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Review

Stem cells from glomerulus to distal tubule: a never-ending story?

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Stem cells: present and future
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Abstract

The growing interest of research in the field of renal stem cells and kidney regeneration aims to get results that allow its clinical application, favoring the birth and development of regenerative medicine.

Nephrogenesis requires differentiation into epithelial cells of a population of progenitor mesenchymal cells. Since this process ends at 36-38 weeks of gestational age, it is quite likely to imagine that such a population disappears in the human kidney after birth. However, several studies have identified in different parts of the adult kidney cells having the characteristics of stem cells that would be involved in renal regenerative processes. They may be classified as resident mesenchymal/epithelial progenitors and often share the same genetic and epigenetic profile as progenitor stem cells active during embryonic life, thus suggesting a common origin.

Current literature includes two lines of thought: one attributes to stem cells a fundamental role in renal regeneration processes while the other sustains the intervention of other mechanisms.

The aim of this review is to report on progress made in research in the field of kidney regeneration starting from the past century and arriving at the present, with an analysis of scientific works that have produced the most important results in this field.

Keywords

Stem cells, CD24, CD133, kidney, regeneration.

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Introduction

Growing interest in renal stem cells (SCs) has to do with their possible role in repair processes subsequent to acute insults to the kidney or chronic renal insufficiency.

The definition of SCs requires the presence of two characteristics: self-renewal, which is to say the capacity to undergo numerous cycles of cell division and remain undifferentiated, and their potential to create different kinds of differentiated cells or their precursors.

Their totipotency, that is, their capacity to differentiate into all embryonic and extra-embryonic tissues, is a prerogative of the earliest cells and progressively decreases in the course of development. Embryonic SCs are those of early derivation which, when isolated and cultivated, are pluripotent: they can differentiate into the three germ layers that make up the embryonic matrix of all body cells.

Adult SCs maintain their characteristics of unlimited self-renewal and are multipotent, being able to differentiate into several kinds of tissue cells while at the same time maintaining a reduced clonogenicity compared to the previous ones. Their direct descendent is a progenitor SC (PSC) that appears more differentiated: it has no or limited self-renewal, but maintains the capacity to generate one or more kinds of cells of the original tissue [1, 2].

SCs are housed in specific, richly vascularized "niches" that modulate their behavior and provide them with nutritional support [3].

Nephrogenesis requires differentiation into epithelial cells of a population of progenitor mesenchymal cells. Since this process ends at 36-38 weeks of gestational age [4, 5], it is quite likely to imagine that such a population disappears in the human kidney after birth. However, several studies have identified in different parts of the adult kidney cells having the characteristics of SCs or PSCs that may be classified as resident mesenchymal/epithelial progenitors which often share the same genetic and epigenetic profile as PSCs active during embryonic life, thus suggesting a common origin [6, 7].

Current literature includes two lines of thought: one attributes to SCs or PSCs a fundamental role in renal regeneration processes while the other sustains the intervention of other mechanisms.

The aim of this review is to report on progress made in research in the field of kidney regeneration starting from the past century and arriving at the present, with an analysis of scientific works that have produced the most important results in this branch.

There was once a kidney unable to regenerate…

Renal regeneration processes consist of the replacement of cells irremediably damaged by a pathological process by others that repair the damaged structures and improve renal functions.

Historically, and for a long period of time, the kidney was considered an organ with a defined number of cells destined to decrease and never able to renew itself following injury.

The first studies in favor of the presence of an elevated regenerative capacity of the kidney date back to the 20th century, but already in 1887 Podwyssozki imagined that an extended cell regeneration process may take place at the level of the renal tubule following a pathological process. He suggested that the regenerated cells came from "within" and derived from the surviving cells [8].

However, SCs were mentioned only in 1928, when Hunter wrote that in the normal adult kidney following acute kidney injury (AKI) caused by mercury chloride the tubules were completely relined by new and atypical cells that markedly differed from the original epithelial cells in size and shape and presented a staining reaction analogous to that of the embryonic epithelium [9]. Reference to the presence of supposed SCs had no follow-up throughout almost the entire 20th century, and most of the literature in the second half of the century contained reports on experiments on humans and experimental animals that described regenerative phenomena following AKI caused by heavy metals or salts. In such reports it is

stated that the renewal process begins with the proliferation of the unaffected cells or those not lethally affected, and that growth factors from systemic or local sources are involved through an autocrine mechanism (action on the same cells that produce them), a paracrine mechanism (action on neighboring cells), or an endocrine mechanism (action on distant cells) [10].

Only in 1994 [11] did a study on an experimental model of AKI advance the hypothesis that the surviving epithelial cells of the renal tubule may dedifferentiate into progenitor cells participating in a genetic program for repair of the damaged nephrons. In this model, vimentin, a marker of the mesenchymal cells, but not epithelial cells, and the proliferating cell nuclear antigen (PCNA), the marker of mitogenesis, were expressed in a large number of surviving renal tubule cells, thus suggesting that tubular regeneration was associated with a dedifferentiation of the epithelial cells into a less differentiated cell phenotype.

Confirmation of this hypothesis came a few years later, in 1999. The same authors found that the neural cell adhesion molecule (NCAM), expressed in the metanephric mesenchyme but not in the adult kidney, was present in large amounts in the proximal tubules five days after post-ischemic hyperfusion of the rat kidney. The molecules slightly expressed in the epithelial cells of the intact renal tubule are thus present in the post-ischemic tubule following hyperfusion and bear witness to the return to a phenotype that goes back to the stage of renal vesicles and presumably mediates the mitogenic response of the dedifferentiated cells and consequently the tubule renewal processes [12].

From the foregoing arose at the beginning of this century the possibility that cells with PSC characteristics may be an active part of renewal processes after a kidney insult, but it was thought that they were not present at first, but derived from processes of dedifferentiation and the return of mature cells to an embryonic phenotype.

While studies from the beginning of this century up to the present do not exclude dedifferentiation as playing an important role in maintaining the integrity of the damaged nephron, they support the hypothesis of SCs with different stages of immaturity and consequently with a different potential for regeneration and differentiation present in other parts of the nephron.

Over the years, the concept of SCs being present in the adult kidney grouped in clusters in specific niches or scattered among the mature epithelial cells has been delineated: physiologically, they may have the task of renewing the cells that conclude their life cycles and, in situations where there is kidney injury with cell necrosis, they may play and important role in kidney renewal phenomena.

Then bipotent renal progenitors arrive… from the glomerulus to the distal tubule

PEC stem cells

Sagrinati et al. (2006) were the first to isolate, by means of specific markers in the kidney of healthy adults, PSCs defined by the expressions CD24 and CD133 among the parietal epithelial cells (PECs) of Bowman's capsule [13]. CD133 is expressed in the hematopoietic cells and different types of adult SCs, while CD24 is a surface antigen expressed in the human metanephric mesenchyme. The former was described in some small groups of SCs in the interstice of the normal adult kidney co-expressed with the fetal kidney embryonic marker PAX2 [14].

The markers CD24+ CD133+ PECs were present in the cytoplasm and membrane of the PECs facing the glomerulus in the urinary pole and were also positive for CD106 (also known as vascular endothelial adhesion protein 1, VCAM1), a surface antigen characteristic of cell elements able to differentiate in mesoderm lines such as the mesenchymal cells or the adult PSCs, besides for other antigens with the same characteristics as CD106, such as CD104, CD54 and CD144; they were instead negative for the endothelial markers and those of the podocytes.

Once purified and placed in culture, these PEC SCs revealed self-renewal and multipotent characteristics since they were capable of generating cells of the proximal and distal tubule and other lines of mesoderm derivation (osteoblasts, adipocytes and cells with neuronal characteristics).

To demonstrate their nature as SCs, the PECs CD24+ and CD133+ (but not CD24- and CD133-) injected into an immunodeficient mouse presenting AKI produced not only regeneration of the renal tubules, but also an improvement in kidney function with the reduction to normalization of the BUN values in the affected mice.

A study performed a short time afterwards (2007) supported the hypothesis of the CD24+ and

CD133+ PECs constituting a niche in the urinary pole of Bowman's capsule as a residue of the initial stages of nephrogenesis [15]. It demonstrated the presence, in the kidneys of fetuses with a gestational age from 7.5 to 17 weeks coming from voluntary abortions, of CD24+ and CD133+ SCs located primarily in the renal vesicles and the S-shaped bodies which later, following the nephron lengthening process, remained limited only to the urinary pole of Bowman's capsule. These were abundantly represented at 8 to 9 weeks only to decrease progressively and leave only a small cluster in the adult.

Once purified, the embryonic CD24+ and CD133+ SCs showed self-renewal and multipotent characteristics: after growing in culture they were capable of producing tubular cells of the different parts of the nephron (including the collector ducts) as well as endothelial and stromal cells, adipocytes and osteocytes. When injected into an immunodeficient mouse with AKI, they showed a major capacity to regenerate the renal tubules than did the adult ones: if the transplantation was composed of embryonic SCs, approximately 15% of the regenerated tubular cells were represented by donor cells, against 6% if the transplantation had been with adult cells. Moreover, if they were injected early they were capable of preventing or limiting kidney injury.

Podocyte stem cells

The results of later studies (2009) led to the discovery that the CD24+ and CD133+ PECs are presumably involved in regeneration phenomena of the glomeruli, and in particular of the podocytes, as well as in those of the tubules [16].

In reality, the study of markers specific to the podocytes (nestin, podocalyxin), has led to the demonstration that the PECs presumably represent a more or less differentiated cell class in a precise sequence in Bowman's capsule and with a different hierarchy. By means of confocal microscopy and flow cytometry it was possible to distinguish three different populations (**Fig. 1**): the first, CD24+ CD133+ PDX-, is found in the urinary pole of Bowman's capsule and does not express podocytary markers (PDX-); the second, CD24+ CD133+ PDX+, located between the urinary and vascular poles, expresses them (PDX+); the third, CD24- CD133- PDX+, situated in the vascular pole of the glomerulus contiguous to the CD24+ CD133+ PDX+ on one side and to the podocytes on the other, has the phenotype characteristics of the differentiated podocytes, since it is without progenitor markers but expresses high levels of podocytary ones.

The PDX- subgroup presents self-renewal and multipotent characteristics since it is capable of producing both podocytes and tubular cells: after injecting it into mice affected by focal segmental glomerulosclerosis (characterized by podocyte depletion and tubular damage) induced by adriamycin, already at 7 days a renewal of kidney function and a reduction of proteinuria was observed and at 28 days the glomerulus and tubulo-interstitial histological picture had greatly improved.

The PDX+ fraction produces podocytary cells only, but it has shown a very low clonogenicity and an inability to repair podocyte damage.

The third population is composed of cells which, like the podocytes, are postmitotic and cannot be cloned or amplified in culture. They, in agreement with previous observations, are analogous to the podocytes in size, shape and phenotype, but their function remains unknown [17, 18].

The CD24+ CD133+ PDX- PECs thus appear to behave as bipotent renal progenitors since they are capable of originating podocytary as well as tubular cells and represent a potential resource for use in repairing glomerulus as well as tubular damage.

Proximal tubule stem cells

In 2011, another important step forward was made by another group of researchers [19] who demonstrated that the CD24+ CD133+ PSCs are to be found also in the proximal tubules of the healthy adult kidney: they are scattered among the mature epithelial cells. This time, the marker used was aldehyde dehydrogenase, already known as a SC marker, [20] and probably involved in maintaining the SCs through conversion of retinol into retinoic acid [21].

In the study, two subgroups of cells positive to the selected marker were identified: one with high and the other with low positivity. The immunohistochemical investigation demonstrated that the first group was composed of scattered epithelial cells CD24+ CD133+. The authors thus hypothesized that these cell elements also derive from the CD24+ CD133+ PDX+ cells of Bowman's capsule and are progenitors both of podocytes of the vascular tubular cells

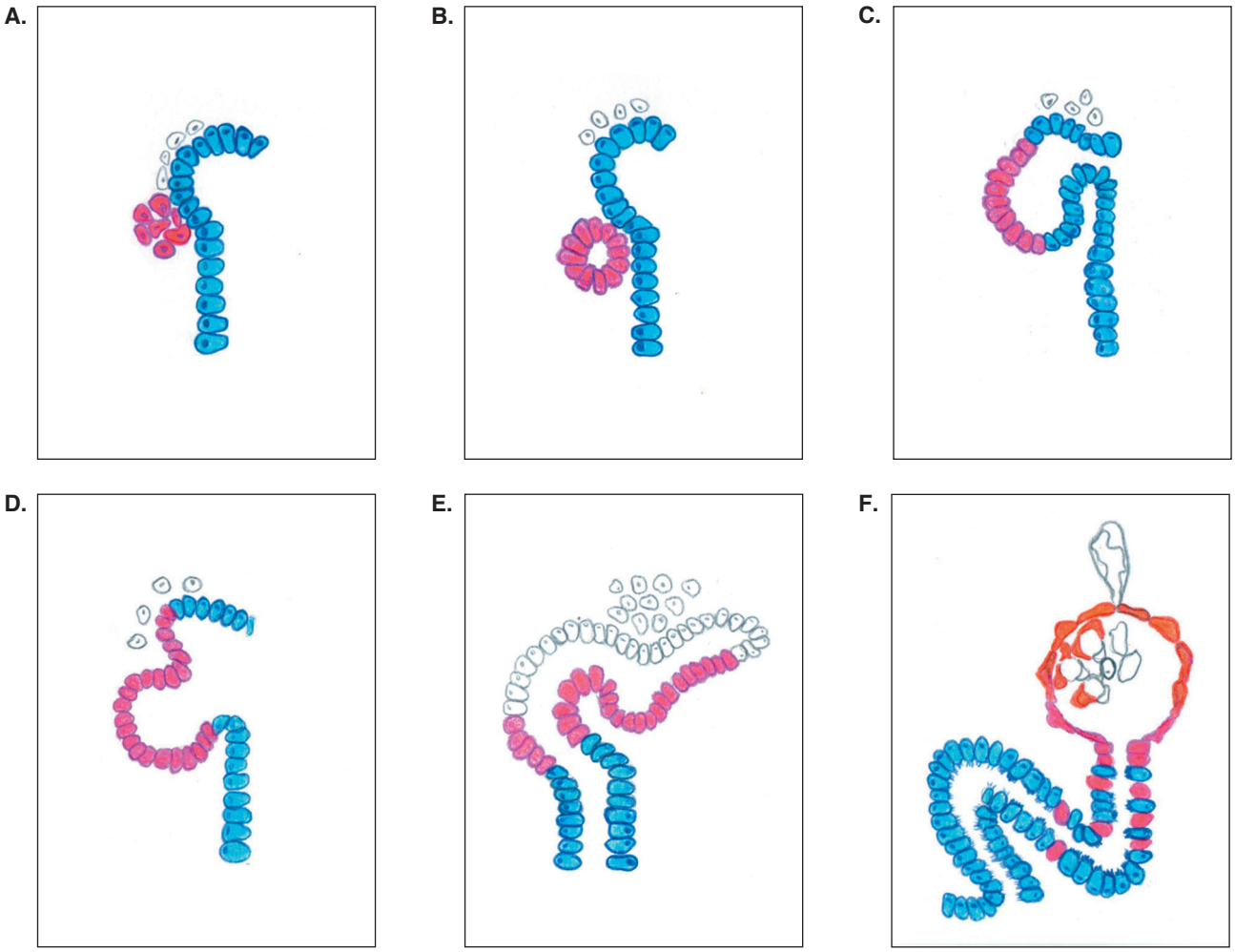

Figure 1. CD24+ CD133+ renal stem cells: from the fetus to the adult: **A-F**. CD24+ CD133+ renal progenitors (pink), located primarily in the renal vesicles and the S-shaped bodies, following the nephron lengthening process, remained limited only to the urinary pole of Bowman's capsule [15] and are represented by only a small cluster in the adult (**A-F**). In the mature nephron (**F**) it is possible to recognize three different populations (hierarchical distribution): the first (pink) CD24+ CD133+ PDX- in the urinary pole; the second (pink/orange) CD24+ CD133+ PDX+ between the urinary pole and vascular pole; the third (orange) CD24- CD133- PDX+ in the vascular pole (not able to be cloned or amplified in culture) [16]. Finally it's possible to recognize scattered CD24+ CD133+ renal progenitors among epithelial cells of the mature prossimal and distal tubules [19, 23].

and the tubular cells with which they are closely contiguous in the tubular-glomerulus junction. In this zone, as hypothesized in previous studies [22] glomerulus-tubular phenotype aspects (i.e. microvilli in the parietal cells) appear to co-exist and the tubular progenitors appear to have aspects intermediate between the renal progenitors of Bowman's capsule and the most highly differentiated tubular cells.

Their clonogenicity appears to be particularly lively in pathological situations involving tubular injury when replacement of necrotic cells is required. In the same study, the histological findings from biopsies of patients presenting acute tubular necrosis in clinical remission showed long lines of CD24+ CD133+ and vimentin+ cells in the context of areas of tubular regeneration, a probable confirmation of the fact that CD24+ CD133+ tubular progenitors are an active part of tubular renewal.

At the same time, the presence of other common markers, both CD24+ CD133+ tubular progenitors and their siblings of Bowman's capsule such as cytokeratin 7 and 19 and vimentin, suggest a common phenotype that coexpresses epithelial and mesenchymal markers.

Interesting is the presence in cells with a high expression of the aldehyde dehydrogenase marker of a transcriptional profile associated with resistance to apoptosis, a characteristic

strengthened also by positivity to the antiapoptotic gene *BCL2* also present in the PEC progenitors.

Distal tubule stem cells

Just one more year went by before the usual CD24+ CD133+ SCs gave signs of life in the distal tubule as well.

Angelotti et al. (2012) characterized the CD24+ CD133+ population using CD106 (also known as VCAM1) and succeeded in isolating two subgroups, CD106+ and CD106-, of which the first in the urinary pole of Bowman's capsule and the second not only in the proximal tubule, but also in the convolute distal one and precisely where it is in close contact with the vascular pole [23]. While the former subgroup placed in culture was capable of generating both podocytes and tubular cells, as previously mentioned [13], the latter originated tubular cells alone. Both, when injected into immunodeficient mice with AKI, improved the kidney from the structural and functional standpoints, but only the subgroup CD106- was able to reduce the severity of the renal insufficiency already on the third day, as shown by the lowering of the BUN. This fact is presumably related to the capacity of the CD106+ SCs to act on the glomerulus as well as on the tubular function owing to their characteristic as bipotent progenitors capable of regenerating podocytes and tubular cells: confirmation of this comes from the results of biopsies of patients with acute or chronic kidney injury in which the CD24+ CD133+ CD106- SCs were represented (on the total number of tubular cells) in significantly higher amounts compared to the CD24+ CD133+ CD106- SCs in the tubules with regenerative aspects (22% vs 6% in the acute processes; 26% vs 12.3% in the chronic forms).

Referring to the work by Lazzeri et al. [15], on the basis of which the SC niche of the urinary pole of Bowman's capsules composed of a residue of the primitive nephrogenesis, it is proper to hypothesize that with the progressive lengthening of the S-shaped bodies, some SCs remain included in the proximal and distal tubule (but not in the collector tubules that do not derive from the metanephric mesenchyme) up to complete development and represent the scattered CD24+ and CD133+ tubular progenitors of the mature nephron.

Renal stem cells and their location in the different parts of the nephron are presented in **Fig. 2**.

But are stem cells real?

Although the regenerative competence of the progenitor system, defined by some authors as "renopoietic" [22], appears evident in the many studies conducted, many doubts are still expressed concerning its actual participation in renal renewal, and probably only genetic tagging and tracing techniques capable of following the entire cell line derived from it may provide proof that this population truly performs this task.

Quite recently, scientific studies that use these techniques, not applicable in humans but widely used in experimental animals, have raised these doubts and propose dedifferentiation once again, starting from mature cells as the probable main regeneration mechanism.

Already in 2008 Humphreys et al. stated that tubular renewal takes place only through the cortical nephron [24].

They used a transgenic mouse in which the label of the cells derived from the metanephric mesenchyme that express gene *Six2* remained persistently from their origin in the mesenchymal cup of the embryo to the adult kidney and is inherited by the offspring. In this way all the cell lines from Bowman's capsule to the mouth of the distal tubule in the collector were labeled, while the interstitial cells and the collector tubule were unlabeled since they did not derive from the metanephric mesenchyme. Following induction of AKI, the renewal of damaged nephrons was the task of resident epithelial cells that represent the direct offspring of the embryonic population Six2+, while the contribution of non-tubular cells, including under this name both those of the renal medulla and other extra renal SCs (e.g. hematopoietic cells), the participation of which was supposed in the past, was denied. Whether or not the intrinsic cells are epithelial cells that survived injury or are residual stem/progenitor cells of the Six2+ population of the mesenchymal cup was elucidated in a later study by the same author (2011): using a DNA analog-based lineage analysis capable of following the cell lines deriving from the cortical tubular cells, it was observed that the cell division was casual (stochastic) and more frequent among the mature epithelial cells affected by, but surviving, the pathological process [25]. On the contrary, there was no evidence of PSCs in rapid division. The cells considered responsible for the regeneration were investigated for the marker Kim1 (Kidney

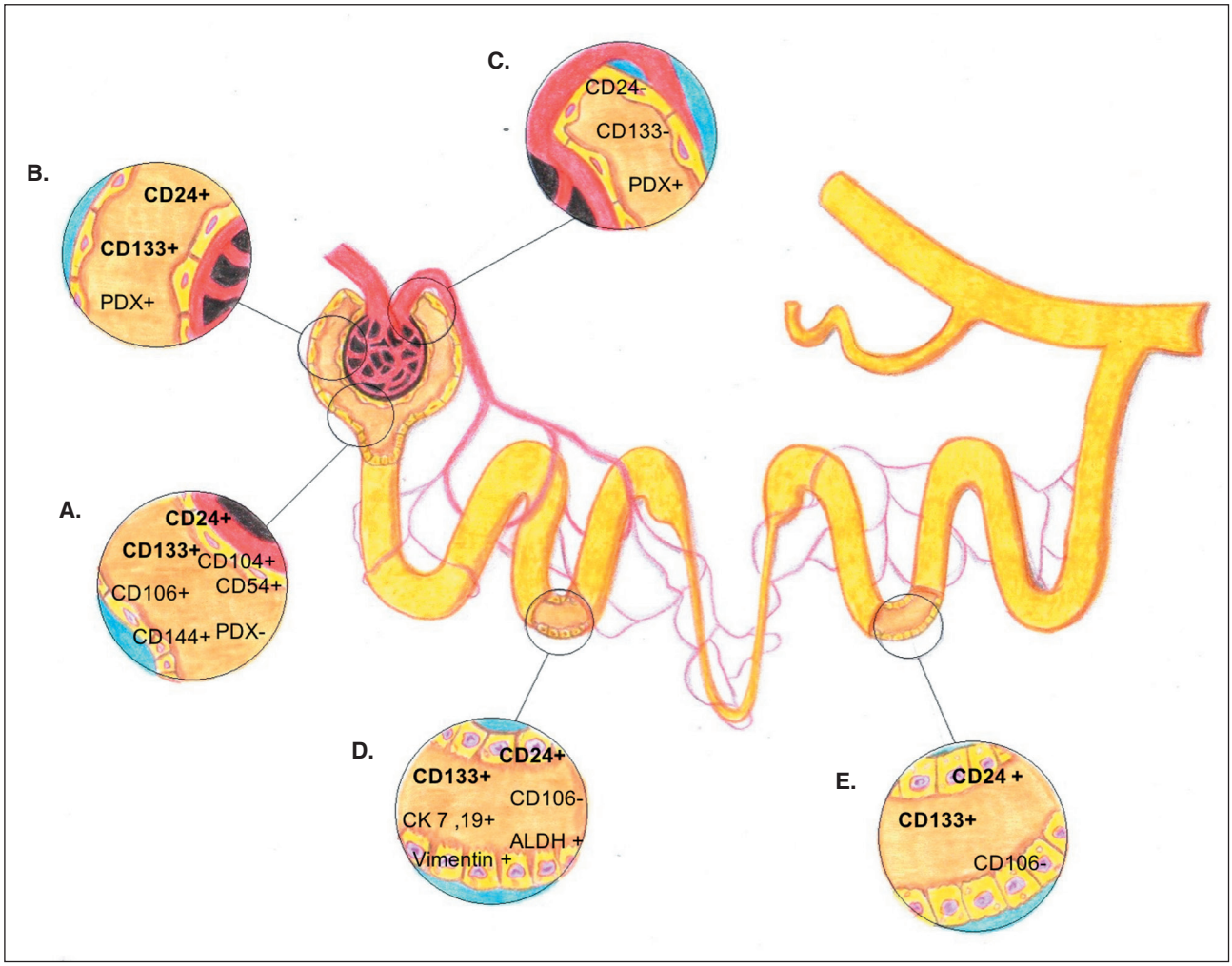

Figure 2. Renal stem cells and their location in the different parts of the nephron. **A.** Urinary pole of Bowman's capsule [17,18]. **B.** Area between the urinary and the vascular pole [17, 18]. **C.** Vascular pole of Bowman's capsule: cells not able to be cloned or amplified in culture [13, 14]. **D.** Proximal tubule [20-22]. **E.** Distal tubule.
CK 7, 19: cytokeratin 7 and 19; ALDH: aldehyde dehydrogenase [13, 15].

injured molecule-1), a receptor strongly induced in the damaged proximal tubular cells, for Pax2, expressed during kidney development and expressed again in the dedifferentiated cells, and for Na/K/ATPase, receptor of the mature tubular cells: while the first two were co-expressed in approximately 75% of the dividing cells, the third was significantly reduced or absent, a demonstration of the fact that most proliferating cells were at the same time damaged and dedifferentiated.

The authors concluded that epithelial proliferation following an ischemic insult takes place owing to the differentiated cells, and prevalently those that survived the damage.

Berger et al. [26], using a transgenic mouse specific for PECs (PEC-rtTA mouse), obtained the marker also of a population of rare scattered tubular cells (STC) located nearby, to demonstrate their common transcriptional origin. The marked STCs increased significantly following ischemic insults, thus showing a high proliferative index: they could not represent a pool of stable SCs since they were no longer present on recovery.

A very recent work by Endo et al. [27] agrees with the previous ones since with another transgenic mouse model (*Ndrg1*$^{CreERT2/+}$*:R26ECFP*) it showed that the proximal tubules were repaired by their very proliferation: the novelty of this report lies in having proved that tubule regenerative capacity is insufficient to prevent the CKD that often follows AKI. To this end, they employed the complexity index [28] of the proximal S-shaped tubule (relationship between the tubular sections and the number of glomeruli in the histological samples) and found it significantly reduced, thus indicating

that the renewed tubules were considerably shorter since their regenerative capacity failed to repair the injury completely.

A further advance in research in recent years is represented by the finding of markers considered those of the SCs in the terminal tubular cells proliferating after AKI. On continuing their previous studies, Humphreys' group [29], by means of a marker (SLC34a1) present only at the apex of the differentiated terminal tubular cells (and absent in the undifferentiated SCs), succeeded in finding the presence of labeled cells in segments S1, S2 and the first part of S3 of the tubule. The subsequent clonal analysis performed in the different AKI phases showed that they had contributed only to regeneration and that the self-duplication mechanism was the only one involved. The totality of proximal tubular cells were labeled prior to AKI and remained labeled afterwards, despite their abundant proliferation, and this excluded the contribution of SCs. As previously observed [25], the cells proliferating after AKI assumed the expression of vimentin, PAX2 and Kim1, but what is of greater interest in this study is the finding of upregulation of markers CD24 and CD133 revealed by the quantitative PCR of the levels of the corresponding m-RNA. Here, it is to be recalled that owing to the absence of suitable antibodies [30, 31] the study of these markers in the mouse had not been possible up to then.

A year before, Smeets et al. found CD24+ CD133+ cells perfectly assimilable to those described by Lindgren and Angelotti which, in a very small number of "normal" human kidney histological samples (from nephrectomized carcinoma patients), became predominant in biopsies of patients in the reperfusion stage following kidney transplantation or with recurring focal sclerosis of the glomerulus. The STCs co-expressed CD44 and vimentin as well as other dedifferentiation markers and, under the electron microscope, showed a less differentiated phenotype (scarce cytoplasm, few mitochondria, immature brush-border) [32].

In the attempt by the same authors to revive the same cells in rats in a model of AKI determined by monolateral ureteral obstruction, they studied CD44+ and vimentin+ cells, comparing them to human CD24+ and CD133+ (not having the antibodies for these markers): they were present in large quantities in the injured kidney but absent in the healthy one.

The end of the story… but is it really over?

Most scientific papers in favor of the existence of SCs in humans agree that only the results of genetic tagging techniques may confirm their real nature. In the last few years there has been a flurry of studies that employ this technique in experimental animals: they find the presence of proliferating cells only after AKI and not in the healthy kidney and tend to deny the pre-eminent role of SCs in tubular renewal processes.

But what conclusions can we draw? Basically, two possibilities emerge.

1. The first acknowledges a leading role in cell regeneration of a system of progenitor cells that extend from Bowman's capsule to the distal tubule. Such a system is composed of scattered SCs resistant to apoptosis, capable of proliferating, migrating, differentiating and replacing damaged cells. Their presence in small numbers is confirmed by studies on samples of a healthy human kidney [23, 33]. This would be enriched in pathological processes in proportion to the gravity of the injury through an exuberant proliferation of CD24+ CD133+ cells, as observed in biopsies of patients presenting acute and chronic tubular damage [16, 19, 23]. Their provenance from an embryonic cluster left over from the first stages of nephrogenesis, located in the urinary pole of Bowman's capsule, from which they can separate during the elongation of the nephrons, is an interesting hypothesis [15]. Their nature as real SCs is confirmed by their capacity to proliferate in culture and maintain a stable phenotype different from that of the differentiated cells, and to "take root" if transplanted in experimental animals. The improvement in the biopsic picture (presence of clear signs of tubular regeneration, reduction of fibrosis) together with that of kidney function (reduction of BUN, proteinuria, the albuminuria/creatininuria ratio) in the experimental model [15] and their lively representation in human biopsic pictures of AKI or CKD may open up new perspectives in the field of regenerative medicine and favor recovery from acute and chronic diseases [13, 16, 19, 23].
2. The second is based prevalently on experimental genetic tagging studies and suggests that following a kidney injury the cells of the proximal tubular epithelium encounter a dedifferentiation process towards the scattered tubular cell phenotype capable of proliferating, migrating,

differentiating and replacing the damaged cells [25-27, 29]. This phenotype expresses surface markers such as vimentin, annexin A3, src-suppressed C-Kinase substrate, CD144, CD133 and CD24, some of which were defined in the past as the markers of tubular progenitor cells [29]. In reality, they would appear to be reprogramming or dedifferentiating markers of the tubular epithelial cells towards a repairing, antiapoptotic and regenerative phenotype, a condition defined as acquired "stemness" [34]. Every STC then enters the cell cycle and undergoes a limited number of cell divisions to replace the cells previously lost in the course of the renewal process following the injury.

At the end of our story we realize that it is not really the end: other chapters will surely be added, in which the two hypotheses advanced will probably reach a common ground. And we hope that this will be the case since the prodigious commitment in the field of research into kidney regeneration has as its goal the obtaining of results that allow its clinical application, favoring the birth and development of regenerative medicine, of which much is being said but the practical application of which still appears distant.

Declaration of interest

The Authors declare that there is no conflict of interest.

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