Therapeutic use of human renal progenitor cells for kidney regeneration

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1 **Strategies for the therapeutic use of human renal progenitor cells for kidney**
2 **regeneration**
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14 **Abstract**

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16 In the human kidney, the repair ability is limited and, after repeated injury, a maladaptive
17 response may be triggered, characterized by fibrosis and loss of function. In particular, the
18 transcription patterns that characterize nephrogenesis of foetal renal progenitors are only
19 partially activated during adult repair. Data from the literature support the role of segment
20 restricted progenitor resident cells supporting renal healing of adult kidneys. In this review, we
21 will discuss the evidence for the localization and involvement of functional human renal
22 progenitor cells in renal repair, and discuss the controversial issue of a fixed or plastic
23 population induced by the microenvironment. We will also discuss the strategies supporting the
24 generation of renal progenitor cells from pluripotent stem cells or directly from differentiated
25 cells. Finally, pre-clinical data on the potential therapeutic use of human foetal cells, adult
26 progenitors or adult renal cells will be addressed.

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31 **Introduction**

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Nowadays, kidney diseases are considered a research and public health priority, as their incidence is increasing worldwide with augmented morbidity and mortality[1]. Acute kidney injury (AKI) is characterized by an acute loss of kidney cells and functions, resulting from ischemic, toxic or inflammatory insults, and associates with in-hospital morbidity and mortality, despite the supportive therapies. In addition, acute insults or chronic injury may lead to the progressive loss of nephrons and renal functions, requiring renal replacement therapy by dialysis or transplantation.

The development of acute and chronic renal disease is strictly correlated with an impaired ability of renal repair to regain function after injury. Neo-nephrogenesis does not occur in the adult human kidney. However, the kidney retains some regenerative potential. Several studies have provided evidence to suggest that repair activities in adult kidneys are supported by resident nephron cells surviving injury, with a dedifferentiated phenotype and a clonal proliferative potential[2, 3]. However, the transcription patterns that characterize nephron generation, known to be present in foetal renal progenitors, are only partially activated during adult kidney repair, indicating differences and weaknesses of the repair process[4]. Consequently, the repair ability in adult tissue is limited and, after repeated injury, results in a maladaptive response, ensuing in fibrosis and loss of function. In fact, it is well established that patients with AKI or subclinical rejections may develop chronic kidney disease (CKD)[5]. Finally, chronic renal failure is characterized by a loss of regenerative processes.

In the present review, we will examine the data relative to the presence, the isolation and the *in vitro* generation of renal progenitor-like cells (RPCs), focusing on results obtained in humans, and discuss their potential therapeutic applications.

A. Presence of progenitors or progenitor-like cells (RPCs) in the renal tissue

RPCs in the foetal kidney

In all mammals, the development of mature nephrons involves the differentiation and organization of RPCs of mesenchymal origin. From the embryonic metanephros, reciprocal inductive interactions between the metanephric mesenchyme and the ureteric bud are required for kidney formation[6]. The cap mesenchyme represents the niche where renal progenitors

undergo mesenchymal-to-epithelial transition[7, 8]. These committed progenitors sequentially form pre-tubular aggregates, renal vesicles and C- and S-shaped bodies, leading to the glomerular and renal tubule compartment[6].

Embryonic progenitors represent the prototypes of the renal cell population that are able to proliferate and differentiate towards different types of nephron epithelia. Therefore, defining the cell programs involved in renal progenitor function may provide important knowledge for the mechanisms of renal regeneration in the adult kidney. Several transcription factors and markers have been identified to characterize RPCs in the developing kidney, and most of our knowledge derives from mouse studies. In particular, RPCs in the mouse embryo were first characterized as a multipotent population expressing Six2 with self-renewing capability[8]. In addition, fate mapping in the mouse embryo led to the identification of several genes marking progenitors and involved in their maintenance and differentiation[9], including the Sall1[10], EYA1[11], OSR1[12], CITED1[7] genes and the transcriptional factors PAX2[13] and WT1[14]. Other markers were reported as being involved in segment specification, such as the Wnt target gene *Lgr5* responsible for generating the thick ascending limb of Henle's loop and the distal convoluted tubule[15], or Notch2, which appears to regulate the shift from self-renewal to differentiation, specifying proximal tubules[16].

In humans, foetal RPCs have been characterized to a lesser extent. Many results were obtained from an extensive gene-expression profile analysis in Wilm's tumour, as this paediatric tumour is characterized by loss of terminal progenitor differentiation[17]. The population expressing foetal renal progenitor markers Six2, Wt1, Cited1, and Sall1 was shown to co-express NCAM and FZD7[18]. By contrast, the CD133 marker appeared to be expressed by Epcam^{+} cells with a more differentiated phenotype[18]. Indeed, these two surface markers, NCAM and CD133, were successfully exploited for the isolation of RPCs from the foetal renal kidney. NCAM1 has been reported to facilitate selection for a nephron lineage that includes SIX2-positive cap mesenchyme cells with *in vitro* clonogenic and stem/progenitor properties[19]. While NCAM1 is absent in the normal adult human kidney, CD133 is expressed from the vesicle state onwards[20,21], and is maintained in scattered cells in the adult renal tissue[22].

In the adult kidney, the nephrogenic mesenchymal progenitor population disappears, maybe due to the loss of its niche[23]. RPCs are only induced in the nephrogenic pathway until the 34th week of gestation[19], and no equivalent cell types can be identified in the adult kidney.

Even during repair, only a partial reactivation of certain foetal transcriptional programs occurs, such as induction of Pax2, BMI-1 and Six2 [4, 24].

RPCs in the adult kidney

Even though the ability of whole nephron regeneration is lost in the human adult kidney, organ homeostasis and the repair process require cells with progenitor-like characteristics. This population is required for the continuous replacement of cells lost during physiological processes. In fact, it is estimated that about 6000 cells, which derive from different nephron segments, are lost every hour in the urine (Prescott). In addition, repair after acute injury requires repopulation of tubular compartments. Initial investigations in the stem cell field supported the search for residual stem cells involved in these processes, whereas subsequent studies failed to identify cells with multipotent nephron-generating ability.

Murine studies

The presence of RPCs and their possible role in renal regeneration has been investigated thanks to lineage tracing and fate-mapping techniques. The involvement of nephron resident cells in tubule regeneration was first demonstrated by Humprehy and colleagues[25]. Using genetic label of mesenchyme-derived renal epithelial cells, they could exclude the role of interstitial or bone marrow derived stem cells in repair after acute injury[25]. In a subsequent study, double tracking of tubular cells following acute ischaemic damage showed a random proliferation of tubular epithelial cells. These results have been attributed to a proliferation of all cells within a specific tubular segment. However, they do not exclude the possibility that a subpopulation of cells with progenitor properties may proliferate and repair the tubular segments, as suggested by other reports[2].

Using rainbow mice, the group of Dekel clearly identified that cells with segment-specific clonal and extensive proliferation ability can support kidney epithelia self-renewal throughout an entire lifetime[2]. Indeed, single colour-expressing cell colonies were detectable along kidney segments at 6 months after cell tagging, thus indicating the presence of clone-forming cells[2]. In addition, segment-specific cells with clonogenic ability also contributed to the repair of specific nephron segments, suggesting that nephron regeneration is granted by segment-committed clonogenic RPCs.

From all these observations, it can be inferred that the process of regeneration occurring in renal tubules relies on resident intratubular cells with a limited segment specific regenerative potential and with clonogenic and proliferative ability, thus representing a "functional" population of renal progenitor cells. However, the identity of these cells remains elusive, as they may possibly derive either by a pre-determined population of RPCs, or by a plastic phenomenon in which cells transiently acquire a RPC phenotype.

Recently, a subpopulation of proximal tubular cells ("scattered tubular cells", STC) was identified and genetically labelled. STCs appeared as an injury-inducible population, as their number did increase when labelled during ischaemic injury[27]. Although these data do not exclude a concomitant activation of renal progenitors in the regenerative process, they underline the capability of tubular differentiated cells to acquire a progenitor-like function and suggest that the plasticity of the tubular phenotype might be involved in this process (review Berger).

In contrast to the tubules, the glomerular regenerative potential is quite limited. RPCs identified in Bowman's capsule appeared to contribute to podocyte regeneration, migrating onto the glomerular tuft, only during infancy to adolescence, whereas this process was not observed in adult life[28]. In addition, using label retention, intratubular slow cycling populations have been confirmed in proximal and distal tubules and in the thick ascending limb of the Henle's loop[29] as well as in the papilla[30]. Furthermore, by expression of stem-cell markers, specific populations of progenitors have been detected as nFatC1+ cells in proximal tubules[31] and as Lgr5 or c-kit expressing cells in the thick ascending limb of Henle's loop and distal convoluted tubules[15,32]. It is possible that distinct populations of segment progenitors might be characterized by different marker expression (Table I).

Human studies

In human tissue, using the AC133 antibody, which recognizes a glycosylation-dependent epitope of prominin (CD133), expressed by haemopoietic stem cells[33,34], a discrete population of CD133+ cells was detected in different segments of the nephron, namely the Bowman's capsule, proximal and distal tubules, as well as in the inner medullary papilla region, including Henle's loop and the S3 limb segment[35-42] (Table I). CD133+ cells co-expressed the mesenchymal marker vimentin and cytokeratins 7 and 19, not expressed by the bulk of tubular epithelial cells[37,39] (Table II). In the Bowman's capsule, CD133+CD24+ cells localized at the

urinary pole, whereas cells expressing differentiation markers of podocytes increased toward the vascular pole[40]. In proximal tubules, CD133+ cells were mainly located in the tubular plicae where proximal convoluted tubules make hairpin turns[3], and co-expressed megalin and aquaporin-1[36,41]. In the distal nephron, small clusters of CD133+CD24+ cells were detected in the portion of the distal convoluted tubule that makes contact with the vascular pole of the glomerulus, and these cells also co-stained for the thiazide-sensitive Na/Cl cotransporter[41]. At variance, CD133+ cells in the medulla lacked expression of differentiation markers[36]. Moreover, the CD24+, CD133+, or vimentin-expressing scattered cells observed within the proximal tubules were shown to co-express known markers of scattered cells, such as annexin 2, claudin-3 and 7, S100A6, and CXCR4[37], as well as collagen 7A1 and the tight junction protein claudin-1[3] (Table II). As most experiments have been performed on normal portions of kidneys derived from tumour-bearing patients, it has been suggested that CD133 expression can be induced. Recently, CD133-expressing cells (from 3 to 12%) have also been shown to be present in normal renal tissue of pre-transplant biopsies from native donors of all ages, indicating their presence at a physiological level[3]. However, this result conflicts with the observation that scattered tubular cells are absent in normal juvenile tissue in rodents[37].

A detailed analysis of scattered intratubular cell morphology, by electron microscopy, indicated a striking difference with respect to adjacent 'normal' proximal tubular cells, as they appeared smaller, lacked brush border microvilli and showed fewer mitochondria[37]. The lack of mitochondria in scattered cells has been further confirmed by electron microscopy using gold-conjugated vimentin antibodies, as well as by double staining of renal tissue with CD133 and mitochondrial markers[3]. In the light of the role of CD133 in glucose uptake[42,43], the CD133-expressing population may possess a particular anaerobic metabolism, which could explain their ability to survive following injury[3]. Indeed, Bourseau- Guilmain et al.[42] showed that CD133 inhibits endocytosis of the transferring receptor, thus resulting in inhibition of iron uptake and consequently of mitochondrial activity. In turn, CD133 expression has been related to glucose uptake in glioma cells[43]. Taken together, these data lead to the proposal that CD133 might be involved in the regulation of glycolytic metabolism. Indeed, in an explant model in which human renal tissue was subjected to ischaemia reperfusion damage, the CD133+ scattered cells did not undergo necrosis and remained anchored to the basal membranes, whereas the detached cells were negative for these markers[3]. This observation supports the notion of a physiologically distinct population with high resistance to damage, rather than an injury-induced

population. It does not address, however, whether the CD133+ cells are a fixed population or a transient functional state modulated by microenvironmental stimuli.

RPCs in renal pathology

Whatever their origin, the CD133 population appears to play a role in acute tissue repair of the tubular compartment. Conversely, their activation in glomeruli appears to be related to glomerular diseases[20]. Loverre et al. first reported that CD133+ cells were significantly increased in patients with delayed graft function resulting from an acute injury, compared to the pre-transplant biopsy or to patients with early graft function[44]. Three other studies reported an increase of tubular CD133+ cells with respect to normal tissue, after acute tubular necrosis in transplanted patients or in proteinuric glomerular diseases[3,37,41]. The involvement of these cells in renal repair is strongly supported by the evidence that almost all proliferating cells in acute tubular injury biopsies were CD133/CD24 positive. In addition, these cells co-expressed the KIM injury marker[37]. Interestingly, the number of CD133+ cells in patients with acute tubular necrosis appeared to be a predictive marker of tissue recovery[45].

The presence of cells expressing progenitor markers was also reported in chronic tissue injury. Stripes and clusters of proliferating tubular CD133+ cells were detected in biopsies of patients with chronic tubule-interstitial damage due to urinary obstruction or focal segmental glomerulosclerosis (FSGS), suggesting the activation of mechanisms for tissue repair[3,41]. It would be interesting to understand whether the loss of the regenerative ability in chronically damaged human tissue could possibly be ascribed to cell exhaustion, or to mechanisms of cell senescence. Interesting, blocking the G2/M phase of the cycle was related to a senescent secretory phenotype occurring in tubular cells after repeated injury[46].

The involvement of progenitor-like cells with enhanced proliferative ability also occurs when abnormal proliferation underlines pathological settings, such as in the glomerular tuft during acute glomerulonephritides and in cells forming cysts in polycystic kidney disease. Indeed, proliferation of CD133+ cells was reported in glomerular hypercellular lesions, and (pseudo)crescents leading to Bowman's space obliteration occurring in collapsing glomerulopathy and crescentic glomerulonephritis, and within glomerular tip lesions at the urinary pole of the Bowman's capsule[20,47]. Similarly, in polycystic kidney disease, all cyst-lining cells strongly expressed CD133[48].

1　　　The results of the above-mentioned studies in human renal tissue clearly indicated the

2　existence, in different nephron segments, of cells that can be defined as adult RPCs by a

3　functional definition of progenitor cells, as they are able to proliferate, participate in tissue

4　homeostasis and repair, and survive to injury. In addition, from a phenotypic point of view, they

5　lack a differentiated phenotype and express stem-related markers. It seems plausible that adult

6　human CD133+ RPCs represent the same population described as segment-restricted

7　progenitors in mice. The debate on the fixed nature of renal progenitors or on the induction of

8　their phenotype is of difficult answer, as no lineage tracing can be applied to human studies.

9　However, cellular plasticity is the most likely explanation. This has been suggested for organs

10　other than kidney, such as the lung, bone marrow and pancreas. Indeed, increasing body of

11　evidence supports the argument that the stem/progenitor-related phenotype is not static but

12　rather a dynamic process characterized by continuous cycle and regulated by microvesicle-

13　related microenvironmental stimuli (Quesemberry Leukemia. 2014 Apr;28(4):813-22.).

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16　**B. RPC cultures**

17　　　Most of the data supporting the phenotypic and functional characteristics of RPCs have

18　derived from the analysis of their behaviour and phenotype once cultured and isolated. Many

19　different RPCs with repair ability have been isolated from adult mouse tissue using stem-

20　related characteristics such as marker expression, BrdU label retention, side population

21　labelling or clonogenicity[49-53]. These cell lines showed variability in marker expression, origin,

22　potency and differentiation ability. More homogeneous and unifying results were obtained in

23　humans, using the strategies depicted in the following paragraphs.

24

25　*Isolation and characterization of RPCs from human renal tissue*

26　　　We first isolated and characterized cells expressing the AC133-detected CD133 stem

27　cell marker from the human renal cortex[54]. CD133^{+} cells were subsequently isolated from the

28　Bowman's capsule of human glomeruli[35], as well as from the inner medulla[36,38]. These CD133^{+}

29　populations, isolated from different nephron segments, shared similar phenotypic properties, as

30　they all expressed foetal renal markers, such as Pax-2, Wt-1*,* and CD24, along with several

31　mesenchymal stem cell markers, such as CD29, CD73 and CD90, with minor variations in

32　expression levels, possibly related to cell culture conditions. This was further confirmed by

analysis of a gene expression profile, which clustered CD133+ cells from glomeruli and tubules against epithelial differentiated cells[55]. However, some genes were differentially expressed, including VCAM- 1, also known as CD106, which was expressed approximately 300-fold more in CD133+CD24+ cells obtained from glomerular outgrowths than from tubules[41]. Functionally, CD133+ RPCs were clonogenic, and, most importantly, differentiated into tubular cells of different nephron segments and glomerular podocytes after appropriate stimulation[35,54]. When injected within Matrigel *in vivo* in immunodeficient mice, RPCs also generated tubular structures expressing nephron segment markers[36,54]. In addition, $CD73^+/CD133^+$ RPCs isolated from the inner medulla were shown to synthesize and release erythropoietin under hypoxia[56].

As CD133 cultures were generated by cell sorting, they should represent the CD133 expressing cells in the renal tissue. However, it has been noticed that total cell cultures of kidney cells may express CD133 at fairly high levels[57]. A possible explanation is the selective ability of CD133+ RPCs to survive in culture. Another possibility is that CD133 could also be acquired by tubular cells due to culture-induced dedifferentiation. This has been recently excluded for podocytes that did not participate in the generation of CD133+ cells cultured from urine[58] but it could be a possible mechanism in tubular-derived CD133+ cell culture. In any case, plasticity of cultured CD133+ RPCs may provide insights in the mechanisms of kidney repair and could be exploited for renal regeneration.

Other selection strategies based on functional assays were utilized to identify and select RPCs from human renal tissue. For example, cells were sorted based by means of a functional assay that which evaluates the stem-related activity of ALDH[39]. ALDH^{high} cells expressed CD133 and vimentin appeared were similar in appearance to CD133- sorted cells[39]. Another functional assay commonly used for stem cell isolation is the ability to growth as spheres. The so-called nephrospheres contained cells at different levels of maturation[59]. Selection of the quiescent cells within the nephrospheres using retention of the PKH_{26} dye identified a self-renewing population capable of multiple differentiations into cells with epithelial, endothelial and podocytic characteristics. Interestingly, these cells also expressed a $CD133^+/CD24^-$ phenotype[59].

A different approach was proposed by the group of Dekel, who exploited the neo-expression of the foetal marker NCAM, by a fraction of human renal cells in culture[57]. Indeed, this marker is absent in normal adult renal tissue, but it can be reactivated after ischaemia-

reperfusion injury[60]. NCAM+ RPCs were shown to be enriched in renal progenitor/stem transcriptional factors (SIX2, SALL1, PAX2, and WT1 and Oct4) and to present a mesenchymal phenotype. In addition, NCAM positive cells showed enhanced functional properties related to stemness (clonogenicity, sphere-forming ability and tubulogenic differentiation on single-cell grafting in chick chorioallantoic membranes and mice) [57].

All the described cell lines of human RPCs possess a limited life span and undergo senescence over time. It is reasonable to suggest that no robust, long-term culture system currently exists that is capable of maintaining potent, clonal expansion of adult progenitors over long periods of time under defined conditions.

Isolation of RPCs from urine

It has been shown that freshly voided urine[66] and urine from the upper urinary tract[67] contain multipotent stem cells. In addition, CD133/CD24 RPCs have been isolated and cultured from the urine of normal subjects as well as from patients with kidney disorders[58]. Urine-derived RPCs were similar to tissue-derived RPCs at a phenotypic and functional level, and differentiated into both tubular cells and podocytes. In addition, urine-derived CD133/CD24 RPCs were used for the diagnosis of glomerular genetic disorders[58].

The release of foetal urine into the amniotic fluid represented the rationale in the search for RPCs in the amniotic fluid. Da Sacco *et al.*[68] have successfully isolated, from the amniotic fluid, a sub-population of metanephric mesenchyme-like cells committed to nephron lineages, and capable of differentiating into podocytes.

Urine-derived cells may represent an attractive source of autologous cells for cell therapy. In addition, they could be exploited as diagnostic tool for genetic glomerular disorders.

Isolation of renal mesenchymal stromal cells from renal tissue

Cells with a mesenchymal origin and multipotent properties are known to be present in a population adjacent to endothelial cells of the microvasculature, on the basis of marker expression (CD146+ NG2+ CD140b+), and may possibly contribute to tissue support[61]. Renal mesenchymal stromal cells (MSCs) isolated from the mouse kidney, differentiated *in vivo* into renin-producing cells[62], indicating a commitment towards renal cell types. Interestingly, a recent paper from Little's group showed that MSC-like cells from mouse collecting ducts can be isolated from adult murine kidneys due to a phenomenon of epithelial-mesenchymal transition

1 after long-term culture *in vitro*[63]. The obtained cells give rise to a long-term self-renewing MSC

2 population, which shows the characteristic pan-mesodermal differentiation. In addition, renal

3 MSCs possess the ability to integrate into aquaporin 2–positive medullary collecting ducts

4 when injected into the kidneys of neonatal mice, unlike bone marrow-derived MSCs[64]. The

5 differentiation of renal MSCs into renal tissue is supported by data showing the expression of a

6 selected pattern of genes in renal MSCs, compared with MSCs of marrow origin, possibly

7 related to a memory of tissue origin[64]. This suggests that renal MSCs may display organ-

8 specific regenerative capacities that are highly superior to those of MSCs from unrelated

9 organs, which could thus be exploited for therapeutic applications[64].

10 In human kidneys, resident MSCs were investigated in glomeruli, based on their vascular

11 nature, and characterized as CD133-/CD146+ cells[65]. Glomeruli-derived MSCs displayed self-

12 renewal capability, clonogenicity, and the ability to differentiate into the different glomerular cell

13 types (endothelial, mesangial and epithelial cells). Evidence that glomerular MSCs do not

14 derive from the bone marrow has been provided by detection of MSCs derived from donors in a

15 sex-unmatched transplant[65].

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18 **C. Modulation or induction of an RPC phenotype**

19 *Micro-environmental stimuli for RPC modulation*

20 As described above, the nature of the cells involved in kidney homeostasis/regeneration

21 is unclear, and further studies are required to define the mechanisms involved in their possible

22 activation[69]. According to the idea of a plastic phenotype of RPCs, it can be speculated that the

23 progenitor phenotype could be a consequence of a cell cycle state, or rather it may result from

24 the modulation of growth and differentiation of resident cells by external stimuli. Interestingly,

25 soluble signals released by a diseased kidney were shown to induce the generation of

26 scattered tubular cells in the contralateral kidney in mice, supporting the hypothesis of cell

27 reprogramming by the microenvironment [27,70]. Knowledge of the activating mechanisms

28 involved in the modulation of the RPC phenotype could be relevant, both in terms of a

29 pharmacological approach for local cell modulation, and in terms of possible *ex vivo* treatment

30 of cells before their *in vivo* injection.

A hypoxic environment appears to activate RPC properties. Hypoxia was reported to increase proliferation, clonogenicity and CD133 expression in RPCs isolated from the inner medulla[36]. Indeed, consistent HIF-1 activation was shown in these RPCs, possibly related to the hypoxic condition of the tissue of origin. The mechanism of hypoxia-related acquisition of progenitor properties may rely on the hypoxia-induced expression of the stem cell transcription factor OCT4A and its regulation by micro-RNA 145[36]. In addition, in cortical RPCs, a comparative analysis between RPCs and differentiated tubular cells identified two microRNAs involved in the regulation of stemness and differentiation of RPCs. In particular, miR-1915 targeted the progenitor factors CD133 and PAX2, and miR-1225-5p targeted toll-like receptor 2 (TLR2) expression, which is a key gene of damage signalling, which is up-regulated in RPCs[71]. In addition, Wnt signalling has been linked to RPC maintenance in the mouse, as renal cells with cloning ability showed increased activation of this pathway[2].

RPC generation from iPS or direct generation of inducible-RPC

Recent advances in the stem cell field could facilitate the generation of RPCs with nephrogenic potential (metanephric mesenchyme-like progenitors) from somatic cells, through the generation of inducible pluripotent stem cells (iPS) or directly from embryonic stem cells. The obvious advantage in the use of iPS is the possible generation of autologous cells and the lack of ethical issues. IPS were successfully shown to generate entire renal structures as well as renal organoids (ref). Moreover, two recent investigations refined the methods for human intermediate mesoderm progenitor generation from iPS using small chemical inhibitors, obtaining high efficiency and rapidity[73,74]. Araoka *et al* [73] successfully generated human intermediate mesenchymal progenitors (80% efficiency in 5 days) from iPS using retinoids and an activator of the Wnt pathway. The resulting cells showed the ability to differentiate into multiple cell types, and to form renal tubule-like structures. Similarly, Lam *et al*.[74], by using retinoic acid and Wnt activator plus FGF2, produced intermediate mesenchymal cells within 3 days. In addition, intermediate progenitors, if treated with FGF9 and activin, further differentiated into Six2+ metanephric mesenchyme progenitors. The generation of autologous cells with nephrogenic potential is highly promising in regenerative medicine of the kidney. However, tumorigenic potential of these cells and the scarce proliferation of iPS-differentiated cells still represent a significant limitation for their application.

An alternative approach for RPC generation is the direct reprogramming of renal cells into progenitors. The group of Little successfully identified, by means of sequential screening, a cocktail of six genes (SIX1, SIX2, OSR1, EYA1, HOXA11, and SNAI2) able to reprogram human tubular cells into metanephric mesenchymal progenitors[75]. These progenitors showed the capacity to contribute to the nephrogenic compartment of a developing embryonic kidney *ex vivo*. Although this study represents a huge step towards the generation of progenitors for *in vivo* use, improved optimization of transfection, including the use of non-integrating or inducible vectors, as well as establishing ideal growth conditions is still required for therapeutic applications.

D. Therapeutic use of RPCs in experimental models of kidney injury

Cell therapy is clearly the most promising tool to provide regenerating cells/stimuli to the renal tissue (Table III, IV and Figure 1). In zebrafish, Diep and colleagues[76] clearly showed that transplanting RPCs with nephrogenic potential generated multiple nephrons. In the fish the mesenchymal nephron progenitor population persists supporting de novo nephrogenesis. In human, this potential is not present in the adult and therefore RPCs do not generate new nephrons, but rather may support the process of nephron repair. However, a mesenchymal nephron progenitor population could facilitate the creation and integration of new nephrons.

Cell therapy with embryonic/ foetal RPCs

Human RPCs isolated from foetal kidneys were shown to exert a therapeutic effect in acute and chronic renal injury. CD24+/CD133+ RPCs isolated from human foetal tissue ameliorated renal function after glycerol-induced AKI[21]. Using this model, the effects were comparable to those of adult RPCs. Human foetal nephron progenitor NCAM+ cells were tested in the 5/6-nephrectomy kidney injury model characterized by loss of renal mass and progression. In this model, cells were administered directly into the renal parenchyma in three consecutive injections, and were shown to engraft and integrate in diseased murine kidneys, with resulting beneficial effects on renal function and arrest of disease progression[19]. Metanephric mesenchyme-like RPCs generated by pluripotent stem cells also showed a

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considerable differentiation potential *in vitro* and *in* vivo in the embryonic kidney[72]. Recently, RPCs derived from human iPSCs were tested in a murine model of AKI. Interestingly, they not only restored renal function but also showed a robust engraftment into damaged tubuli (Imberti).

Cell therapy with adult RPCs

Pioneer experiments in acute models of tubular or glomerular damage showed a potential therapeutic effect of the administration of adult rat/murine RPCs of different type and origin [49,51-53,77] (Table III). Similarly, administering adult CD24+/CD133+ RPCs from the Bowman's capsule, cortical tubular compartment, and inner medulla to immunodeficient mice with rhabdomyolysis-induced AKI was shown to have therapeutic benefits[35,41,54,78] (Table IV). In these experiments, a single dose of CD133+ cells (ranging from $5x10^5$ to $1x10^6$ cells) was administered, after induction of renal damage. This cell therapy improved renal function, as shown by the rapid decrease of creatinine and blood urea, together with amelioration of tissue histology[35,41,54,78]. These results correlated closely with those commonly obtained using MSCs, and comparison of MSCs with medulla-derived CD133+ cells demonstrated an equivalent therapeutic effect[78]. However, a bio-distribution study showed that CD133+ cells preferentially localized to the kidney region, with less cell dispersion in other parenchymal organs, with respect to MSCs, when analysed by optical imaging 48 hours after injection[78]. Similarly, administration of CD133+CD24+ cells isolated from the Bowman's capsule promoted renal amelioration in a model of FSGS, characterized by podocyte injury, reducing proteinuria and improving chronic glomerular damage[40]. This effect was restricted to undifferentiated CD133+CD24+PDX- cells, whereas no effect was observed using partly differentiated CD133+CD24+PDX+ renal cells, or CD133 negative cells[40]. Moreover, CD133+CD24+ cells isolated from urine similarly differentiated into podocytes and reduced proteinuria in the adriamycin model[58].

Using the same glycerol-induced AKI model used for CD133+ RPCs, the therapeutic effect of NCAM+ RPC overexpressed markers of fetal RPCs was compared with that of the total human kidney epithelial cell culture, uniformly positive for CD24/CD133. Both populations displayed a therapeutic effect, with no difference between NCAM+ and NCAM- cells[57].

This observation may suggest a possible effect for cell therapy using renal epithelial cells, as reported below. By analogy mature tubular epithelial renal-like cells, obtained from differentiation of spermatogonial derived embryonic-like stem cells, protected against acute and subsequent chronic damage *in vivo* in a model of kidney ischaemia[79]. However, the he administration of human CD133 negative cells did not provide benefit in tubular or glomerular acute damage[35,40,41]. As the CD133 negative population was not fully characterized, it can not be determined whether the lack of a therapeutic effect may be related to the activity of fibroblasts or rather of renal epithelial cells.

Mechanisms of action of RPCs

An intriguing point of discussion in renal regenerative medicine is the mechanism of effect of the administered cells. While the effect ascribed to MSCs is mainly related to paracrine delivery of soluble mediators and bioactive vesicles containing genetic material (mRNA and microRNA),[80] mechanisms of both paracrine stimulation and tissue integration have been described for human RPCs. Detection of the injected adult RPCs in the kidneys of AKI mice was reported in both proximal and distal tubules[35,41,54,]. Analysis of cell integration after 11 days showed that around 7% of the total number of tubular cells co-expressed the labelling dye and tubule markers[35]. Similarly, in a model of podocyte damage, CD133+CD24+PDX- cells generated new podocytes, representing of all podocytes after 7 days 11.08 ± 3.3%, and maintaining their engraftment after 45 days[40]. Interestingly, the ability to differentiate into podocytes was restricted to Bowmann's capsule isolated RPCS, that also differentiated into tubular cells, whereas tubular derived RPCS cells did not[41]. However, RPCs isolated from inner medulla showed a limited persistence within the murine tissue at 1 month after the injection[78]. High tissue integration has been also reported for foetal RPCs directly injected into the renal parenchyma in a 5/6 nephrectomized kidney[19]. Tissue analysis showed three distinct patterns of engraftment: initially, RPCs were located within existing mouse tubules or within the interstitium, and after 3 months they tended to organise into large mature-appearing epithelial tubules with a patent lumen, resembling segment nephrogenesis[19].

In addition, RPCs were described to release a large variety of growth factors and vesicles that may contribute to tissue repair. In particular IL-15, a kidney specific factor involved in renal

differentiation[81], was selectively released by CD133+ cells[78]. RPC-derived extracellular vesicles may also be involved in the process of repair, as they promoted *in vitro* survival and proliferation in target epithelial cells[82].

The data from the literature and also from our laboratory indicate a variable number of RPC engraftment within the tubules. Weather the cell engraftment in the kidney entirely accounts for repopulation of tubular cells is questionable as no more than 10-11% human cells are detected within regenerating tubules and the evidence for their differentiation in mature epithelial cells are weak. The relevance of RPC integration would require dedicated long term experiments. In fact, cell integration both in AKI and in CKD models was assessed only up to 2 weeks. We suggest a combined mechanism of paracrine support and integration in the effects of RPCs observed in pre-clinical studies. It is conceivable that the engrafted RPCs may coordinate the endogenous repair.

It could be hypothesized that a paracrine mechanism, as described for MSCs, could be sufficient for a therapeutic effect in AKI. This was also confirmed for kidney-derived MSCs as the therapeutic effects were recapitulated by administration of kidney-MSC-derived extracellular vesicles[83,84]. Indeed, in acute damage, activation of resident responsive regenerative cells could be sufficient to support repair. At variance, the integration mechanisms of RPCs find a specific need in CKD, as regenerating responsive cells could be exosted and the integration of new functional cells to regain function would be required.

Therefore, in acute settings, the use of MSCs appears clinically more applicable than that of RPCs, which show limited cell sources and expansion, undefined culture conditions, and poorly accessible or inadequate autologous tissue. In chronic settings, RPCs that are committed to the generation of nephron cells would represent the ideal cell type to obtain clinically-relevant beneficial effects. In addition, the use of patient-derived RPCs, from tissue or from urine, could have significant relevance in CKD as a possible autologous therapy that does not require immunosuppression. It would be of interest to test the effect of adult RPCs in a model of CKD characterized by nephron loss such as the 5/6 nephrectomy used for foetal RPCs[19]. In addition, data on the application of RPCs of adult and foetal sources should be obtained, and comparatively analysed.

1   Differential results may also relay on different modalities of cell delivery. Is it well

2   established that i.v. injected cells may be entrapped in the lungs and liver and thus release

3   pacrine factors from this distal sites. In addition, i.v. or intra-arterially delivered cells need to

4   overcome two basement membranes to reach the tubular compartment. This appears

5   achievable in acute injury, when those barriers are partly damaged due to tissue injury. At

6   variance, during chronic injury, an intra-parenchymal application may be necessary for

7   localization of injected cells within the nephron. Indeed, intra-parenchymal delivery was chosen

8   to administer RPCs or differentiated tubular cells in models of murine CKD (Table III and IV).

9   This route of administration presents the advantage to require a low number of cells and to

10   avoid cell dispersion in undesired organs. In contrast, it might be associated with adverse

11   events such as local necrosis, thrombosis and limited distribution of the cells within the kidney.

12   *Cell therapy with adult renal-derived cells*

13   Interestingly, a therapeutic effect of administering adult differentiated cell populations

14   isolated from normal kidneys has been reported by a number of studies. Human renal cultures

15   enriched in erythropoietin-positive cells were administered to the renal parenchyma in a model

16   of chronic renal damage[85]. Results showed that these cells improved renal function and

17   reduced renal fibrosis and inflammation, unlike erythropoietin-depleted cells. However, cells did

18   not survive up to 12 weeks, suggesting a rapid paracrine effect. Other studies indicated the

19   therapeutic effect of a tubular cell-enriched renal cell population[86]. Indeed, intra-parenchymal

20   injection of isolated and cultured epithelial cells to mice with established chronic kidney injury

21   improved renal function, extended survival and reduced the severity of kidney tissue pathology.

22   This epithelial cell subpopulation was mainly composed of cells from the tubular and collecting

23   duct and was depleted of glomerular, vascular and erythropoietin-producing cells. Similarly, a

24   rat tubule-enriched epithelial cell population promoted regeneration in diabetic rats when

25   injected intra-parenchymally, and stabilized disease progression up to 1-year post-treatment[87].

26   The mechanism ascribed to the therapeutic effect of the intra-parenchymal injected cells in

27   CKD mice kidney was the activation of a regenerative response process. In particular, after 3 to

28   6 weeks, a number of genes were upregulated including the pluripotency-associated SOX2

29   gene and the RPC gene CD24[88]. These data could possibly support the regenerative capacity

30   of differentiated cells, as proposed by Kusaba et al[26].

The strategy of intraparenchymal injection of adult renal cells is under investigation in a phase I trial in human patients suffering from CKD, using expanded autologous renal cells (Neo-Kidney Augment™ Cellular Therapy; ClinicalTrials.gov Identifier: NCT01846715). Human cells were isolated by biopsies from patients and expanded following a described protocol that allowed generation of an injectable large number of cells[89]. The obtained bioactive renal cells embedded into gelatine hydrogels were intra-parenchymally administered to patients (5 to date). The results of this trial have not yet been published. It will certainly be of interest to acquire knowledge on the mechanisms involved in the therapeutic effects of the administered cells, taking into account a possible, although limited, cell dedifferentiation into RPCs.

Conclusions and perspectives

While foetal cells with nephron-forming activity have been characterized in terms of molecular profile, the nature of the cells involved in kidney homeostasis/regeneration is unclear[67], and further studies are required to understand the mechanisms involved in their possible activation. Adult human RPCs isolated from different nephron compartments appear to display progenitor-like properties, and provided a therapeutic effect in models of acute and chronic renal damage[35,40,41,54,76]. In acute pre-clinical models, their therapeutic efficacy was comparable to MSCs[76], which, in turn, may benefit from more readily-available sources and reduced immune compatibility issues (Figure 1).

Although adult RPCs successfully engrafted within the renal tissue in acute models, their effect in a model of CKD with nephron loss needs to be established. Indeed, as they are committed to the kidney cell progeny, they could obviate the potential development of inappropriate cell types, and they would represent an ideal cell type to obtain clinically-relevant beneficial effects in patients with renal failure. However, the source of these cells is limited and they possess a short life span, undergo senescence over time and have a restricted nephrogenic potential.

Foetal-like RPCs with nephrogenic potential seem to be the ideal cell type for cell-based therapy in CKD. The in vitro generation of renal progenitors by iPS differentiation is of particular interest to overcome ethical issue and to get autologous tissue. However, it not only requires

complex and long differentiation passages, but it also has the limitation that once differentiated, progenitors do not proliferate in culture. The direct reprogramming of renal cells into induced RPCs may overcome this issue and generate expandable progenitors with high transfection efficacy. However, at present, no long-term culture system has proven to be capable of maintaining potent expansion of human adult progenitors over an extended period. Finally, adult renal cells unexpectedly appeared to be of therapeutic potential, and could be considered as a possible simple alternative for kidney regeneration. Increasing information on the nature of this population, including the presence of RPCs, and on the mechanism of action, is required.

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Key points: RPC characterization

- Embryonic progenitors represent the prototype of the renal cell population, with nephrogenic potential towards all the different types of nephron epithelia.

- In the adult kidney, the nephrogenic mesenchymal progenitor population is absent.

- Adult RPCs appear as segment-restricted progenitors. Whether they are a fixed population or rather a transient functional state modulated by microenvironmental stimuli is still unclear.

- Human CD133+ cells with phenotypic and functional progenitor-like properties are present in all nephron segments as scattered distinct cells with a dedifferentiated phenotype, and survive and proliferate after damage.

- Adult RPC cultures have been obtained by different nephron compartments or by urine and show homogeneous characteristics *in vitro* although distinct integration potential in pre-clinical models, possibly due to segment commitment.

- All the cell lines of human RPCs possess a limited life span and undergo senescence over time.

Key points: Acquired knowledge and limitations of RPC therapy

- **Pro**: Concerted efforts have been employed in developing the technology to generate metanephric mesenchyme progenitors by differentiation of pluripotent stem cells or by direct reprogramming of renal cells.
  Con: Maldifferentiation, oncogenic potential and expansion limits may impair their application.

- **Pro:** Adult human RPCs show therapeutic potential in acute tubular and glomerular damage due to both paracrine mechanisms and differentiation.
  Con: No differences are observed in pre-clinical models with respect to MSCs, although these cells lack integration potential. MSCs may benefit from more readily-available sources and reduced immune compatibility issues.

- **Pro**: Adult and foetal RPCs show long-term engraftment in glomeruli and tubules, in pre-clinical models of CKD and possibly a low risk of maldifferentiation. The generation of

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patient-derived RPCs could have significant relevance in chronic settings as a possible autologous therapy that does not require immunosuppression.

Con: Knowledge of the differentiation, integration potential and persistence of the different cell types is still required.

- **Pro**: Adult sources of renal cells were shown to be beneficial in CKD and are currently undergoing a phase 1 clinical trial using an intra-parenchymal injection of autologous cells.

  Con: Cells derived from CKD patients may lose regenerative ability, and additional reprogramming technology could be required. Mechanisms involved should be investigated more fully.

- The administration modalities for cell therapy with RPCs needs to be defined. As tubular localization requires the passage trough glomerular or tubular basal membrane, a local administration appears more feasible.

  Pro. Reduced cell number required, reduce extra-renal localization

  Con. Possible local damage (necrosis or thrombosis), local rather than general diffusion of cells.

Table I. Identification of RPCs in renal rodent or human tissue using different markers or functional tools. Localization within nephron districts and evidences of *in vivo* function are indicated.

	Identification tool	Tissue location	Tissue function	*In vitro* culture	Refs
Murine/rat tissue	Scattered cells in PEC-rtTA mice	BC, PT, DT	Proliferation after injury	n.d.	27,28
	Sca-1	interstitium	-	multipotency	53
	BrdU retention (short chase)	PT, TL-HL, DT, CD	Proliferation after injury	epithelial diff.	29
	BrdU retention (long chase)	papilla	Proliferation/ migration after injury	multipotency,	30
	Side population	PT	-	multipotency	52
	Lgr5	TL-HL and DT	-	n.d.	15
	nFatC1+	PT	apoptosis-resistance proliferation and repair	n.d.	27 31
	c-kit	TL-HL and DT		multipotency	32
human tissue	CD133+/CD24+	BC, PT, HL, DT	apoptosis-resistance proliferation	multipotency	3, 36-41

Abbreviations: BC: Bowmann's capule, PT: proximal tubules, TL-HL: thick segment of the Henle's loop, HL: Henle's loop, DT: distal tubules, CD: connecting ducts.

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4 **Table II**. Localization of CD133+ RPCs in different nephron districts, and marker co-
5 expression.

Nephron district	Tissue location	Markers	Co-expression of scattered cell markers	Co-expression of segment markers	Res
Bowmann's capsule	Tubular pole	CD133+/CD24+/ PDX -	Vimentin Collagen 7A Claudin-1	no	21 39 40
Proximal Tubules	Tubular plicae	CD133+/CD24+/C D106+	Vimentin CK7, CK19 Collagen 7A Claudin-1, 3 and 7 annexin 2, S100A6 CXCR4	Megalin Aquaporin-1	3 36 37 39 41
Henle's loop	Segments and loop	CD133+ Nestin+	-	no	36 38
Distal tubules	In contact with the glomerular vascular pole	CD133+/ CD24+	-	Thiazide-sensitive Na/Cl cotransporter	41

6 Abbreviations: PDX: podocalixin, CXCR4: C-X-C chemokine receptor type 4 , CK: cytokeratin.
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3 **Table III.** Cell therapy using rodent renal-derived cells in pre-clinical models of AKI and CKD.
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	Cell type	Model	administration	Benefit	Mechanism	ref
adult RPCs	S3-derived tubular RPCs	IRI / cisplatin	subcapsule	Improvement of renal function	Tubular integration/ Paracrine	49, 75
	Multipotent RPCs	IRI	intra-arterially	Improvement of renal function	Tubular integration No paracrine	51
	Tubule-derived side population cells	adriamycin	I.V. and intra-parenchymal	Reduced albuminuria	Paracrine	52
	kidney-derived MSCs	IRI	I.V.	Improvement of renal function and of angiogenesis	Paracrine EV release	83, 84
	Sca-1 interstitial cells	IRI	Intra-parenchymal	Improvement of renal function	Tubular integration	53
Adult renal cells	Human EPO+ cells	IRI and gentamycin	Intra-parenchymal	Improvement of renal function- Reduction of fibrosis	Paracrine	85
	Rat renal cells	5/6 neph.	intra-parenchymal	Reduction of disease progression	Tubule Integration/ Paracrine	86
	Rat renal cells	Diabetes	intra-parenchymal	Reduction of disease progression	Tubule Integration/ Paracrine	87
ESC-differentiated cells	Tubular differentiated-spermatogonial stem cells	IRI	Intra-parenchymal	Improvement of function- reduced development of fibrosis	Paracrine	79

5 Abbreviations: MSCs: mesenchymal stromal cells, IRI: ischemia-reperfusion injury, 5/6 neph.:
6 5/6 nephrectomy, EPO: erythropoietin, I.V.: intravenously.
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Table IV. Cell therapy using human renal-derived cells in pre-clinical models of AKI and CKD.

	Cell type	Model	administration	Benefit	Mechanism	ref
Foetal RPCs	Foetal CD133+/CD24+ RPCs	AKI (glycerol)	i.v.	Improvement of renal function	Tubular integration	21
	Fetal NCAM+ RPCs	CKD (5/6 neph.)	Intra-parenchymal	Prevention of disease progression	Tubular integration	19
Adult RPCs	Bowmann's capsule CD133+/CD24+/PDX- RPCs	FSGS (adryam.)	i.v.	Improvement of function-reduced fibrosis	Podocytic/ Tubular integration	40
		AKI (glycerol)	i.v.	Improvement of function	Tubular integration	35
	Cortical CD133+/CD24+/ CD106+ RPCs	FSGS (adryam.)	i.v.	Improvement of function	Tubular differentiation only	41
		AKI (glycerol)	i.v.			
	Cortical CD133+ RPCs	AKI (glycerol)	i.v.	Improvement of function	Tubular differentiation	54
	Medullary CD133$^+$ RPCs	AKI (glycerol)	i.v.	Improvement of function	Tubular integration/ paracrine	36,78
	Urine CD133+/CD24+ RPCs	FSGS (adryam.)	i.v.	Improvement of function	Podocytic/ Tubular integration	58
	NCAM+ RPCs	AKI (glycerol)	i.v.	Improvement of function	-	19
Adult renal cells	Renal CD24-/CD133- cells	FSGS (adryam.)	i.v.	No improvement of renal function	-	35
		AKI (glycerol)	i.v.			40 41
	Bowmann's capsule CD133+/CD24+/ PDX+ cells	FSGS (adryam.)	i.v.			40
	NCAM- cells from total epithelial cell culture	AKI (glycerol)	i.v.	Improvement of function	-	19
iPS-derived RPCs	iPS-derived RPCs	AKI (Cisplatin)	i.v.	Improvement of function	Tubular differentiation	ref

Abbreviations: PDX: podocalixin, FSGS: focal segmental glomeruloscerosis, induced by administration of adriamycin (adryam.). 5/6 nephrectomy: 5/6 neph. IRI: ischemia-reperfusion injury.

Legend for Figure

Figure 1. Potential strategies of cell therapy for kidney regeneration. Abbreviations: GPSC, germline cell-derived pluripotent stem cells; iPS, induced pluripotent stem cells; ESC: embryonic stem cells; EPO: erythropoietin.

Biographies:
For each author please provide the correct current main job title and
affiliation for the print version of your article, as well as a brief (just 50-
100 words) biography detailing career, training and research interests for the
online version.

Review criteria:

We searched for original articles in the PubMed database published between 1990 and 2015 containing the serach terms: "renal regeneration", "renal repair", "kidney stem cells", "kidney progenitors", "iPS", "kidney injury", "cell therapy", "preclinical models", "CD133", alone and in combination. All papers identified were English-language, full-text articles. Papers were prioritized on the basis of relevance and we apologize to authors of manuscripts that might have been missed.