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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 xeno-transplantation 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

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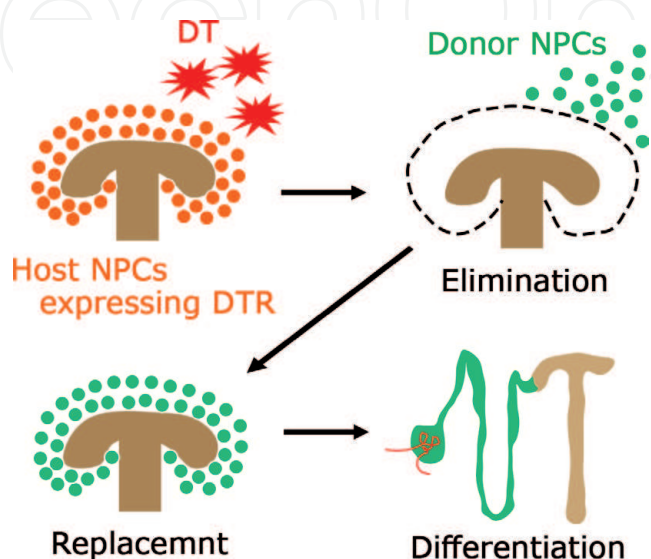
Figure 1.
Schematic of the drug-induced cell elimination system to exchange native NPCs with exogenous NPCs.

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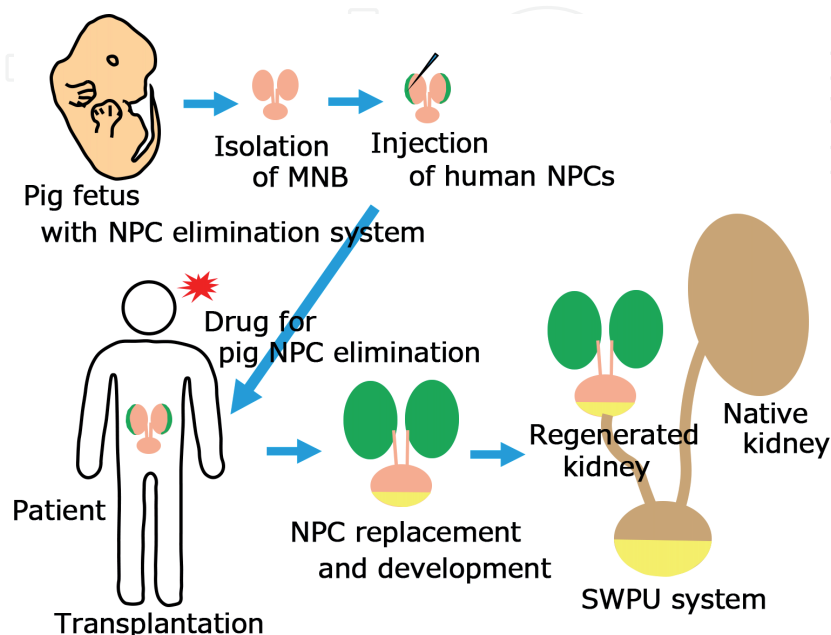
Figure 2.
Schematic of our novel xeno-regenerative therapeutic strategy for kidney regeneration.

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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