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Regeneration of Glomerular Podocytes by Human Renal Progenitc

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Abstract

Depletion of podocytes, common to glomerular diseases in general, plays a role in the pathogenes glomerulosclerosis. Whether podocyte injury in adulthood can be repaired has not been establishe Here, we demonstrate that in the adult human kidney, CD133+CD24+ cells consist of a hierarchic population of progenitors that are arranged in a precise sequence within Bowman's capsule and ex heterogeneous potential for differentiation and regeneration. Cells localized to the urinary pole tha expressed CD133 and CD24, but not podocyte markers (CD133+CD24+PDX− cells), could reger both tubular cells and podocytes. In contrast, cells localized between the urinary pole and vascula that expressed both progenitor and podocytes markers (CD133+CD24+PDX+) could regenerate o podocytes. Finally, cells localized to the vascular pole did not exhibit progenitor markers, but disp phenotypic features of differentiated podocytes (CD133−CD24−PDX+ cells). Injection of CD133+CD24+PDX− cells, but not CD133+CD24+PDX+ or CD133-CD24− cells, into mice witl adriamycin-induced nephropathy reduced proteinuria and improved chronic glomerular damage, suggesting that CD133+CD24+PDX− cells could potentially treat glomerular disorders characteri by podocyte injury, proteinuria, and progressive glomerulosclerosis.

Glomerular diseases account for 90% of ESRD at a cost of $20 billion/yr in the United States.[1] Tl traditional glomerular disease classification encompasses a bewildering array of descriptive patho entities and their clinical counterparts. However, converging evidence suggests that podocyte depl

secondary to toxic, genetic, immune, infectious, oxidant, metabolic, hemodynamic, and other mechanisms, is a common determining factor that can result in a broad spectrum of clinical syndr As long as the podocyte loss is limited, restitution or repair is possible. By contrast, 20 to 40% podocyte loss results in a scarring response, until at greater than 60% podocyte loss glomeruli bec globally sclerotic and nonfiltering. These stages of podocyte depletion are accompanied by corresponding degrees of proteinuria and, as an increasing proportion of glomeruli become involv by measurable reduction in the clearance function of the kidney.[1–3] To what extent podocytes can replaced at all during adult life, and if so, how and at what rate, is still unclear. Indeed, mature podocytes have limited capacity to divide *in situ* and display all phenotypic and functional feature highly specialized, terminally differentiated cells.[4,5] A potential mechanism for podocyte replacer might be stem cell migration from the bone marrow, as described in some models.[6–8] However, it been established that the developmental source of podocytes are resident renal progenitors.[4,5,9,10]

Recently, on the basis of CD24 expression, a surface molecule that has been used to identify diffe types of human stem cells,[11–13] CD133 (a marker of hematopoietic and other types of adult tissue cells[13,14]), and the polycomb group protein Bmi-1 (a transcription factor that is critical for maintenance of stem cell self renewal[15,16]), we have identified a subset of cells in adult human ki that exhibit stem cell phenotypic and functional features and can regenerate tubular cells of differ portions of the nephron, *in vitro* and *in vivo*.[17–19] These CD133+CD24+ renal progenitors are physically located within Bowman's capsule, the only place in the kidney that appears to be contig with both tubular cells as well as glomerular podocytes. In this study, we investigated whether CD133+CD24+ renal progenitors can also regenerate podocytes through their division and migrat along Bowman's capsule toward the glomerular tuft. We demonstrate here that CD133+CD24+ re progenitors are a heterogeneous and hierarchical population of progenitor cells arranged in a preci sequence within the Bowman's capsule of adult human kidneys, which exhibit a heterogeneous differentiative and regenerative potential for either tubular renal cells or podocytes.

RESULTS

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Heterogeneous Expression of Renal Progenitors and Podocyte Markers among Ce Bowman's Capsule

To evaluate the possible existence of a hierarchical relationship between CD133+CD24+ renal progenitors and podocytes, we first analyzed the expression of the renal progenitor markers CD13 CD24; markers that specifically stain podocytes in the context of adult healthy human glomeruli, as nestin;[20] and complement receptor-1 (CR1)[21] or podocalyxin (PDX), which stains podocytes a endothelial cells,[22] in glomerular structures of adult human kidneys by using laser confocal microscopy. On the basis of the expression of CD133, CD24, PDX, nestin, and CR1, we demonst by confocal microscopy that CD133+CD24+ renal progenitors are a heterogeneous and hierarchic population of undifferentiated and more differentiated cells that are arranged in a precise sequenc within Bowman's capsule (Figure 1). Three distinct populations of cells were identified. A subset more undifferentiated cells expressing CD133 and CD24 in the absence of CR1, nestin, and PDX localized at the urinary pole of Bowman's capsule (Figure 1). A transition population expressing

CD133 and CD24, as well as nestin, CR1, and PDX, usually localized between the urinary and the vascular pole (Figure 1). Finally, more differentiated cells expressing neither CD133 nor CD24, b exhibiting the podocyte markers, localized at the vascular pole of Bowman's capsule and were contiguous with fully differentiated podocytes (Figure 1). A list of the different markers of renal c used in the study is given in Table 1.

Figure 1.

Heterogeneous expression of renal progenitors and podocytes markers by cells of Bowman's capsule in adult human kidney. (A) Triple-label immunofluorescence for CD133 (red), CD24 (blue), and PDX (green) showing co-expression of CD133 and CD24 on one subset ...

Table 1.

Different markers of renal cells used in the study and their localization in adult healthy human kidneys

Identification of CD133+CD24+PDX−, CD133+CD24+PDX+, and CD133−CD24−PDX+ Cells in Adult Human Kidneys

To recover each of the three populations, we took advantage of the surface expression by these cel CD133 and/or PDX. PDX is considered a podocyte marker but, at least in human kidney, its expr has also been described in some parietal cells of Bowman's capsule,[23] and on a subset of endothel cells.[22] Therefore, total renal cells were first depleted of CD45+ cells (leukocytes)[17,18] and then analyzed for the contemporaneous expression of CD133, CD24, PDX, and CD31 (an endothelial marker) by triple-label immunofluorescence. FACS analysis demonstrated that CD133+CD24+ ce were 1 to 4% of total renal cells, as previously reported,[17–19] whereas CD133+CD24+PDX+ cell were 0.2 to 0.7% of total renal cells. In addition, none of these two populations expressed the endothelial cell marker CD31.[17,18] These results suggest that CD133+CD24+ cells contain a tran population co-expressing markers of both renal progenitors and podocytes (CD133+CD24+PDX+ a more undifferentiated population that does not express PDX, in agreement with the findings obt by confocal microscopy (Figure 2A). To provide further support to this possibility, we separated P and PDX− cells by immunomagnetic cell sorting, which allowed recovery of the two populations a purity of more than 98% (Figure 2B). The assessment of renal progenitor markers on PDX+ cell revealed that a relevant percentage (4 to 8%) of these cells co-expressed CD133 and CD24. More CD133+CD24+PDX+ cells did not express CD31, whereas they consistently co-expressed the podocyte marker WT-1 (Figure 2B, left). These findings provide additional evidence for the existe of a rare, distinct population characterized by an intermediate phenotype between renal progenitor differentiated podocytes. Of note, CD133+CD24+ cells also represented 1 to 5% of PDX- cells (Figure 2B, right). In previous studies, we demonstrated that the combined surface expression of C and CD24 in healthy adult human kidneys is a selective property of renal progenitor cells of Bowm capsule.[17–19] Thus, CD133+CD24+PDX+, CD133+CD24+PDX−, and CD133−CD24−PDX+

populations were directly purified from total renal cell suspensions by means of immunomagnetic techniques and analyzed by real-time quantitative reverse transcription PCR (RT-PCR). CD133+CD24+PDX− cells expressed the renal progenitor markers CD133 and Bmi-1,[17–19] but n podocyte-specific markers nephrin, podocin, and Wilms' tumor antigen-1 (WT-1). CD133+CD24+PDX+ cells expressed both renal progenitor and podocyte markers. CD133−CD24−PDX+ cells did not express renal progenitor markers but high levels of podocyte-specific transcripts (Figure 3, A through C). Taken together, these results suggest that CD133+CD renal progenitors represent a heterogeneous population, including a subset of cells (CD133+CD24+PDX+) that displays initial signs of podocyte commitment.

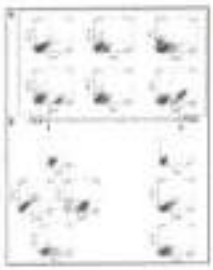

Figure 2.

Identification of CD133+CD24+PDX−, CD133+CD24+PDX+, and CD133−CD24−PDX+ cells in adult human kidney cell suspensions. (A) After depletion of CD45+ cells, total renal cells ...

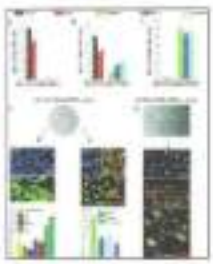

Figure 3.

Phenotypic characterization and distinct differentiative properties of clonal progenies of CD133+CD24+PDX− and CD133+CD24+PDX+ cells. Assessment of mRNA levels for CD133, Bmi-1, nephrin, WT-1, and podocin ...

Clonally Expanded CD133+CD24+PDX− Cells Generate *In Vitro* Tubular Cells and Podocytes, whereas CD133+CD24+PDX+ Cells Can Generate Only Podocytes

The functional characterization of the three populations provided definitive evidence that CD133+CD24+PDX− cells represent a multipotent population, whereas CD133+CD24+PDX+ ce represent transition cells showing features of podocyte progenitors and the CD133−CD24−PDX+ are fully differentiated podocytes. Indeed, CD133+CD24+PDX− cells could be cloned, maintaine culture, and expanded in a medium that allows the growth of undifferentiated renal progenitors [microvascular endothelial growth medium (EGM-MV)].[17–19] More importantly, clonally expand CD133+CD24+PDX− cells could not only be differentiated toward the tubular lineage if cultured the tubular differentiating medium [renal epithelial cell growth medium (REGM+HGF)],[17,18] but toward the podocyte lineage if cultured in the podocyte-maintaining medium, VRAD (Vitamin D3 retinoic-acid-supplemented DMEM)[24] (Figure 3D). Differentiation toward tubular cells resulted i acquisition of binding properties of Lotus tetragonolobus agglutinin (LTA), a specific property of proximal tubular epithelia (Figure 3D). Real-time RT-PCR demonstrated strong upregulation of ot markers of different portions of the nephron, such as aminopeptidase A and the Na/Gluc1 cotransporter, γ-glutamyltransferase, the Na/H exchanger, aquaporin-1, aquaporin-3, or the thiazic sensitive Na/Cl transporter (Figure 3D). By contrast, when the same clones of CD133+CD24+PD cells were cultured in VRAD, they began to express the podocyte markers nephrin, WT-1,

synaptopodin, podocin, PDX, and anti-glomerular epithelial protein 1 (GLEPP-1) at both mRNA protein levels, as demonstrated by real-time RT-PCR and confocal microscopy, respectively. Differentiation of CD133+CD24+PDX− cells associated with a progressive downregulation of CI expression, as already reported.[17] Of note, when single cell suspensions obtained from ten clones recloned by limiting dilution, the resulting second-round subclones displayed the same capacity fo multidifferentiation as the original clone, providing additional evidence that CD133+CD24+PDX- cells are indeed multipotent and exhibit self renewal in culture. On the other hand, CD133+CD24+PDX+ cells could not be expanded or cloned in EGM-MV, whereas if plated in VI medium they generated clones comprised of a maximum of 800 to 1000 cells that could never be subcloned, suggesting that these cells did not display self-renewal capacities. Confocal microscop real-time RT-PCR demonstrated that CD133+CD24+PDX+ clones showed the phenotype of trans cells co-expressing CD133, CD24, WT-1, synaptopodin, GLEPP-1, and nephrin (Figure 3E). Accordingly, clones could never be obtained from CD133+CD24+PDX+ cells in the presence of REGM+HGF medium, suggesting that these cells cannot generate tubular cells. Finally, CD133−CD24−PDX+ were unable to generate clones even in VRAD medium and survived in cul for only a few days, further confirming their nature as terminally differentiated cells. These findin suggest that CD133+CD24+PDX− cells in Bowman's capsule represent an uncommitted populatio cells with extensive self-renewal potential that can generate both tubular cells and podocytes. By contrast, CD133+CD24+PDX+ cells do not display the potential to differentiate in tubular cells, suggesting that they represent a progenitor already committed toward the podocyte lineage.

Only CD133+CD24+PDX− Cells Reduce Proteinuria and Improve Glomerular and Tubular Injury in Severe Combined Immunodeficiency Mice Suffering from Nephro Syndrome

The ability of CD133+CD24+PDX− and CD133+CD24+PDX+ cells to regenerate injured renal c was then assessed in a model of adriamycin-induced renal injury, which resembles focal segmenta glomerulosclerosis, a human disorder characterized by podocyte depletion and tubular damage.[25] this end, CD133+CD24+PDX−, CD133+CD24+PDX+ cells, or saline were injected into adriamy treated Severe Combined Immunodeficiency (SCID) mice. As an additional control, adriamycin-treated SCID mice were injected with a mixture of CD133−CD24− renal cells. Because persistent podocyte depletion induces proteinuria, urinary albumin/creatinine ratio levels were measured in mice 7 d after adriamycin injection. Mice with adriamycin-induced nephropathy showed high urir albumin/creatinine ratio levels that were unaffected by injection of saline, CD133−CD24− cells, o CD133+CD24+PDX+ cells (Figure 4A). By contrast, injection of CD133+CD24+PDX− cells stro reduced proteinuria, as reflected by significantly lower urinary albumin/creatinine ratios (Figure 4 Similar results were obtained when single clones of CD133+CD24+PDX− cells were used (Figur Because adriamycin-induced nephropathy is a chronic disorder characterized by persistent protein and progressive glomerulosclerosis with tubulointerstitial injury, we also tested the effect of CD133+CD24+PDX− cell treatment over long periods of time. As shown in Figure 4B, treatment repeated injections of CD133+CD24+PDX− cells significantly reduced urinary albumin/creatinin ratios at all time points analyzed. In chronically injured kidneys, the improvement of proteinuria induced by injection of CD133+CD24+PDX− cells was also associated with reduced glomerular

tubulointerstitial injury, as demonstrated in periodic acid-Schiff (PAS)-stained sections at day 28 (Figure 4, C through E). In saline-treated mice, glomerulosclerosis was significantly increased in association with reduction of glomerular surface area, whereas relative interstitial volume was expanded. Moreover, the degree of tubular atrophy, as characterized by a decrease in the height of tubular epithelial cells, loss of brush border, and vacuolization, was aggravated in the saline-treate group as compared with CD133+CD24+PDX− cells at day 28 after adriamycin injection (Figure 4 through E).

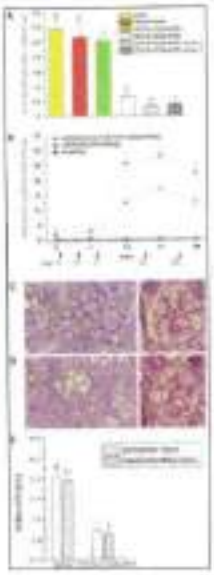

Figure 4.

CD133+CD24+PDX− cells, but not CD133+CD24+PDX+ or CD133-CD24− cells, reduce proteinuria and improve glomerular and tubulointerstitial injury in SCID mice affected by adriamycin-induced nephropathy. ...

CD133+CD24+PDX− and CD133+CD24+PDX+ Cells Exhibit Different Regenerative a Differentiative Potential in SCID Mice Suffering from Nephrotic Syndrome

To further investigate their ability to regenerate injured podocytes and tubular cells, CD133+CD24+PDX−, CD133+CD24+PDX+, and CD133−CD24− renal cells were labeled with red fluorescent dye PKH26 before their injection into adriamycin-treated SCID mice. Labeled CD133+CD24+PDX− cells localized to glomerular structures, where they acquired the podocyte-specific markers synaptopodin, WT-1, nephrin, and podocin (Figure 5, A through F). In addition, double-label immunohistochemistry for human HLA-I antigen and podocin confirmed the engraft of CD133+CD24+PDX− cells into the glomerular structures (Figure 5, G through I). Furthermore relevant numbers of labeled CD133+CD24+PDX− cells were also observed in tubular structures labeled with LTA (Figure 5K). Identical results were obtained when single clones of CD133+CD24+PDX− cells were used. By contrast, when labeled CD133+CD24+PDX+ cells wer injected, only rare red-labeled podocytes were observed (Figure 5L). Finally, red labeling was nev observed in mice injected with CD133−CD24− renal cells (Figure 5M), with saline solution (Figu), or in healthy mice injected with CD133+CD24+PDX− cells (Supplementary Figure 1). Quantita of the number of PKH26-positive cells expressing markers of differentiated podocytes or tubular was performed on sections stained with podocin or LTA, respectively. On day 7 after injury, the number of cells that showed PKH26 labeling was equal to 11.08 ± 3.3% of all podocytes, and to 7 1.9% of all proximal tubular cells in mice treated with CD133+CD24+PDX− cells, whereas no tu cells and only 0.8 ± 0.4% of all podocytes were replaced by CD133+CD24+PDX+ cells. After 45

number of CD133+CD24+PDX− cells engrafted in the kidney of mice with adriamycin-induced nephropathy remained similar (Supplementary Figure 2), thus confirming the different regenerativ differentiative potential of CD133+CD24+PDX− and CD133+CD24+PDX+ cells.

Figure 5.

Distinct regenerative potential of CD133+CD24+PDX−, CD133+CD24+PDX+, or CD133−CD24− cells for podocytes and tubular cells in SCID mice affected by adriamycin-induced nephropathy. (A) Representative ...

DISCUSSION

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Recent insights have defined a unified concept of glomerular diseases in which podocyte injury or is a common determining factor, which suggests the need for rational clinical efforts to allow pod preservation.[1,3] Mature podocytes are postmitotic cells that can undergo DNA synthesis to a limit degree but do not proliferate because they arrest in the G2/M phase of the cell cycle.[1,3] However, most adult epithelia, replacement of damaged or dead cells is maintained through the presence of stem/progenitor cells.[26] Unless the epithelial stem/progenitor cells are permanently damaged, mos epithelia are able to repair their tissues after injuries.[26] Although glomerular disorders represent th most prominent cause of ESRD, remission of the disease and regression of renal lesions have bee observed in experimental animals and even in humans.[27] This shows that remodeling of glomerul architecture is possible, which would imply regeneration of the injured podocytes and reconstituti the glomerular tuft. However, the inability of the podocyte to proliferate and replace injured cells suggests the existence of potential stem/progenitor cells within the adult glomerulus.

In this study, we provide the first evidence that podocytes can be regenerated from a resident population of renal progenitors localized within the parietal epithelium of Bowman's capsule of th human renal glomerulus. On the basis of expression of the renal progenitor markers CD133 and C as well as of the podocyte markers PDX, nestin, synaptopodin, and CR1, we demonstrated by con microscopy and FACS analysis that CD133+CD24+ renal progenitors are a heterogeneous and hierarchical population of undifferentiated and more differentiated cells that are arranged in a prec sequence within Bowman's capsule. A subset of more undifferentiated cells expressing renal prog markers in the absence of podocyte markers localized at the urinary pole of Bowman's capsule. A demonstrated by clonal analysis, these cells could act as bipotent progenitors for both tubular cell podocytes *in vitro* and *in vivo* and exhibited self-renewal potential. A transition population expres both renal progenitors and podocyte markers was localized between the urinary and the vascular p of Bowman's capsule and exhibited differentiative properties only toward the podocyte lineage an limited potential of clonogenicity and amplification (Figure 6). Finally, more differentiated cells, t did not express renal progenitor markers but exhibited high levels of the podocyte specific marker localized at the vascular pole of Bowman's capsule, contiguous to podocytes. These cells shared v podocytes all of the properties of postmitotic cells and could not be cloned or amplified in culture consistent with their nature as terminally differentiated podocytes, thus showing agreement with

previous studies that there are capsular parietal cells localized at the vascular pole of the glomerul analogous in size, shape, and phenotype to visceral podocytes, but whose function and role were unknown.[23,28,29] The discovery that CD133+CD24+ renal progenitors represent a potential sourc podocyte replacement provides the basis for a novel concept that podocyte injuries can be repaire principle by a resident stem cell compartment.[4] In addition, the results of this study provide an intriguing explanation for the genesis of crescents and pseudocrescents, which are known to reflec uncontrolled proliferation of parietal epithelial cells in response to injury.[30,31] Indeed, it is tempti speculate that CD133+CD24+ renal progenitors proliferate in an attempt to replace injured podoc but if regeneration occurs in a dysregulated manner it can generate hyperplastic lesions that can le renal progenitor depletion, scar formation, and nephron loss.

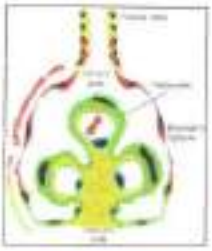

Figure 6.

Schematic representation of the hierarchical distribution of CD133+CD24+PDX− and CD133+CD24+PDX+ cells within human glomeruli. CD133+CD24+PDX− renal progenitors (red) are localized ...

Finally, the demonstration that CD133+CD24+ renal progenitors can regenerate injured podocyte suggests that these cells might provide a novel tool for cell therapy of proteinuric renal disorders. However, only CD133+CD24+PDX− cells displayed the potential to regenerate podocytes and tul cells and functionally improve glomerular injury, which suggests that these cells can behave as bi progenitors. By contrast, CD133+CD24+PDX+ cells did not induce improvement of glomerular function and rarely generated podocytes, suggesting that these cells display a very limited engraft capacity in agreement with their lack of self-renewal potential. Finally, CD133−CD24− cells did engraft within injured human kidneys at all and did not induce functional improvement of glomer injury, which is consistent with their nature of terminally differentiated cells. Taken together, thes results suggest that CD133+CD24+PDX− renal progenitors may be ideal for stem-cell-based kidn regeneration because of their broad differentiation potential, which allows replacement of both podocytes and tubular cells because of their inherent organ-specific identity. Although we cannot exclude the possibility that the engraftment observed might at least in part be related to cell fusion exchange of PKH26 dye between cells, the observation that CD133+CD24+PDX− renal progenit reduce proteinuria is of potential clinical utility. Indeed, several studies have examined the possibi that bone-marrow-derived stem cells might be used for renal repair.[32–36] However, their beneficia can be offset by their abnormal local differentiation into adipocytes accompanied by glomerular sclerosis.[33] In conclusion, the results of this study provide the first demonstration that glomerular injury can be repaired by using resident renal progenitor cells and suggest that the kidney might contain a "renopoietic system" (Figure 6) with a bipotent progenitor localized at the urinary pole Bowman's capsule, where it can initiate the replacement and regeneration of glomerular and tubul epithelial cells.

CONCISE METHODS

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Antibodies

The following antibodies were used: anti-CD24 mAb (SN3), anti-WT-1 mAb (F6), and anti-nephr mAb (C17) (Santa Cruz Biotechnology, Santa Cruz, California); anti-human HLA-I mAb (W6/32 (Sigma-Aldrich, Saint Louis, Missouri); anti-CD133/2 mAb (293C3) and PE-conjugated anti CD1 mAb (293C3) (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany); anti-synaptopodin mAb (C (Progen, Heidelberg, Germany); anti-podocin pAb (Alpha-Diagnostic, San Antonio, Texas); anti-ı pAb (Chemicon, Temecula, California); anti-CD31 mAb (WM-59), anti-CR1 mAb (E11), PE-conjugated mouse anti-IgG2b (MPC-11), and anti-IgG1 (E11) (BD Biosciences, San Diego, California); anti-PDX mAb (222328) and PE-conjugated anti-PDX mAb (222328) (R&D Systems Minneapolis, Minnesota); anti-GLEPP-1 mAb (5C11) (BioGenex, San Ramon, California); and a IgG2a (HOPC-1) and PE-conjugated goat anti rat IgG (H+L) (Southern Biotech, Birmingham, Alabama). Alexa Fluor 633-labeled goat anti-mouse IgG1, Alexa Fluor 488-labeled goat anti-mou IgG2a, Alexa Fluor 488-labeled goat anti-mouse IgG1, Alexa Fluor 488-labeled goat anti-rabbit I Alexa Fluor 546-labeled goat anti-mouse IgG2b, and Alexa Fluor 546-labeled goat anti-mouse Ig were from Molecular Probes (Eugene, Oregon).

Tissues

Normal kidney fragments were obtained from 15 patients who underwent nephrectomy because o renal tumors, in accordance with the recommendations of the Ethical Committee of the Azienda Ospedaliero-Universitaria Careggi in Florence, Italy.

Confocal Microscopy

Confocal microscopy was performed on 5-μm sections of frozen renal tissues, or on cells cultured chamber slides by using a LSM510 META laser confocal microscope (Carl Zeiss, Jena, Germany described.[37] Staining with FITC-labeled LTA (Vector Laboratories, Burlingame, California) was performed as described.[17]

Immunomagnetic Cell Sorting and Flow Cytometry

To obtain PDX+ and PDX− cells, cortex from normal kidney fragments was minced and digested collagenase IV (750 U/ml; Sigma) for 45 min at 37°C, then depleted of leukocytes using anti-CD MicroBeads (Miltenyi), as described previously.[17,18] The CD45− fraction was collected and analy by flow cytometry. PDX+ and PDX− cells were isolated by incubating the CD45− fraction with anti-PDX mAb (R&D Systems) and using rat anti-mouse IgG2a+b MicroBeads (Miltenyi) as the secondary antibody. The separation was performed by magnetic cell sorting using LS columns (Miltenyi), and the efficiency of separation was evaluated by cell labeling with goat anti-rat IgG (PE (Southern Biotech). The recovered fractions were used for flow cytometric analysis. Flow cytometric analysis of surface molecules was performed as described.[17,18] To assess the expressic cytoplasmic WT-1, after incubation with anti-CD133/2 mAb, cells were fixed for 15 min with formaldehyde (2% in PBS), permeabilized with PBS containing 0.5% BSA and 0.5% saponin, an incubated with the specific mAbs. Each area of positivity was determined by gating on the same c

stained with isotype-matched mAbs. A total of 10^4 events for each sample was acquired. Because combined surface expression of CD133 and CD24 in healthy adult human kidneys is a selective property of renal progenitors cells of Bowman's capsule,[17–19] CD133+CD24+PDX+, CD133+CD24+PDX−, and CD133−CD24−PDX+ populations were directly recovered from total cell suspensions. To recover CD133+CD24+PDX+, CD133+CD24+PDX−, and CD133−CD24−P populations, total renal cells were first depleted of CD45 and CD31 and then labeled with PE-conjugated anti-PDX mAb, followed by magnetic labeling with anti-PE Multisort microbeads the anti-PE Multisort Kit (Miltenyi), according to the manufacturer's instructions. To obtain CD133+CD24+PDX+ and CD133−CD24−PDX+ cells, magnetic beads were removed by using Multisort release reagent, and cells were treated with a second magnetic separation for CD133 (Cl Cell Isolation Kit, containing the anti-CD133/1 mAb, clone AC133, also used for hematopoietic s cell sorting). By contrast, to obtain CD133+CD24+PDX− cells, PDX− cells were directly treated a second magnetic separation for CD133. The purified cell fractions consisted of more than 98% (CD133+CD24+PDX+, CD133+CD24+PDX−, or CD133−CD24−PDX+ cells. To recover CD133−CD24− cells, total renal cells depleted of CD45 and CD31 were also sequentially deplete CD133 and CD24 by magnetic cell sorting. The purified cell fractions consisted of more than 99% CD133−CD24− cells.

Cell Cultures and *In Vitro* Differentiation

Cells were plated in EGM-MV (Lonza Ltd., Basel, Switzerland) with 20% FBS (Hyclone, Logan, Utah) or in VRAD medium[24] containing DMEM-F12 (Sigma) supplemented with 10% FBS, vita D3 100 nM (Sigma), and all-trans retinoic acid (100 μM; Sigma). Generation of clones was achie by limiting dilution in 96-well plates and in four-chamber glass slides (VWR International, West Chester, Pennsylvania). Tubulogenic differentiation was induced as described elsewhere with RE((Lonza Ltd.).[17,18] For podocyte differentiation, cells were treated for 3–7 d with VRAD medium.

Real-Time Quantitative RT-PCR

Taq-Man RT-PCR was performed as described.[37,38] CD133, Bmi-1, WT-1, nephrin, podocin, PD Na/Cl transporter, Na/Gluc1 cotransporter, aminopeptidase A, γ-glutamyltransferase, Na/H excha aquaporin-1, and aquaporin-3 quantification was performed using Assay on Demand kits (Applie Biosystems, Warrington, United Kingdom).

Immunohistochemistry

Double immunohistochemistry for HLA-I and podocin was performed as detailed elsewhere.[17,18] Briefly, after a 30-min preincubation with normal horse serum (Vectastain ABC kit), sections wer layered for 30 min with anti-human-podocin pAb, followed by biotinylated anti-rabbit IgG horse antibody, and the avidin-biotin-peroxidase complex (Vectastain ABC kit), and 3-amino-9-ethylcarbazole (AEC) (red color) as peroxidase substrate. Sections were subsequently exposed t anti-HLA-I mAb (Sigma), followed by biotinylated anti-mouse IgG horse antibody and the avidin biotin-peroxidase complex (Vectastain ABC kit). Vector SG (bluish-gray color, Vector Laboratori was used as a chromogen. Colocalization of AEC and vector SG in the same cell resulted in a fina

purple/dark blue color.

Xenograft in SCID Mice Model of Adriamycin Nephropathy

Adriamycin nephropathy was induced in female SCID mice (Harlan, Udine, Italy) at the age of 6 by a single intravenous injection of adriamycin (doxorubicin hydrochloride, 6 mg/kg in PBS, Sigr on day 0 in the tail vein. Animal experiments were performed in accordance with institutional, regional, and state guidelines and in adherence to the National Institutes of Health Guide for the C and Use of Laboratory Animals. On day 1, and again on days 4, 9, 18, and 25 after adriamycin injection, two groups of mice received intravenous administration as follows: group 1, saline (n = mice); and group 2, PKH26-labeled CD133+CD24+PDX− cells (n = 60 mice; 0.75×10^6 cells/d). Twelve mice were killed at each time point after adriamycin injection (day 7, 14, 21, 28, and 45) f each group.

Additional groups of mice were treated with saline (n = 12), CD133−CD24− (n = 6), CD133+CD24+PDX+ (n = 6), CD133+CD24+PDX− (n = 12), or with clonally expanded PKH26-labeled CD133+CD24+PDX− cells (n = 3 mice for each clone) (0.75×10^6 cells/d at day 1 and 4 adriamycin injection). A total number of five distinct clones obtained from three different donors used. Mice were killed at day 7 after adriamycin injection. All of the organs of the mice were exa for cells trapping or engraftment. After injection, a limited number of cells were entrapped in the l whereas no cells were observed in the other organs.

As an additional control, PKH26-labeled CD133+CD24+PDX− cells were injected in healthy mic = 6 mice; 0.75×10^6 cells/d on day 1, and again on days 4, 9, 18, and 25). Mice were killed at day

In all mice, urinary albumin and creatinine in 24-h urine were determined with Albuwell M kit (Exocell, Philadelphia, Pennsylvania) and Creatinine Assay kit (Cayman Chemical, Ann Arbor, Michigan). Normal range of urinary albumin or creatinine in our experiments was calculated in ei additional untreated mice per day. Kidneys were collected from all animals.

Analysis of Renal Morphology

For analysis of mouse renal morphology, kidney sections of 5-μm thickness of animals killed at d were fixed in ethanol and stained with PAS reagent (Carlo Erba, Milan, Italy). Twenty high-powe fields (400×) of renal cortex were randomly selected for assessing tubular (atrophy, casts, and vacuolization) and interstitial changes (fibrosis and inflammation) and graded from 0 to 5 (tubulointerstitial area in the cortex was graded as follows: 0, normal; 1, area of interstitial inflammation and fibrosis, tubular atrophy, and vacuolization involving <10%; 2, lesion area betw 10 and 20%; 3, lesion area between 20 and 30%; 4, lesion area between 30 and 40%; and 5, lesior involving >40% of the field). Fifty randomly selected glomeruli were assessed for glomerular dan (well developed exudative, mesangial proliferation and glomeruli hypertrophy) and graded as foll 0, normal; 1, slight glomerular damage of the mesangial matrix and/or hyalinosis with focal adhes involving <10% of the glomerulus; 2, sclerosis of 10 to 20%; 3, sclerosis of 20 to 30%; 4, sclerosi 30 to 40%; and 5, sclerosis >40% of the glomerulus. All scoring was performed in a blinded mann

Statistical Analysis

The results were expressed as mean ± SEM. Comparison between groups was performed by the Mann-Whitney test or by ANOVA for multiple comparisons (ANOVA for repeated measures), as appropriate. $P < 0.05$ was considered to be statistically significant.

DISCLOSURES

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None.

Acknowledgments

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Notes

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See related editorial, "Parietal Epithelial Cells Regenerate Podocytes," on pages 231–233.

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