Regenerating the kidney using human pluripotent stem cells and renal progenitors

Journal: *Expert Opinion on Biological Therapy*

DOI: 10.1080/14712598.2018.1492546

Regenerating the kidney using human pluripotent stem cells and renal progenitors

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Key words: iPSCs, kidney, regeneration, stem cells, renal progenitor cells

Abstract

Introduction

Chronic kidney disease is a major healthcare problem worldwide and its cost is becoming no longer affordable. Indeed, restoring damaged renal structures or building a new kidney represent an ambitious and ideal alternative to renal replacement therapy. Streams of research have explored the possible application of pluripotent SCs (embryonic SCs and induced pluripotent SCs) in different strategies aimed at regenerate functioning nephrons and at understanding the mechanisms of kidney regeneration.

Areas covered

In this review, we will focus on the main potential applications of human pluripotent SCs to kidney regeneration, including those leading to rebuilding new kidneys or part of them (organoids, scaffolds, biological microdevices) as well as those aimed at understanding the pathophysiological mechanisms of renal disease and regenerative processes (modeling of kidney disease, genome editing). Moreover, we will discuss the role of endogenous renal progenitors cells in order to understand and promote kidney regeneration, as an attractive alternative to pluripotent SCs.

Expert opinion

Opportunities and pitfalls of all these strategies will be underlined, finally leading to the conclusion that a deeper knowledge of the biology of

pluripotent SCs is mandatory, in order to allow us to hypothesize their clinical application.

Highlight box

- Human pluripotent stem cells (SCs) include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). These cells can provide useful tools either to therapeutic purposes either to investigate disease pathophysiology and mechanisms, including renal diseases and kidney regeneration.
- ESCs have the advantage to be relatively quick to obtain and to be not anymore subject to licensing/royalty to be paid. Anyway, some major concerns, such as ethical issues, the high risk to degenerate in neoplasms and immunocompatibility, still remain open
- iPSCs have the great advantage of harboring the same genetic background of the individual they are derived, thus representing an ideal tool to study the effects of genetic variants in the pathogenesis of diseases. The main risks connected with the use of iPSCs are represented by tumorigenicity and immunogenicity, the presence of an epigenetic memory, technical and economical problems related to their long turnaround time and the presence of loyalties.
- Human pluripotent SCs has two main fields of application in kidney regeneration: they can be used to build "a new kidney" or part of it by the mean of studies on organoids, scaffolds, organ-on-a-chip and blastocyst

complementation, or they can be used to investigate the mechanisms of kidney regeneration trough disease modeling and gene editing.

- Renal progenitor cells represent an attractive alternative either to study or to modulate kidney regeneration, providing important advantages in the field.
- A deeper knowledge of the biology of pluripotent SCs is mandatory, in order to allow us to hypothesize their clinical application.

1. Introduction

Chronic kidney disease (CKD) is a major healthcare issue worldwide. Recent studies report that at least 10% of the adult population in western countries suffers from a variable level of CKD [1-4], probably representing only the "tip of the iceberg" of one of the wounds of the 21st Century. Irrespective of the nature of the disease that leads to the loss of kidney function, CKD can progress toward end-stage renal disease (ESRD), requiring dialysis or kidney transplantation to allow patients to survive. Considering that kidney diseases are frequently clinically silent and CKD is largely undiagnosed, it is easy to understand how this issue could potentially assume catastrophic proportions. Moreover, the social, economic and healthcare burden to sustain long-term renal replacement therapies (RRT) is tremendous and probably no longer affordable worldwide [4, 5].

It is in this context that kidney regeneration has received an impressive push forward. Indeed, restoring damaged renal structures or building a new kidney represent an ambitious and ideal alternative to RRT. Adult human kidney has a limited number of nephrons, determined during embryonic development by a multitude of genetic and environmental factors, that could not be modified after the 36th week of gestation [6-9]. This is commonly believed to be due to the exhaustion of a population of nephron progenitor cells [6, 8, 9]. This inherent incapability of total kidney regeneration in humans poses a detrimental limit to the opportunity of regenerative medicine in nephrology. Notwithstanding this, streams of research have tried to overcome this border

and some ways toward the application of strategies for kidney regeneration seem to be at least plausible. All of them are based on the use of pluripotent stem cells (SCs).

2. Human pluripotent stem cells

Irrespective of the species, pluripotent SCs are cultured cells sharing with the blastocyst-stage embryo the capability to generate an entire body [10]. From a functional point of view, they possess two principal properties: a high proliferative ability and a broad differentiation capacity. The first one, also known as self-renewal, refers to the ability to extensively replicate without undergoing differentiation or senescence, while the second consists in the property of differentiate into more than one mature somatic cell type from each of the three embryonic germ layers (namely, ectoderm, mesoderm and endoderm) [7, 11-13]. In mammals, including humans, pluripotent SCs progressively exhaust during embryonic development, probably giving rise to tissue-restricted stem/progenitor cells, usually referred to as committed stem/progenitors cells. The capability to persist after fetal life depends on the functional features of the cell population and on the regenerative potential of the organ they belong with, that could allow stem cell niches to build and to maintain their function.

Based on their functional properties, pluripotent SCs can be long-term cultured *in vitro*. When exposed to determined culture conditions (e.g deprivation of growth factors that maintain pluripotency), pluripotent SCs undergo stochastic differentiation. Consequently, they give rise to embryoid bodies *in vitro* while by implantation into an immunodeficient animal host, these cells generate teratomas [7, 11, 14]. Both embryoid bodies and teratomas contain cell types owing to the three embryonic germ layers, thus proving the pluripotency of the cell they originate from. In addition, direct differentiation by exposing pluripotent SCs to growth or inhibitors factors that have been demonstrated to specifically regulate progressive steps of embryonic development is an alternative option to obtain the mature cell type of interest, in particular of kidney cells [7, 14, 15].

Human pluripotent SCs include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Indeed, the properties of these two cell populations as pluripotent SCs have been unequivocally demonstrated. These cells can provide useful tools either to therapeutic purposes either to investigate disease pathophysiology and mechanisms.

2.1 Embryonic stem cells

ESCs represent the culprit of pluripotent SCs. They are primary cultures of human embryonic cells from the inner cell mass of the blastocyst, about 5 after fertilization [16]. They are separated and grown in culture after derivation

from embryos [11] and can be induced to differentiate into a broad range of cell types [17].

They surely provide a hopeful strategy for kidney regenerative therapies (Figure 1). They are capable of differentiation into different kidney mature cell types. The first report of ESCs use to obtain renal cells was provided in *Xaenopus leavis*, by exposing the ectoderm to mesoderm-inducing factors (e.g. retinoic acid, activin A) [18-20]. This strategy allowed authors to obtain cells of the pronephros, including glomerular and tubular cells [18, 21, 22]. Similar studies were subsequently performed in mammalian metanephric kidney, including the human one, with success in obtaining renal progenitor cells from embryonic bodies [7, 23-26]. Indeed, most protocols of human ESCs differentiation to kidney cells have been translated from studies in mice, including strategies for differentiation of ESCs in nephron precursor population and even direct differentiation in more mature kidney cells, bypassing nephron precursors [23, 27-33]. These studies allowed the authors to generate populations of podocyte- and renal tubular epithelial-like cells, whose phenotypic and functional properties anyway still remain to be clearly stated [7, 34, 35].

ESCs can be relatively quickly obtained (around 20 weeks) and are not anymore subject to licensing/royalty to be paid. Therefore, they represent a relatively easy-to-obtain cellular tools. Anyway, some major concerns still remain open talking about ESCs. Firstly, ESCs-based methods are inherently burden with ethical issues that have significantly limited their use, at least in some countries. Secondly, cells and tissues derived from ESCs are at high risk

to degenerate in neoplasms, especially teratomas [36]. Lastly, ESCs-derived differentiated cells are allogeneic in nature and they inevitably suffer from all the issues related to allografts and immunocompatibility (acute and chronic rejection, graft *versus* host disease, although the latest has been reported only in case of ESCs transplantation for bone marrow regeneration).

2.2 Induced pluripotent stem cells

iPSCs are somatic cells reprogrammed to acquire an ESC-like functional status [10]. iPSCs technology was first described in 2006 [37]. Briefly, somatic cells are transiently induced to overexpress embryonic transcription factors (OCT4, SOX2, KLF4, c-MYC in the initial work) that lead the cells to acquire a phenotypic and functional status similar to that of ESCs.

iPSCs can be obtained by vector integration methods (e.g. retroviral vectors, lentiviral transgene integration) or integration-free methods (e.g. plasmids, Sendai virus, synthetized RNAs, proteins) that are less likely to persist after reprogramming [37-39]. iPSCs can be classified into primed and naive. Naive iPSCs model the inner cell mass of the pre-implantation blastocyst, whereas primed iPSCs resemble cells derived from post-implantation epiblasts. In general, naive iPSCs are easier to maintain and differentiate, but need to be obtained using chemically defined conditions. The difference between naïve and primed iPSCs, as well as their species of origin, could potentially influence the results of the studies performed and pose important challenge for their clinical use.

Although the efficiency of the reprogramming is low, iPSCs can be extensively expanded in culture, giving rise to various types of cells and tissues [40]. Like ESCs, iPSCs can generate derivatives of all the three germ layers after introduction into pre-implantation embryos. Following *in vitro* differentiation, iPSCs are able to generate cells that resemble renal progenitors and their progeny, such as podocytes and tubular epithelial cells (Figure 1) [34, 41-44].

iPSCs have the great advantage of harboring the same genetic background of the individual they are derived. As a natural consequence, they represent an ideal tool to study the effects of genetic variants in the pathogenesis of diseases [14]

A major concern of iPSCs is the presence of an epigenetic memory, that is to say a series of methylation/demethylation sites that are typical of the specific differentiated cell type and of the tissue/organ of origin. This feature could affect not only the ability of iPSCs-derived mature cells to reliably recapitulate the disease pathophysiology, but could also affect the differentiation capability, since it has been demonstrated that iPSCs preferentially differentiate back to the cell type of origin [45, 46]. This last issue may be probably at least in part overcome by a better knowledge of the molecular and genetic networks regulating the differentiation process from iPSCs and tissue specific progenitors or mature cells. Moreover, it has been demonstrated that reprogrammed cells (iPSCs) differ from pluripotent SCs in the activation/inactivation of genetic loci previously thought to be highly stable. However, a great advantage in using iPSCs for understanding kidney regeneration could derive from generating them from kidney cells. Indeed,

iPSCs have been generated from cells of renal origin (mesangial, tubular epithelial cells, urine-derived renal epithelial cells) [34, 47-50], providing indisputable advantages for cell therapy and kidney regeneration. Indeed, kidney-derived iPSCs would retain not only the genetic background but also the renal peculiar epigenetic memory of the cell of origin.

The main risks connected with the use of iPSCs are represented by the accumulation of somatic mutations that can result in tumorigenicity and immunogenicity. With regard to malignant transformations, some studies reported critical activation of oncogenes in iPSCs [36]. However, since cellular reprogramming is clonal in nature, it seems plausible to attribute the events of malignant transformation to genetic alterations that occur at low frequency in the starting population and that are inherent to it [39]. On the other hand, patient-derived iPSCs are not immunogenic in nature, but the accumulation of genetic mutation must be considered as a potentially confounding event with regard to immune tolerance [10, 39].

iPSCs pose technical and economical problems, since the turnaround time to obtain cultures is about one year *per* patient and the technology is protected by patent and its use requires the payment of loyalties, at least for commercial strategy[10]. This issue could at least in part be overcome by the building of banks of allogenic iPSCs that can provide clinical grade iPSCs within shorter time. On the other hand, since iPSCs are derived from adult individuals, the main ethical issue related to their use is privacy.

3. Exploring kidney regeneration: potential applications of human pluripotent stem cells

As already mentioned, the adult human kidney is not able to generate new nephrons. As a consequence, pluripotent SCs have long been considered as the only cultivable cells capable of neo-nephrogenesis. Therefore, they represent the most studied tool in kidney regenerative medicine (Figure 1).

Since the first description of cellular reprogramming and iPSCs technology development, questions about the differences existing among iPSCs and ESCs arose. Indeed, these two cell types differ in gene expression profiles, DNA methylation and pluripotent potential (i.e. differentiation ability), as assessed by different groups by microarrays studies, targeted bisulfite sequencing and assays of differentiation efficacy, respectively [17, 39]. Notwithstanding this, it is plausible that these differences are shades not very easy to assess and that iPSCs and ESCs have at least partially overlapping phenotypes, depending on the source of cells, the laboratory conditions used to obtain the cultures and even stochastic events, including genetic imprinting. Indeed, ECSs and iPSCs show exceptional similarities that could maybe be explained considering that are both obtained by laboratory manipulation [39, 51-53]. Therefore, the selection of ESCs or iPSCs clones suitable for medical application appears to be critical. With regard to kidney regeneration, pluripotent SCs must differentiate into kidney rather than other mature cells, be able to reproduce the spatial and

anatomical complexity of the organ, and then induced to acquire functionality [54]. Indeed, two major obstacles reside in kidney regeneration by human pluripotent SCs: first, the kidney contains a multitude of different cell types that has to be someway reconstructed and second, the organ begin to develop late in embryonic development, that is to say far away from the state of pluripotency [54].

Notwithstanding this, attempts to pave the way for pluripotent SCs to regenerate the kidney have been made. Indeed, recent advances in the SCs field, together with a great push of nanotechnologies, have enabled the *in vitro* generation of complex structures resembling whole kidneys, termed organoids, or part of them, like scaffolds and engineered glomerular filtration barriers. Besides this, the identification of endogenous repair and regeneration strategies in injured kidney is another option that has to be taken into consideration in order to find strategies to be exploited therapeutically in kidney regeneration (Figure 1).

3.1 Building a new kidney with human pluripotent stem cells

3.1.1 Organoids

The term "organoids" refer to suspensions of human pluripotent SCs that self organize in culture to form small organs and tissue arrangements [55-57]. Therefore, a kidney organoid is a miniaturized and simplified version of a

kidney produced *in vitro*, that shows a realistic renal microanatomy. The methodological and conceptual advance concerning organoids prompted researchers to use them not only for classical developmental experiments but also for regeneration studies.

The first attempts to create kidney organoids for studying regeneration, however, obtained avascular fetal-like kidney tissues [55]. This obstacle was successfully overcome by using a dissociation/reaggregation method of mouse kidney cells that showed to be able to integrate into living recipients, to generate vascularized glomeruli and to perform nephron-specific functions [58]. The same authors then used this strategy to create chimeric kidney organoids from mixed suspension of murine embryonic kidney cells and human amniotic fluid SCs [59]. The ability to generate vascularized nephrons from single-cell suspensions marked a step forward on the way of replacing renal function by tissue-engineered kidneys.

Important advances toward the generation of patient-specific organoids as tools for studying human kidney development, modeling disease, developing new drugs and evaluating novel regenerative strategies derived from the development of efficient protocols for the differentiation of human pluripotent SCs into kidney organoids. Indeed, when induced to differentiate in renal epithelial cells, human pluripotent SCs spontaneously organize into structures resembling different segment of the nephron, from glomeruli to collecting ducts [6, 42, 60, 61]. In particular, the exposure of iPSCs to different levels of Wnt agonist allowed authors to obtain pluripotent SCs of the metanephric mesenchyme and of the ureteric bud, giving rise to nephron structures that

recapitulate the most important developmental steps of the embryonic kidney [60]. These structures showed functional properties such as a spatial and temporal pattern of expression of markers specific of different segments of the nephron [60]. Almost simultaneously, kidney organoids have been created from human ESCs [61]. Human pluripotent SCs-derived organoids contain all the components of the fetal human kidney (nephrons, collecting ducts, endothelial cells, interstitium) [60, 61].

From a conceptual point of view, kidney organoids have two main principal functions concerning regenerative nephrology: to establish the similarity between human fetal kidney cells and the corresponding cells contained within the organoids by the mean of morphological analysis and gene expression profile assays; to detect protocols for obtaining the differentiation of cells present within the organoids into the specific renal cell lineages of interest [62].

The development of these protocols paved the way for the possibility of patient-specific disease modeling and drug screening, at a further level than the cellular one, as well as for the replacement of renal tissue by bioengineering approaches. However, organoids are not a kidney. The complex large-scale organization of the organ, that is crucial for some of its functional properties, cannot be fully reproduced, making these structures far away to be considered as kidney replacing. Anyway, these organoids can be suitable for other medical purposes, such as compounds and drugs screening.

3.1.2 Scaffolds

Pluripotent SCs-derived organoids have provided interesting information about the capability of self-organization in similar-kidney structures. Notwithstanding this, this strategy could be inefficient in generating a sufficient number of cells for kidney regeneration purposes. Therefore, alternative solutions have been set up. In this view, "re-cellularization" of biologic or artificial scaffolds with appropriate combinations of specific renal cell types is an attractive hypothesis. These cells can be either isolated from pluripotent SCs-derived organoids, that are separated and expanded in culture, or differentiated *in vitro* from their specific tissue/cell of origin [62, 63]. Scaffolds can be made of purified silk, 3D-printed polymer arrays, decellularized kidneys and extracellular matrix [63]. Independently on the kind of scaffold, this approach requires the appropriate set-up of vascular structures (here including the correct development and localization of endothelial, smooth muscle and pericytes cells, as well as interstitial cells) to allow the scaffolds to be served by blood flow and the structures to be consequently functional [64, 65]. Finally, these newly generated structures should be communicate with collecting ducts to allow the urine stream to flow and kidney to fulfill all its functions [66]. The ultimate goal is to obtain synthetic kidneys that can be transplanted to a host [62].

3.1.3 Engineered glomerular filtration barrier and renal tubules

The glomerular filtration barrier (GFB) is a highly specialized structure interfacing with blood and responsible for its filtration. The integrity of the GFB is guaranteed by a correct anatomical and functional organization among its three principal constituents: the glomerular endothelial cell, the basement membrane, and the podocyte. Since many kidney disorders determine the loss of the GBF integrity, rebuilding a functioning GFB is a goal of regenerative medicine and represents another strategy for modeling kidney diseases [67].

The podocyte is a post-mitotic cell incapable of proliferation and cell division [68, 69]. It has been demonstrated that podocytes can be replaced by a progenitor cell, localized within the Bowman's capsule, although this regeneration is limited [68-71]. The possibility to obtain a fully differentiated podocytes is therefore essential for modeling the GFB and studying kidney disorders and regeneration. In a recent study, iPSC-derived podocytes were put in the context of an engineered GFB that recapitulates the properties of the human glomerular capillary wall, to which mechanical forces resembling pulsatile blood flow were applied [72]. Human iPSCs-derived podocytes produced proteins of the glomerular basement membrane and showed foot processes that confer the permselectivity typical of the "native" GFB. By inducing podocytes damage, the authors recapitulated the morphological and phenotypic features of focal segmental glomerulosclerosis [72]. This strategy could be used for the modeling of many others kidney disorders, with particular attention to podocytopathies (genetic, toxic, infectious), adding to the cellular and molecular level the complexity of the GFB [67].

In parallel to the development of artificial GFB, a significant number of studies reported on the generation of devices mimicking the structure of kidney tubules. Many studies used artificial microchip coated with primary lines of human tubular epithelial cells [73-78]. Upon the exposure to microfluidic shear stress, bioartificial renal tubules showed to recapitulate some of the main phenotypic and functional features of renal tubules, such as solute reabsorption and secretion and cellular polarization. These first studies introduced a new tool for exploring kidney regeneration, with particular regard to the tubular compartment. Indeed, it is plausible to hypothesize that artificial microdevices could be coated with patient-derived human pluripotent SCs, thus allowing tubular diseases (like acute kidney injury, AKI) to be reliably recapitulated and drugs efficacy and nephrotoxicity to be tested in a personalized manner.

3.1.4 Blastocyst complementation

The generation of transplantable kidneys is among the ultimate goals of regenerative nephrology due to a shortage of donor organs, that represents a critical obstacle to the expansion of transplantation programs. Notwithstanding this, the complex 3D cellular and tissue interactions required for organogenesis are quite difficult to recapitulate *in vitro*. Blastocyst complementation is a method used to overcome these obstacles. Briefly, it consists in generating organs *in vivo* by injecting pluripotent SCs (either ESCs or iPSCs) into blastocyst-stage embryos (mainly, but not exclusively, rodents).

This finally leads to the generation of chimeric embryos in which pluripotent SCs contribute to the generation of host tissues and organs [79]. Interspecies blastocyst complementation is a variation of the classical technique in which the recipient host is genetically manipulated to carry DNA mutations that prevent the development of a target organ [80]. Ideally, the injection of donor- derived pluripotent SCs would developmentally compensate for the defect and form the missing organ. This strategy had been initially used for the reconstitution of bone marrow but was subsequently applied to the generation of entire organs (e.g. pancreas, heart, eye) [81]. The resulting organs are composed almost entirely of cells derived from donor, even if the blastocyst complementation involves different species.

To the aim of kidney organogenesis, this technique has been used in few works that arose the possibility of widening the opportunities of kidney regeneration.

In the very first experiment, wild-type mouse pluripotent SCs were injected into Sall1 knockout mouse blastocysts in which kidneys did not developed because of the genetic defect [79]. This led to the generation of kidneys entirely formed by the injected mouse-derived cells, except for structures not under the influence of Sall1 expression (such as collecting ducts and microvasculature), thus rescuing bilateral renal agenesis [79]. Unfortunately, the resulting chimeric animals did not survive until adulthood for reasons that are not completely clear [79]. Following the first experiments performed in rodents, the research shifted to generate chimeric animals between human pluripotent SCs and larger animals (e.g. pigs), in order to obtain organs sized as closer as possible to that of humans. To this aim, the potential of human pluripotent SCs

to survive into the blastocyst of pigs and cattle have been tested and gave very preliminary but promising results [82]. Obviously, more studies are needed to set up the experimental conditions that are proper to the scope of generating xeno-kidneys suitable for kidney replacement purposes.

Important ethical issues regarding the manipulation of blastocysts and the generation of viable humans-animals chimaera mark this strategy. Moreover, the experiments are technically difficult to perform and few laboratories have the resources necessary to pursue them. As a matter of fact, xeno-generated organs need to be extremely "pure" to avoid immune system rejection after transplantation, thus raising important questions about how to technically obtain "pure" organs of interest after generating the chimeric animals. Finally, blastocyst complementation in some organs could be incompatible with life. Therefore, besides a very theoretical interest, this strategy, although promising, is far to be considered for clinical application.

3.2 Understanding kidney regeneration trough modeling of renal diseases

3.2.1 Modeling of renal diseases using pluripotent stem cells

One of the main opportunities of human pluripotent SCs is the development of "disease in a dish", that is to say laboratory models of human kidney diseases, that can be used to understand the mechanisms of diseases (Figure 1). They represent an important complement to mouse models, which may not fully recapitulate human genotypes and phenotypes and are technically and

economically costly to generate. ESCs and iPSCs carrying mutations in genes responsible for inherited kidney diseases have now been generated (e.g. autosomal dominant and recessive polycystic kidney disease, Alport syndrome) [10].

iPSCs can be obtained from patients with kidney diseases. Harboring mutations clearly responsible for the disease, patients-specific iPSCs do not require genome editing to recapitulate the pathophysiology of the disease. To this aim, a multitude of iPSCs cell cultures have already been obtained and can be used to compare *in vitro* properties with clinical features of patients [10]. As an example, they have been extensively used in assessing the process of cystogenesis in autosomal dominant polycystic kidney disease (ADPKD) and the role of policistin-2 mistrafficking in the cilium in the pathogenesis of the disease [44]. These studies demonstrated that the cystic phenotype is less than 100% penetrant even in presence of truncating mutations [10].

Moreover, some of these mutations could be specie-specific and these cell cultures hold not only the mutation of interest but also other variants that could eventually act as modifiers. Therefore, the use of iPSCs permits to skip at least some issue related to the reproducibility of data obtained from animal models, including mice, making them highly indicated in genotype-phenotype correlation studies following whole-exome sequencing population screening [83].

In addition, human iPSCs can be used for drug discovery. Being at the same time specie-specific and phenotypically diverse, cell culture obtained from

iPSCs are amenable tools to test the effects of new compounds and to evaluate efficacy, toxicity and pleiotropic effects [10, 83]. For all these purpose, the limiting step seems to be the development of reliable *in vitro* assays to test the feature of interest.

Finally, human pluripotent SCs can serve as tools to identify new biomarkers for renal disorder or to validate existing ones. Their role is quite consolidated in other organs and tissues (e.g. heart, central nervous system, liver) [10]. In the nephrology field, this approach would be of particular interest for AKI. Indeed, the identification of up-/down-regulation of specific gene/molecules in response to AKI would be of benefit in the early identification of AKI and in the preclinical evaluation of drugs/molecules to treat patients, to avoid nephrotoxicity and to promote tubular regeneration. In addition, the assessment of specific phenotypic features in cell cultures obtained from pluripotent SCs carrying mutations in genes responsible for inherited kidney diseases could also be considered as biomarkers. As an example, cysts development in cell cultures obtained from iPSCs could predict the pathogenicity of genetic variants in ADPKD and the development of the phenotype before the clinical onset of the disease, acting as a biomarker for disease progression and, potentially, for disease severity and prognosis assessment [10]. This could conceptually be useful also for other renal disorder, such as nephrotic syndrome and focal segmental glomerulosclerosis [10].

3.2.2 Gene editing for kidney regeneration

Recent years have seen a significant increase in genome editing techniques applied to human cells. Indeed, genome editing provides the possibility to efficiently introduce a variety of genetic alterations to the cell of interest, ranging from single-nucleotide modification to whole gene addiction or deletion, all with high degree of target specificity. Irrespective of the specific genome editing strategy used (Zinc-finger Nucleases (ZFN), Transcription activator-like effector nucleases (TALEN) and CRISPR-Cas9) these techniques can be applied to human pluripotent SCs and permit to correct the disease- causing gene mutations if applied to patient-derived iPSCs or to introduce specific mutations into non-disease affected wild-type iPSCs (Figure 1) [81].

Recently the CRISPR-Cas9 technology has attracted much attention and gained wide usage in gene editing both in ESCs and in iPSCs owing to its simplicity in design and ease of use. However, a major challenge in the application of this technology is the possibility of off-target effects [86]. For this reason, this technologies needs to be continuously improved.

Gene editing of human pluripotent SCs has three principle possible applications:

1. Elucidating gene function. The cells that have undergone genome editing should contain only the intended change in an otherwise isogenic background, thus providing the most stringent test of gene function. Freedman et al. [87] produced kidney organoids from human pluripotent SCs knocked out for podocalyxin by CRISPR-Cas9 in order to investigate its role during kidney organogenesis. Comparing the results obtained with wild-type organoids, the

authors concluded that podocalyxin is essential for organoid differentiation, with particular attention to podocyte junctional organization.

2. Modeling human diseases. Patient-derived iPSCs are widely used to modeling monogenic disorder because they are derived from the patient, easily manipulated, clonally expanded and differentiated toward the cells of interest. The application of gene editing allows creating isogenic controls with the rescue of disease causative gene mutations, avoiding the confusion with genetic background or epiphenomena resulting from possible line-to-line variations. Recently, to better recapitulate disease phenotypes, human iPSC- derived 3D organoids have been developed to study the cell-cell interaction in a cellular context that mimics human physiology [88, 89]. In particular an in *vitro* organoid model for ADPKD was performed applying CRiSPR-Cas9 genome editing system to introduce biallelic truncating mutations in *PKD1* or *PKD2* in human pluripotent SCs, providing a good and promising approach to model cystogenesis [87]. Furthermore, patient-derived iPSCs in addition to enabling personalized diseases modeling could be used as potential high- throughput drug-screening platforms.

3. Gene therapy. Ideally, gene editing would lead in the future to new cell therapy strategies for the treatment of renal diseases, either by promoting the application of non-immunogenic patient's own iPSCs genetically corrected target mutations for kidney repair and regeneration, either by generating sources for organ replacement therapy, combining genetically corrected human iPSC platform with 3D organoids. In addition, gene editing could also allow allogenic iPSCs to be considered from cell therapy from unmatched

donor. Moreover, it is also possible that gene editing might also enable to generate a "universal donors", that is to say human pluripotent SCs with increased graft immunocompatibility.

4. Alternative options for kidney regeneration: renal progenitors cells

The kidney is a complex organ. This complexity includes the existence of distinct functional compartments, distinct segments within each compartment and distinct cell types within each segment. Moreover, a well-defined three-dimensional structure involving the correct interaction between renal resident cells, vasculature and interstitium is crucial for the proper assumption of the functional features. As a consequence, this complexity influences the regenerative properties of the tissue. Indeed, in contrast to many other organs the diversity of cell types of the kidney requires an extensive variety of differentiated cells to be generated in order to ameliorate disease or injury.

Understanding the mechanisms of endogenous kidney regeneration and repair represents another important goal of regenerative nephrology. In other organs, the discovery of endogenous stem/progenitors systems fueled identification of innovative treatment strategies up to regenerative medicine and tissue engineering [90, 91]. In the kidney, the identification of endogenous stem/progenitor systems remained a challenge until very recently.

As previously stated, mammals are not capable of neo-nephrogenesis. Anyway, the existence of kidney regeneration is suggested by clinical and experimental evidence [92]. In recent years, conclusive evidence for the existence of renal progenitors has been reported in lower vertebrates such as fish, insects and in mammals [92-94]. Because of their limited differentiation potential these cells have been referred to as renal progenitor cells (RPC).

In humans, CD133+CD24+ renal epithelial cells have been demonstrated to represent a hierarchical population of RPC, containing parietal epithelial cells and a scattered population of tubular epithelial cells, that represent about 2-4% of total renal cells [92, 95]. They are marked by the co-expression of the cell surface markers CD133 and CD24 that permit to recover them from tissue and to be grown in culture, so that their functional properties can be assessed *in vitro*. These cells have been shown to possess self-renewal potential, resistance to senescence, ability to grow in culture as spheres and the capability to differentiate *in vitro* into several types of renal epithelial cell, such as podocytes and tubular epithelial cells, as well as into adipocytes, osteoblasts, endothelial cells and neuronal cells [68, 95]. These observations lead to the conclusion that CD133+CD24+ renal epithelial cells may represent a multipotent stem cell population. The recent observations that amgiomyolipomas in tuberous sclerosis derive from a multipotent cancer stem cell that originate from renal epithelium confirmed the hypothesis that the renal epithelium may have differentiation capacity that goes beyond the epithelial phenotype, which was considered to be its only possible lineage based on lineage-tracing experiments performed in mouse models of AKI [96-98]. Consistently, CD133+CD24+ renal

epithelial cells exhibit cellular plasticity and stem-like properties, such as multipotency [92, 95]. Their behavior, such as the differentiation toward epithelial cells instead of adipocytes or endothelial cells, is probably influenced by niche-specific factors that could profoundly differ in healthy or disease (e.g. tumor) microenvironment, as well as in *in vitro* assays or in transplantation experiments [96].

Unlike iPSCs, RPC are not generated by the forced expression of SCs genes, but can instead be directly isolated from adult human kidneys. Therefore, they are frequently referred to as "endogenous SCs", and can represent further possible tools or target for alternative treatment for kidney regeneration.

4.1 Renal progenitors cells as tools for kidney regeneration

The injection of RPCs into immunopermissive hosts could represent an interesting strategy for kidney regeneration.

Several independent studies demonstrated that adult renal progenitors had therapeutic effects in immunodeficient mice with rhabdomyolysis-induced AKI [16, 95, 99-102]. In all these models, cell therapy improved measurable renal function and structural injury. Afterwards, the administration of $CD133^{+}CD24^{+}$ cells isolated from the Bowman's capsule improved renal outcomes in a model of FSGS that is characterized by podocyte injury [68]. $CD133^{+}CD24^{+}$ cells that were isolated from urine also differentiated into podocytes and reduced proteinuria in mice with adriamycin-induced nephropathy [103].

Multiple studies have demonstrated the therapeutic potential of RPCs isolated from fetal kidneys in acute and chronic renal injury. In mice with glycerol-induced AKI, injection of human fetal $CD133^{+}CD24^{+}$ RPCs improved renal function comparably to adult RPCs [104]. In the 5/6 nephrectomy model of kidney injury, human fetal nephron NCAM-1^{+} progenitors injected directly into the renal parenchyma engrafted and integrated, with consequent improvement of renal function and slowing of disease progression [105]. In one study, the therapeutic effects of NCAM-1^{+} nephron progenitor cells and of cultured human $CD133^{+}CD24^{+}$ renal progenitors in a mouse model of glycerol-induced AKI were comparable [106].

The exact mechanisms by which human RPCs exert beneficial effects when used for cell therapy are not completely elucidated, probably including both paracrine stimulation of adjacent cells and tissue integration, even with different persistence in each model [68, 95, 102, 107].

Although potentially promising, the use of renal progenitors for cell therapy of kidney injury has also several drawbacks. Sources of RPCs are limited, and tissue from which autologous RPCs can be obtained is poorly accessible or insufficient for cell isolation. However, it is possible to retrieve autologous RPC from the patient's urine, a technology potentially offering an autologous CKD therapy that should not require immunosuppression [103].

Outcomes of cell therapy might also depend on the modality of cell delivery to the injured compartment. Cells that are delivered intravenously or intra-arterially may become trapped in other organs, although this does not seem to happen for RPC [68]. Intraparenchymal delivery has the advantages of requiring few cells and avoiding cell dispersion in off-target organs, but might be associated with adverse events, such as local necrosis, thrombosis and a limited distribution of the cells within the kidney.

Finally, it is possible that either the disease process itself or external factors (e.g. drugs) may influence the ability of cells to regenerate injured kidneys [71, 108]. Therefore, the exact effects of this possibly confounding factors needs to be clearly identified, allowing us to enhance the therapeutic potential of these therapies.

Although the possibility to generate new functional renal tissue by cell therapy represents one of the main goals of regenerative nephrology, there is a great lack of knowledge about the differentiation processes and how to control them in order to allow injected cells to be functional. Indeed, the possible application of cell therapy could not still be envisaged until strictly controlled differentiation protocols are developed and many molecular mechanisms are understood.

4.2 Renal progenitor cells for modeling kidney regeneration

The choice of a validated tool of investigation represent a major challenge to understand kidney regeneration and find new drugs for modulating it. RPCs can be easily amplified, maintained in culture and induced to differentiate into all epithelial cell subtype. Therefore they represent an efficient and innovative tool to this purpose (Figure 2).

Cultures of RPCs can be obtained from human healthy tissues using immunomagnetic sorting for CD133 and CD24 (Figure 2) [68, 95, 104]; in addition, the expression of CD106 allows us to specifically separate tubular-committed or podocyte-committed progenitors [99]. Several studies documented the involvement of dysregulated proliferation and migration of RPC at different stages of their differentiation toward mature podocytes in the generation of glomerular hyperplastic lesions, highlighting that RPC regenerative capacity after injury must be strictly regulated in order to

prevent an inefficient or excessive response that can determine a failure in replacing the loss podocytes [109, 110]. Therefore, RPC can be used as a useful tool to study the pathophysiology of kidney diseases and mechanisms of repair and regeneration. However, the inaccessibility of the human RPCs from the affected patients obstacles their application for personalized diseases modeling and drug screening in the context of the individual's overall genetic and epigenetic background. Recently Lazzeri et al. [103] developed a method to isolate and amplify RPCs from renal cells that naturally are loss in the urine of patient with renal disorders. In particular, urine-derived RPCs display an identical phenotype and functional properties to tissue-derived RPCs (Figure 2). Indeed, the isolation of urine-derived RPC from patients affected by genetic kidney disease would be a valuable instrument to achieve a proper functional study aimed to clarify the role of the identified variant in the pathophysiology of the disease [103]. Moreover, the advent of high-throughput techniques of sequencing has amplified the identification of variants of unknown clinical significance and the need of appropriate test to establish their pathogenicity [111]. Patient-specific RPCs may be also usefully for testing patient-specific safety and efficacy of drugs. Moreover, RPCs can be isolated also from the urine of pre-term neonates [112]. These cells show high differentiation ability, potentially broadening the potential of RPCs for regenerative kidney repair.

4.3 Renal progenitors as targets for kidney regeneration

At different with other pluripotent stem cell populations, RPC have the advantage to represent also possible targets to boost kidney regeneration (Figure 2). Indeed, recent studies demonstrated that the RPC-mediated kidney regeneration can be modulated through many molecules, such as the chemokine SDF-1 blockers [113], Notch signaling inhibitors [70], the glycogen synthase kinases 3-α and -β (GSK3s) inhibitor BIO [69], retinoic acid [71], as well through several drugs as Interferon [114], Steroids [115], renin-angiotensin-aldosterone system inhibitors [116] and Leptin [117]. All these pharmacological agents can enhance renal progenitor differentiation into podocytes favoring glomerular regeneration or block RPC hyperactivation.

In particular the beneficial effects of SDF-1blockers, Notch signaling inhibitors and renin-angiotensin-aldosterone system inhibitors are attributable to the reduction of abnormal RPC proliferation [70, 112, 116], whereas the beneficial effects of the glycogen synthase kinases 3-α and -β (GSK3s) inhibitor BIO, retinoic acid, Interferon, Steroids and Leptin are attributable to the enhance RPC differentiation into podocytes [69, 71, 114-116]. Finally, very recently Lazzeri et al. [118] demonstrated that the treatment of mice with HDAC (Histone deacetylases) inhibitors following AKI stimulate the proliferation of RPC enhancing the recovery of renal function and a better reconstitution of tubular integrity.

5. Conclusions

Understanding the molecular and cellular mechanisms of kidney regeneration is important for the development of new therapeutic strategies aimed at reversal and/or attenuation of kidney damage. Ideally, this should support attempts to therapeutically enhance kidney regeneration in order to prevent irreversible nephron loss and CKD. The realization of miniaturized organ-on-chip devices, which combine biological and engineering approaches represent another possibility to restore kidney function.

6. Expert Opinion

Human pluripotent SCs represent indisputable tools to improve our knowledge. Nevertheless, the use of these cells still presents some issues and limitations:

1. Efficiency: efficient protocols for renal lineage-specific differentiation of pluripotent SCs and progenitors usually recapitulate embryonic development stages by a step-wise generation of progenitors (from intermediate mesoderm to metanephric mesenchyme). It is possible that one of the main limitations in the generation of functional kidneys from pluripotent SCs is an incomplete definition of the kidney cell lineage specification programs, both *in vivo* and *in vitro*. This can only be overcome by the definition of robust protocols to generate nephrogenic cells.
2. Similarly, the lack of clearly defined indicators of success in obtaining pluripotent stem cell and in their differentiation is troubling. Moreover, molecular and morphological phenotyping has not been exhaustive and functionality has rarely been analyzed.

3. Safety: oncogenic transformation risk must be clearly assessed. Indeed, patient-derived iPSCs are expected to be fully immunocompatible with the patient they have been obtained from. Therefore, every cells derived from iPSCs is invisible to the host immune system and this could theoretically rise questions about the potential neoplastic risk evolution; negative effects on nephrogenesis and renal growth or kidney injury (by the release of unidentified molecules) should also be assessed.
4. Comparisons: Many studies performed on human pluripotent SCs demonstrated that ESCs and iPSCs while essentially similar, exhibit significant variability in terms of gene expression profiles and epigenetic factors. These differences can be attributed either to the cell lines them-selves, either to laboratory and cultures condition. Either way, they influence the differentiation ability of the cells and make comparisons between cells in disease modeling not fully reliable.
5. Reliability in disease modeling: kidney diseases are complex and the pathophysiology of the progression is probably influenced by multiple factor related not only to kidney but also to other organs and functions. *In vitro* cell cultures have the intrinsic limit of not reproducing this complexity and can probably provide information only at a cellular and molecular level.

All these issues underline the need of a deeper knowledge of the biology of pluripotent SCs, in order to shorten the distance that separate their use from clinical application, that must remain the main goal of regenerative nephrology.

Funding

This article is supported by the Tuscan Region to the Meyer Children's Hospital "Programma attuativo regionale Fas-FSC (SMART)" and from the Tuscan Association for the Study of Childhood Renal Diseases (A.Ma.R.T.I.).

Declaration of Interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Reviewer Disclosures

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose

Figure legend

Figure 1. Schematic representation of the possible applications of human pluripotent stem cells to kidney regeneration.

Once human pluripotent stem cells have been obtained from patients (iPSCs) or from blastocyst (ESCs), they can be cultured *in vitro* and induced to differentiation into the cell type of interest. Afterwards, these cells can be used to generate organoids, to repopulate scaffolds or to generate biological microdevices or for blastocyst

complementation. In addition, cell cultures derived from human pluripotent stem cells can be used for kidney diseases modeling (e.g. drug screening, gene editing).

iPSCs, induced pluripotent stem cells; ESCs, embryonic stem cells.

Figure 2. Schematic representation of the possible applications of renal progenitor cells cells to kidney regeneration.

Renal progenitor cells can be obtained from kidney tissue or from fresh urine samples. Renal progenitor cells can be cultured *in vitro* and induced to differentiate into the cell type of interest. Afterwards, these cell cultures can be used for understanding the pathophysiology of kidney regeneration and to unravel potential mechanisms to enhance it (e.g. drug screening, gene editing).

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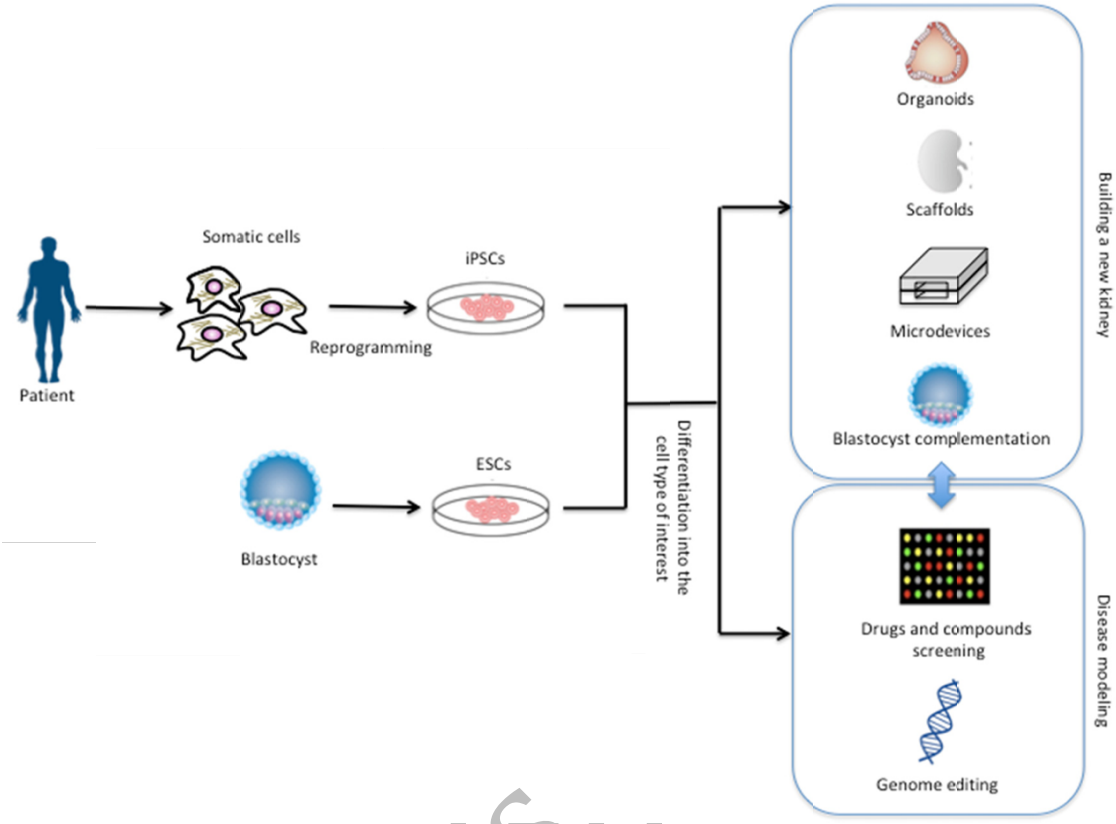

Figure 1

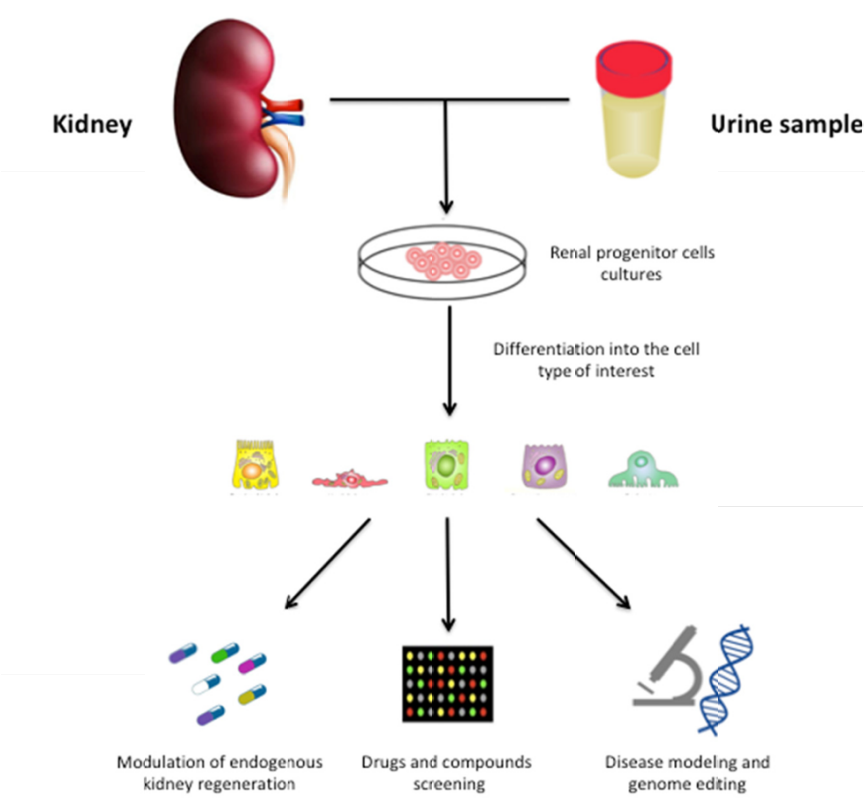

Figure 2