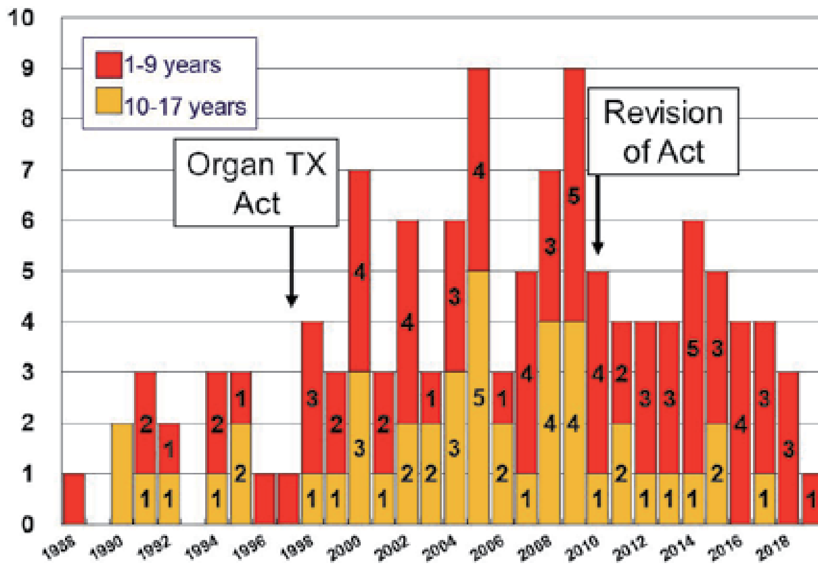

Figure 6.
Pediatric heart transplantation abroad from Japan.

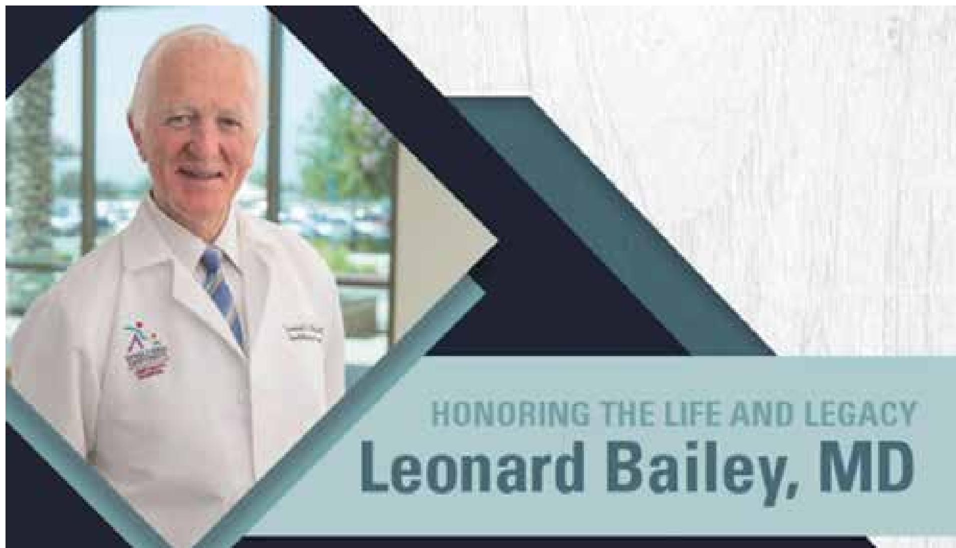

Figure 7.
The panel of Professor Leonard Bailey's memorial service.

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Section 2

3D-Bioprinting and Decellularization

Chapter 5

Tissue-Specific Bioink from Xenogeneic Sources for 3D Bioprinting of Tissue Constructs

Sriya Yeleswarapu, Shibu Chameettachal, Ashis Kumar Bera and Falguni Pati

Abstract

3D bioprinting brings new aspirations to the tissue engineering and regenerative medicine research community. However, despite its huge potential, its growth towards translation is severely impeded due to lack of suitable materials, technological barrier, and appropriate validation models. Recently, the use of decellularized extracellular matrices (dECM) from animal sources is gaining attention as printable bioink as it can provide a microenvironment close to the native tissue. Hence, it is worth exploring the use of xenogeneic dECM and its translation potential for human application. However, extensive studies on immunogenicity, safety-related issues, and animal welfare-related ethics are yet to be streamlined. In addition, the regulatory concerns need to be addressed with utmost priority in order to expedite the use of xenogeneic dECM bioink for 3D bioprinted implantable tissues for human welfare.

Keywords: 3D bioprinting, xenogeneic tissues and organs, xenogeneic decellularized extracellular matrix (dECM), dECM bioink

1. Introduction

The field of tissue engineering centers on development of tissues that are capable to regenerate and has a capacity to restore the damaged organs both structurally and functionally [1, 2]. Scaffolds that are developed to serve this purpose should be able to provide cell attachment sites and allow cell proliferation and migration while maintaining its structural and mechanical integrity [2]. Along with this, the placement and uniform distribution of cells in the scaffold play a major role to determine its functional efficiency [3]. This precise positioning of multiple cell types in an organized manner can be achieved with 3D bioprinting [4]. Plenty of natural materials, such as gelatin [5, 6], alginate [7–9], collagen [10, 11], and synthetic materials like polycaprolactone (PCL) [12–16] and polyethylene glycol (PEG) [17–22], come in handy while printing a structure. Although the above-mentioned natural materials are biocompatible, disadvantages such as mechanical instability, limited degradability, restricted cell proliferation, and differentiation challenged researchers to investigate more on natural materials [23–25]. As a result, human organ/tissue specific extracellular matrix (ECM) emerged as a best source to develop a functional tissue in laboratory conditions [23, 26, 27]. Yet, the major

limitation for this best material is its availability [28–30]. The next alternative source of ECM is to use from other species that are anatomically, physiologically, and metabolically similar to the recipient such as nonhuman primates (like apes, monkeys, and porcine) [31–33]. However, due to the risk of infections from nonhuman primates to human patients and organs from apes, baboons are abandoned, and hence pig became a suitable candidate as an organ donor for humans [33]. There is growing interest of xenogeneic ECM material as printable bioink (biomaterial formulation used for bioprinting) in the field of bioprinting due to easy access and the availability in required quantity. A process termed decellularization allows maximum removal of cellular content while retaining the ECM components from the native animal tissue to reduce the chance of immune rejection when implanted in the patient [29]. The first ever reported *in vivo* study of decellularized tissue was reported in 1991 by Krejci et al. [34], where human decellularized skin was used in mouse model. In 1995, Badylak's group used decellularized xenogeneic small intestinal submucosa for Achilles tendon repair [35]. Later, a number of decellularized ECM (dECM)-based devices are introduced, e.g., human dermis, porcine urinary bladder, porcine small intestine submucosa, and porcine heart valves [36] (**Figure 1**; for details refer to **Table 1**). In the recent past, there are several preliminary reports demonstrating the use of animal-derived dECM in the form of bioinks for developing functional tissues [27, 37]. Not only high cellular viability, these dECM-based constructs also showed enhanced differentiation and proliferation of cells into specific cell types when embedded in tissue-specific ECM [23, 27, 38]. Apart from the need to develop a fully functional construct, the foremost reason for not implanting these structures into human beings is due to high risk of xenotoxicity. Other species, being the source of material for the scaffold that has to be transplanted into human, have to undergo several stringent laws and clear all the clinical trials and ethical concerns. In this book chapter, discussion on the status of xeno-sourced dECM-based bioprinting, including the few reported preclinical studies, is included. The processing steps for dECM preparation and associated

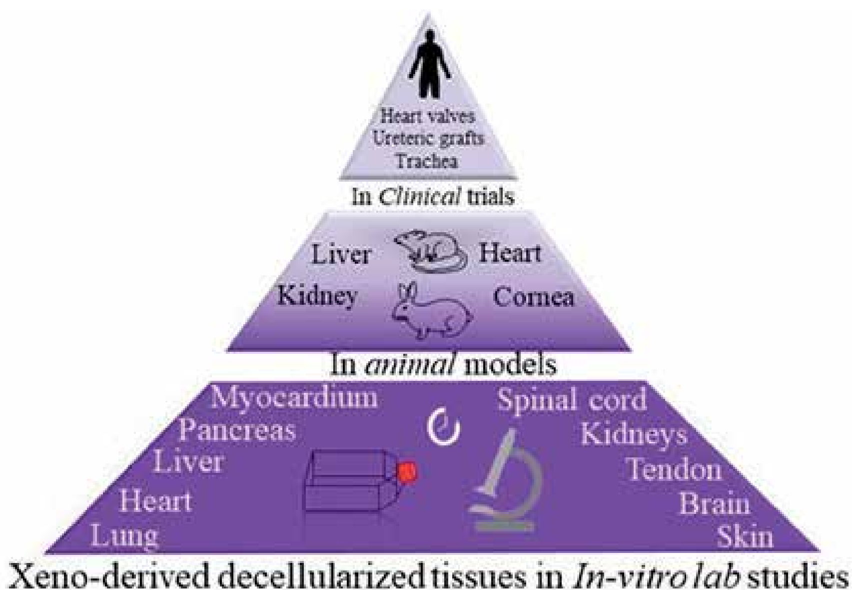

Figure 1.
An upright triangle representing number of decellularied xeno-transplants that are being tested at various stages viz in vitro lab experiments, animal and human trials.

Source	Tissue	Cell types	Recipient	Result	Reference
Porcine	Pericardium	Human sheath synoviocyte, human adipose derived stem cells	*In vitro* culture	• Production od synovial fluid with hyaluronic acid	[126]
Porcine	Myocardium	Porcine adipose derived stem cells, rat adipose derived stem cells	Rat myocardial infarction model	• Stem cells expressed endothelial marker • Increased vascular formation in the myocardial tissue	[127]
Porcine	Myocardium	Human embryonic stem cells	*In vitro* culture	• Myocardial maturation	[128]
Porcine	Liver	Rat endothelial cells	*In vitro*, culture and *in vivo* porcine model	• Clinically relevant vascularized bioengineered liver	[129]
Balb/c Mice	Liver	Balb/c Mice derived mesenchymal stem cells	*In vitro* culture	• Maturation of hepatic like tissue	[130]
Rat	Liver	Adult rat hepatocyte	*In vitro* culture	• *In vitro* maturation of liver with albumin secretion, urea synthesis and cytochrome P450 expression	[131]
Porcine	Liver	Second trimester human fetal liver cells-hepatocytes, stellate cells	*In-vitro*, culture and *in vivo* porcine model	• *In vitro* maturation of liver with albumin secretion, normal metabolic parameter	[132]
Rat	Heart	Rat neonatal cardiocytes, rat aortic endothelial cells	*In vitro* culture	• Increasing of left and right ventricular pressure • Contraction after 8 days of *In vitro* culture	[133]
Mice	Heart	Human induced pluripotent stem cell-derived multipotential cardiovascular progenitor cells	*In vitro* culture	• Engineered heart tissues exhibited spontaneous contractions, generated mechanical forces • Drug responsive	[134]
Mice	Lungs	Mesenchymal Stromal cells derived from bone marrow of adult male mice	*In vitro* culture	• Matrix from decellularized fibrotic lungs support prolonged growth of cells • Decellularized lungs that are diseased can significantly affect the cell growth and differentiation	[135]
Porcine	Kidney	Immortalized murine hematopoietic support endothelial cell line	*In vitro*, culture and *in vivo* Yorkshire porcine	• Unseeded implanted scaffolds sustained blood pressure, renal ultrastructure maintained	[136]

Source	Tissue	Cell types	Recipient	Result	Reference
Porcine	Kidney	*Mice* embryonic stem cells	*In vitro* culture	• Reseeded scaffold showed HGF and VEGF levels similar to native kidney	[137]
Porcine	Pancreas	Human amniotic fluid derived stem cells	*In vitro* culture	• Acellular pancreas supported stem cell and pancreatic islets growth • Could serve as a platform for bioengineering pancreas to treat diabetes mellitus	[138]
Female ICR mice	Pancreas	Acinar AR42J and beta MIN-6β cell lines	*Mice* model	• Strong up-regulation of insulin gene	[139]
Rats	Spinal cord	Acellular scaffolds for in-vivo, NIH3T3 cells for in-vitro studies	*In-vitro* culture and *in vivo* Sprague-dawley Rats	• Induce the regeneration of injured nerves (*in vivo*) • Enhanced adhesion and proliferation of cells (*in vitro*)	[140]
Porcine	Brain	iPSC derived neural progenitor cells (NPCs)	*In-vitro*, culture and *in vivo* mice model	• NPC expressed neural markers in brain matrix gel (*in vitro*), • Formation and assembling of larger microscale fibril like structure in gel (*in-vivo*)	[141]
Porcine	Skin	Human dermal fibroblasts	*In-vitro* culture	• Gene ontology showed skin morphogenesis, epidermis development	[142]
Porcine	Cornea	Acellular Cornea	*In-vitro* rabbits	• In-vivo good biocompatibility, • Translucent cornea within 8 weeks • Implants integrated into rabbit cornea without rejection signs	[143]
Porcine	Cornea	Rabbit corneal keratocytes, epithelial, endothelial	*In vitro* culture	• Epithelial cells showed high expressions of CK3, spindle shape keratocytes displayed vimentin	[144]
Porcine	Cornea and limbus	Acellular scaffolds	*In vivo* rabbit model	• Corneal transparency and epithelial integrity with no graft rejection • Basal epithelial cell matured to limbal epithelial cells	[145]

Source	Tissue	Cell types	Recipient	Result	Reference
Porcine	Myocardium Slice	Acellular patch	Acute myocardial infarction rat model	• Firm attachment and integration with the infarcted region • Neovascularization within 1 week, contraction of left ventricle wall and cardiac functional parameters improved significantly	[146]
Porcine	Liver	Hepatoblastoma ($HepG_2$)	*In-vivo* rodent model	• Intact liver capsule with porous acellular lattice structure with cell supportive behaviour • No immunogenicity observed	[147]
Porcine	Heart valves (Synergraft^{TM})	Acellular scaffolds	Human study 4 male children	• 3 Children died of graft rupture • Severe inflammation • Significant calcific deposits • No cell repopulation of porcine matrix	[56]
Bovine	Ureter graft	Acellular scaffolds	Human study, 9 patients	• Acute and chronic transmural inflammation • Graft failure with aneurysmal dilation and thrombosis in complex arteriovenous conduits	[148]
Human (allograft)	Trachea	Patient epithelial and MSC derived chondrocyte	Human study	• Immediate functional airway • No immunogenic reaction	[149]

Table 1.
Various decellularized xeno derived organs that are used in in vitro, animal and human studies.

benefits in terms of immuno-compatibility, possible immunological reactions during xenotransplantation, importance of xenografts, ethical concerns, and regulatory restrictions are also discussed.

2. Immunogenicity against dECM

Xenotransplantation may be the best way to alleviate the burden of allograft organ shortage from the last decade. The most enormous barrier to xenotransplantation is the immunological rejection which de-emphasizes this technique. The profound immunological rejection happens by both antibody-mediated immune response as well as cell-mediated innate or adaptive immune response. Several carbohydrate antigens have been identified that could act as targets for human natural

antibodies to inhibit immune rejection; these include Galα1-3Galβ1-4GlcNAc (referred to as α1,3Gal), Hanganuziu-Deicher (H-D) antigen, Tn, Forssman antigen, Sda antigen, etc. [39, 40]. Two antibody-mediated processes are hyperacute rejection (HAR) and acute humoral xenograft rejection (AHXR), which attack mainly the vascular system of graft tissue. HAR is mediated by natural antibodies against α-1,3Gal epitope, present in vascular endothelium of mammals except for humans, or their most recent ancestors, the Old World monkeys [31, 41]. α-1,3Gal epitope is expressed in other organisms, because of increased human interaction with these animals; anti α1,3Gal is being developed in human sera. When it binds to its antigen determinant site of anti α1, 3Gal, it activates the complement system and coagulation system to reject the graft within minutes to hours. HAR is histologically characterized by the presence of interstitial hemorrhage edema and thrombosis in small blood vessels. The depletion of α1,3Gal antibody or complement inhibition may be the best strategies to prevent HAR. But early attempts to reduce antibody by injecting a competitive antagonist of α1,3Gal antigen were unsuccessful [42] because AHXR can reject graft with a very low concentration of α1,3Gal antibody after several days or weeks. On the other hand, non-alpha Gal antigens Hanganuziu-Deicher (H-D) antigen and Sda antigen are present in vascular endothelium and on the surface of erythrocyte of all mammals except humans. The antibody against these H-D and Sda antigens is responsible for HAR and AHXR reaction via activation of complement (classical pathway) and coagulation system in α1,3Gal transferase gene knockout (GalT-KO) pigs [40, 43, 44]. The complement can also be activated via alternative pathway by islets transplantation and cause instant blood-mediated inflammatory reaction (IBMIR), resulting in an early rejection of transplanted islets [45]. The most successful approach to prevent antibody-mediated xenograft rejection is (i) transgenic pigs that express human complement regulatory protein that inhibits antibody-mediated complement activation [46] and (ii) pigs with a knockout α1,3Gal transferase gene [47, 48]. The elimination of α1,3Gal epitope extended the survival of xenograft to 2–6 months [43]. On the other hand, combination of both strategies at a time has increased the graft survival. Recently significant prolongation of graft survival was documented more than 900 days in a pig-to-baboon cardiac xenograft from α1,3Gal transferase knockout, which express human complement regulatory protein CD46 and human thrombomodulin (GTKO.hCD46.hTBM) [49, 50]. The strength of cellular rejection of xenotransplantation remains uncertain, because of difficulty in avoiding HAR and AHXR.

Xenografts are more prone to rejection when compared to allografts due to the antibodies produced by T-cells dependent activated B-cells. Inclusion of T-cell suppressive treatment significantly prolonged the survival rate (>400 days) of xenograft, where natural antibody-mediated immune rejection was suppressed [49–51]. The initial immune reaction by HAR and AHXR produced pathogen-associated molecular patterns (PAMPs) which activate the innate immune system, such as NK cells, macrophages, and neutrophils. Overcoming these barriers needs severe and sustained exposure to immune-suppressive drugs, which is very much harmful to host tissue.

All biologists are focusing on cells and intracellular contents and their regulation to escape from immune reaction, but the scenario has changed after Hauschka and Konigsberg's work in 1966 [52]. It was reported that only the ECM can differentiate myoblast to myotube formation. As the ECM has inbuilt tissue-specific matrix composition and topological cues, it may be an ideal scaffold for the use in tissue engineering. Both antibody-mediated and innate immune responses trigger by the specific receptor present on their respective target cells and inflammatory molecules like TNF, IFN, and different cytokines released upon activation of specific

cells. Decellularization is the best strategies to evade immune reaction by removing cells as well as receptors present on their surface membrane. Unfortunately, the implantation of decellularized allograft into a human produced the mixed type of result of compatibility and recipient immune response. In spite of all the hurdles, some early clinical success of ECM scaffold was achieved [53, 54], but a low level of immune reaction was identified by some group. The heart and lung xenotransplantation working group in the National Heart, Lung, and Blood Institute (NHLBI) has identified xenogeneic immune response against ECM to be a major problem to use in clinical medicine [55]. Cryopreserved human allografts are extensively used in cardiac valve reconstruction; immunologic response of these allografts has been investigated by several groups to activate the anti-HLA antibody. Hawkins et al. reported that HLA class I and II antigens reduced by 99% in the decellularized human allograft, and postoperation reactive antibody levels of HLA class I or II did not increase in children up to 12 months [56]. The inhibition of the immunomodulatory effect of decellularized tissue is obtained mainly by the removal of predominantly alpha-gal epitope along with other non-gal antigen in vascular endothelium and by removal of MHC class I and II molecules during decellularization. Although the donor-derived MHC class I became undetectable at the time of decellularization, it again reached measurable value following implantation (host-derived MHC class I) and is vascularized with host tissue [57, 58]. The underlying mechanism of decellularization on host immune response remains to be determined. Due to low or zero levels of MHC class I and II, T-cell proliferative response as well as B-cell activation is inhibited, and the anti-inflammatory effect can be seen *in vitro*, which results in the reduction of IL-2 and IFN-γ as well. As there is no MHC class antigen-presenting receptor, T cell does not recognize the foreign antigen, and T-cell-mediated immune response is suppressed. But the elevation of IL-10 fails to conclude the underlying mechanism because it has the only source from activated T cell, B cell, and macrophages [58]. It is reported that M2 phenotype in the graft prevents rejection of the xenogeneic donor tissue; however, the mechanism of macrophage activation to release IL-10 remains unknown. Till now, it is not well understood which protein and in which way decellularized xenogeneic material promotes immune reaction. The decellularized tissue may expose new protein, and the decellularization protocol may also have a significant impact on the response of human mononuclear cells [59]. Rieder et al. [60] reported that decellularized vascular wall elicited more immune cell proliferation than native equivalent, and hence, it proved the above hypothesis. It also hypothesized that opsonization would be the way of inflammation response and can occur through preformed antibody or binding of unspecific plasma protein to the surface. In genetically modified organism, (pig) alpha-gal epitope is knocked out, and it does not elicit immune response in decellularized tissue, but in unmodified xenogeneic tissue, some amount of alpha-gal antigen may be retained, and that could be enough to stimulate immunogenic response. However, further study is needed to find out the mechanism of immune response with regard to decellularized matrices.

2.1 Strategies to resolve immune reaction against xenogeneic DECM

Xenogeneic dECM has a huge potential to be used in tissue engineering and regenerative medicine; some early enthusiastic studies in animal and clinical trials using decellularized tissues resulted in severe inflammatory reaction, fibrous overgrowth, and tissue destruction [61–64]. Despite all these immunological reactions, in recent years xenogeneic biomaterials are being used in abdominal surgery [65–67]. There have been some early studies, where glutaraldehyde cross-linking in native matrix inhibits immune response by the modification of surface area of tissues

that inhibit the interaction with peripheral blood mononuclear cell (PBMC) and in turn T-cell activation [68]. But the problem of glutaraldehyde fixation is that it can change the tissues' topology and promote their degradation by calcification [69]. The natural cross-linking product quercetin, a plant flavonoid pigment, may be more effective, which increases mechanical strength and reduces immunogenicity [70].

3. Importance of xenografts in dECM-based bioprinting

Organs in the human body are extremely complex structures consisting of multiple cell types arranged in defined spatial organization, with varied ECM composition. It is due to this balanced and organized compositions that organs achieve perfect functionality [71]. Any disruption to this native structure alters the functionality of the organ drastically. The demand for organ transplantation is increasing exponentially due to the rise in traumatic injuries and changes in lifestyle, while the supply of organs increased marginally over time. The demand for organ transplantation is estimated to further rise with the advancements in diagnostics leading to early detection of diseases [72]. Researchers all over the world have been striving hard to find alternative strategies to reduce this gap for many years, using a combination of many materials along with cells [73]. As a result, researchers developed comparatively simple organs using tissue engineering approaches, such as artificial skin [74], cartilage [75], and trachea [76] that display a part or nearly full functionality of the particular tissue. Xenotransplantation is another promising approach that was started in early 1920s and has a potential to serve as a temporary measure to save patient's life in the absence of allogenic organ [77]. Nevertheless, the barriers such as graft failure due to immune reaction [63] and infections from the graft to the patient prevent the acceptance of xenotransplantation as a treatment option. Consequently, an emerging technique, 3D bioprinting, revolutionized the field of tissue engineering and regenerative medicine exhibiting its potential to develop complicated organs [78]. To fabricate a scaffold, this technique uses materials that are biocompatible and cells that are tissue-specific, while the best biomaterial to develop a tissue that eventually goes to human body is the material derived from that specific tissue, viz., ECM, as it can provide reseeded cells with local tissue environment [23]. This property of tissue-derived material can anchor cells and provides sufficient biochemical and mechanical cues allowing them to proliferate and differentiate to those tissue-specific lineages which ultimately aid in complex tissue formation [79, 80]. Ideally, autologous tissues are expected not to illicit an immune response after implantation, thus reducing the chance of organ rejection. However, due to the lack of sufficient autologous tissue, allogeneic tissues are chosen for transplantation. Allogeneic tissues also suffer from rejection from the host due to antibody-mediated rejection or T-cell movement into the allograft [81]. Genetic dissimilarity between donor and recipient turns out to be the main cause to induce immune response and eventually rejection of the graft [81]. Hence, the process of decellularization when applied on allogeneic tissues reduces the amount of genetic material, thereby allowing graft survival in the host [82]. But, the final yield of material after all the processing of tissue is very low and is insufficient for printing a higher volume 3D structure. Because of which, considering patient's own tissue or tissue from the same species for development of bioink is not practical. The very next alternative that researchers explored was to obtain tissue source from other species and use its matrix as a bioink for tissue development [23]. The concept of using other species (porcine) tissue as a source of material for humans emerged due to the anatomical and physiological similarities between both the species [83, 84]. Apart from the cellular content, organs are rich in the noncellular component, i.e.,

ECM [85]. In almost all the tissues, ECM proteins are produced by the resident cells [85, 86]. Many macrolevel molecules, growth factors, and fibrillar proteins in varied quantities constitute this considerable volume of the tissue [85]. Polysaccharides and proteins such as glycosaminoglycans (GAGs), hyaluronan, collagen, fibronectin, laminin, and elastin are the major ECM components in an organ [85]. These ECM components allow cell adhesion and cell migration, provide biochemical and mechanical properties, and impart elasticity that helps cells to obtain morphological orientation and physiological functionalities. Of all the ECM components mentioned, collagen is the most abundant protein which almost covers 30% of the protein content present in multicellular organisms [85, 86]. In vertebrates, as many as 28 different types of collagen are recognized with 46 distinct polypeptide chains, and the sources of collagen are abundantly available from marine animals to animals that live on land [87]. The main role of this profoundly available protein is to provide mechanical strength, maintain cellular adhesion, and support migration and other cellular functionalities that direct mature tissue formation [85]. To develop tissues like bone [88], skin substitutes [89], small intestine tissue [90], skeletal muscle tissue [91], collagen that is extracted from xenogeneic sources has been used extensively in research works. Elastin is another ECM component that connects with collagen to provide elasticity to the tissue. It is due to this close association; elastic nature of tissue is being maintained. To develop constructs *in vitro*, along with the exposing cells to abundant proteins, enough mechanical properties are to be provided [85]. Hence, it is necessary to include elastin components into the engineered scaffold which imparts mechanical properties to the tissue. By combining the proteins, viz., different types of collagen and elastin, a reasonable amount of work has been done on blood vessel engineering, heart valve development, tissue-engineered vascular grafts, musculoskeletal tissues, cartilage, and skin engineering [92]. The other fibrous protein that contributes for organization of ECM and is responsible for cell functionality such as cellular attachment is fibronectin. Scaffolds that are functionalized with fibronectin enhanced properties such as cell adhesion [93, 94], promoting elastin deposition [95]; cellular migration responsible for tumor metastasis [96, 97] has been reported in literature. When it comes to engineering a tissue *in vitro* using 3D bioprinting, the material should be biocompatible as well as print friendly. Components of ECM such as collagen, elastin, and fibrin were explored for them to be used as bioinks either separately or in combination with one another in 3D bioprinting technology. The potential of collagen as bioink was displayed for developing human skin model with keratinocytes and fibroblasts [98], cartilage tissue engineering [99], 3D collagen-based cell blocks that exhibited osteogenic activity [100], and osteochondral mimicking structures [101] and in bone regeneration applications [102]. The use of fibrinogen as a bioink was also reported for developing cartilage [103, 104] and vascular grafts [105]. The immune response to xenogeneic collagen in human models was reported to be not adverse, and in most of the cases, the presence of antibodies for xeno-derived collagen was due to by-products during acceptance of implanted graft by host [106]. It is also reported *in vitro* experiments conducted with collagen and elastin derived from porcine and bovine did not trigger immune cells nor trigger proliferation of isolated B and T cells [107]. Nonetheless, to mimic native tissue environment for enhanced cellular functionality, a combination of all the proteins and macromolecules is required. Hence, instead of using all the macromolecules separately in varied amounts, researchers started using ECM of the tissue for tissue engineering and 3D bioprinting applications (**Figure 2**), thereby providing all the necessary cues to the reseeded cells in essential amounts. For better acceptance of the 3D printed structure with ECM, decellularization of animal tissue is done to remove the maximum cellular content prior to 3D printing process. This reduces the chances of xenogeneic

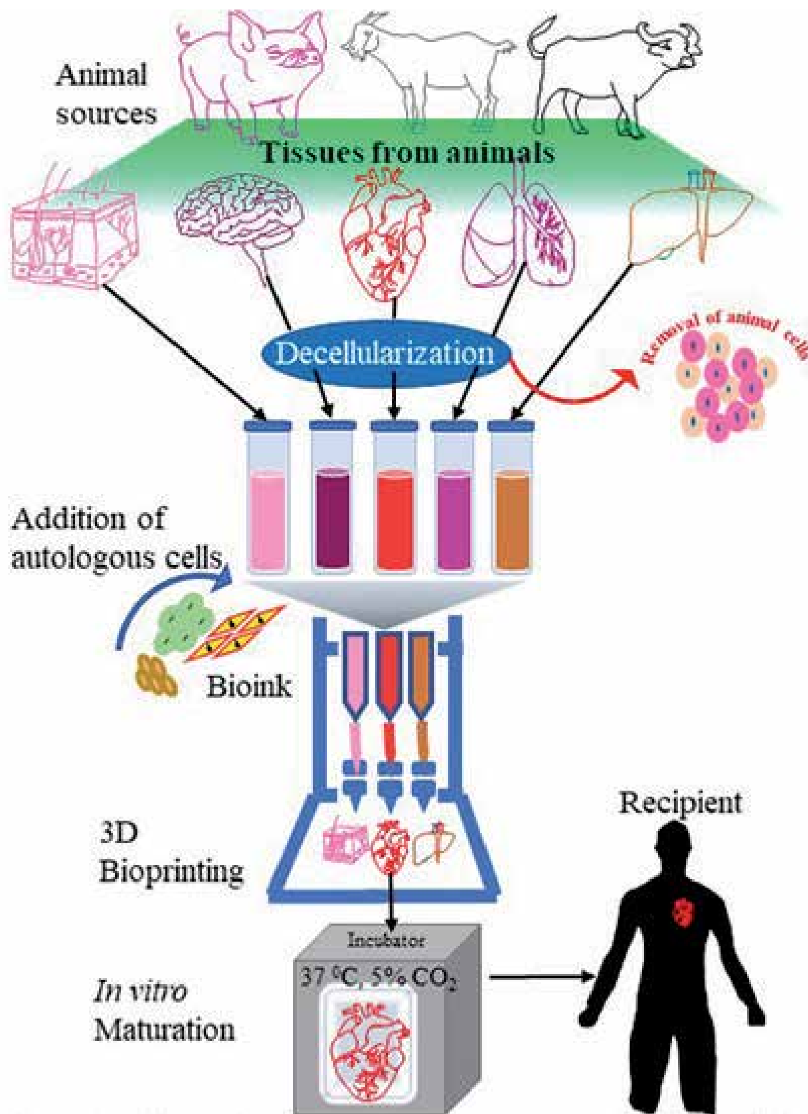

Figure 2.
Schematic representing the process of 3D bioprinting, in vitro maturation and transplantation of tissues developed from animal tissue derived decellularized extracellular matrix.

rejection in human body. In the next section, the use of dECM as a bioink for 3D printing applications is discussed.

4. Current status of the xenografts application in bioprinting

The process of decellularization dates to 2000s, wherein organs such as skin, vascular tissue, and bladder were decellularized. In 2014, it was first shown that after decellularization process, the ECM that is devoid of cellular material could be used as a bioink for 3D printing applications [23]. In the recent past, almost all the organs have been subjected to the process of decellularization and used for 3D bioprinting. With 3D bioprinting of decellularized organs such as the heart, liver, cartilage, adipose tissue, skeletal muscle, skin, etc., researchers have demonstrated the potential of dECM-based constructs in terms of cell compatibility, cell

attachment, migration, and proliferation. Decellularized heart matrix derived from porcine showed an enhanced expression of myosin heavy chain [23] and expression of transcription factors by cardiac progenitor cells [108]. The functionality of 3D engineered heart, developed from decellularized rat heart, was also demonstrated in one study [109]. Similarly, decellularized liver matrix from porcine exhibited consistent secretion of urea and albumin up to 14 days of culture [110] and higher levels of markers suggesting hepatocyte maturation [27]. Early adipogenic marker and lipoprotein lipase were notably observed in human-derived decellularized adipose tissue [23]. However, there is a need of further *in vitro* experiments on decellularized matrices, to completely replicate the complex geometry of the organs. With the current state of art, the *in vitro* models can be tested for immune response in animal models. For any biological material that is being implanted should contain as less as 50 ng/mg of DNA content for not eliciting the immune response in host body. To ensure this low level of nucleic acid content, the process of decellularization of xeno tissues must be stringent and harsh. Detergents such as SDS and Triton X served as chemical agents to remove the maximum DNA content from tissues in decellularization process. Using chemical treatment, acceptable level of DNA content was achieved in almost all the tissues decellularized so far. Apart from DNA nuclear material, Gal epitopes present in animals are also found to be responsible for acute implant rejection [23]. There are few reports from literature wherein 3D dECM scaffolds have been implanted in animal models to understand the host response. In one study, scaffolds that were fabricated using decellularized adipose tissue derived from porcine were implanted into mice. Due to significant reduction in DNA content and gal epitopes, the ECM grafts showed no signs of inflammation or necrosis. Also, there was formation of neo-adipose tissue with mature adipocytes supporting adipogenesis and acceptance of a xenograft [111]. Porcine-derived skin was also subjected to decellularization to show its potential in skin tissue engineering. Using chemical such as trypsin/EDTA and Triton X, the decellularized skin matrix was digested to form bioink, and a skin substitute was printed. This, when implanted into the wound of 10 mm in mice, accelerated wound healing was observed when compared to control groups. Further, immunofluorescence staining showed early differentiation markers for epithelial tissue and CD-31 signifying re-epithelialization and vascularization, respectively [112]. The reported results exhibit the acceptance of xeno-derived dECM-based 3D bioprinted scaffolds by the host tissue. This is made possible due to the stringent chemicals and enzymes involved in decellularization process. Nevertheless, much more studies and experiments both *in vitro* and *in vivo* must be done for using these scaffolds as replacement of deceased parts in the human.

5. Regulatory facets of xeno dECM-based tissue transplantation

Although the prospective benefits are unquestionable, the use of xenogeneic products in human health care raises a number of issues; hence it has to be controlled strictly by the regulatory bodies to avoid complications. The duty of regulatory bodies is to regulate the indiscriminate use of animal-sourced material intended for human health application. The challenges include (1) the potential risk of transmission of infectious agents from source animals, (2) informed consent related issues, and (3) animal welfare issues [113].

From the preclinical testing, the regulations are made strict for the human welfare before use in clinical trials. In general, enough studies have to be performed for safety characterization of therapeutic agents including the efficacy or the activity and the toxicity or undesired effects to the host system. This type of potential clinical

risks constitutes an important component of an FDA regulation. Transfer of animal microorganisms to the recipient with the graft during xenograft transplantation is another major concern for regulatory authorities [114]. There are reports that HIV, hepatitis B and C, Creutzfeldt-Jakob disease, and rabies can be transmitted between humans during transplantation. It is also proved that contact between animals and humans during animal husbandry and from pets or food products can lead to zoonotic infections. So, the use of animal cells, tissues, and organs in any forms keeps the public health at risk with known and unknown infections. Hence it is advised to go for thorough screening for all kind of possible zoonotic infections by following the standard protocol [113]. Moreover, the risk of these microorganisms or virus getting adapted to human-to-human transmission is also a major factor that has to be considered, which might be a concern for general population [115]. When it comes to cross-species whole organ transplantation, there is unavoidable transfer of endogenous retrovirus that is existing in the genome of all porcine cells into the patient receiving the organ. However, there exists no documentation regarding the transfer of these viruses in humans who are exposed to pig organs [116], probably due to the lack of long-term observation.

Preclinical studies provide valuable insight into the safety issues before being used in the human volunteers. Animal welfare is a major concern during the application of xenogeneic products in humans. Since animals' welfare is a major ethical issue, it is considered by regulatory bodies before approving any product of animal origin for clinical use.

Also, during the clinical trial stages or in long term, the volunteers or the patients and the close contacts should be educated about the chance of infectious disease risks and about how to manage those risks. Moreover, such counseling should also be continued for long term as some infection may take years to get manifested. Also, lifelong surveillance is advised by FDA irrespective of the status of the implant or graft or other xenotransplantation product.

Conversely, 3D bioprinted *in vitro* organs and tissues that are being developed using dECM are expected not to pose potential threat to recipients. This is because the cell and nucleus materials are being removed from the tissue using harsh chemicals during the process of decellularization. However, the regulatory bodies ensure that xenotransplantation is allowed only when there are evidences that show near-zero chance of recipient getting infected and informed consent, and acceptance for lifelong postoperative care from the patient was collected [116]. Nevertheless, stringent regulations will be required from regulatory bodies to monitor the pros and cons for a longer duration.

6. Ethical and safety concerns

There are numerous challenges and hurdles being faced for translating xenogeneic products to the clinical level. Though the potential of tissue- or organ-derived bioink for 3D bioprinting is getting proved and accepted, to reach human level it must overcome ethical concerns apart from dealing with technological and regulatory challenges. The opinions expressed on ethics behind using xeno-derived material for humans are based on the source of material and the consequence after transplants, which are already mentioned in the regulatory facets [117]. There are few groups who argue that the primary idea of using animal organ into human is unethical, while few claiming that the detrimental outcomes after the transplant are unacceptable [118]. The apprehension on the outcomes of the xenotransplantation seems valid as there are reports in the literature suggesting that patients who received the animal organs survived only for a short span [77]. The use of animal

organ in patient started in the twentieth century. Organs such as liver, heart from baboon [119], and kidney from chimpanzee [120] were transplanted to patients who survived for a very short lifespan ranging from 20 to 195 days after the implantation [77]. Immune rejection is the primary reason for failure of the graft [77]. Apart from immune response from the host, there are insufficient scientific evidences about the risk of transmission of pathogens that are passive in animal species [117]. Though it is proven that these microorganisms that are existing in animal species are not harming them, it could be fatal when they enter other species [117]. It is ethical to have an informed consent from the patient, not only regarding the transplantation but also about all the further complications that could arise due to the foreign material being placed inside [117, 121]. With xeno-organ transplantation, the risk of animal virus and microorganisms entering human body is expected to rise [121]. Apart from this, there are a lot many unknown viruses that are hosted by animal species whose effects are not at all predictable [117]. Hence, the recipient should also be informed about the risks and preventions that he/she must take posttransplantation, restricting his freedom [121, 122]. Further, to increase the success rate of transplants, recipients are constantly under the influence of immunosuppressant drugs, which would enhance his chances of other infections [117]. However, immunological reactions are not reported much after using dECM 3D bioprinted constructs. Additionally, one has to justify whether the amount that is being spent on xenotransplantation research for translation to clinical level is really worth, as it can help a relatively smaller group of people. Furthermore, for animal welfare, there are animal-related ethical issues which are considered important similar to human ethical issues [123]. Some groups believe that, the use of animals to fulfill human needs is strongly unethical, while few accept that if the benefits surpass the degree of suffering of animals, then there is no harm to use animal organs for saving human life [124]. Almost all the vertebrates suffer and perceive pain in a similar way [121]. Producing transgenic animals for organ transplantation also received criticism, as during this process, much more pain and suffering is imposed on animals due to multiple experiments in succession. In order to reduce the chance of viral transmissions, these transgenic animals are quarantined and kept in isolation [121]. Hence, the supports for animal welfare argue that the animals that undergo genetic engineering technique will be deprived of its natural habitat and are forced to live in a secluded place with pain and agony [117]. Will this suffering of an animal be the guarantee that its organ is successfully put into use remains as an unanswered question. Apart from ethics, religious feelings also come into play. A pig that is considered to have similar genetic and physiological traits similar to human [125] is considered unclean in many religions but is considered as a versatile model in biomedical research. On the other hand, if the benefits and safety of xenotransplantation is proven for human well-being, dealing with animal ethics could be vindicated. Nevertheless, how well the community approves and agrees to the use of transgenic animal organs for transplantation to serve humans is yet to be understood.

7. Future perspective

We believe that the severity of some disease conditions will be able to justify the use of xenogeneic therapeutic options, but the risk and benefits must be evaluated and concluded at the earliest. The most important concern, infectious disease transmission, including the chance of latent viral infections, must be studied in a larger picture including all possible disease transmissions. Though studies are limited, severe immunological reactions are not reported by using decellularized bioinks till date indicating its future potential in regenerating organs and tissue. Large

population studies are required to rule out the possibilities of rejection. A well-defined animal source is also required as species close to humans are not preferred. The animal husbandry conditions must be defined and should start dedicated farms isolated from other animals and be monitored regularly to avoid unexpected or non-listed diseases. Moreover, an unquestionable monitoring system for animal welfare conditions is also important during the raise in the use of xeno-products in human.

8. Conclusion

The tissue-derived decellularized extracellular matrice bioink is the latest trend in the field of 3D bioprinting. The 3D bioprinted constructs from xenogeneic dECM are yet to be studied and analyzed extensively. However, the immune response to xenogeneic collagen, the major dECM-derived bioink component, in human models is not induced by any complicated immune reactions in the host. Though studies are in progress, the 3D bioprinted constructs with xenogeneic dECM bioink are least studied for safety and efficacy despite immune reactivity studies. The animal welfare-related issue is untouched. The initial studies using xenogeneic decellularized matrices are tempting; therefore it is worth to speculate that 3D bioprinting with xenogeneic dECM can revolutionize the field of regenerative medicine.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 6

Optimization of a Decellularization/Recellularization Strategy for Transplantable Bioengineered Liver

Quanyu Chen, Xiaolin You, Jiejuan Lai, Shifang Jiang, Hongyu Zhang and Lianhua Bai

Abstract

The liver is a complex organ that requires constant perfusion for the delivery of nutrients and oxygen and the removal of waste in order to survive. Efforts to recreate or mimic the liver microstructure via a ground-up approach are essential for liver tissue engineering. A decellularization/recellularization strategy is one of the approaches aiming at the possibility of producing a fully functional organ with in vitro-developed construction for clinical applications to replace failed livers, such as end-stage liver disease (ESLD). However, the complexity of the liver microarchitecture along with the limited suitable hepatic component, such as the optimization of the extracellular matrix (ECM) of the biomaterials, the selection of the seed cells, and development of the liver-specific three-dimensional (3D) niche settings, pose numerous challenges. In this chapter, we have provided a comprehensive outlook on how the physiological, pathological, and spatiotemporal aspects of these drawbacks can be turned into the current challenges in the field, and put forward a few techniques with the potential to address these challenges, mainly focusing on a decellularization-based liver regeneration strategy. We hypothesize the primary concepts necessary for constructing tissue-engineered liver organs based on either an intact (from a naïve liver) or a partial (from a pretreated liver) structure via simulating the natural development and regenerative processes.

Keywords: tissue engineering, decellularization, recellularization, thrombogenicity, hemocompatibility, partial hepatectomy transplantation

1. Introduction

The liver is the largest internal organ in the human body, accounting for approximately 2–5% of the total body volume [1, 2]. Physiologically, the liver possesses over 500 different functions [3] and any severe damage could be life-threatening, such as that caused by ESLD, including acute liver failure and chronic liver disease.

In modern times, the failure of solid organs, such as ESLD caused by injury or disease, has become a major challenge in clinics [4]. According to the U.S. Centers for Disease Control and Prevention (https://www.cdc.gov/), in 2014, 38,170 people died of ESLD. Currently, orthotopic liver transplant (OLT) is an ideal therapy for ESLD. However, a shortage of liver organ donors severely limits OLT usage. The Department of Health and Human Services in the United States has estimated that (https://optn.transplant.hrsa.gov) 22 people on the National Transplant Waiting List die each day, while one person is added to the waiting list every 10 min. Additionally, people fortunate enough to receive an organ transplantation have to suffer from the lifelong use of immunosuppressants against chronic rejection. Therefore, new technologies are eagerly needed to create a transplantable liver [5]. Tissue engineering is a mixed field that aims to fabricate functional organs in vitro [6]. Over the decades, great progress has been achieved in the laboratory, and even some livers have been used in clinics [7]. Tissue engineering by using a decellularization/recellularization strategy, which maintains the architecture, vascular system, and ECM components, has been shown to be a promising tool for solid organs, such as liver.

Liver tissue engineering by using decellularization/recellularization strategy (**Figure 1**) involves biomimicking the architecture and physiological features of the native liver. The procedure generally needs three major components: a scaffolding platform, seed cells, and a 3D microenvironment. Despite the numerous advances over the years, it is still an enormous challenge to fabricate a liver organ [8]. Generating liver organ-specific 3D structure scaffold to keep as much as original biochemical, physiochemical, and biomechanical ECM microenvironment is the one of the main hurdles in liver engineering field [9]. Such physiological 3D structure also plays a remarkable role in influencing seeded cell long-term survival and complex liver tissue mass formation [10]. To achieve this, scientists have been working with different scaffolding systems for liver tissue engineering. Studies have shown that a construction strategy based on a combination of a decellularized naïve liver matrix and recellularization with seed cells has led to constructs that match human organs in size and structure. However, the present constructs still only fulfill

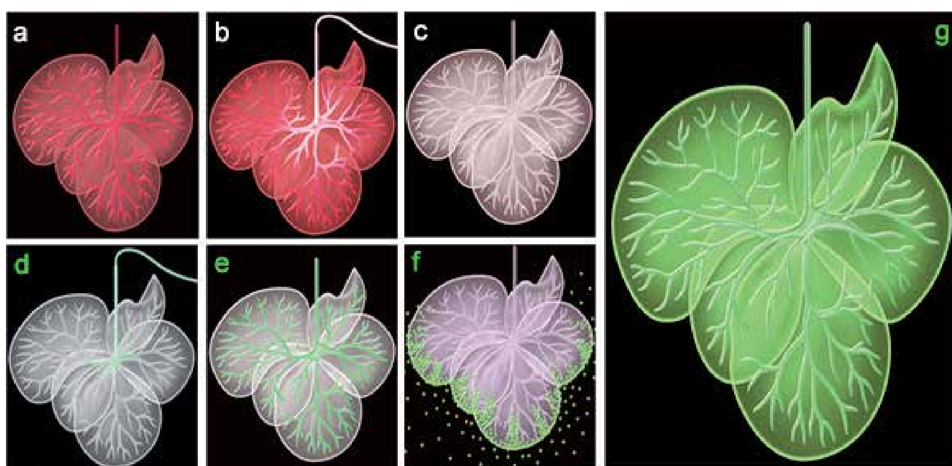

Figure 1.
The decellularization/recellularization strategy in liver tissue engineering. Mammals donor-derived livers undergo a process of decellularization to obtain decellularized liver scaffolds (DLS) (step a–c), and then recellularized seed cells are placed onto the scaffolds (step d–f). Finally, the recellularized scaffolds are placed into 3D culture conditions in a bioreactor to construct liver-like tissues or organs with an overall structure and vasculature (step g).

partial functions of the liver. The preservation of a functional ECM during decellularization, cellular differentiation [11], and a lack of endothelial-lying vascular networks limits the long-term functional integration of constructs after in vivo transplantation. As techniques continue develop, some methods with the potential to overcome these challenges should be explored in the near future, which will further boost the development of a tissue-engineered liver with improved functions. In this chapter, we have tried to focus on the possibility of liver tissue engineering by using a decellularization/recellularization strategy and to describe the current advancements made in the field to address a possible clinical transplantation.

2. Decellularization-based scaffold biomaterials

The term “biomaterials” traditionally means a nonliving substance used for a medical purpose. As the technology of biomaterials developed, the definition expanded to include substances to control the biological environment of cells and tissues for increased compatibility with a host to allow for colonization, proliferation, and differentiation of cells while maintaining their specific morphologies, configurations and avoiding immunological rejection. Based on the increasing knowledge of ECM biology, scaffold biomaterials can be grouped as synthetic materials, natural materials, or a decellularized matrix [12]. Moreover, modifications have been made to enhance the biologically active signals of scaffolds, leading to improved cell attachment, survival, and tissue formation [13, 14].

Biomaterials with required properties have been well studied from synthetic materials. For instance, a nanofibrous matrix made of poly and poly-embedded growth factors was transplanted into animals and restored cardiac regeneration by promoting vascularization [15]. Zawaneh et al. have reported the design of an injectable synthetic and biodegradable polymeric biomaterial consisting of polyethylene glycol and a polycarbonate of dihydroxyacetone that is easily extruded through narrow-gage needles, biodegrades into inert products, and is well tolerated by soft tissues [16]. Those chemically and biologically modified synthetic materials could result in a better way to mimic and control seed cell responses [17, 18]. Another advantage of synthetic materials is their easier to predict and control the degradation of synthetic scaffolds. However, despite this wealth of knowledge, the ability of synthetic biomaterials to support cell attachment, or induce phenotypic expression is much lower than that of natural biomaterials [19–21]; thus, natural biomaterials have been extensively studied [22].

Natural biomaterials include collagen, alginate, and chitosan. These types of biomaterials are inherently able to facilitate for seed cell attachment, proliferation, and functional differentiation, thus they hold significant promise for liver tissue engineering [23, 24]. However, traditional natural materials have poor inherent bioactivity, acidic byproducts, etc., and alone cannot rebuild the complex architecture of solid organs like liver. Other limitations include their unpredictable degradation kinetics; generally, weak mechanical strength, and risk of evoking an immune response [25], etc. also need to be considered.

Decellularized scaffolds (matrices) being natural biomaterials, which are deprived of cellular components while maintaining their original architecture and vascular system, have been widely studied and used in more complex tissue engineering [26]. In the case of liver tissue engineering, the use of decellularization/recellularization strategy was inspired by a pioneer study of heart tissue engineering from the Ott group in 2008 [27]. After that, liver tissue engineered by using this approach has been fabricated [28–30]. Compared to those derived from other synthetic or natural biomaterial scaffolds, the decellularized liver scaffold (DLS)

mostly preserves the native complex liver ECM components, spatial microstructure, and perusable vascular architecture [31, 32] as more "biocompatible ways" for seed cells attaching and reorganizing on a complex 3D level [33]. Therefore, the DLS might have more favorable advantages than other scaffolds for clinical application although the biocompatibility signal between ECM of the DLS and seed cells is still unclear. Scientists have recellularized stem cells onto the natural 3D DLS and have found that these culturing cells not only survive better in the scaffold structure than their culturing in 2D environment but also differentiate into functional cells as well [34]. Zhang et al. seeded adult mouse liver hepatic stem/progenitor cells onto the DLS that generated from naïve liver (nDLS) and cultured the complex in bioreactor, which formed a liver-like construction. Importantly, the nDLS/cell construction was able to repair a cirrhotic liver and even replace the failure liver [35].

Although many studies have been performed in the DLS field for liver tissue engineering [36–40], unfortunately, because of the nDLS being a lack of "active microenvironmental" support in existing ECM components, the optimization of the nDLS biomaterials become an important procedure for improving the skill of liver tissue engineering. Many protocols have been applied to modify the non-bioactive decellularized scaffolds. The application of a variety of growth factors [41] to promote the survival, proliferation, and differentiation of cells, like insulin-like growth factor 1 thought to promote hepatic cell differentiation from bone marrow-derived mesenchymal stem cells, vascular endothelial growth factor applied to enhance the vascularization of tissue-engineered tissues or organs. Additionally, the complex synergistic and antagonistic actions between different kinds of growth factors in vivo, more attention should be paid to the combined and sequential application of different growth factors. Consideration of optimizing the ECM of nDLS foir its behave like "naïve liver regenerative niche" might be a nice way to induce liver-like tissue formation spontaneously both in vitro and in vivo. Based on this, recently, Yang et al. has presented a very interesting experiment: the authors generated an acellular liver scaffold from pretreated naïve liver. They pretreated a naïve liver by performing a 30–55% partial hepatectomy, and the liver was maintained in vivo for 3–5 days until acute liver regeneration occurred, which allowed for the generation of the scaffold from the regenerative liver (rDLS) (**Figure 2**). These rDLS retain a variety of higher level of supporting growth factors for liver spontaneous regeneration as compared to that of nDLS, including their collagens, growth factors (HGF, TGF-α, IL-6, b-FGF, VEGF), glycosaminoglycans, antithrombotic proteins, and other matrix proteins [42]. Since the novel rDLS possesses a natural liver regenerative microenvironment, so-called "bioactive" ECM, it has shown more efficiency than nDLS in promoting primary hepatocyte survival and antithrombotic activity. Notably, when recellularized the rDLS with intrahepatic stem/progenitor cells and cultured them in 3D environment, a more likely liver organ was formatted as compared to the nDLS recellularized with the same stem/progenitor cells, after transplanted into recipients [42]. This pioneer study demonstrated that "bioactive" scaffolds of the rDLS obtained from a regenerative liver possess an advanced natural "active state niche" as compared to nDLS ("still state niche") for promoting primary hepatocyte survival, resistance to thrombosis, and liver-like organ construction. Other forms of bioactive factors are also involved in liver tissue engineering, like microRNAs, etc. [43, 44]. Furthermore, it needs to be mentioned that the advantage of highly conserved each specific ECM protein of decellularized scaffold among species of which the ECM are recognizable within and between species largely without immune rejection [45, 46] when properly processed to remove cellular antigens that would induce an immune rejection without damaging the ECM.

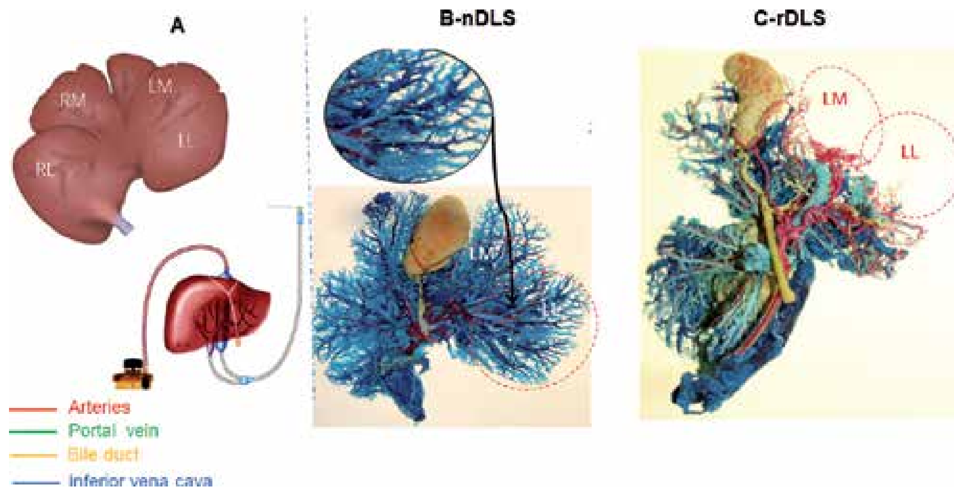

Figure 2.
Generation of a porcine decellularized liver scaffold from naïve livers and livers that had undergone partial hepatectomy (PHx). (A) Perfusion procedure for liver organ decellularization. (B) Blood-vessel tree of a decellularized scaffold from a naïve liver (nDLS). (C) Blood vessel tree of a decellularized scaffold from a partial hepatectomy (PHx) liver (rDLS).

3. Seed cells response to the natural three-dimensional-decellularized liver biomaterial scaffold

Cellular components are an integral part of any tissue engineering. In the case of the liver, it is important to find appropriate cells, such as hepatocytes or stem cells and to seed them into biomaterial scaffolds to regenerate liver tissues or organs [47]. Appropriate seed cells contain parenchymal such as hepatocytes, cholangiocytes, and supportive cells like liver sinusoidal endothelial cells, Kupffer cells, hepatic stellate cells, and pit cells [48]. Hepatocytes account for 60–65% of a liver's cell population [1, 2, 49], which is important for liver tissue engineering. If it is difficult to obtain patient-derived hepatocytes, along with challenging isolation, culture, and the low yields of these cells in vitro [50], stem cells are required for liver tissue engineering [51, 52].

Stem cells are generally grouped as embryonic stem cells (ESCs), somatic stem cells (SSCs), and inducible pluripotent stem cells (iPS) [53]. ESCs have a higher regenerative capacity and can be manipulated to differentiate into other cell types [54, 55]. For liver tissue engineering, ESCs are considered beneficial for the purpose of cell differentiation. For instance, epithelial cells differentiate from ESCs, which could cover the interior of vessels (arteries, veins, and capillaries) of DLS, and the interior of vessels is one of the major players of the angiogenesis process in physiological and pathological conditions involved in thrombus resistant effects. Due to the ethical problems with ESCs, tetratomics and expanded adult human hepatocytes [56], iPS are described as an alternative for adult human hepatocyte differentiation [57–61]. More studies about iPS are under active investigation at present [62], but dozens of publications regarding iPS-derived hepatic lineages have varied from report to report, making it difficult to compare the relative successes of the various modified protocols in enhancing hepatocyte differentiation [63, 64]. Moreover, cultured human hepatocytes often upregulate inappropriate immature markers, such as alpha-fetal protein (AFP). Consequently, any comparisons made to these altered adult hepatocytes may make the candidate immaturely appear more strongly functional than they truly are. Indeed, an examination of published accounts reveals that many protocols lead to fetal hepatocyte-like cells, but in some

cases, the characterization reported is not sufficient to determine the fetal versus mature nature of the resulting differentiated hepatic cells. Given the seemingly fetal nature of iPS-derived hepatic cells produced to date, it is apparent that additional, careful modification of differentiation protocols is still required for further investigation before clinical implementation. Somatic stem cells could overcome the obstacles caused by ECSs, thereby making them more appropriate for liver tissue engineering [65, 66].

SSCs are composed of intrahepatic SSCs and extrahepatic SSCs. Bone marrow-, umbilical-, and fat tissue-derived mesenchymal stem cells are well accepted extrahepatic SSCs [67–69], while oval cells, especially neuro-glial antigen 2 (Ng2)-expressing cells ($Ng2^+HSP$), are currently identified as intrahepatic stem/progenitor cells. Isolation of the $Ng2^+HSP$ should be completed by using a specific protocol [70]. Other sources of SSC behaviors seeded in the DLS have also influenced liver tissue engineering. Several studies have demonstrated that liver-derived mesenchymal stem cell (MSC)-like cells can differentiate into hepatocytes and cholangiocytes in nDLS and that the functional differentiation of MSCs in certain situations could be an alternative approach for an engineered liver organ transplantation in the treatment or replacement of ESLD [35, 71]. Our recently studied animal models have revealed that intrahepatic MSC-like SSCs repaired injured livers better than extrahepatic MSCs [unpublished]. Contrary to past hypotheses, extrahepatic bone marrow-derived MSCs do not seem to directly differentiate themselves into hepatocytes, in particularly in vivo, compared to local (liver) MSC-like cells, such as above mentioned the $Ng2^+HSP$. As the $Ng2^+HSP$ has been demonstrated to have a role in tissue repair [70] and failed liver replacement [35] in liver cirrhosis murine model, we recently further demonstrated that the intrahepatic $Ng2^+HSP$ cells are capable of more efficiency than extrahepatic BM-MSCs in self-renewal and hepatocyte and cholangiocyte differentiations (unpublished) (**Figure 3**). Interestingly, by using the $Ng2^+HSP$, Zhang et al. have successfully reconstituted a liver construct in vitro

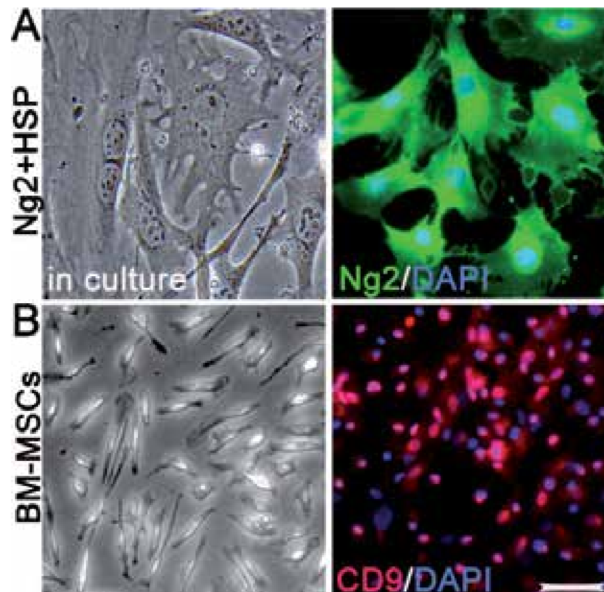

Figure 3.
Murine intrahepatic and extrahepatic mesenchymal stem cells (MSCs). (A) Cultured and immunofluorescently stained of intrahepatic neuro-glial antigen 2 (Ng2)-expressing mesenchymal stem cell (MSC)-like stem/progenitor cells ($Ng2^+HSP$). (B) Cultured and immunofluorescently stained identical bone marrow (BM)-derived-MSCs (BM-MSCs), as visualized by optical microscopy, scale bar = 100 μM.

that is very similar to a naïve liver organ [35]. In addition, the immuno-modulatory, anti-inflammatory, antiapoptotic, and angiogenic properties of the intrahepatic MSC-like Ng2$^+$HSP in the liver still need to be further investigated for liver tissue engineering.

4. Decellularization/recellularization strategy-based liver construction

With the development of decellularization approaches, such as the detergent perfusion technique, whole decellularized scaffolds from liver organs have been produced DLS with an ECM structure and bioactive components being used fabricate bioengineered liver tissues, thus serving as a platform for liver organ bioengineering. Within the past several decades, numerous accomplishments have been driven by the development of these construction strategies. To date, decellularization-based liver construction strategies are constantly advancing such as maintaining complete hepatic vessel networks [72].

Despite the well-conserved macroscopic structure of a liver organ obtained by using decellularization, it is still difficult to avoid some disruption to the ECM composition and ultrastructure through decellularization, which leads to impairment of the natural 3D microenvironment, for example, an impairment of glycosaminoglycans within the ECM by enzymes [73] can cause altered stiffness. Therefore, improved measures for preserving the integrity of the ECM during the decellularization process are required [74, 75]. An functional engineered liver tissue usually uses stem cells or progenitor cells that need to differentiate into multiple kinds of repair cells, which is a challenge to directly seed cells to colonize in relevant sites of DLS to induce their differentiation into specific cell types. Whether an engineering formed liver organ can successfully fulfill its functions depends not only on its physically decellularized scaffold structure but also on an effective recellularization. Therefore, how to populate seed cells like differentiated hepatocytes from different kinds of seed cells or stem cells themselves onto the DLS needs to be carefully considered. In particular, how to manipulate the DLS to enhance the targeted specific colonization of cells to specific areas of DLS such as perfused endothelial cells [76–78], has drawn much attention. To ensure the long-term survival of an engineered liver by allowing exchanges of oxygen, nutrients, and disposal of metabolic waste [79], a functional vascular network and thrombosis after transplantation also needs to be considered. Despite the conservation of the general vascular structure by DLS, the formation of a functional vascular network remains a challenge for liver organ construction. The mainstream strategy to fabricate an engineered liver organ with a functional vascular network includes also the procedure of prevascularization. The initial approaches have been successfully used in spontaneous lineage of endothelial cells in DLS vascular networks after recellularization with stem cells [80–82] to challenge thrombosis after transplantation when exposed to blood, thus leading to localized organ failures [83]. There are two nice approaches showed that endothelialization of vasculature and immobilization of heparin on nDLS could reduce its incidence of thrombosis [84]. More recently, from a pretreated naïve liver obtained rDLS, exhibited except for strong promoting primary hepatocyte survival but also antithrombosis more effect [biomaterials 2018]. Notably, after transplantation guiding the rDLS/cells complex forms complex liver-like tissues (geometries) more effective on rDLS than on nDLS (**Figure 4**), meanwhile combined with better organization of endothelial lineage in rDLS than in nDLS [42]. This suggests that rDLS possesses an advanced "bioactive natural regeneration state niche" relative to the nDLS, which preserves a "still state niche." Therefore, the spontaneous manipulation of the ECM on DLS is a more promising strategy for decellularization-based

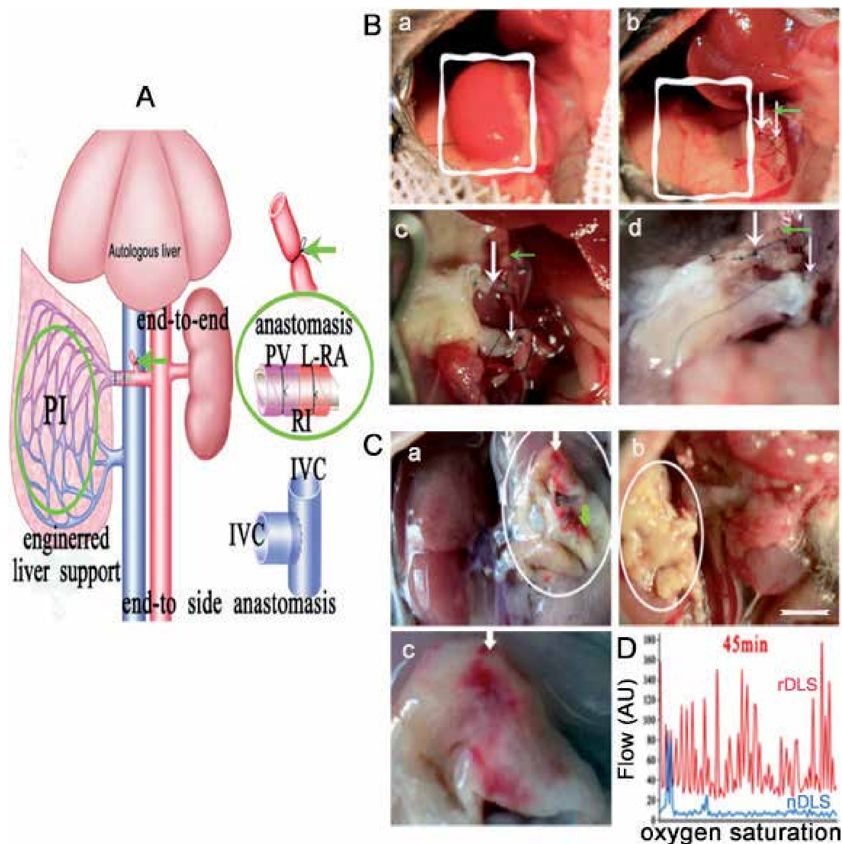

Figure 4.
Comparison of the murine liver-lobule-like tissue construction formation between rDLS and nDLS after portal-renal arterialized auxiliary heterotopic liver transplantation. (A) Schematic of the procedure. The left green cycle indicates the DLS, and the right green cycle indicates the end-to-end anastomosis of the PV (scaffold)-L-RA (recipient). The green arrows in the panels indicate the right-RA. The right bottom cartoon shows the end-to-side anastomosis of the IVC (scaffold)-IVC (recipient). (Ba–d) Exposure of the right-side kidney (the square indicates the kidney) (a). Nephrectomy of the right-side kidney (the square indicates the lack of kidney) (b). The cell-loaded DLS where the kidney was removed (the bold arrow indicates the PV, thin arrow indicates the IVC, and the green arrow indicates a right renal artery (right-RA)). The left-side renal artery (L-RA) was connected to the PV with cross-clamping of the PV and the IVC of the recellularized scaffold (c). The noncell loaded DLS was connected to the recipient by the same procedure as the cell-loaded DLS where the kidney was removed (d). (Ca–c) DLS seeded with Ng2$^+$HSP cells formed a liver-lobule-like construct in rDLS (a and b) after approximately 20–40 days (a, indicated as a cycle), for two lobes with better blood patency (b), represented with a white arrow; there was no visible blood flow in the nDLS loaded with Ng2$^+$HSP cells for the same time (c). (D) Blood flow velocity (flow, arbitrary unit, AU) was measured in rDLS and nDLS at 45 min within 100 s after the operation by a near-infrared-LDF system, scale bar = 50 μM.

liver tissue. In the future, the objective of a decellularization-based liver construction strategy could be based on generating a 3D decellularized biomaterial scaffold with natural "regenerative bioactive niche" for the seed cell attachment, proliferation and differentiation of cells, and developing a transplantable "new" liver in vitro that maintains the structures and functions of a naïve liver.

In summary, compared with other strategies that can only fabricate partial structures, a decellularization/recellularization-based liver tissue engineering strategy enables the construction of the liver structures with complete blood vessel network at a clinically relevant scale, thus becoming a more promising approach for liver tissue engineering. However, in order to provide a promising route for developing a functional bioartificial liver with potential applications for humans by

such strategy, several questions must be answered: (1) Is the use of a decellularized liver matrix the only possible solution? (2) What kinds of cells need to be chosen for recellularization? Extrahepatic cells? or possibly resident stem/progenitors cells? (3) What is the optimal decellularized liver scaffold (DLS)? (4) What is the length of time for incubation in a bioreactor? (5) Would the technique be applicable to a human liver with its extensively sinusoidal surface?

5. Conclusion and challenges

Clearly, decellularization/recellularization through the development of in vitro and in vivo tissue and organ models for liver bioengineering are advancing strategies. This, combined with multidisciplinary team-workers performing focused, systematic studies to address critical questions, is essential for the success of this strategy. The following critical issues might need to be addressed before clinical applications: (1) preservation and modification of a functional ECM structure to better mimic the regenerative niche; (2) selection of effective seed cell sources for recellularization; (3) modification of blood-vessel networks for "endothelialized DLS"; (4) long-term survival by preventing from thrombosis and functions after transplantation; and (5) immune rejection. In the coming years, many new techniques will be explored, which are expected to have the potential to address these challenges.

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Abbreviations

ESLD	end-stage liver disease
ECM	extracellular matrix
3D	three-dimensional
OLT	orthotopic liver transplant
DLS	decellularized liver scaffold
nDLS	the decellularized scaffold that generated from naïve liver
rDLS	the decellularized scaffold that generated from pretreated liver
HGF	hepatic growth factor
TGF-α	transforming growth factor-alpha
IL-6	interleukin 6
b-FGF	fibroblast *growth* factor-beta
VEGF	vascular endothelial growth factor
ESCs	embryonic stem cells
SSCs	somatic stem cells
iPS	inducible pluripotent stem cells
$Ng2^{+}HSP$	neuro-glial antigen 2 (Ng2)-expressing cells
MSC	mesenchymal stem cell

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Section 3

Regeneration

Chapter 7

Controllable Immunosuppression in Pigs as a Basis for Preclinical Studies on Human Cell Therapy

Shin Enosawa and Eiji Kobayashi

Abstract

Along with a growing interest in regenerative medicine, pigs are becoming a popular model for preclinical studies on human cell therapy. Due to pharmaceutical species difference and inability to self-medicate, specific modification and care are necessary in immunosuppressive regimen for pigs. Here, we summarize recent literature on immunosuppression in pigs for experimental transplantation. Based on literature and our own experiences, a practical protocol has been proposed in this report. In early studies of allogeneic organ transplantation, recipient pigs were administered cyclosporine or tacrolimus, and mycophenolate mofetil at slightly higher dose than that in human cases, because of relatively poor effectiveness of the drugs in pigs. Steroids may be effective but sometimes can cause debilitating side effects. Cell transplantation studies follow the basic protocol, but it remains to be clarified whether the smaller graft mass, even if it is xenogeneic, requires the same scale of immunosuppression as organ transplantation. To obtain reliable results, the use of gastrostomy tube and blood trough level monitoring are highly recommended. Nonpharmaceutical immunosuppression such as thymic intervention and the use of severe combined immunodeficient pigs have also been discussed.

Keywords: pig, experimental transplantation, immunosuppression, human cell therapy, regenerative medicine

1. Introduction

The number of preclinical studies conducted using pigs has been increasing, especially in the field of cell therapy [1]. The merits of using pigs include (1) size advantages that enable to mimic clinical procedures; (2) availability of various experimental pigs such as miniature, microminiature, and gene-engineered strains; and (3) worldwide trend of discouragement of using dogs as research models.

However, immunosuppressive treatment has not been established well in pigs. Initially, pigs were used as models for performing allogeneic organ transplantation; now, the hope is to use them as xenogeneic organ donors. In the latter case, immunosuppressive protocols have been designed for primate recipients, while the number of reports mentioning immunosuppressive protocols for xenogeneic transplantation in pigs is unexpectedly few. In addition, insufficient medications are occasionally found in studies conducted by researchers who are not accustomed to organ transplantation.

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We summarize here the pharmaceutical immunosuppressive regimens in experimental transplantation of organs, tissues, and cells in pigs (**Table 1**), as well as highlight the usefulness of thymic intervention and severe combined immunodeficient (SCID) pigs. Finally, we propose an appropriate introductory protocol that will fit human cell and tissue transplantation.

Study design	Pigs	Immunosuppression
In vitro culture, IC_{50} estimation [2]	Unknown	Cys, Tac, Aza, Rap, MMF, MP
Orthotopic allogeneic small bowel transplantation [3, 4]	Large White × Landrace, 26.4 ± 4.7 kg	Tac 0.43 ± 0.14 mg/kg/day, po (keep trough 5–15 ng/ml), MMF 10 mg/kg × 2/day, po
Trough level determination [5]	Yorkshire × Landrace, 22–30 kg	Tac 0.25 mg/kg × 2/day, po, MMF 20 mg/kg × 2/day, po
Orthotopic allogeneic forelimb transplantation [6, 7]	Outbred farm pig, 13–24 kg (6–8-week-old)	Cys 40 mg/kg/day, po (keep trough 100–300 ng/ml) or Tac 1.5 mg/kg/day, po (keep trough 4–8 ng/ml), MMF 500 mg/day, MP and P (tapered)
Orthotopic allogeneic (Class I disparate) kidney transplantation [8–10]	MHC-defined miniature swine, 5–7-month-old	Cys 10–13 mg/kg/day, iv (keep trough 400–800 ng/ml) [8, 9] or Tac 0.15 or 0.30 mg/kg/day, iv (keep trough 20–40 or 45–80 ng/ml) [10]
Heterotopic allogeneic (Class I disparate) heart transplantation [11]	MHC-defined miniature swine, 3–6-month-old	MMF 1.5 g × 2 /day, iv or Cys 10–13 mg/kg/day, iv (keep trough 400–800 ng/ml)
Immortalized human hepatocytes transplantation to liver [13]	Landrace, 6 kg	Tac 0.5 mg/kg/day, im
Human bone marrow mesenchymal stem cell transplantation to myocardium [14]	Domestic pig, 35–40 kg	Cys 5 mg/kg/day, route unknown
Human umbilical mesenchymal stem cell transplantation to cardiac muscle [15, 16]	Yorkshire, size and age unknown	Cys 4–10 mg/kg/day, po, 1–2/day, some cases treated with steroids
Human iPS cell-derived cardiomyocyte sheet to endocardium [18]	Minipig, 20–25 kg	Tac 0.75 mg/kg/day, MMF 500 mg/day, Pl 20 mg/day
Human ES cell-derived retinal sheet to retina [19]	Yucatan minipig	Cys, po, details unknown
Orthotopic allogeneic (Class I disparate) kidney transplantation [20]	MHC-defined miniature swine, 3–6-month-old	Thymectomy and donor thymus transplantation
Human hepatocyte transplantation to spleen [21]	Microminiature pig, 13–16-month-old	Neonatal thymectomy
Human artificial vascular tubes for neck arteriovenous shunt [22]	Göttingen minipigs, 6–7-month-old, ≥15 kg	Thymectomy and splenectomy, and Tac 0.5 mg/kg/day, MMF 60 mg/kg/day, prednisolone 20 mg/day, po

Abbreviations: half maximal inhibitory concentration (IC_{50}); major histocompatibility complex (MHC); cyclosporine (Cys); tacrolimus (Tac); azathioprine (Aza); rapamycin (rap); mycophenolate mofetil (MMF); methylprednisolone (MP); prednisone (P); prednisolone (Pl); per os (po); intravenous injection (iv); intramuscular injection (im).

Table 1.
Summary of immunosuppressive protocols.

2. Role of species differences in the effectiveness of immunosuppressants in vitro

The half maximal inhibitory concentration (IC_{50}) of major immunosuppressants was reported to be higher in mitogen response of pig lymphocytes than that in human lymphocytes [2]. The IC_{50} of cyclosporine, tacrolimus, and azathioprine was 19.1 times [1.72 μg/ml (pig) vs. 0.09 μg/ml (human)], 13.0 times [2.99 ng/ml (pig) vs. 0.23 ng/ml (human)], and 11.0 times [1.43 μg/ml [pig] vs. 0.13 μg/ml (human)] higher in pigs than in human lymphocytes, respectively. The species differences decreased in case of rapamycin and mycophenolate mofetil (MMF); IC_{50} values of rapamycin and MMF were 2.28 times [2.05 ng/ml (pig) vs. 0.90 ng/ml (human)] and 1.45 times [10.75 ng/ml (pig) vs. 7.42 ng/ml (human)] higher in pigs than in humans, respectively. In contrast, the IC_{50} value of methylprednisolone in pigs was only 0.41 times [0.11 μg/ml (pig) vs. 0.27 μg/ml (human)] the value in humans. These results suggest that the blood concentration of calcineurin inhibitors should be kept higher in pigs than in humans to suppress blast formation of lymphocytes in transplantation studies.

3. Immunosuppressive medications in allogeneic transplantation

In allogeneic orthotopic small bowel transplantation, high-dose tacrolimus monotherapy and low-dose tacrolimus-MMF combination therapy were compared using Large White-Landrace pig strain as donors and recipients (both weighing approximately 26 kg) [3, 4]. Tacrolimus dose was controlled to keep trough at 15–25 ng/ml (high-dose group) or 5–15 ng/ml (low-dose group). The average dosage amount of tacrolimus in the high single dose group was 0.3 mg/kg/day intramuscularly from the day of operation to day 6 and 0.61 ± 0.26 mg/kg/day via gastrostomy after day 7. In the low-dose combination group, recipients were administered 0.1 mg/kg of tacrolimus intramuscularly on the day of operation and 0.43 ± 0.14 mg/kg/day (average) of tacrolimus and 10 mg/kg twice a day of MMF via gastrostomy. All recipients in the high single dose group died within 46 days, while 7 out of the 10 recipients in the low-dose combination group survived for more than 60 days; the nontreated controls died within 15 days [3]. The subgroup study of tacrolimus-MMF combined group revealed that the recipients with low trough level of tacrolimus showed better survival, suggesting that higher trough level increases side effects of infection [4]. In general, the protocol consisting of calcineurin inhibitors (cyclosporine or tacrolimus) and MMF suppresses the immune responses of T and B cells, respectively, and the combination treatment leads to effective immunosuppression with low side effects.

A pharmacokinetic study recommended the oral administration of 0.25 mg/kg of tacrolimus and 500 mg of MMF at 12 h intervals to pigs weighing 22–30 kg [5]. MMF dose was calculated to be around 20 mg/kg at each administration. The trough level of tacrolimus was kept at 5–15 ng/ml.

When orthotopic forelimb transplantation was performed between outbred pigs weighing 13–24 kg, recipients were administered cyclosporine or tacrolimus and MMF orally once a day [6, 7]. The desired trough levels were 100–300 ng/ml in case of cyclosporine and 3–8 ng/ml for tacrolimus. A total of 500 mg of MMF was administered, i.e., 21–38 mg/kg. They also used steroids; 500 mg of methylprednisolone was injected intravenously during the operation, and 2.0 mg/kg/day of prednisone was given on the first postoperative day and then tapered by 0.5 mg/kg/day every 3 days to a maintenance dose of 0.1 mg/kg/day until the end of observation period (90 days) [6] or for the first 30 days [7].

The Massachusetts General Hospital group uses genetically defined mini pigs, swine leukocyte antigen (SLA)^{gg} (class I^{c}/class II^{d}) donors, and SLA^{dd} (class I^{d}/class II^{d}) recipients [8–11]. In orthotopic kidney transplantation, 10–13 mg/kg of cyclosporine once a day, administered with a catheter to the external jugular vein, kept the trough level at 400–800 ng/ml [8, 9]. The first 12-day administration resulted in the survival of well-functioning major histocompatibility complex (MHC) class I-disparate kidney grafts for over 90 days regardless of use of steroids. In another study [10], continuous intravenous injection of 0.15 or 0.30 mg/kg/day of tacrolimus treatment kept the drug level at 20–40 or 45–80 ng/ml, respectively, and the first 12-day administration resulted in well-functioning kidney grafts that survived for over 5 months. In addition, the high-dose regimen achieved successful engraftment of MHC class I^{c}/class II^{c}-mismatched kidney. The same group also compared the separate effect of cyclosporine and MMF on the survival of class I-disparate heterotopic heart graft [11]. The treatment protocol of cyclosporine was the same as above [8] and MMF was administered at 1.5 g twice a day through a catheter into the external jugular vein to keep the trough level at 3–5 μg/ml. The survival days of the test heart grafts were 53 ± 7.5 days (mean ± SD) and over 124 days in cyclosporine and MMF groups, respectively. The graft vascular changes were also mild in the MMF group.

4. Immunosuppressive medications in xenogeneic transplantation

Because pigs are hoped to be a xenogeneic donor, the major objectives in pig experiments include establishing xenogeneic antigen-free pigs and developing strategies for long-term survival in nonhuman primates [12]. In such studies, immunosuppression is almost equivalent to human clinics using tacrolimus, MMF, and antibody remedies. Pig recipients in xenotransplantation appear in the preclinical studies of human cell and tissue therapy.

In a short-term experiment of intrahepatic transplantation of human hepatocyte cell line, 0.5 mg/kg of tacrolimus was injected intramuscularly for 7 days [13]. When pigs received xenogeneic human-lined hepatocytes, the recipients survived D-galactosamine-induced hepatic injury. Human albumin appeared in the recipient serum 2 days after transplantation but disappeared at day 7, suggesting that the cells survived only for a few days.

Intramyocardial transplantation of mesenchymal stem cells had been actively investigated in not only basic research but also clinical practice. Because of the size advantage, pigs were used for preclinical studies to explore proof of concept by mimicking clinical procedure. Human bone marrow-derived mesenchymal stem cells labeled with radioactive indium (^{111}In) were transplanted in porcine myocardium via catheter inserted from a femoral artery and traced by whole body scanning for 6 days [14]. Recipient pigs were orally treated with 5 mg/kg of cyclosporine from 3 days before to 6 days after cell transplantation. Immunosuppressed pigs retained the radioactivity far longer than nontreated controls. In another study, pigs received human umbilical mesenchymal stem cells in artificial cardiac infarct area and were administered 5 mg/kg of cyclosporine orally twice a day, from the day before to 8 weeks after cell transplantation [15]. In a similar study, 10 mg/kg of cyclosporine was administered orally, twice a day from 3 days before to 8 weeks after the cell transplantation [16]. As a more sophisticated approach, cardiomyocyte sheet transplantation is being undertaken [17, 18]. When cell sheets consisting of human induced pluripotent stem (iPS) cell-derived myocytes were transplanted onto the epicardium of minipigs (weighing 20–25 kg) mimicking clinical trial, 0.75 mg/kg/day of tacrolimus, 500 mg/day of MMF, and 20 mg/day of prednisolone were administered from 5 days before to 8 weeks after the transplantation [18].

Transplantation of human embryonic stem cell-derived retinal pigmented epithelium was also performed in pigs [19]. Although immunosuppression was precarious, cyclosporine was added in the feed and their blood level 2 h after administration was only 1 pg/ml, and the graft tissue was detectable after 3 months. Because retina is known as an immune-privileged site, additional immunosuppression may not be necessary.

5. Nonpharmaceutical immunosuppression: thymic intervention and SCID pigs

Studies on genetically defined minipigs by Massachusetts General Hospital group emphasize the role of thymus in the establishment and maintenance of immunological tolerance. As quoted above [8], the 12-day administration of cyclosporine induced the long-term engraftment of MHC class I-disparate kidneys, but not if the thymus was removed. They also stated that old recipients tend to be difficult to establish tolerance [9]. These observations lead to the concept of tolerance induction by thymic transplantation. In this, unlike conventional thymic tissue transplantation, the donor thymus is transplanted by vascular anastomosis that assures immediate and perfect function of the thymus. Three weeks after the complete removal of thymus, the recipient was transplanted with MHC fully-disparate donor thymus in the neck region and infused continuously with 0.15 mg/kg/day of tacrolimus (trough level, 30–40 ng/ml) for 12 days. After 3–4 months, the recipient accepted a kidney from the thymus donor without immunosuppression [20].

Previously, we reported the effectiveness of thymectomy on the acceptance of xenogeneic human hepatocytes and artificial vascular tubes [21, 22]. Upon hepatocyte transplantation, the blood human albumin levels were higher in neonatally-thymectomized microminiature pigs than in nonthymectomized controls [21]. In another study, thymus and spleen were removed from Göttingen minipigs aged 6–7 months ($\geq$ 15 kg), followed by the administration of 0.5 mg/kg/day of tacrolimus, 60 mg/kg/day of MMF, and 20 mg/day of prednisolone [22]. Seven days after the removal, an artificial vascular tube made from human fibroblasts was transplanted in between a carotid artery and a jugular vein to form an arteriovenous shunt. While the shunt was obstructed completely by thrombus 2 weeks after the operation in pigs without the removal of thymus and spleen, the shunt was functional in pigs with thymectomy and splenectomy, even though the immunosuppressive treatment administered was equal.

Finally, we would like to refer briefly to the availability of SCID pigs in preclinical study on human cell therapy. According to a well-constructed review [23], there are 11 SCID pig strains so far; one was naturally found and others were genetically modified. The mutated genes in these strains are *ARTEMIS* (a gene encoding a nuclear protein that is involved in V(D)J recombination and DNA repair), *interleukin 2 receptor gamma chain (IL2RG)*, *recombination-activating genes (RAG)1*, and *RAG2*. Three strains have double mutations, namely *RAG1* and *2*, *RAG2* and *IL2RG*, and *ARTEMIS* and *IL2RG*. In accordance with gene function, each strain lacks specific immune-competent cell lineages such as T, B, and natural killer (NK) cells. Human cell transplant experiments were reported as iPS cell teratoma formation [24] and ovarian cancer engraftment [25], both of which did not focus on preclinical study on human cell therapy. SCID pigs need the highest antibacterial care because of their vulnerability to infection. Indeed, they have been reported to survive for only 6 months at the longest [23]. In addition, as general features of mutant pigs, there are diversities in phenotypic severity and small litter size. If

these difficulties can be overcome, SCID pigs will be useful experimental animals for preclinical study on human cell therapy.

6. Conclusion

Due to the lack of identifiable sign of rejection, immunosuppression in cell transplant experiment is hard to control. Successful protocol is established only based on case-by-case experiences. Here, we suggest an introductory regimen of immunosuppression in human cell and tissue transplantation into pigs using tacrolimus and MMF. Preliminary doses are 0.5 mg/kg of tacrolimus orally and 40 mg/kg of MMF orally, and the administration should start 3 and 5 days before transplantation, respectively. Drugs can be administered by mixing in the powdered feed before transplantation; however, after transplantation, it should be given through a gastrostomy tube to assure the dosage in order to not be affected by appetite. Periodical examination of drug trough levels is indispensable and should be reflected in subsequent dose. Steroids should be carefully tested because their immunosuppressive dose has a risk of side effects such as gastrointestinal ulcer and systemic over immunosuppression. In addition, unavailability of exogenous steroid monitoring makes dosage control difficult. If surgical skill is available, the combination of thymectomy and splenectomy is recommended. Since the graft mass of cell and tissue transplantation is far smaller and not fully vascularized than organs, the recipients may need less immunosuppression. Data accumulation and optimization are desired in this field.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 8

A Novel Strategy for Xeno-Regenerative Therapy

Toshinari Fujimoto, Takashi Yokoo and Eiji Kobayashi

Abstract

The shortage of organs for transplantation is of critical importance worldwide. Xenotransplantation or xeno-embryonic organ transplantation can stably supply organs and is considered to be an established alternative treatment. Regenerative medicine is another option, and recent advances in stem cell research have enabled the reproduction of miniature organs, called organoids, derived in vitro from human induced pluripotent stem cells. However, the in vitro production of large and complex organs that can efficiently function in vivo is not yet accomplished. We proposed a novel strategy for xenotransplantation in which a chimeric kidney is constructed by injecting human nephron progenitor cells into a porcine embryonic kidney, thereby eliminating pig nephron progenitor cells and allowing transplantation into a human and long-term survival. In this chapter, we discussed advantages and pitfalls of xenotransplantation and xeno-embryonic kidney transplantation. Recent attempts of human organoids and blastocyst complementation were reviewed. Finally, we proposed our novel xeno-regenerative therapeutic strategy.

Keywords: kidney regeneration, xeno-embryonic kidney transplantation, organogenic niche method, nephron progenitor cell replacement system, induced pluripotent stem cells

1. Introduction

Currently, the only definitive treatment for end-stage organ failure is transplantation. However, the global scarcity of organs is a critical challenge, necessitating that novel alternatives be developed. Xenotransplantation is a revolutionary therapy that can supply organs stably. In recent years, gene editing techniques, such as CRISPR/Cas9, have been developed to produce animals that generate organs at low risk of rejection and infection. Given that our understanding of xenogenic immune barriers has expanded, xenotransplantation may have a promising outlook. Presently, the strong antigenicity of xenogenic organs is the main barrier to xenotransplantation and has resulted in the development of methods of xeno-embryonic transplantation that use less antigenic organs. Although embryonic organs are prematurely transplanted, they can mature in vivo in a self-sustaining manner to perform their function. Xeno-embryonic organs, therefore, have some utility as a scaffold for the regeneration of human organs.

Regenerative medicine is anticipated to be a promising alternative when tackling the problem of a shortfall in organ availability. Recent advances in stem cell research have enabled the reproduction of miniature organs called organoids, which are derived in vitro from human induced pluripotent stem cells (iPSCs). However, we

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have not yet achieved in vitro reproduction of large and complex organs that function efficiently in vivo. Because kidneys comprise a three-dimensional and complex combination of various cell types that must perform homeostatic and endocrine functions, in vitro regeneration of the kidneys is particularly challenging compared with other organs. To overcome this challenge, we sought to use xeno-embryonic kidneys as a scaffold for development of human progenitor cells. By transplanting exogenous nephron progenitor cells (NPCs) into the metanephric mesenchyme of a xenogenic fetus, we aimed to regenerate whole neo-kidneys from the transplanted NPCs via their xenogenic development program. Specifically, we propose a novel xenotransplantation strategy wherein a chimeric kidney is constructed by injecting human NPCs into a porcine embryonic kidney and transplanted into a human after eliminating pig NPCs.

In this chapter, we discussed advantages and pitfalls of xenotransplantation and xeno-embryonic kidney transplantation. Recent attempts of human organoids and blastocyst complementation were reviewed. Finally, we proposed our novel xeno-regenerative therapeutic strategy.

2. Kidney xenotransplantation

Xenotransplantation is a revolutionary therapy used to solve the problem of organ shortage. The concept has existed for more than 100 years, with the first kidney xenotransplantation performed in 1906. In this procedure, a pig kidney was heterotopically transplanted into a patient with renal failure but had to be removed after 3 days because of vessel thrombosis [1]. Subsequent attempts at renal xenografting failed, and the practice was abandoned. However, xenotransplantation re-emerged as an option following the development of powerful immunosuppressive agents. In 1964, the kidney of a chimpanzee was successfully transplanted into a patient with renal failure and functioned for 9 months before the patient ultimately died of pneumonia [2]. Nonhuman primates were often used as a xenograft source at this time because the similarities between species produced good outcomes. However, this practice was abandoned because of the relative scarcity of nonhuman primate sources, concerns about disease transmission, and ethical issues. By contrast, pigs are almost limitlessly available as a transplant source, and their kidneys are similar in size and physiological function to those of humans. At present, porcine kidneys are therefore considered a suitable xenotransplantation source [3].

Nevertheless, using porcine kidneys in xenotransplantation presents some problems that must be addressed. The most critical problem is the presence of α-galactose-1,3-galactose (Gal). This galactose moiety is added to cell surface sugars in swine by α-1,3-galactosyltransferase (GalT), whereas primates, including humans, do not inherit GalT and possess anti-Gal antibodies as natural antibodies [4]. When a pig kidney is transplanted into a primate, anti-Gal antibodies bind to the Gal antigen expressed on porcine vascular endothelial cells, activate human complement, and cause hyperacute vascular rejection that immunosuppressants alone cannot prevent. Recently, genetically modified pigs with low risk of rejection potential were created with gene editing technology; a representative example is the GalT-knockout (KO) pig [5]. Given that these transgenic pigs do not express Gal, the anti-Gal antibody in primates does not react with them. Pigs expressing human complement regulatory proteins (e.g., human CD55 and CD46) that suppress human complement activation have also been reported [6, 7]. Other attempts have been made to overcome the risk of coagulation dysfunction by introducing human coagulation-regulatory genes, such as thrombomodulin, into pigs [8]. Moreover, pigs are now available that have various combinations of these genetic

modifications, and research is ongoing as to the optimal combination for transplantation. Actually, it is technically possible to produce pigs with multiple KO genes and multiple transgenes simultaneously [9, 10]. One such example is the double gene KO pig including GalT-KO that expresses three human complement regulatory genes and two anti-inflammatory genes; however, this combination might not yet be complete [10].

Improved immunosuppressive regimens are also contributing to the progress seen in xenotransplantation of pig kidneys. There has been particular interest in blockade inhibiting the CD40/CD154 pathway, with anti-CD40 or anti-CD154 antibody therapy contributing significantly to a prolongation of renal xenograft survival [11, 12]. In 2019, a GalT-KO kidney expressing CD55 was transplanted from pig to rhesus macaque and had the longest survival of life-sustaining xenograft to date (499 days) [13]. However, although hyperacute rejection by Gal antibodies has been largely overcome, late antibody-mediated injury by non-Gal antibodies remains a problem. Another problem that needs to be resolved is recipient death from infection due to strong immunosuppression [12].

The possibility of zoonotic infection cannot be ignored in xenotransplantation. Given that pigs can be bred in pathogen-free environments, the risk of acquiring zoonotic infections is lower than that of primates. However, the risk of porcine endogenous retrovirus (PERV) that integrates along chromosomes cannot be removed by this approach. In previous research, it was reported that PERV can infect human cells in vitro [14]. In 2015, CRISPR/Cas9 succeeded in knocking out 62 copies of the PERV pol gene in porcine cells [15], and in 2017, a PERV-free pig was produced [16].

As shown in this section, the measures taken against rejection and infection mean that kidney xenotransplantation is rapidly approaching clinical reality.

3. Xeno-embryonic kidney transplantation

The use of xeno-embryonic transplantation may broaden the organ pool. This approach seems to benefit from a lower risk of rejection compared with adult organ transplantation, making a potentially invaluable therapeutic resource. Although an embryonic organ is transplanted prematurely, it can mature in a self-sustaining way in vivo to become functional. Xeno-embryonic organs may be particularly useful as a scaffold for the regeneration of human organs.

Metanephroi have generally been used for embryonic kidney transplantation [17] because this form is already committed to becoming a kidney. When transplanted into a recipient, the metanephroi is free to differentiate and mature into a whole kidney. The transplanted metanephroi promotes angiogenesis, encouraging host blood vessel infiltrating, thus resulting in glomeruli that are composed of host-derived vasculature [18]. The developed metanephroi produces urine, and anastomosing the ureter of the metanephroi and the ureter of the host has been shown to prolong the survival time of host anephric rats [19]. Moreover, the developed metanephroi acquires endocrine function, producing both renin and erythropoietin [20, 21].

Conveniently, the metanephroi is a fetal organ that may have low immunogenicity, potentially making it especially suitable for transplantation. Contrasting with adult grafts that already have the donor vessels, the avascular metanephroi is only vascularized by host vessels after it is transplanted. Thus, humoral immunity to donor endothelial cells is less likely to occur when using the metanephroi for transplantation [22]. Additionally, we can expect a reduced expression of donor antigens, such as HLA class I and II, on a developing metanephroi graft when compared to an adult graft [23].

The ultimate size of the developed metanephroi appears to be imprinted during the early stages of embryonic development. Considering human clinical application, pigs are an ideal resource for metanephric transplantation as with adult kidney xenotransplantation. In the case of allogenic porcine transplantation, the metanephroi on embryonic day 28 (E28) has been successfully transplanted into a nonimmunosuppressed recipient pig and shown to differentiate into a mature kidney without rejection [24]. Allogenic adult kidney grafts are easily rejected without immunosuppression. Transplants originating from pig embryos on E27 to E28 all exhibited significant growth and full differentiation, while those harvested on E20 and E25 failed to develop and only differentiated into few glomeruli and tubules, together with other derivatives, such as blood vessels, cartilage, and bone [25]. This indicates that metanephroi that are too immature may be incompletely pre-programmed and may differentiate into nonrenal structures. However, age-dependent graft growth and survival in allogenic rats was shown to be optimal from E15 and worsened progressively for metanephroi obtained on E16 to E21. The developed metanephroi obtained on E15 showed maturation of renal elements and no sign of rejection, whereas those obtained on E20 had a poor renal architecture and a dense lymphocytic infiltrate [26]. Importantly, there appears to be an optimal window for harvesting metanephroi to obtain good transplantation outcomes.

Successful xeno-metanephric transplantation has been reported previously. In an important study, E28 pig metanephroi or adult kidneys were transplanted into recipient rats with and without immunosuppression. Those transplanted into nonimmunosuppressed rats showed tissue rejection, whereas those transplanted into hosts treated with CTLA-4-Ig underwent growth and differentiation. By contrast, adult kidney grafts showed disturbed morphology, necrotic tissue, and a high degree of lymphocyte infiltration, even when hosts were treated with CTLA-4-Ig [25].

The immune advantage of metanephroi over developed adult kidneys has been demonstrated by direct comparison of xenotransplantation into host animals treated with immunosuppressants. Next, it will be necessary to study the xenotransplantation of pig metanephroi into nonhuman primates.

In the case of xenotransplantation of pig islets, embryonic islet tissues are regarded as a choice for xenotransplantation with several advantages including reduced immunogenicity, long-term proliferative potential, and revascularization by host endothelium. However, the embryonic implants exhibit a delayed insulin response to glucose in vivo (>3 months) and limited effect on improvement of blood glucose level [27]. Fetal and neonatal pig islets have the higher expression of GAL and will be more susceptible to xenorejection than adult pig islets [28, 29]. Therefore, adult pig is regarded as the primary donor source of islet xenografts, which can supply an adequate amount of viable islet cells and start functioning immediately after implantation.

4. Kidney organoids derived from pluripotent stem cells (PSCs)

The field of stem cell research is growing at a rapid pace. The reproduction of organoids derived from human iPSCs in vitro is already possible in several organs, including the optic cup, intestines, and liver [30–32]. Although embryologic kidney development is complicated, the reproduction of kidney organoids has been reported. Kidneys arise from metanephroi, which develop via the reciprocal interaction between the metanephric mesenchyme, containing NPCs and stromal progenitor cells, and the ureteric bud. Takasato et al. reported simultaneously inducing metanephric mesenchyme and ureteral buds from human iPSCs to

produce kidney organoids. The generated organoids contained nephrons associated with a collecting duct network surrounded by renal interstitium and endothelial cells [33]. Taguchi et al. also reported the successful differentiation of human iPSCs into NPCs and ureteric buds in vitro, by repeating the development of the metanephric kidney [34, 35]. Additionally, they reconstructed kidney organoids with higher-order structures, containing embryonic branching morphogenesis, by reaggregating NPCs and ureteric buds derived from mouse PSCs and stromal progenitor cells from mouse embryos. However, a method for differentiating human iPSCs into stromal progenitor cells is yet to be established. Furthermore, neither of the developed kidney organoids have a urine drainage system, and both are too small to function in vivo. Therefore, generating functional kidneys in vitro remains a challenge before this research has translational potential.

5. Blastocyst complementation

As an alternative to in vitro directed differentiation of iPSCs, previous studies have considered methods of regenerating solid organs from transplanted exogenous cells to function in vivo by borrowing a xenogenic development program. One such method is blastocyst complementation. When PSCs are transplanted into blastocysts, which are early animal embryos, chimeras containing blastocysts and PSCs are formed. When PSCs are injected into blastocysts that have undergone genetic manipulation not to generate a target organ, the missing organ is formed from the injected PSCs by systemic chimera formation. Using the method of blastocyst complementation, kidneys derived from mouse iPSCs have been regenerated in sall1 knockout mice that lack kidneys [36]. Successful kidney regeneration has also been derived from mouse iPSCs in sall1 knockout rats [37]. Therefore, this generation mechanism appears to have interspecies compatibility. The renal lineage cells were derived from the injected PSCs, whereas nonrenal lineages such as blood vessels and stromal cells in kidneys were chimeric for both blastocyst cells and PSCs. Recently, mouse PSC-derived vascular endothelial cells were regenerated into *Flk-1* knockout mice, lacking a key gene for vascular endothelial development [38]. By simultaneously disrupting Flk-1 and genes required for genesis of the target organ, rejection-free organs could be generated from patient-specific iPSCs. The size of the regenerated organ will be affected by the size of the host animal blastocyst. Successful allogenic blastocyst complementation has been shown to regenerate large organs in pancreas-deficient pigs [39].

Given that human iPSCs fundamentally lack the ability to form chimeras, blastocyst complementation cannot be applied directly to humans. Inducing the expression of anti-apoptotic genes could give some chimera-forming ability to human iPSCs [40, 41], but the long-term safety would require clarification because these are also recognized oncogenes. Another issue is that basing this method on systemic chimera formation leads to the serious ethical concern of chimera formation in host gametes or neural tissue other than the target organs. The introduction of the heterologous cells during insemination must be thoroughly considered for the loss of the personal identity of a living being [42]. If these problems can be resolved, such a method that can produce organs that function in vivo would be highly significant.

6. Organogenic niche method and NPC replacement system

We have developed an organogenic niche method that utilizes a xenogenic development program. In this method, exogenous organ progenitor cells are transplanted

into the region of the xenogenic fetus where the target organ develops. The transplantation of progenitor cells into host tissue matched by developmental stage may be critical for efficient cell grafting. In our experiments, we first injected human mesenchymal stem cells (hMSC) expressing glial cell line-derived neurotrophic factor into the embryonic rat site where budding of the ureteric bud occurred. Second, the transplanted host rat embryo was grown in a whole-embryo culture system [43]. Third, the transplanted hMSCs were integrated into the metanephroi and differentiated into tubular epithelial cells, interstitial cells, and glomerular epithelial cells [44]. Fourth, we transplanted the developed metanephroi into recipient rats. Using this approach, the metanephroi integrated with the vessels of recipient rats and the vascularized nephrons (derived from hMSCs) regenerated. The neo-kidney derived from hMSCs also produced urine by filtering the host blood, and the level of urea nitrogen and creatinine in the urine was higher than that of the host serum [45], and it also secreted human erythropoietin in response to host anemia [46]. Thus, we successfully regenerated human cell-derived neo-kidneys with in vivo function. As described, instead of PSCs, we used stem cells or progenitor cells that have limited potency. These cells were only locally transplanted into embryos at mid-to-late gestational ages, thereby ensuring that chimera formation only occurs in the kidney and avoiding any potential ethical concerns.

Existing native host cells inhibit the engraftment of transplanted donor cells. We recently developed a new method combining an organogenic niche with eliminating host NPCs to increase the efficiency of donor cell engraftment [47]. In this method, we used transgenic mice in which the diphtheria toxin receptor (DTR) was specifically expressed on Six2-positive NPCs (Six2-iDTR transgenic mouse). Rodents such as mice and rats naturally lack the DTR, so Six2-positive NPCs selectively undergo apoptosis with the administration of diphtheria toxin. When donor mouse NPCs are transplanted into host mouse metanephroi, they became chimeric with the existing native host NPCs, and contribution rate of the donor cells was 30% of cap mesenchyme cells. Administering diphtheria toxin eliminated the host mouse NPCs and allowed 100% replacement with donor mouse NPCs that could generate neo-nephrons [47] (**Figure 1**). In this way, we succeeded in achieving full replacement with heterogeneous donor rat NPCs. Importantly, we revealed that nephrons derived from rat NPCs could connect to the host mouse collecting ducts, even when nephrons and collecting ducts were heterogeneous. Next, we examined the possibility of in vivo regeneration of interspecies kidneys

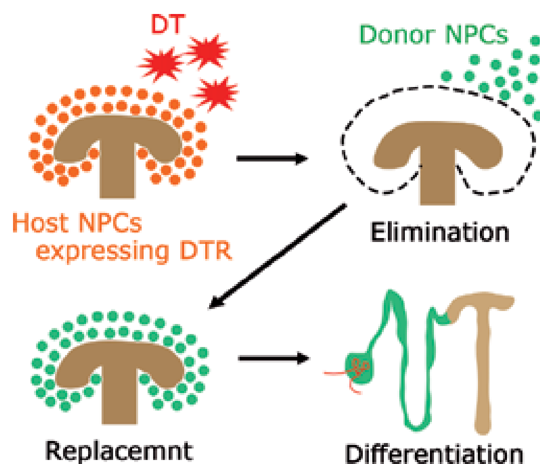

Figure 1.
Schematic of the drug-induced cell elimination system to exchange native NPCs with exogenous NPCs.

using NPC replacement. In subsequent research, we successfully regenerated rat nephrons using the Six2-iDTR mouse metanephroi as a scaffold in recipient rats receiving immunosuppressive therapy. We showed that neo-kidneys were vascularized by blood vessels originating from the recipient rats using the species-specific antibody for detection. Furthermore, we injected fluorescent-labeled dextran into the recipient rats, and the accumulation of dextran in Bowman's space of neo-glomeruli and in the lumen of neo-tubules was confirmed. Our findings confirmed that neo-kidneys were incorporated into blood circulatory system of recipients, resulting in functional neo-glomeruli filtration [48].

In the future, we aim to use this system of kidney regeneration with pig fetuses as the bioreactor and human iPSC-derived NPCs as the cell source. It is not difficult to supply a cell source because protocols for inducing NPCs from human iPSCs have already been developed [33, 34]; additionally, expansion of NPC culture is possible [49, 50]. A fundamental problem with our proposals is that human cells permanently express DTR and can undergo apoptosis when treated with diphtheria toxin. Therefore, the DTR system cannot be applied directly to humans. Recently, we developed a new transgenic model to ablate NPCs using an alternative drug that does not affect human cells (unpublished data).

7. Stepwise peristaltic ureter system

Although transplanted metanephroi can produce urine, they lack a urine excretion pathway, gradually become hydronephrotic, and cease functioning. Neo-kidneys regenerated using metanephroi as a bioreactor may also follow the same mechanism. Recently, we developed a urine excretion strategy for embryonic kidneys generated by stem cell methods.

The ultimate size of a metanephric graft is determined by the size of the source animal. Pigs are therefore considered a suitable resource from this perspective. To eliminate the potential for rejection, we transplanted metanephroi from cloned pig fetuses into syngenic hosts. All transplanted metanephroi differentiated successfully into mature kidneys, growing to 5–7 mm in length by 3 weeks. After 5 weeks, metanephroi grew to more than 1 cm and retained urine in the developed ureters, and after 8 weeks, they grew to about 3 cm and started to develop hydronephrosis as urine production increased [51]. Ureteral primordia start peristalsis during the embryonic stage and normally excrete urine into the bladder, and this sustained urine excretion may be important for normal development.

To delay the onset of hydronephrosis and to promote the growth of metanephric grafts, we transplanted metanephroi with ureters and a bladder (MNB) into a recipient animal. After 4 weeks, hydronephrosis occurred in the group with normal metanephroi transplants but not in the group with MNB transplants. In the MNB group, urine retention in the bladder was observed. Histopathologic examination also showed more pronounced tubular luminal dilation and interstitial fibrosis, and greater reductions in the number of glomeruli, in the metanephroi group than with the MNB group. Moreover, urine volumes and urinary levels of urea nitrogen and creatinine were higher in the MNB group than in the metanephroi group.

Furthermore, we demonstrated the generation of a urine excretion channel in MNB by using the stepwise peristaltic ureter (SWPU) system. Briefly, at an appropriate time, we connected the host ureter to the MNB graft containing urine produced by the metanephroi. The SWPU system allowed for continuous urine drainage from the developed bladder of the MNB into the recipient bladder via the recipient ureter. Even 8 weeks after transplantation, the MNB showed no hydronephrosis and had maintained mature renal structures, such as glomeruli and renal

tubules. The levels of urea nitrogen and creatinine were much higher in the urine from the MNB than in the sera of recipients. Finally, the SWPU system significantly prolonged the lifespan of anephric rats in the MNB group compared with the nontransplanted group.

In a previous study, researchers demonstrated that they could create a urinary pathway by directly connecting the ureter of the transplanted metanephroi to the ureter of the host (ureteroureterostomy) to prolong the short-term survival of anephric rats [19]. However, the SWPU system is more efficient than ureteroureterostomy in terms of preventing hydronephrosis and allowing maturation of the metanephroi. Surgery for the SWPU system is also easier than that for uretero-ureterostomy because the bladder of the MNB expands with urinary retention. Furthermore, we can join two metanephroi to a host ureter using the SWPU system, whereas it is difficult to connect two metanephroi to the host ureter. In a previous study, it was reported that the survival time in anephric rats correlated with the total volume of the grown metanephroi [52]. It is possible that the SWPU system is more effective than the conventional method in prolonging survival time for this reason.

Assuming that MNBs can be used as a scaffold for kidney regeneration before transplantation into patients with renal failure, we investigated the effects of host renal failure on the structure and activity of the transplanted MNB. Uremic conditions were reproduced using a 5/6 renal infarction rat model, and 4 weeks after transplantation, the developed bladder was successfully anastomosed to the host ureter. At 8 weeks after transplantation, histological analysis showed the presence of mature glomeruli and tubules in the groups with and without renal failure. There were also no differences between these groups in terms of survival in anephric host rats, indicating that the grafts were responsible for prolonging host survival, even under renal failure conditions [53]. The results of this study demonstrate that a transplanted MNB can grow and function effectively, even under uremic conditions.

The use of MNB as a kidney regeneration scaffold can provide new treatment for patients with renal failure. We assume that the SWPU system will be applicable to human neo-kidneys regenerated via the NPC replacement system, using a pig MNB as a scaffold to establish the urinary excretion pathway. In brief, human iPSC-derived NPCs may be injected into the metanephroi of porcine fetuses that

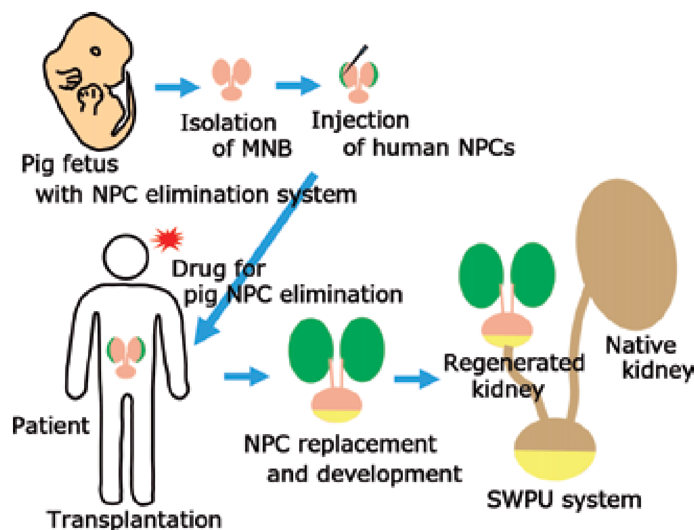

Figure 2.
Schematic of our novel xeno-regenerative therapeutic strategy for kidney regeneration.

are genetically manipulated to have an NPC elimination system. Human nephrons may then regenerate in porcine metanephroi by eliminating the porcine NPCs and replacing them with human NPCs. The MNB that has human kidneys will be transplanted into patients with end-stage renal disease, and an excretion pathway will be constructed. In this case, the regenerated kidneys will be of human origin, but the ureters and bladder of MNB will be of porcine origin (**Figure 2**). Although further investigation is required, we assume that replacing nephrons, which are the main targets to rejection, could decrease antigenicity.

8. Regenerative potential of iPSCs derived from patients with renal failure

The use of iPSCs generated from patients holds promise for tailored therapy that uses patient-derived cells, tissues, or organs. In clinical settings, it is desirable to use patient-derived iPSCs as the cell source for neo-kidneys to circumvent immune rejection. However, because uremia can reduce the function of stem cells, it may be problematic to use stem cells derived from patients in renal failure. Previous studies have shown that uremia causes many toxic effects, including reduced proliferation capacity, abnormalities of differentiation, and angiogenic dysfunction in stem cells [54, 55]. We previously reported that gene and protein expression of p300-/CBP-associated factor was significantly suppressed and that in vivo angiogenesis activation was decreased in hMSCs derived from patients with end-stage renal disease (ESRD) [56]. However, there have been no reports about the biological properties of iPSCs derived from patients with ESRD. In our recent study, iPSCs derived from patients with ESRD could differentiate into NPCs as efficiently as iPSCs derived from healthy controls. Moreover, NPCs derived from patients with ESRD showed the potential to become mature and vascularized nephrons in vivo, similar to the process in healthy control [57]. These findings suggest that iPSCs from patients with ESRD may still be a useful cell source for kidney regeneration.

9. Conclusions

In this chapter, we have described several potential alternatives to allotransplantation, focusing on our novel xeno-regenerative therapeutic strategy for kidney regeneration. Although there are issues to be overcome with the treatment alternatives that are being developed, recent advances in genetic recombination technology and stem cell research may make them available in clinical practice. We have addressed the development of genetically modified pigs that possess an NPC elimination system and have performed experiments with NPCs derived from human iPSCs. To date, each step of our proposed strategy for kidney regeneration has been accomplished successfully in rodent models. This includes the regeneration of kidneys derived from transplanted NPCs via NPC replacement, the transplantation of regenerated kidneys into host animals, and the construction of a urine excretion pathway (i.e., the SWPU system). Looking to the future, we aim to complete a series of studies to allow transplantation from pig to human, which should facilitate the translation of our strategies to clinical settings.

Conflict of interest

The authors declare no conflict of interest.

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Chapter 9

Pigs as Models of Preclinical Studies and In Vivo Bioreactors for Generation of Human Organs

Yujiro Kawai, Shugo Tohyama, Hideyuki Shimizu, Keiichi Fukuda and Eiji Kobayashi

Abstract

Pigs are valuable and essential large animal models for human medical applications, including for stem cell therapy. Moreover, substantial effort has been made to directly engraft genetically engineered pig organs in the human body and to use pigs as in vivo bioreactors for the growth and development of human cells, tissue, or organs. However, engraftment of human cells in pigs has not yet been achieved. Although severe combined immunodeficient pigs have been developed, which can accept human biological materials, these pigs do not have practical value at present owing to difficulty in their care. To overcome these current limitations, we have proposed the generation of operational immunodeficient pig models by simply removing the thymus and spleen, enabling the long-term accommodation of human tissue. In this review, we summarize research progress on xenotransplantation animal models that accept human cells, tissues, or organs.

Keywords: regenerative therapy, transplant, bioreactor, immune tolerance

1. Introduction

Organ transplantation is often the only possible treatment for a patient with organ failure. The organs are donated from either living or deceased donors, and thus the number of transplantable organs is limited and insufficient to meet the clinical demand. Consequently, some illegal or unethical transplantations along with transplant commercialism and tourism have emerged, representing a worldwide problem.

The discovery of the potential of pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced PSCs (iPSCs), to regenerate tissues or organs offers new hope to overcome this situation. Human ESCs [1] and iPSCs [2] are now widely used to generate tissues or organs, and techniques for the in vitro production of specific cell types have been developed [3]. However, these strategies still have several limitations for clinical application, including the size, maturity, function, and risk of tumor formation after transplantation [4].

To solve these problems, large animal models for the transplantation of human PSC-derived cells, tissues, or organs are required. In this review, we summarize the animal models currently used in the development for xenotransplantation and highlight the efficacy and prospects of pig models to accept human tissues or organs.

2. Xenotransplantation in small animals (mouse and rat)

Immunosuppression is a key requirement for an animal to accept human tissues or organs, as a functional immune system will result in the host animals rejecting the human grafts. The nude mouse was the first immunosuppressed animal model developed in 1962 [5]. Nude mice lack T cells and can therefore accept human tumor cells. Subsequently, severe combined immunodeficient (SCID) mice were developed in 1983, which lack both B cells and T cells [6]. McCune et al. [7] successfully transplanted a human fetal thymus, liver cells, and lymph node into SCID mice, resulting in the differentiation of human T cells and B cells. However, the rate of engraftment of human cells in these mice was low due to maintenance of their natural killer (NK)-T cell activity. Gerling et al. [8] developed NOD/SCID mice by crossbreeding SCID mice with NOD mice, a diabetes model due to autoimmunity in the pancreas, which also show low NK-T cell activity and macrophage function [9]. Combining the low activity of NK-T cells and macrophages in NOD mice with the lack of B cells and T cells in SCID mice, the use of NOD/SCID mice improved the engraftment rate of hematopoietic stem cells [10]. Ito et al. [11] produced NOG mice as a crossbreed of NOD/SCID mice and gamma(c)(null) mice, which completely lack NK-T cells, and achieved a dramatically improved engraftment rate of human hematopoietic cells.

We previously reported the successful transplantation of rat cells into SCID mice [12]. Isolated hepatocytes obtained from the rat liver were injected into urokinase-type plasminogen activator (uPA)/SCID mice, in which urokinase-type plasminogen accumulates specifically in the native liver causing the damaged liver. The mice served as bioreactors to allow the transplanted rat hepatocytes to proliferate in the mouse host, resulting in more than 95% of cells in the mouse liver being of rat origin. Oldani et al. [13] successfully developed a mouse-rat chimeric liver, which was transplanted in rats. They injected hepatocytes isolated from Lewis rats into C57Bl/6$^{Fah-/-Rag2-/-Il2rg-/-}$ mice to create chimeric livers, which were transplanted into rats with or without immunosuppression. Without immunosuppression, the recipient rats died from acute rejection, whereas rats with immunosuppression survived for more than 112 days and maturation of rat bile ducts was observed 4 months after transplantation. We also demonstrated that the nude rat model could serve as an in vivo bioreactor. Liver grafts from Syrian hamsters were transplanted into nude rats that administered several immunosuppressive agents, including tacrolimus and mycophenolate mofetil (MMF). After auxiliary xenogenic partial liver transplantation, regeneration of the liver graft was observed, and its weight increased from pre-transplant to 7 days after transplantation [14].

These immunodeficient mouse models, including SCID, NOD/SCID, and NOG mice, are useful for research on regenerative medicine using human PSCs, allowing for evaluation of teratoma formation to confirm the differentiation of the cells into the three germ layers [1]. In addition, these models are widely utilized for evaluation of tumorigenicity in human PSC-derived cells after transplantation [4], since human PSC-derived cells or tissues have a risk of tumor formation from contamination of undifferentiated PSCs [15, 16]. Small animals such as mice and rats are widely applied as models in cell transplantation research owing to their ease of handling. However, small animals have limitations in terms of the number of cells that can be transplanted and evaluation of therapeutic efficacy, that is, a human clinical application might require the transplantation of several hundreds of million cells, which is impossible to accomplish in small animals. Moreover, large animal models are required for accurate evaluation of the efficacy of cell transplantation.

Furthermore, large animal models are expected to play roles as bioreactors for functionally mature human tissues or organs.

3. Xenotransplantation in middle and large animals (monkey and pig)

Chong et al. [17] transplanted human ESC-derived cardiomyocytes into the hearts of pig-tailed macaques as a nonhuman primate model. The main advantage of this model is that the hearts are much larger (37–52 g) than those of mice (0.15 g), rats (1 g), and guinea pigs (3 g), which allowed for the transplantation of 1×10^9 cells into the infarcted myocardium and subsequent engraftment. The macaques were administered methylprednisolone, cyclosporine, and abatacept (a CTLA4 immunoglobulin) to prevent immune rejection. The efficacy of human ESC-derived cardiomyocytes in the infarcted hearts of pig-tailed macaques was demonstrated, and maturation of the transplanted ESC-derived cardiomyocytes was observed [18]. However, compared to an adult human, pig-tailed macaques are still relatively small (5.2–12.6 kg), and the heart is much smaller than that of a human (300 g).

Pigs are a suitable animal for preclinical studies and in vivo reactors in terms of their size and anatomy that correspond well to those of humans. To establish an immunosuppressed state that allows for transplantation of human PSC-derived cells or tissues into host pigs without rejection, SCID pigs were also developed [19]. Suzuki et al. [19] generated cloned pigs by serial nuclear transfer using fibroblasts with disruption of the X-linked interleukin 2 receptor subunit gamma (*IL2RG*) gene, as this mutation is known to cause X-linked SCID in humans. The SCID pigs accepted human cells, indicating their potential in preclinical studies and as in vivo reactors with human PSCs. However, raising these pigs is a technical challenge; among the 31 cloned piglets produced, only four survived for over 1 year. In addition, SCID pigs must be raised under meticulous hygiene conditions, which impose a further cost for their establishment and maintenance. Therefore, it is not practical to use SCID pigs as models in preclinical studies and in vivo reactors.

Total thymectomy is an alternative strategy to create immunosuppressed pigs that can accept human cells. Binns et al. [20] first proposed the concept of achieving immunosuppression by performing thymectomy in neonatal pigs in 1972. Microminiature pigs (MMPs) are smaller than domestic or ordinary miniature pigs and are thus suitable model animals for preclinical studies [21]. To develop immunodeficient MMPs, we performed thymectomy in neonatal pigs, which were transplanted with human hepatocytes that could engraft in the pig liver without any immunosuppressive agents [22]. To further improve the immunodeficient pig model, we performed splenectomy along with the thymectomy in 6–7-month-old miniature pigs and administered several immunosuppressive agents, including tacrolimus, MMF, and prednisolone, via a stomach tube [23]. This so-called operational immunodeficient miniature pig (OIDP) model allowed for the successful implantation of artificial human vascular tubes created by a three-dimensional bioprinting. Moreover, the human tube was inserted between the carotid artery and jugular vein to act as a shunt, and blood flow was observed for 3 months without immune rejection.

As mentioned above, establishment of a chimera is a potential strategy for growing human tissues or organs in large animals. Matsunari et al. [24] demonstrated that blastocyst complementation can be applied to large animals by creating chimeric pigs. Specifically, they generated embryos from clones of porcine somatic cells, which showed an apancreatic phenotype, and their complementation

with allogenic blastomeres resulted in the development of a functional pancreas. Wu et al. [25] reported a successful pig-human chimera that was created by introducing human PSCs into fertilized pig eggs. Therefore, when combined with blastocyst complementation, human organs can be created in a human-pig chimera; however, these methods are associated with serious ethical and legal problems. Alternatively, the introduction of human-derived cells to pig fetuses can lead to immune tolerance, allowing for the acceptance of human PSC-derived tissues or organs.

4. Immune tolerance induction for xenotransplantation

Immune tolerance is defined as a lack of an immune response against particular antigens. In general, the immune system has tolerance to self-antigens and only responds to non-self-antigens, which is a challenge for transplantation, as the grafted cells or tissues are rejected and not able to survive in the host body. The phenomenon of immune tolerance was first described in 1945 in which anastomosis in the placenta was observed in twin calves, and they accepted each other's skin grafts [26]. Hasek et al. [27] subsequently confirmed this phenomenon in chicken and duck by producing parabiosis in fertilized eggs. In 1953, Medawar et al. [28] established actively acquired tolerance by implanting a live antigen in the fetuses of mice or embryonic chicks. Using this method, Binns et al. [29] also tried to create immune tolerance in pigs by implanting bone marrow cells or lymphocytes from another pig into fetal pigs, resulting in prolonged survival of skin graft in the treated pigs.

In addition to these examples, induction of immune tolerance to human cells or tissues has been attempted in other animals. Kenneth et al. [30] transplanted human mesenchymal stem cells into fetal sheep early in gestation. Despite the xenogeneic condition, the human mesenchymal stem cells engrafted and survived in multiple tissues for up to 13 months after transplantation. These strategies of injecting human cells into a fetus were proven to result in immune tolerance to human cells after birth.

As MMPs have emerged as suitable candidates for immune tolerance induction to accept human cells, tissues, and organs owing to their useful applications in preclinical studies and in vivo reactors, it may be possible to create MMPs with immune tolerance to human cells by injecting a human antigen into pig fetuses without requiring the need to create human-pig chimeras [31].

5. Conclusions

Our newly developed OIDPs can accept human cells, tissues, and organs derived from human PSCs. These models will allow for long-term observation after the transplantation of human PSC-derived cells or tissues to better evaluate the safety and efficacy of the procedure. Moreover, if human cells, tissues, and organs are transplanted into piglets, they will grow in vivo along with the growth of the host pig. These grafts will then mature and be of suitable size with appropriate function for human application. Therefore, pigs can be suitable models for preclinical studies and serve as in vivo bioreactors for developing human tissues or organs (**Figure 1**). Transplantable MMPs without immunosuppressive agents are expected to be developed in the near future as promising and valuable animal models for researchers, which can dramatically promote regenerative medicine and organ transplant therapies with human PSCs.

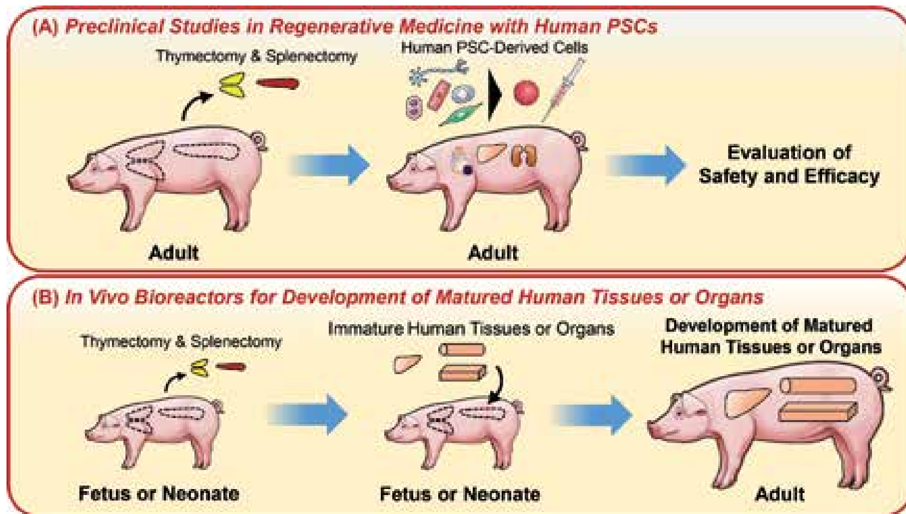

Figure 1.
Schema of pigs as models of preclinical studies and in vivo bioreactors. (A) Adult operational immunodeficient miniature pigs (OIDPs) are useful for preclinical studies in regenerative medicine with human PSCs, enabling evaluation of the safety and efficacy of cell transplantation. In particular, after transplantation of human PSC-derived spheroids or organoids into the OIDPs, the risk of tumorigenicity can be evaluated. (B) Fetal or neonatal OIDPs are also useful as in vivo bioreactors, facilitating the efficient in vivo growth of immature human tissues. After immature human PSC-derived tissues or organs are transplanted into OIDPs, they will mature along with the growth of the host.

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Recently, remarkable progress has been made in the area of preclinical xenotransplantation experiments. Surprisingly, a heterotopic heart from the gene-editing pig continued to beat for almost 2.5 years, when implanted in the monkey abdomen, and a pig life-supporting kidney could also function for over 1.3 years in monkeys. Concerning islets, islets from gene-editing pigs could work for more than one year in monkeys. It is noteworthy that one group reported a survival of adult wild-type pig islets of over 600 days. On the other hand, the progress in these preclinical trials strongly affected not only the xenotransplantation study itself but regeneration studies to use pigs as a scaffold to foster human induced pluripotent stem cells.

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