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CURRENT BIOENGINEERING AND REGENERATIVE STRATEGIES FOR THE GENERATION OF KIDNEY GRAFTS ON DEMAND

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Abstract

Currently in the USA, one name is added to the organ transplant waiting list every 15 min. As this list grows rapidly, fewer than one-third of waiting patients can receive matched organs from donors. Unfortunately, many patients who require a transplant have to wait for long periods of time, and many of them die before receiving the desired organ. In the United States alone, over 100,000 patients are waiting for a kidney transplant. However, it is a problem that affects around 6% of the word population. Therefore, seeking alternative solutions to this problem is an urgent work. Here we review the current promising regenerative technologies for kidney function replacement. Despite many approaches being applied in the different ways outlined in this work, obtaining an organ capable of performing complex functions such as osmoregulation, excretion or hormone synthesis is still a long-term goal. However, in the future the efforts in these areas may eliminate the long waiting list for kidney transplants, providing a definitive solution for patients with end-stage renal disease.

Keywords: kidney disease; kidney engineering; blastocyst complementation, stem cells, kidney regeneration, decellularization, metanephros

Introduction

Currently, many patients are suffering degenerative processes or injuries which end in specific irreversible organ failure. In many instances, therapeutic options are limited to supportive measures and preventing further damage [1], but transplantation represents the ideal method of restoring full physiological organ function [2]. Paradoxically, the effectiveness of this treatment has used up many organs for transplant, and their availability has been the main limitation of the technique [3,2]. In the USA, one name is added to the organ transplant waiting list every 15 min [4]. While this list grows rapidly, fewer than one-third of waiting patients can receive matched organs from donors [5]. For this reason, many patients who require a transplant have to wait long periods of time and a lot of them die before receiving the desired organ [6]. Specifically, patients with advanced renal disease are habitually obliged to resort to renal replacement therapies alternative to transplant, such as haemodialysis, due to the long waiting list for a kidney. Nevertheless, more patients either die or are removed from the waiting list because of the progression of pathophysiological conditions such as coronary artery disease during prolonged haemodialysis [7]. Moreover, these techniques fail to meet the functional endocrine and reabsorption demands of normal kidney function [2], affecting the patient's quality of life [8] and entailing a very high cost for public sanitary services. Currently, these costs could reach up to €1,518 million for countries like Spain or £1.2 billion in the United Kingdom [9, 10]. In the USA alone, more than 400,000 patients are suffering from end-stage kidney disease and the waiting list for a kidney extends to 100,000 individuals [11,12]. However, it is a problem that affects

around 6% of the word population [13]. Thus, the global prevalence of chronic kidney disease is rising at an alarming rate, correlated with the high increase in prevalence of obesity, which is associated with type II diabetes and renal failure [14]. Even in Spain, a leading country in the field of transplantation, today approximately 129 (incidence) and 1039 (prevalence) patients per million habitants still require renal replacement therapies [9]. However, even in the event of getting a transplantable kidney, around 20% of recipients will experience an episode of acute rejection within 5 years of transplantation, and approximately 40% of recipients will die or lose graft function within 10 years after transplantation [15].

Regenerative medicine has garnered considerable attention in recent years because it has the potential to provide the ultimate treatment for various diseases by generating new organs for transplantation. However, the development of an organism involves not only differentiation of cells, but also their morphogenesis and appropriate patterning to form the architectural context of tissues and organs [16]. Thus, mammalian cells, as part of multicellular organisms, function in tissue units that contain several types of cells, which together form an organ. Essentially, to function adequately, cells need to communicate with each other and their microenvironment, by means of growth factors, morphogens, cell adhesion molecules and mechanoreceptors. Specifically, human kidney exhibits a remarkable architectural complexity, coupled with the presence of at least 30 different specialized cells [17]. Thus, recapitulation of complex functions such as glomerular filtration and reabsorption and secretion of solutes are dependent on a three-dimensionally

integrated kidney structure, which is why cell therapies with individual cells are inefficient in restoring kidney function [18].

However, the idea of generating a functional kidney graft in vitro on demand would extend the option of kidney transplantation to more patients. Furthermore, the use of autologous cells could eliminate the need for lifelong immunosuppressive therapy. In this line, the field of renal bioengineering is exploring new frontiers basing on biotechnology, bioengineering, stem cells and regenerative medicine in an attempt to obtain a renal organ able to function as well as a native kidney. Here, we review the latest developments in regenerative medicine strategies for generation of kidney grafts on demand, with a main focus on (a) stem cells; (b) blastocyst complementation; (c) decellularization/recellularization technology; (d) bioprinting in 3D; (e) renal device; (f) xenoembryos, and (g) transplantation of embryonic kidneys.

Stem Cells

Pluripotent stem cells (PSC) generally include both embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC), which have the potential to differentiate into any cell type and self-assemble into heterogeneous tissues or organs. Through direct differentiation, PSC have originated several cell types or tissues, such as hepatic [19], neural [20], cardiac [21], pancreatic [22] and blood tissues [23]. In the case of the kidney, recent progress has generated human nephron progenitor cells, also including intermediate mesoderm and

metanephric mesenchyme cells [24]. By sequential application of chemicals or growth factors, there are studies that differentiated *in vitro* PSC and generated cells with ureteric bud-committed intermediate mesoderm fate with the potential to assemble spontaneously [25] and which could generate renal structures such as nephrons and proximal tubules [26]. The co-culture of embryoid bodies (which contains nephron progenitors) with a mouse embryonic spinal cord (an inducer of kidney tubulogenesis), resulted in the formation of tubular renal structures with the characteristic markers of renal structures [26]. However, no mechanism by which to generate a vascular system around these renal structures is known, so urine output could not be demonstrated.

However, the potential tumorigenicity of PSC is one limiting step in the future clinical application of this methodology. In this case, adult stem cells (ASC) receive great interest, as they are clinically safe, not being tumorigenic [24, 27]. These ASC have been isolated from many human tissues such as the intestines, muscles, skin, blood, nerves, heart, liver, dental pulp, adipose tissue, umbilical cord blood, amniotic fluid and endometrial tissue [28]. The ASC can divide by asymmetric division leading to two types of daughter cells, one of which is an identical parent cell mother involved in the process of self-renewal, while the other results in a transient cell amplification that proliferates to produce the various differentiated cell types required to maintain tissue homeostasis [28]. In the kidney, renal ASC are located in specific regions in the adult organ, such as in tubular epithelial cells [30], Bowman’s capsule [31], the renal papilla [32] and the S3 segment of the proximal tubules [33]. Using S3-segment ASC, the reconstitution of a 3D kidney-like structure *in vitro* has been

described [34]. However, as in the case of PSC, although the reconstructed kidney structure possesses glomeruli, proximal tubules, Henle's loop, distal tubules and collecting ducts, it had no functional vasculature. Thus, non-vascularized kidney structures did not produce urine. These results suggest that tissue-specific stem cells may only have the ability to reconstitute the minimum unit of its organ of origin by differentiating into specialized cells in the correct niche [24].

However, although it is difficult to build a complex organ like the kidney using techniques based on individual cells, these cells may be a promising cellular source for kidney repair and regeneration. Furthermore, these cells could be used in the different gene-editing platforms that have recently emerged to increase homologous recombination efficiency. Thus, DNA nucleases and CRISPR/Cas9 have emerged as potential tools for gene editing to generate kidney disease animal models [35, 36] or to generate human reporter PSC lines that may help us in the dissection of the molecular cues that organize the renal differentiation and evaluate its maturity [37, 38].

Blastocyst Complementation

At blastocyst, the initial embryonic stage 5 days after fertilization, the injected PSCs become synchronized with the development of inner cell mass, generating a chimeric body. Thus, injection of normal ESC into a deficient blastocyst results in the formation of a normal chimeric organism known as a

blastocyst complementation phenomenon. This method uses the chimera-forming ability of PSCs that are injected into a xenoblastocyst, which lacks potential to form any particular cell lineage. Thus, these cells lines are exclusively derived from exogenous normal PSC, which assume the role that deficient cells cannot accomplish due to the lack of any functional gene [39]. Employing this methodology for the first time, in 1993 Chen et al. injected normal ESC into the blastocyst unable to develop mature B or T lymphocytes [40]. Thus, somatic chimeras were generated with foreign ESC-derived mature B and T lymphocytes. However, this blastocyst complementation system was applied to reconstruct several different tissues and organs such as thymic epithelium [38], germ cells [41], heart [42], pancreas [43, 44], liver [45] and kidney [46]. Recently it has been reported that rat iPSC injected into a pancreatogenesis-disabled mouse blastocyst produced a normal chimeric mouse with almost entirely rat pancreas that produced insulin and whose pancreas islets improved hyperglycaemia when transplanted into a diabetic rodent model [43, 44, 47]. Similarly, blastocysts with a deficient blastocyst in fumarylacetoacetate hydrolase injected with normal mouse iPSC produced chimeric mice with a iPSC-derived liver whose hepatocytes had a proliferative capacity characteristic of normal hepatocytes [45]. These studies indicated that progeny derived from PSC could occupy and develop in a vacant developmental niche, a fact that could be used along with demonstrated interspecific blastocyst complementation to in vivo-generation of organs derived from donor PSC using a xenogeneic environment [24, 46, 47].

In the case of the kidney, deficient mice blastocyst in spalt-like transcription factor 1 (Sall1; a transcription factor essential in renal organogenesis) injected with mouse iPSC resulted in mice with kidneys almost entirely originated from the injected iPSC [44]. This is because, although the $Sall1^{+}$ metanephric tissues are exclusively derived from iPSC, $Sall1^{-}$ tissues such as ureteric bud and nervous and vascular system are derived from the host. This may constitute an obstacle, as it could promote a tissue rejection response [39]. In fact, when normal rat iPSC where injected into Sall1-deficient mice blastocysts, the expected results were not achieved and the progeny did not possess kidneys derived from the rat iPSC [48]. Consequently, these findings could be an obstacle to the notion of generating a functional human kidney through blastocyst complementation employing a xenogeneic environment. Of course, this does not exclude that blastocyst complementation remains one of the most promising strategies for obtaining a whole functional kidney.

Decellularization/Recellularization technology

Natural or organ-derived biological scaffolds composed of extracellular matrix (ECM) are used for a variety of reconstructive surgical applications and are increasingly used in regenerative medicine strategies [49]. The biocompatibility with natural materials is obviously excellent [50] and it is important to note that the ECM is a secreted product of cells whose composition and ultrastructure are determined by several factors that influence the phenotype of these cells, such as mechanical forces, biochemical milieu, oxygen requirements, pH and the

gene expression patterns [51]. Likewise, the ECM influences the behaviour and phenotype of the resident cells [49, 51]. That is to say that cell attachment, migration, proliferation and three-dimensional spatial arrangement are strongly affected by matrix composition (collagen, fibronectin, laminin, glycosaminoglycans and growth factors). The ECM plays a central role in mammalian development and physiology [51]. In fact, the amino acid sequence and quaternary structure of many components of ECM such as collagen are highly conserved across species. This sequence homology could function as a constructive scaffold in mammalian recipients, rather than inciting a destructive inflammatory reaction.

Decellularization is a technique for obtaining natural scaffolds that could be used for recellularization [52, 53]. This methodology generally involves the perfusion of detergents, enzymes or other cell-lysing solutions through the organ vasculature to remove the cellular components while preserving the 3D architecture and biochemical composition of native ECM. It has been reported that decellularized cadaveric scaffolds can provide a niche for stem cells to differentiate into an appropriate cell type that contributes to whole organ generation [54]. Employing this strategy, Ott et al. developed a functional artificial rat heart using a heart scaffold that retained its three-dimensional geometry and vasculature, whereby cardiac cells were perfused for recellularization [53]. This experiment results in a contractile myocardium. This methodology of decellularization before recellularization has also been employed to develop transplantable liver and lungs using mature hepatocytes and alveolar epithelial cells, respectively [55, 56].

Following this strategy, several attempts were made to regenerate a kidney, as it is known that ECM plays a crucial role in kidney development and repair [2, 24]. After decellularization, kidney scaffolds have been shown to preserve the glomerular and tubular architecture and the vascular network [57]. This structuration retains renal-specific biochemical and biophysical cues that are able to modulate cell proliferation and differentiation, with a regional-specific effect on stem cell behaviour. In 2013 Song et al. reported successful whole kidney regeneration, which may produce urine after transplantation [15]. They used cadaveric kidneys from rats, pigs and humans to produce acellular renal scaffolds by decellularization solution perfusion. Then, these scaffolds were repopulated by perfusion of endothelial and epithelial cells, leading to the formation of viable renal tissues. However, it remains unclear how cells become properly differentiated and assembled into vascularized nephrons to produce urine. But although bioartificial organs generated by decellularization strategies are associated with massive thrombi, in regenerative medicine this approach may represent a hopeful solution to the shortage of donors in the field of organ transplantation.

Bioprinting in 3D

Three-dimensional (3D) bioprinting is based on depositing living cells together with supporting biomaterials into precise positions to build biological structures or organs in 3D [58, 59]. However, this technology is still in its infancy and in

order to obtain a whole organ by 3D bioprinting it is necessary to develop novel supporting biomaterials, which support the growth of living cells, and high spatial resolution devices that translate into three dimensional complex geometries the appropriate component to build complex biological structures composed of vascular and nervous systems [58, 59]. However, studies that have used this technology to generate structures like vessels, bone, cartilage, skin, nerves, muscle, adipose tissue and tumours have been published [60]. Nevertheless, the kidney is a spatially heterogeneous organ and for that reason, fully recapitulating *de novo* its intricate architecture and complex composition through scaffold engineering technologies like three-dimensional bioprinting would be a technically difficult task, if not impossible, with the current level of technology [61]. The main obstacle to the generation of a kidney is the current inability to mimic the kidney ECM and deposit the many and various renal cell types in the correct arrangement [57]. At this point, in accordance with the previous point is interesting to say that decellularized kidney ECM used as supporting material could provide a kidney-specific instructional cue to the printed cells, so that they would behave properly, generating an organ de novo [15, 57]. However, although this technique is promising, as anything could be built if the level of technology available were sufficient, there is still a long way to go.

Renal device

Current renal substitution therapy with haemodialysis or haemofiltration has been the only successful long-term ex vivo organ substitution therapy to date [62]. However, the limited removal of metabolic waste products in kidney patients on dialysis leads to high morbidity and mortality [63]. Between regenerative medicine and renal replacement therapy, the tissue engineering of a bioartificial kidney as a renal tubule assist device represents a novel possible solution to create a structure to replace a kidney function [2, 24]. This device consists of a bioengineered structure that contains a hybrid "living membrane" with functional proximal tubule epithelial cells supported by an artificial functionalized hollow fibre membrane, which demonstrated absorptive, metabolic, endocrine functions and active organic cation transport [24, 63]. Renal cells grow in monolayers until confluence and perform different reabsorption and secretory functions due to the presence of specific active transporters presents in the living proximal tubule renal cells. Although these transport functions are less efficient than those in native proximal tubules, it has been demonstrated that the combination of a synthetic haemofiltration device and a renal tubule cell therapy device containing porcine renal tubule cells in a perfusion circuit successfully replaces filtration, transport, metabolic and endocrine kidney functions in acutely uremic dogs [62]. This technology has already been the subject of several clinical trials [64, 65], so it could mean that in a not-too-distant future this technology could be a solution for patients with end-stage renal disease, given the shortage of kidneys for transplantation.

Xenoembryos

Taking advantage of an organogenic niche as a developing embryo, it has been shown that if stem cells are injected, they can be integrated into the embryo development programme and become part of the newly generated structures [24, 39, 48, 54]. Taking advantage of this mechanism, it has been reported that microinjection of human mesenchymal stem cells (hMSC) into the site where the metanephros (embryonic kidney) will develop allows these cells to integrated into the developed metanephros and morphologically differentiated to tubular epithelial cells, interstitial cells and glomerular epithelial cells [66]. Recently, it was demonstrated that a xenogeneic foetus can provide a niche in which hMSC can undergo mesenchymal-to-epithelial transition and differentiation of nephrons can proceed [48]. During this process the metanephroi were developed in an embryo that was grown in a whole embryo culture system after the injection of hMCS [54]. At this point, if metanephroi are recovered and transplanted into the omentum, the recipient organism develops a vascular system to connect this embryonic organ, allowing it to grow and form functional nephrons [67]. Thus, the new kidney formed contains a human nephron and the vasculature from the host. Furthermore, this neo-kidney was capable of producing urine by filtering the recipient's blood and secreted human erythropoietin in anaemic recipient animals [67]. Through this strategy, whole functional kidney could be generated from hMSC, although the kidney formed has a chimeric structure. Nevertheless, in the future it may be possible to transplant the humanized metanephroi into human omentum, allowing them to continue to develop and nourish the vascular system generated from human receptor. Finally, if we used transgenic animals for a suicide-inducible gen, we would be able to eliminate the xenogeneic tissue [48]. Future studies that use

large animals like pigs might provide a novel direction for regenerating donor kidneys with a suitable size and function for transplantation [24].

Transplantation of embryonic kidneys

For years, xenotransplants have been considered as a possible solution to the organ shortage, but rejection and zoonoses have limited the application of this kind of treatment [68, 69]. However, transplantation of kidney precursors into adult hosts showed that intact embryonic kidneys are able to attract the formation of a vascular system from the host to ensure a blood supply, undergoing maturation and exhibiting functional properties while avoiding rejection from non-immunosuppressed hosts [18, 70]. This finding, together with the production of specific pathogen-free animals [71], could provide a novel solution for kidney need [18, 72]. As leverage against PSC, metanephros cells are already committed to a genetic programme of renal development and "knowing" the destination cell type and how it should be assembled [3], similarly to if the primordia remained undisturbed within the embryo [73]. It has been reported that metanephroi of both human and pig origin transplanted into mice could differentiate into functional nephrons that produce a dilute urine [72]. Through this strategy, it was reported that survival of total nephrectomized rats can be increased by prior metanephros transplantation and ureteroureterostomy [74]. In addition, Yokote et al., reported that if metanephroi were transplanted beside bladders (developed from cloacae) and this was connected to the host ureters, hydronephrosis could be avoided and

metanephroi could fully grow, producing and excreting urine through the recipient ureter [75]. Furthermore, new kidneys developed from metanephroi provide not only an excretion function, but also an endocrine function, synthesizing renal hormones like renin and erythropoietin [76, 77]. In rats with adenine-induced renal failure, the renin activity of metanephroi contributes to raising arterial blood and suppresses the progression of vascular calcification by significantly reducing vascular calcium and phosphorus content [78]. Xenotransplanted embryonic kidney also provides a niche for endogenous mesenchymal stem cell differentiation into erythropoietin-producing tissue [79]. In this regard, using metanephroi from suicide-inducible metanephros donors would enable us to eliminate the xenotissue, leaving only autologous EPO-producing tissue. For this reason, long-term immunosuppression therapies would not be required and ethical concerns could be mitigated [79].

One important issue in this field is that the influence of the insertion site of the kidney is not indifferent. Matsumoto et al. reported that renin production was greater in metanephroi transplanted into the paraaortic area, where the developing kidney is exposed to hydrostatic pressure from the aorta, although there were no site-specific differences in erythropoietin production [77]. To date metanephroi have been transplanted into different sites such as the anterior eye chamber [80], intrarenally [80-84], intra-abdominally [85] or intraomentally [84, 86], but all these experiments were performed through open surgery. To our best knowledge, our recent studies [87-89] were the only experiment to tackle embryonic kidney transplantation through laparoscopic surgery. In this work, we developed a new minimally invasive laparoscopic procedure to transfer

metanephroi into the retroperitoneal fat [87-89], where only one endoscope trocar was inserted into the abdominal cavity and kidney precursor was aspirated into an epidural catheter that was introduced through the epidural needle and inserted into the hole performed in fat tissue. Around 50% of rabbit metanephroi that were allotransplanted through this method grew and differentiated, presenting normally developed glomeruli, proximal and distal tubules and collecting ducts [87]. This kind of laparoscopic surgery, rather than open laparotomy may help move the process to higher animals whose management is more difficult, but whose nephron structure and size closely approximate human nephrons [3]. Our group carried out a preliminary study in goat to provide a better test of the procedure feasibility for clinical application [90]. Following this protocol, we showed that six weeks post xenotransplantation of a 15 day-old rabbit metanephros, it grew.

However, even in a more favourable future situation, where the organ supply and demand could be balanced using xenotransplants from regenerative medicine, the ability to physically distribute the organs to patients in need and produce these organs in a way that allows adequate inventory control and quality assurance might compromise the technique [88-90]. To this end, organ cryopreservation will be indispensable and to date, only Bottomley et al. [91] have evaluated the cryopreservation of whole metanephroi immediately after thawing, but only under in vitro conditions. Other cryopreservation studies were performed on human embryonic stem cells, but in these cases the experiments were performed on individual cells instead of the entire transplantable metanephros [92, 93]. We recently tested in vivo the effect of long-term

cryopreservation of metanephroi. Briefly, we vitrified metanephroi following the minimum essential volume method using Cryotop® as device and VM3 as vitrification solution. This in vitro process showed a survival rate of over 80% of the metanephros cells. So, when it was transplanted in vivo, similar grown rates were observed between fresh and vitrified 15-days-old-metanephroi [88], whose capacity for angiogenesis was preserved. Also in nascent kidneys from vitrified metanephroi, mature glomeruli were developed, whose histomorphometry analysis showed that vitrification has no significant effect on glomerular perimeter, when compared to the corresponding values in the control kidneys. Furthermore, the expression of renin and erythropoietin were also similar in vitrified new kidneys and control kidneys.

If metanephros-developed kidneys could grow large enough to address a urinary tract connection surgery, transplantation of metanephroi could lead to a definitive solution to the shortage of kidneys, being an inexhaustible source of these organs. Therefore, the addition of growth factors or substances that might favour the angiogenic action of metanephroi, to connect the host vascular system, should be checked in an attempt to obtain good sized functional kidneys.

Conclusions

Progress was made toward the ultimate goal of developing functional kidney grafts in vitro or in vivo on demand. However, kidney regeneration is

considerably more complex than regeneration of other organs due to its complex functional architecture and the lack of understanding of the molecular mechanisms underlying stem cell differentiation to renal cells. This review has summarized the recent research in bioengineering and regenerative medicine to reconstruct a functional transplantable organ that accomplishes the native kidney functions. Although many approaches are being implemented in the different ways outlined in this work, obtaining an organ capable of performing complex functions such as osmoregulation, excretion or hormone synthesis is still a long-term goal. In addition, if the artificial kidney is achieved, the organ must be able to survive and function in the long term. Our group, in addition to having developed a laparoscopic method for transplanting metanephroi into large organisms, which can approximate the technique for clinical trials, has made a substantial contribution to the development of a biobank of kidney precursors as an unlimited source of kidneys, facilitating sanitary and inventory control and the distribution of organs. More efforts in the field of bioengineering, regenerative medicine and biotechnology are necessary to elucidate the mechanisms able to develop a functional renal structure capable of fulfilling the functions of a native kidney. The idea is that the long waiting list for a kidney transplant in patients with end stage renal disease will be eliminated, providing a definitive solution to these patients. We believe that the efforts in these areas will bring results in the future and will enable this idea.

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Disclosures

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