

Extracellular Matrix Scaffolds As a Platform for Kidney Regeneration

Andrea Peloso¹, Riccardo Tamburrini², Lauren Edgar², Bettina Wilm^{3,4}, Ravi Katari², Laura Perin⁵, Patricia Murray^{3,4}, Giuseppe Orlando²

¹ General Surgery, Fondazione IRCCS Policlinico San Matteo and University of Pavia, Pavia, Italy

² Wake Forest School of Medicine, Winston Salem, USA

³ Department of Cellular and Molecular Physiology, Institute of Translational Medicine, University of Liverpool, Liverpool L69 3GE, UK

⁴ Centre for Preclinical Imaging, University of Liverpool, Liverpool L69 3GE, UK

⁵ GOFARR Laboratory for Organ Regenerative Research and Cell Therapeutics, Saban Research Institute, Children's Hospital Los Angeles; Department of Urology, University of Southern California, Los Angeles, USA

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Corresponding author:

Giuseppe Orlando, MD, PhD, Marie Curie Fellow
Department of Surgery, Section of Transplantation
Wake Forest School of Medicine
Winston Salem, USA
Tel: +1 336 7166903
Fax: + 1 336 7135505
Email: gorlando@wakehealth.edu

Abstract

Chronic and end stage renal disease (ESRD) have reached pandemic levels and pose a substantial public health burden. Unfortunately, available therapies lack efficacy in preventing progression to its end stage phase. Regenerative medicine promises to restore function of diseased organs among which the kidney, through two possible approaches: firstly, the maximization of the innate ability of tissues to repair or regenerate following injury; secondly, the *ex vivo* bio-fabrication of the organ in question. When regenerative medicine is applied to the setting of chronic or ESRD, it is intuitive that the development and employment of strategies to enhance renal repair, promote the generation of new nephrons in the damaged kidney, or manufacture transplantable kidneys, could have a major impact on the management of this pandemic. Among the different regenerative medicine technologies currently under development, the so called cell-on-scaffold seeding technology (CSST) – whereby cells are seeded in- or onto supporting scaffolding biomaterials – seems to offer the quickest route to clinical application. In this review, we illustrate the state of the art of investigations in the field of CSST aiming at restoring kidney function.

1. Introduction.

As of March 6th 2016, 121,576 patients are registered on the organ transplant waiting list in the US (<https://optn.transplant.hrsa.gov/>, accessed on April the 15th 2016). This number increases by 5% every year, with one more patient being added to the list every ten minutes. In 2015, 30,973 transplants were performed and they were made possible by the generous consent granted from families of 15,064 donors. On average, 21 people die every day, while waiting for a transplant. Moreover, because transplantation can often be the solution to many of the diseases associated with aging, the hidden demand is estimated to be far beyond current levels. It therefore becomes evident that the lack of donor organs is considered a major health crisis that dramatically impacts the finances of any given country, if we consider that the market for organ failure treatments is estimated at about \$80 billion per year. Importantly, this critical situation has been a major driving force behind the rise of regenerative medicine (RM) in the past decades. This term refers to a field within the health sciences that aims at repairing, regenerating or replacing functionally impaired human cells, tissues, or organs to ultimately restore or establish normal function (Katari et al., 2015). The process of regenerating body parts can occur *in vivo* or *ex vivo*, and may require cells, natural or artificial scaffolding materials, growth factors and gene editing, or combinations of these elements (Orlando et al., 2011). Given the immense potential that RM has shown to meet the most urgent needs of organ transplantation, RM is becoming a field of major interest and research investment for the transplant community (Orlando et al, 2013; Orlando and Walker, 2014; Rogers et al., 2015), as well as for related specialties of health sciences like nephrology (Morales et al., 2014).

Chronic and end stage renal disease (ESRD) have reached pandemic levels and pose a substantial public health burden (Liyanaige et al., 2015). Unfortunately, etiology and pathophysiology are unclear and treatment is often inadequate. Ideally, patients with chronic kidney disease should receive treatment strategies aimed at counteracting disease causes and mechanisms and enhancing intrinsic processes of repair and regeneration. For patients with ESRD, the best treatment option is kidney transplantation,

which nevertheless, is dramatically limited by an inadequate supply of transplantable grafts and by the heavy toxicity related to lifelong immunosuppression.

Tissue engineering is a subfield of RM that aims at restoring function of diseased organs mainly through two possible approaches: firstly, the maximization of the innate ability of tissues to repair or regenerate following injury; secondly, the *ex vivo* bio-fabrication of a diseased organ. When tissue engineering is applied to the setting of chronic or ESRD, it is intuitive that the development and employment of strategies to enhance renal repair, promote the generation of new nephrons in the damaged kidney, or manufacture transplantable kidneys, could have a major impact on the management of this pandemic.

2. Tissue engineering approaches to kidney repair, regeneration and replacement

The technologies that are currently being developed in order to meet the ultimate objectives of kidney tissue engineering – namely, repair, regeneration and replacement of terminally diseased kidneys with new functioning ones – may be scholastically subtyped in five categories: cell-on-scaffold seeding technology (Salvatori et al., 2014), developmental biology, stem cell, 3D printing and kidney-on-a-chip technology (Table 1).

2.1. Cell on scaffold seeding technology (CSST)

This technology is centered upon the idea to regenerate the cellular compartment of a given tissue or organ, via the seeding of cells on- or into supporting scaffolding material. The rationale for this lies on the evidence that ECM's molecular, physical and architectural characteristics are critical in the determination, differentiation, proliferation, survival, polarity, welfare and migration of the cells of any given tissue. In other words, cells do well only when they reside in their natural niche represented by the innate ECM that Mother Nature has engineered for them.

Scaffolds may be either synthetic or natural. Natural scaffolds are obtained from animal (including human) organs through a process called decellularization, whereby the cellular compartment of the organ in question is destroyed and cell remnants are cleared from the remaining extracellular matrix (ECM) scaffold. The rationale for using natural scaffolds lies on the evidence that the ECM is the *sine qua non* for the life of multicellular animal organisms, as it defines the physical and chemical interactions that control cellular physiology and fate, and provides mechanical and structural support to cells and tissues (Hynes, 2009). As the synthetic scaffolds lack these critical characteristics, ECM scaffolds represent the ideal platform for CSST (Badylak et al., 2011; Badylak et al., 2012).

2.2. Developmental biology

The overarching goal of developmental biology technologies is the fabrication of tissues and organs through the recapitulating *ex utero* of the different steps of organ ontogenesis that occur *in utero*. To date, our understanding of organogenesis is based on mammalian animal models, on the assumption that the

conservation of genes across species allows for deducing knowledge of human development from animal models (Little MH 2016). However, while the ability of animal models to be predictive for humans remains to be demonstrated (Shanks et al., 2009), organ development is an extremely complex process whereby primordial cells differentiate, migrate, proliferate and grow, in an extremely fascinating and spatial-temporally controlled manner. Moreover, organogenesis is orchestrated by physical, molecular and cellular cues that unfortunately remain largely unknown, and takes place in an environment – the uterus – whose architecture and physiology cannot be reproduced by any of the currently available bioreactors. Nevertheless, recent investigations have shed light on some developmental mechanism regulating the preferential induction of collecting duct versus kidney mesenchyme progenitors, and have ultimately led to the generation of functioning renal organoids with potential future applications like nephrotoxicity screening, disease modelling and as a source of cells for therapy (Takasato et al. 2015).

2.3. *Stem cell*

The conceptual foundation of this technology lies on the ability of stem cells to give rise to cells of the same type, as well as to cells with a different phenotype. Stem cells can be identified at all stages of life of any given biological entity, including humans, however their *stemness* potential weakens from the earlier stages of life throughout senescence. Embryos contain stem cells with the highest *stemness* potential and have the formidable ability to generate all cells of the mature organism. On the other hand, adult stem cells are specific to a given tissue and their *stemness* is limited to the ability to generate only the cells of the tissue in question. Some stem cells can also differentiate into cells outside their normal repertoire of differentiation for the location where they are found, so giving rise to tissues and organs other than the one in which they reside; for example, bone marrow stem cells can differentiate into bone, cartilage and adipose tissue. In the past decade, a groundbreaking technology has identified a new type of stem cell with a huge potential for clinical application – namely, induced pluripotent stem cells – that can be generated from adult, already committed cells through gene reprogramming (Takahashi and Yamanaka, 2015). Finally, stem cells are also characterized by remarkable anti-inflammatory and immune-modulatory properties for which their use is being proposed in a myriad of clinical settings whereby the mechanisms of disease are inflammatory or immune-mediated.

The working hypothesis that justifies the stem cell approach to kidney repair and regeneration is that, in the case of acute or chronic reversible damage of a given tissue, the damage may be repaired through the activation of the dormant stem cells residing within a specific niche of the tissue in question. In the case of kidney bioengineering, stem cells may represent a valuable source of cells for the reconstitution of the cellular compartment of any tissue.

2.4 *3D printing*

3D bioprinting is an emerging technology that facilitates the layer-by-layer precise positioning of biological materials, biochemicals and living cells, with the ultimate goal of fabricating viable and functioning tissues and organs destined to replace terminally diseased counterparts (Murphy and Atala, 2014; Peloso et al., 2015). 3D bioprinting has already been used for the precise construction of

microfluidic devices in the setting of organ-on-a-chip (see below), as well as for the generation and transplantation of multiple tissue types, including multilayered skin, bone, vascular grafts, tracheal splints, heart tissue and cartilaginous structures. Although 3D printing of an anatomically and physiologically complex organ such as a kidney is currently beyond our capabilities at the required scale, one approach that seems to be promising is the generation of ‘mini-tissue’ building blocks that contain all of the functional components of the kidney and can theoretically be combined in a series of repeating functional units connected via a vascular and tubular network. Mini tissues can be fabricated and assembled into the larger construct by rational design, self-assembly or a combination of both. For instance, Organovo has recently disclosed the 3D printing of kidney proximal tubular tissue using multiple cell types among which fibroblasts, endothelial cells and renal proximal tubular cells. This tissue was fabricated without any ECM and was able to survive *in vitro* for 2 weeks (<http://3dprintingindustry.com/2015/04/01/organovo-announces-its-first-3d-bioprinted-kidney-tissue/> accessed on April 30th, 2016).

2.5 Kidney on-a-chip

Chips are 3D microfabricated patterns and structures that closely mimic organ-specific microarchitecture and function *in vitro* (Peloso et al., 2015). Briefly, chips consist in the patterning of fine channels in microfluidic devices that can be attached to fluid pumps and analytical probes and are seeded with tissue/organ functional cell types. Chip technology has been recently developed to replicate the complex organ-specific cellular and structural organization of a given tissue or organ, in order to fully recapitulate its physiology and function, and represents a major advancement from 2D culture methodology. A kidney-on-a-chip can theoretically mimic the structural, mechanical, transport, absorptive, and physiological properties of the human kidney (Wilmer et al., 2016), yet the goal of fully recapitulating the human kidney structure and function is still a long way away. A system that is capable of fully mimicking all key aspects of kidney function will require both tubular and glomerular components together with a functional vascular network, combined with proper compartmentalized fluid flow. This technology is discussed in more detail by Nieskens and Wilmer in this issue of European Journal of Pharmacology.

3. CSST applied to kidney bioengineering

To date, CSST has been the main technology implemented in the manufacturing of body parts that could be eventually implanted in more than 200 patients, as well as in the experimental attempt to generate complex modular organs like the kidney (Orlando et al., 2013; Montserrat et al., 2016). CSST using ECM based scaffolds seems therefore to offer the quickest route to clinical application probably because the 3D framework of the innate ECM scaffolds holds all cues necessary for cells to be viable and functional. As a corollary, the native ECM of a given organ should represent the ideal template for all regenerative medicine and tissue engineering technologies that aim to replicate the cellular niche as *sine qua non* for tissue and organ bio-fabrication.

Renal ECM is an interlocking meshwork of glycosaminoglycans, nidogen/entactin, and fibrous proteins like collagens, fibronectins, and laminins. Soluble signals, including cytokines, growth factors, and chemokines, are embedded within the ECM and together with fibrous proteins orchestrate cellular behaviors, including cell survival, proliferation, migration, and differentiation. The renal ECM is a very dynamic structure and different compartments show site-specific unique compositions of extracellular macromolecules designed to support the underlying functional needs of distinct nephron segments (Petrosyan et al., 2016). Over a decade of research in kidney tissue engineering has shown that ECM scaffolds can be successfully and consistently produced from virtually all species including humans, are completely acellular and virtually non-immunogenic, maintain their architecture and essential molecular composition, lack cell membrane molecules, are able to determine cell phenotype and induce genes of renal development, possess remarkable angiogenic properties as demonstrated by the ability to induce vessel formation in the chorioallantoic membrane, are biocompatible *in vitro* and *in vivo*, and, when repopulated with renal cells, are able to show some function (Peloso et al, 2015; Petrosyan et al., 2015; Petrosyan et al., 2016). Moreover, when acellular porcine renal ECM scaffolds are implanted in pigs, the framework of the innate vasculature remains well preserved and is able to sustain physiologic blood pressure (Orlando et al., Ann Surg 2012).

3.1 Rodent models

In 2009, Ross et al. (Ross et al., 2009) were the first in reporting that the infusion of a detergent based solution throughout the vascular pedicle allows the complete clearance of the cellular compartment of rat kidneys, while the innate ECM structure and architecture remain quite preserved. The decellularization protocol was based on 5-days of continuous perfusion with ionic and non-ionic detergents, sodium chloride, deionized water and DNase through the renal artery at a pressure of about 100 mmHg. The measures of outcome evaluated by authors were the complete decellularization of the native kidney in combination with the preservation of the 3D framework of the ECM, as well as of its basic components like collagen IV and laminin. This was assessed with some straightforward histological tests which confirmed the absence of any cell or cell debris, while serial staining showed the presence of a solid network of collagen and laminin fibers. No information about the quantitation of DNA, collagen, laminin, elastin or glycosaminoglycan (GAG) after decellularization was provided, and it was not determined whether any detergent remained within the acellular scaffolds. Moreover, authors seeded scaffolds with embryonic stem cells, by direct anterograde injection through the artery and direct retrograde injection through the ureter. Interestingly, primitive precursor cells attached, populated and proliferated ubiquitously within the glomerular, vascular, and tubular structures. Very importantly, these authors made the fundamental observation that these cells had changed their phenotype by the end of the first week. In fact, the evidence that the ECM of a given organ is able to induce the expression of genes critical for the development of the organ in question, is of paramount relevance in regenerative medicine and is consistent with Bissell's early intuition of the "dynamic reciprocity" between the ECM on the one hand and the cytoskeleton and the nuclear matrix on the other hand, that represents one of the milestones in the history of ECM biology (Bissell et al. 1982). On the wake of these successful preliminary investigations, in 2012 the same group provided evidence that pluripotent precursor cells seeded into rat renal ECM acellular scaffold vasculature differentiate into endothelial cells, which in turn follow a normal ontogeny as they remodel the laminin and collagen of their basement membranes (Ross et al.

2012). These authors first tested vascular cell endothelialization by endothelial specific BsLB4 lectin and anti-VEGFR2 (Flk1) antibodies; then, the remodeling of the matrix basement membranes from rat to mouse (referred to as “murinization”) was assessed by a monoclonal antibody specific for mouse laminin β 1 chain. Later, in an elegant study where biocompatible acellular ECM scaffolds could be obtained through continuous infusion of SDS (sodium dodecylsulfate) into rat kidneys for only 17 hours, Remuzzi’s group observed the loss of cell pluripotency and the start of differentiation towards the meso-endothelial lineage (Bonandrini et al. 2014).

This work paved the way for different protocols to optimize the decellularization process, as well as to reconstitute the parenchymal and vascular compartment of the kidney in the rat model. A milestone paper appeared in *Nature Medicine* in 2013 by Ott and co-workers, who reported the successful seeding of acellular rat scaffolds with rat neonatal kidney cells to reconstitute the parenchymal cell compartment, and with human umbilical venous endothelial cells to reconstitute the endothelium and allow implantation (Song et al., 2013). These authors combined antegrade seeding of endothelial cells with retrograde seeding of the kidney cells under the application of a transrenal pressure gradient in an *ad hoc* designed bioreactor. Cells were able to spread throughout the 3D framework of the scaffold, repopulate half of the glomeruli and could be found in different segments of the nephrons across the scaffolds while expressing tissue-specific markers. Furthermore, the partially recellularized kidney scaffolds were assessed for function both *ex vivo* and *in vivo*. *Ex vivo*, authors demonstrated some degree of filtration capacity when compared to acellular ECM scaffolds and cadaveric kidneys; moreover, parameters including vascular resistance, albumin retention and glucose reabsorption were lower in the regenerated kidneys when compared with intact kidneys procured from dead rats. *In vivo*, the group grafted the regenerated kidneys orthotopically into recipient rats and showed that urine-like solution could be produced although at lower levels than in native kidneys; furthermore, creatinine, urea and albuminuria were lower than in native kidneys but higher than in decellularised kidneys in control animals. This study, just like Ross’s first paper, represents a milestone in the literature, which is of interest for the following three reasons: first, because for the first time it reported on the development of a dedicated technology that may be applied to regenerate – or bioengineer – both cellular compartments (endothelium and parenchymal) of the kidney; second, because for the first time, authors very bravely attempted to address the question of how to restore the function of renal organoids both *in vitro* and *in vivo*; third, because it emphasized the role of bioreactors as well as of seeding strategies aimed at reconstituting the cellular compartment (Peloso et al., 2015).

Function was studied by the group at Shandong University, China (Guan et al., 2015), which also assessed the amount of critical growth factors as a measure of outcome of their decellularization method. Wertheim’s group evaluated growth factors as well, while attempting the optimization and critical evaluation of decellularization strategies to develop renal extracellular matrix scaffolds as biological templates for organ engineering and transplantation (Caralt et al, 2015). Moreover, for the first time his group reported on the use of human iPSC-derived endothelial cells and tubular epithelial cells to repopulate their biomatrix obtained with Triton/SDS-based solution.

A pressure gradient to recellularize rat scaffolds was also attempted by Hachisuka et al. (Hachisuka et al., 2015). These authors placed the scaffolds in a custom-built device that could remove air to create a vacuum. Recellularization was done through direct injection of cells in the artery and the ureter. By applying different pressure gradients, an increase of engrafted cells was observed in both the vessels and

the parenchyma, although seeding of the parenchyma seemed to be less efficient. Seeded endothelial cells survived and proliferated during 72-hour perfusion culture, and vascular resistance increased along with increased cellularity of the kidney scaffolds.

3.2 Porcine models

Pigs are known as the most common animal source of clinical xenografts (Baptista et al., 2009) and therefore represent the ideal model for clinically relevant investigations in the field of kidney – and, more broadly, organ – bioengineering and regeneration. In the attempt to identify a new potentially inexhaustible source of organs for transplant purposes, the pig may represent that invaluable source both as a source for xenografts, as well as a source for ECM scaffolds (Salvatori et al, 2015). In 2012, Orlando et al. reported for the first time the use of porcine kidneys as a source of renal ECM scaffolds for kidney bioengineering investigations (Orlando et al., 2012). He showed that ECM scaffolds can be successfully and consistently produced from 25 kg female Yorkshire pig kidneys. Such scaffolds are acellular, maintain their 3D architecture and molecular composition, show an intact and patent innate vascular tree, and are biocompatible both *in vitro* and *in vivo*. In fact, when cells are seeded on fragments of ECM, they attach and remain viable for 1 week. When acellular scaffolds are implanted in pigs and kept *in situ* for 2 weeks, animals tolerate the scaffold well. Importantly, for the first time Orlando et al. provided evidence that acellular scaffolds are able to sustain physiological blood pressure despite the absence of muscle cells and endothelium in the vasculature. Moreover, it was also noticed that the framework of the innate vasculature is resilient and able to respond to modifications of the applied pressure, when the scaffold is perfused with solutions. This information is of critical importance in view of translation, and suggests that ECM-based scaffolds may be the scaffold of choice in organ bioengineering. In fact, synthetic scaffolds lack the innate vasculature, and no technology is currently capable of reproducing the vascular network of any given tissue or organ. Of note, when porcine acellular scaffolds were procured and analyzed with H&E staining, as expected, the whole vasculature was filled with thrombi due to the lack of endothelium (Orlando et al., 2012). Later on, the same group from Wake Forest published a study showing that 0.5% SDS was the most effective solution for decellularizing porcine kidneys (Sullivan et al. 2012).

In an attempt to develop strategies to recellularize acellular ECM scaffolds, it is important to understand how the ECM interacts with cells. To address this fundamental question, McNeill et al. derived a method to develop region-specific ECM biomaterials (namely, ECM sheets, ECM hydrogels, and solubilized ECM) for stem cell culture from the three regions of the porcine kidney – cortex, medulla, papilla (O'Neill et al., 2013). The objective of the study was to determine if there were region-specific effects of kidney ECM on the growth and metabolism of kidney-derived stem cells (KSC), how these effects depend on the preservation of ECM structure versus composition alone, and if these effects extend to exogenous (non-kidney) stem cells, such as mesenchymal stem cells (MSCs). Interestingly, when KSC were seeded on renal ECM and control ECM from other organs, it was found that ECM biomaterials derived from the kidney affect the growth and metabolism of KSC with regional specificity. In particular, there is a significant degree of recognition and specificity between adult kidney stem cells and their extracellular environment. Stem cells showed significantly higher proliferation and higher metabolic activity in kidney ECM when compared to KSCs in ECM from other organs. In addition, KSCs showed

lower proliferation and higher metabolic activity when cultured in papilla ECM (kidney stem cell niche) compared to medulla and cortex ECM.

Guan et al. (Guan et al., 2015) reported a new method for repopulating ECM renal scaffolds, consisting in multiple injections with a 28G needle throughout the porcine kidney. More recently, the group at Wake Forest (Abolbashari M et al., 2016) demonstrated the feasibility of seeding porcine primary renal cells into whole-renal porcine ECM scaffolds by multiple injections with 23G needles, after pre-conditioning of the scaffold prior to seeding by 1-day flushing with a specific culture medium. Briefly, renal cells were suspended in media at a concentration of 10×10^6 cells/ml and then delivered into the cortical region of the renal scaffolds using a 23G needle. The injections started in the periphery of the kidney in the upper pole. The distance between the different injection sites was 2.5 mm with a depth of 5 mm. 0.5 ml (5 million cells) of the cell suspension was injected at each site of both the anterior and posterior surfaces of the upper pole of the kidney. In total, 400×10^6 cells were injected into each kidney, and, at completion of the seeding process, the -seeded scaffolds were incubated statically for 30 min before being subjected to perfusion culture for up to 28 days. During the study, the organoid seemed to be viable and exert function. Reportedly, this method grants repopulation of approximately 40-50% of the upper pole of the kidney. The same group also attempted the regeneration of the endothelium using antibodies to bound endothelial cells that were conjugated to the ECM in order to improve retention of endothelial cells (Kap KO et al., 2014).

As the detergents currently used to obtain ECM scaffolds could be damaging to the microstructure of the renal tissue and may undesirably solubilize the endogenous growth and signaling factors, some authors have tried to optimize the method in order to minimize the damage to the ECM. The group in Provo, USA (Pooornejad et al, 2016), recently illustrated an automated decellularization method for porcine kidneys combining physical and chemical steps. Briefly, freezing/thawing, incremental increases in flow rate under constant pressure, applying osmotic shock to the cellular membranes, and low concentrations of SDS were used to decrease SDS exposure time during the decellularization process from 36 to 5 h, which preserved the microstructure while still removing 99% of the DNA. Importantly, GAGs were almost totally preserved, leading to an enhancement of cell-ECM interactions. Human renal cortical tubular epithelium cells grew more rapidly when cultured on the ECM obtained from the improved decellularization process and also demonstrated more *in vivo*-like gene expression patterns. Of note, a better preservation of GAGs was also reported by Wang et al. (Wang et al., 2015), whose studies also addressed the topic of residual detergent within the scaffolds as well as of whether porcine endogenous retroviruses can be detected within the scaffolds.

3.3 Non-human primate models

Rhesus monkey kidneys have been proposed by Tarantal's group as an ideal platform for investigations aiming at assessing the role of ECM scaffold in the field of kidney regeneration, repair and bioengineering. In their seminal studies, Tarantal et al. have demonstrated that decellularized sections of rhesus monkey kidneys of all age groups provide a natural ECM with sufficient structural properties with spatial and organizational influences on human embryonic stem cell migration and differentiation

(Nakayama et al., 2010; Nakayama et al., 2013; Batchelder et al., 2015), and that the age of the donor is a critical factor in repopulation efficiency (Nakayama et al., 2011). Interestingly, ECM allows formation of nephron structures, stores several residual growth factors that may be beneficial in scaffold recellularization, and retains antimicrobial proteins that may be beneficial for *in vitro* culture as well as downstream host integration (Nakayama et al., 2011).

3.4 ECM scaffolds derived from the human kidney

It is envisioned that, when research will deliver the technology to allow fabrication of renal organoids with CSST, a patient affected by ESRD will have one of his kidneys removed robotically. This kidney will be thereafter processed in order to obtain healthy cells, as well as an acellular scaffold that will serve as template for the bioengineering of his or her new kidney. This vision has justified the use of discarded kidneys as a platform for kidney bioengineering (Katari et al., 2014; Gifford et al., 2015). Only in the US, up to 2,600 kidneys that were initially procured for transplant purposes are eventually discarded due to poor biopsy, surgical damage, long preservation, etc. (Orlando et al. 2013). Orlando and colleagues were the first to conceptualize the use of human discarded kidney as a source of ECM scaffolds for CSST research, and to show feasibility of application of CSST to the human kidney (Orlando et al., 2013; Peloso et al., 2015; Petrosyan et al., 2015). Investigations showed that SDS-based decellularization yields acellular ECM scaffolds, as in the case of the rodent, porcine and non-human primate models. However, the most relevant difference between human and non-human ECM scaffolds is that human scaffolds consist of diseased or damaged matrix, which raises concerns about its usefulness. However, as evidence of fibrosis reversibility has been provided in a myriad of clinical settings, the use of discarded kidneys has become increasingly popular in tissue engineering, as demonstrated by the plethora of primary papers on the topic released in the past 5 years. In the case of the human kidney, the work of Orlando's group has been fundamental in showing that the ECM intrinsic to human organs seems to be an extremely useful biomaterial for whole organ bioengineering for several reasons, among which is the evidence that they maintain the framework of the innate vasculature which is critical for implantation *in vivo*. Preliminary investigations have shown that ECM scaffolds can be successfully and consistently produced from the human kidney, and display the following key features: they maintain their architecture; their gross molecular composition and vasculature is preserved; they lack immunogenic cell membrane proteins; and they retain numerous bioactive growth factors that are stored in the 3D structure of the ECM, as demonstrated by the fact that they induce angiogenesis in a chorioallantoic membrane assay (Orlando et al., 2013; Peloso et al., 2015). Importantly, when multipotent stem cells are seeded on such scaffolds, cells attach well, are able to migrate throughout the 3D framework of the scaffold, remodel the ECM, and show the ability to mount an inflammatory response and promote angiogenesis (Petrosyan et al., 2015).

4. Challenges to the development of transplantable kidney organoids using acellular ECM scaffolds

Despite recent progress, CSST has to overcome numerous challenges before it can be translated to the clinic.

First, we need to better understand how the human kidney develops, as well as the mechanisms with which the kidney repairs itself after damage. Worldwide, kidney development has polarized the attention

of countless research teams as well as of funding institutions and agencies. However, due to the inherent difficulties associated with investigating the development of human organs, our knowledge of kidney development is based mainly on small animal models. On the other hand, investigations aiming at revealing the mechanisms through which the kidney repairs itself are often conducted on patients who are experiencing different degrees of renal impairment, so bypassing the biases that may derive from non-human models.

Second, when the *ex vivo* engineering (synonymous with fabrication) of a transplantable kidney organoid is considered, current investigations are being conducted without any criteria *vis a' vis* of the nutrients, oxygen and energy supply required by the new organ to develop. There is basically no information about how much oxygen, nutrients and energy a developing kidney needs to complete its maturation and be considered viable. Indeed, a critical step will be to achieve the maturation of the seeded scaffolds in custom made bioreactors, yet long term maturation that recapitulates kidney ontogenesis (up to 3 months) has never been reported and is burdened by a high risk of super infection of the bio-system.

Third, reports on the characterization of the different renal ECM scaffolds are scattered, measures of outcome are inconsistent, and a unifying vision is missing. Therefore, it will be of paramount importance for investigators in the field to unite forces, share experiences and combine assets, specializations and visions.

Fourth, strategies to recellularize ECM scaffolds are still at their early stages, and results are far too preliminary. Moreover, scalability to more clinically relevant models seems hard at the moment.

Fifth, it remains unclear which cells should be used – progenitor cells with different degrees of stemness, adult cells, iPS, etc. – to repopulate acellular scaffolds in order to regenerate the parenchymal and vascular compartments. While progenitor cells have a huge potential given their ability to generate different cell lineages, the risk of tumourigenesis cannot be ruled out and represents a significant burden even if investigations are still far from any clinical applicability. Even in this case, coordination and integration of *ad hoc* research is desirable and would certainly foster progress. As a matter of fact, the NIH has established the “Re-Building Consortium” (<https://www.rebuildingakidney.org/>) to support the development and implementation of strategies such as *de novo* repair of nephrons, the re-generation of nephrons, and the *in vitro* engineering of a biological kidney to enhance renal repair and promote the generation of new nephrons after damage.

Sixth, current technology lacks appropriate and efficient bioreactors and mathematical models.

5. Conclusions

CSST promises to meet the most urgent needs of organ transplantation, namely the identification of a potentially inexhaustible source of organs and immunosuppression-free transplantation (Orlando et al., 2013). In fact, the use of autologous cellular material has the potential to obviate the need for lifelong antirejection therapies, while *de novo* organ fabrication *a' la demande* using scaffolds of human or animal origin could, theoretically, provide a limitless supply of transplantable organs for waiting list patients, thus circumventing the challenge of organ shortage. While current technology allows production of natural ECM scaffolds from virtually all animal species, a thorough characterization of the renal ECM is still missing and the reconstitution of the cellular compartment post decellularization remains too far from the realm of the possible. So far, it seems that cells and ECM do not really integrate in a fully functioning

system, as occurs in nature. Research should aim at understanding the mechanisms of interaction between cells and ECM, as well as of the role of growth factors of which the matrix is a reservoir. Acquisition of in-depth knowledge of the developmental mechanisms of the single organs will be crucial for designing appropriate strategies which reproduce, in suitable bioreactors, the key steps of organ ontogenesis.

Conflicts of interest

The authors report no conflict of interest.

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Legend of table 1. The table summarized the state of the art of the five RM technologies as they are being applied to kidney disease.

References

- Abolbashari, M., Agcaoili, S.M., Lee, M.K., Ko, I.K., Aboushwareb, T., Jackson, J.D., Yoo, J.J., Atala, A. 2016. Repopulation of porcine kidney scaffold using porcine primary renal cells. *Acta Biomater.* 29, 52-61.
- Badylak, S.F., Taylor, D., Uygun, K. 2011. Whole-organ tissue engineering: decellularization and recellularization of three-dimensional matrix scaffolds. *Annu Rev Biomed Eng.* 13, 27-53.
- Badylak, S.F., Weiss, D.J., Caplan, A., Macchiarini, P. 2012. Engineered whole organs and complex tissues. *Lancet.* 379, 943-952.
- Batchelder, C.A., Martinez, M.L., Tarantal, A.F. 2015. Natural Scaffolds for Renal Differentiation of Human Embryonic Stem Cells for Kidney Tissue Engineering. *PLoS One.* 10, e0143849.
- Bissell, M.J., Hall, H.G., Parry, G. 1982. How does the extracellular matrix direct gene expression? *J Theor Biol.* 99, 31-68.
- Caralt, M., Uzarski, J.S., Jacob, S., Obergfell, K.P., Berg, N., Bijonowski, B.M., Kiefer, K.M., Ward, H.H., Wandinger-Ness, A., Miller, W.M., Zhang, Z.J., Abecassis, M.M., Wertheim, J.A. 2015. Optimization and critical evaluation of decellularization strategies to develop renal extracellular matrix scaffolds as biological templates for organ engineering and transplantation. *Am J Transplant.* 15, 64-75.
- Gifford, S., Zambon, J.P., Orlando, G. 2015. Recycling Organs - Growing Tailor-Made Replacement Kidneys. *Regen Med.* 10, 913-915.
- Guan, Y., Liu, S., Liu, Y., Sun, C., Cheng, G., Luan, Y., Li, K., Wang, J., Xie, X., Zhao, S. 2015. Porcine kidneys as a source of ECM scaffold for kidney regeneration. *Mater Sci Eng.* 56, 451-456.
- Guan, Y., Liu, S., Sun, C., Cheng, G., Kong, F., Luan, Y., Xie, X., Zhao, S., Zhang, D., Wang, J., Li, K., Liu, Y. 2015. The effective bioengineering method of implantation decellularized renal extracellular matrix scaffolds. *Oncotarget.* 6, 36126-36138.
- Hachisuka, S., Sato, Y., Yoshiike, M., Nakazawa, R., Sasaki, H., Chikaraishi, T. 2015. Enhanced recellularization of renal extracellular matrix scaffold under negative pressure. *Integr Mol Med.* 2, 394-399
- Hynes, R.O. 2009. The extracellular matrix: not just pretty fibrils. *Science.* 326, 1216-1219.
- Katari, R., Peloso, A., Orlando, G. 2015. Tissue engineering and regenerative medicine: semantic considerations for an evolving paradigm. *Front Bioeng Biotechnol.* 2, 57.
- Katari, R., Peloso, A., Zambon, J.P., Atala, A., Orlando, G. 2014. Renal bioengineering with ECM scaffolds generated from discarded human kidneys. *Nephron.* 126, 119.
- Ko, I., Abolbashari, M., Huling, J., Kim, C., Mirmalek-Sani, S.H., Moradi, M., Orlando, G., Jackson, J.D., Aboushwareb, T., Soker, S., Yoo, J.J., Atala, A. 2014. Enhanced re-endothelialization of acellular

kidney scaffolds for whole organ engineering via antibody conjugation of vasculatures. *Technology*. Volume 2: Issue 3.

Little, M.H. 2016. Closing the circle: from organoids back to development. *Development*. 143, 905-906.

Liyanage, T., Ninomiya, T., Jha, V., Neal, B., Patrice, H.M., Okpechi, I., Zhao, M.H., Lu, J., Garg, A.X., Knight, J., Rodgers, A., Gallagher, M., Kotwal, S., Cass, A., Perkovic, V. 2015. Worldwide access to treatment for end-stage kidney disease: a systematic review. *Lancet*. 385, 1975-1982.

Montserrat, N., Garreta, E., Izpisua Belmonte, J.C. 2016. Regenerative strategies for kidney engineering. *FEBS J*. doi: 10.1111/febs.13704.

Morales, E.E., Wingert, R.A. 2014. Renal stem cell reprogramming: prospect in regenerative medicine. *World J Stem Cells*. 6, 458-466

Murphy, S.V., Atala, A. 2014. 3D bioprinting of tissues and organs. *Nat Biotech*. 32, 773-785.

Nakayama, K.H., Batchelder, C.A., Lee, C.I., Tarantal, A.F. 2010. Decellularized rhesus monkey kidney as a three-dimensional scaffold for renal tissue engineering. *Tissue Eng Part A*. 16, 2207-2216.

Nakayama, K.H., Batchelder, C.A., Lee, C.I., Tarantal, A.F. 2011. Renal tissue engineering with decellularized rhesus monkey kidneys: age-related differences. *Tissue Eng Part A*. 17, 2891-901.

Nakayama, K.H., Lee, C.C., Batchelder, C.A., Tarantal, A.F. 2013. Tissue specificity of decellularized rhesus monkey kidney and lung scaffolds. *PLoS One*. 8(5):e64134

Neofytou, E., O'Brien, C.G., Couture, L.A., Wu, J.C. 2015. Hurdles to clinical translation of human induced pluripotent stem cells. *J Clin Invest*. 125, 2551-2557.

O'Neill, J.D., Freytes, D.O., Anandappa, A.J., Oliver, J.A., Vunjak-Novakovic, G.V. 2013. The regulation of growth and metabolism of kidney stem cells with regional specificity using extracellular matrix derived from kidney. *Biomaterials*. 34, 9839-9841.

Orlando, G., Booth, C., Wang, Z., Totonelli, G., Ross, C.L., Moran, E., Salvatori, M., Maghsoudlou, P., Turmaine, M., Delario, G., Al-Shraideh, Y., Farooq, U., Farney, A.C., Rogers, J., Iskandar, S.S., Burns, A., Marini, F.C., De Coppi, P., Stratta, R.J., Soker, S. 2013. Discarded human kidneys as a source of ECM scaffold for kidney regeneration technologies. *Biomaterials*. 34, 5915-5925.

Orlando, G., Farney, A.C., Iskandar, S.S., Mirmalek-Sani, S.H., Sullivan, D.C., Moran, E., AbouShwareb, T., De Coppi, P., Wood, K.J., Stratta, R.J., Atala, A., Yoo, J.J., Soker, S. 2012. Production and implantation of renal extracellular matrix scaffolds from porcine kidneys as a platform for renal bioengineering investigations. *Ann Surg*. 256, 363-370.

Orlando, G., Soker, S., Stratta, R.J. 2013. Organ bioengineering and regeneration as the new Holy Grail of organ transplantation *Ann Surg*. 258, 221-232.

Orlando, G., Walker, S. 2014. Organ Bioengineering, Regeneration and Repair: Where the Future of Organ Transplantation is Taking Shape. *J Transplant Stem Cell Biol.* 1, 1-2.

Orlando, G., Wood, K.J., Stratta, R.J., Yoo, J., Atala, A., Soker, S. 2011. Regenerative medicine and organ transplantation: Past, present and future. *Transplantation.* 91, 1310-1317.

Peloso, A., Ferrario, J., Maiga, B., Benzoni, I., Bianco, C., Citro, A., Currao, M., Malara, A., Gaspari, A., Balduini, A., Abelli, M., Piemonti, L., Dionigi, P., Orlando, G., Maestri, M. 2015. et al. Creation and implantation of acellular rat renal ECM-based scaffolds. *Organogenesis.* 11, 58-74.

Peloso, A., Katari, R., Murphy, S.V., Zambon, J.P., Defrancesco, A., Farney, A.C., Rogers, J., Stratta, R.J., Manzia, T.M., Orlando, G. 2015. Prospect for kidney bioengineering: shortcomings of the status quo. *Exp Opin Biol Ther.* 15, 547-558

Peloso, A., Katari, R., Patel, S., Hemal, S., Zambon, J.P., Salvatori, M., Orlando, G. 2013. Considerations on the development of a model of kidney regeneration in rats. *Exp Rev Med Dev.* 10, 597-601.

Peloso, A., Petrosyan, A., Da Sacco, S., Booth, C., Zambon, J.P., O'Brien, T., Aardema, C., Robertson, J., De Filippo, R.E., Soker, S., Stratta, R.J., Perin, L., Orlando, G. 2015. Renal ECM scaffolds from discarded kidneys maintain glomerular morphometry and vascular resilience, and retains critical growth factors. *Transplantation.* 99, 1807-1816.

Petrosyan, A., Orlando, G., Peloso, A., Wang, Z., Farney, A.C., Rogers, J., De Filippo, R.E., Delario, G., Willis, C.D., Stratta, R.J., Soker, S., Perin, L. 2015. Understanding the bioactivity of stem cells seeded on ECM produced from discarded human kidneys. *CellR4.* 3, e1401.

Petrosyan, A., Zanusso, I., Lavarreda-Pearce, M., Leslie, S., Sedrakyan, S., De Filippo, R., Orlando, G., Da Sacco, S., Perin, L. 2016. Decellularized renal matrix and regenerative medicine of the kidney: a different point of view. *Tissue Eng Part B Rev.* [Epub ahead of print]

Poornejad, N., Momtahan, N., Salehi, A.S., Scott, D.R., Fronk, C.A., Roeder, B.L., Reynolds, P.R., Bundy, B.C., Cook, A.D. 2016. Efficient decellularization of whole porcine kidneys improves reseeded cell behavior. *Biomed Mater.* 11, 025003.

Rogers, J., Katari, R., Gifford, S., Tamburrini, R., Edgar, L., Voigt, M.R., Murphy, S.V., Igel, D., Mancone, S., Callese, T., Colucci, N., Mirzazadeh, M., Peloso, A., Zambon, J.P., Farney, A.C., Stratta, R.J., Orlando, G. 2016. Kidney transplantation, bioengineering and regeneration: an originally immunology-based discipline destined to transition towards ad hoc organ manufacturing and repair. *Expert Rev Clin Immunol.* 12, 169-182.

Ross, E.A., Abrahamson, D.R., St. John, P.L., Clapp, W.L., Williams, M.J., Terada, N., Hamazaki, T., Ellison, G.W., Batich, C.D. 2012. Mouse stem cells seeded into decellularized rat kidney scaffolds endothelialize and remodeled basement membranes. *Organogenesis.* 8, 49-55.

Ross, E.A., Williams, M.J., Hamazaki, T., Tereda, N., Clapp, W.L., Adin, C., Ellison, G.W., Jorgesen, M., Batich, C.D. 2009. Embryonic stem cells proliferation and differentiate when seeded into kidney scaffolds. *J Am Soc Nephrol.* 20, 2338-2347.

Salvatori, M., Peloso, A., Katari, R., Zambon, J.P., Soker, S., Stratta, R.J., Orlando, G. 2015. Semi-xenotransplantation: the regenerative medicine based-approach to immunosuppression-free transplantation and to meet the organ demand. *Xenotransplantation.* 22, 1-6.

Shanks, N., Greek, R., Greek, J. 2009. Are animal models predictive for humans? *Philos Ethics Humanit Med.* 4, 2.

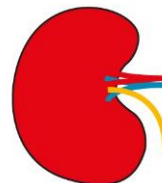
Song, J.J., Guyette, J.P., Gilpin, S.E., Gonzalez, G., Vacanti, J.P., Ott, H.C. 2013. Regeneration and experimental orthotopic transplantation of a bioengineered kidney. *Nat Med.* 19, 646-651.

Sullivan, D.C., Mirmalek-Sani, S.H., Deegan, D.B., Baptista, P.M., Aboushwareb, T., Atala, A., Yoo, J.J. 2012. Decellularization methods of porcine kidneys for whole organ engineering using a high-throughput system. *Biomaterials.* 33, 7756-7764.

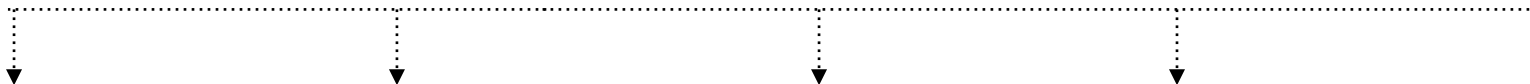
Takahashi, K., Yamanaka, S. 2015. A developmental framework for induced pluripotency. *Development.* 142, 3247-3285.

Takasato, M., Er, P.X., Chiu, H.S., Maier, B., Baillie, G.J., Ferguson, C., Parton, R.G., Wolvetang, E.J., Roost, M.S., Chuva de Sousa Lopes, S.M., Little, M.H. 2015. Kidney organoids from human iPS cells contain multiple lineages and model human nephrogenesis. *Nature.* 526, 564-568.

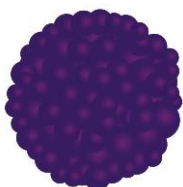
Wilmer, M.J., Ng, C.P., Lanz, H.L., Vulto, P., Suter-Dick, L., Masereeuw, R. 2016. Kidney-on-a-Chip Technology for Drug-Induced Nephrotoxicity Screening. *Trends Biotechnol.* 34, 156-170



Regenerative Medicine Solution for Kidney Bioengineering



Renal Organoids



Key Strengths:

- Self-Organising
- Easy to Generate

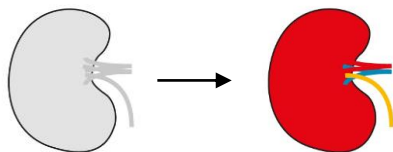
Key Weaknesses:

- Unlikely to provide sufficient filtration
- Difficult to connect to host's excretory system

Potential for translation in next 10 years:

-Moderate

Re-Cellularised Scaffold



Key Strengths:

- Native ECM induces RPC differentiation
- Microarchitecture conserved
- Easy connection to host excretory system

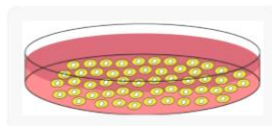
Key Weaknesses:

- Difficult to achieve complete Re-Cellularisation

Potential for translation in next 10 years:

-High

Stem Cell Technology



Key Strengths:

- Autologous SCs can be cultured if Cell Therapy is needed
- SCs can be cultured to understand their growth, development and differentiation
- Useful for Drug Screening

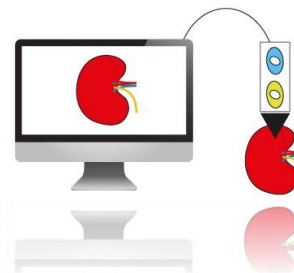
Key Weaknesses:

- Ethical limitation
- Difficult to achieve consistent results in terms of regeneration and repair

Potential for translation in next 10 years:

-Low

3D Printing



Key Strengths:

- ECM and Cells printed together (solves problem of re-cellularisation)
- Consistent Reproducibility

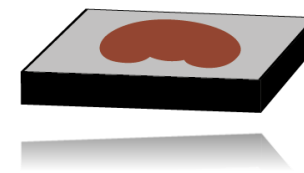
Key Weaknesses:

- Printed ECM unlikely to induce differentiation of RPCs
- Not yet technically feasible

Potential for translation in next 10 years:

-Moderate

Kidney On-A-Chip



Key Strengths:

- Native ECM induces RPC differentiation
- Easy to replicate
- Drug screening

Key Weaknesses:

- Not feasible for connection to host excretory system
- Microarchitecture not conserved
- Sufficient Oxygenation may be difficult and may not be consistent throughout the chip

Potential for translation in next 10 years:

-Moderate