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ORIGINAL PAPER

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Tubular cell proliferation in the healthy rat kidney

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Abstract We searched for morphological evidence to support the hypothesis that stem cells are responsible for renal tubular cell proliferation. The rationale of the study was that if proliferation relies on progenitors, mitotically active cells should be less differentiated than their neighbors. As the retention of the thymidine analog BrdU has been the only approach employed to identify stem cells in the kidney up to now we additionally characterized BrdU-retaining cells. Rat kidneys were fixed by perfusion. Cycling cells identified by mitotic figures or the expression of the proliferating cell nuclear antigen (PCNA) were examined by light microscopy and electron microscopy as well as immunofluorescence for four differentiation markers. Newborn rats were injected with BrdU in order to detect label-retaining cells. After a period of 8, 14 and 35 weeks the kidneys were examined for BrdU by immunofluorescence and the four differentiation markers mentioned above. All cycling cells showed the same degree of differentiation compared to non-cycling cells. Most of the detected label-retaining cells were differentiated. We conclude that cycling cells in tubules of the healthy kidney are differentiated and that the retention of label is not a criterion to identify stem cells in renal tubules.

Keywords Kidney · Mitosis · PCNA · BrdU · Stem cells

Introduction

Acute renal failure is a frequent and life-threatening event. In most cases death of tubular epithelial cells constitutes the major structural injury (“acute tubular necrosis”) and is also the main cause of impaired renal function. The factors, which limit healing, are postinjury alterations in renal hemodynamics, tubular obstruction, inflammation, and the rate of regeneration of tubular epithelia (Schrier et al. 2004). As the basic mechanisms of proliferation of tubular epithelial cells are poorly understood, the latter process is the least accessible to therapeutic intervention. The few existing experimental studies on cell division in the nephron examined the regeneration of epithelia in acute renal failure (Bonventre 2003; Ichimura et al. 2004; Imgrund et al. 1999; Maeshima et al. 2003; Megyesi et al. 1998, 2002; Witzgall et al. 1994).

There are two non-mutually exclusive hypotheses concerning renal tubular cell proliferation. On the one hand, differentiated cells might be capable of division. This might require some degree of prior dedifferentiation (Bonventre 2003; Ichimura et al. 2004; Imgrund et al. 1999; Megyesi et al. 1998, 2002; Witzgall et al. 1994). On the other hand, several recent studies suggest a role of stem cells in the generation of new tubular epithelial cells in the adult (Lin and Igarashi 2003). In some studies the stem cells were resident kidney cells (Maeshima et al. 2003; Oliver et al. 2004). Other studies indicated that bone marrow derived cells contributed to the regeneration of the epithelium after tubular injury (Gupta et al. 2002; Kale et al. 2003; Mengel et al. 2004; Morigi et al. 2004; Rookmaaker et al. 2004).

The only established fact concerning cell turnover in the healthy kidney is that it is very slow in the adult (Witzgall et al. 1994). This lack of information is unsatisfying since data on cell division in healthy kidney would represent an important basis for understanding regeneration mechanisms.

The aim of this study was to test the stem cell hypothesis by a morphological approach. Morphology is essentially adequate for the identification of stem cells since these are by definition at a low level of differentiation. Stem cells give rise to the fast cycling transit-amplifying (TA) cells. Regarding the state of

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differentiation, TA cells are intermediates between stem cells and the postmitotic terminally differentiated cells (Alison et al. 2002). Therefore cycling cells, comprising stem cells and TA cells, should be morphologically different from terminally differentiated cells.

Putative renal stem cells have previously been identified on account of their ability to retain the thymidine analog bromo-deoxyuridine (BrdU) (Maeshima et al. 2003; Oliver et al. 2004). These studies assume that cells, which retain detectable amounts of BrdU in their DNA for long periods of time (so-called "label-retaining cells") due to infrequent divisions, represent stem cells. In order to see whether label-retaining cells in renal tubules could be undifferentiated precursors, and thus might qualify as stem cells, we examined their expression of differentiation markers.

The data obtained with the two approaches suggest that cell proliferation in the healthy kidney relies on the division of differentiated cells. They do not provide support for the stem cell hypothesis.

Materials and methods

Unless otherwise stated we used juvenile male Wistar rats 140–150 g body weight (5-week old). They were fed a standard laboratory diet.

For identification of label-retaining cells we used newborn male Wistar rats. Starting at 7 a.m. on day 4 after birth 0.5 mg of BrdU in 0.05 ml of isotonic saline were injected s.c. eight times at intervals of 8 h. Kidneys were perfusion-fixed 4, 8, 14 and 35 weeks later. The experimental protocol was approved by the Cantonal Veterinary Office of Zurich.

Fixation and tissue treatment

The rats were anesthetized by an i.p. injection of pentobarbital (100 mg/kg body weight) and fixed by vascular perfusion (Dawson et al. 1989). The fixative contained 3% paraformaldehyde (PFA), 0.01% glutardialdehyde (GA) and 0.5% picric acid, dissolved in a 3:2 mixture of 0.1 M cacodylate buffer (ph 7.4, added with sucrose, final osmolality 300 mosmol) and 4% hydroxyl ethyl starch (HES; Fresenius Kabi, Bad Homburg, Germany) in 0.9% NaCl. The kidneys were fixed for 5 min, and then rinsed by vascular perfusion with 0.1 M cacodylate buffer for 5 min.

Light microscopy and electron microscopy

Tissue slices were immersed for at least 24 h in the above fixative supplemented with 0.5% GA, postfixed in 1% OsO_4 and embedded in epoxy resin according to routine procedures. One-micrometer sections were studied by light microscopy after staining with AzurII–methylene blue. For electron microscopy ultrathin sections were contrasted with uranyl acetate and lead citrate.

Immunofluorescence

The following antibodies were rabbit polyclonals: anti-proliferating cell nuclear antigen (PCNA) (Delta Biolabs, Campbell, CA, USA); anti-phosphate/sodium cotransport IIa (NaPi-IIa) (gift from Dr. J. Biber, Institute of Physiology, University of Zurich); anti-megalin (gift from Dr. J. Roth, Institute of Pathology, University of Zurich); anti-PMP70 (70-kDa peroxisomal membrane protein) (Affinity Bioreagents, Golden, CO, USA). The following antibodies were mouse monoclonals: anti-bromodeoxyuridine (BrdU) clone 3D4 (BD Biosciences Pharmingen, San Diego, CA, USA), anti-Na-K-ATPase clone C464.6 (Upstate Biotechnology, Lake Placid, NY, USA), anti-vimentin clone V9 (Chemicon, Temecula, CA, USA).

Two millimeter thick slices of kidney were frozen in liquid propane cooled down to −196°C by liquid nitrogen and cut into 4 μm thick cryostat sections. In all protocols that included detection of PCNA or BrdU the cryostat sections were microwaved for 10 min in 0.01 M citrate buffer at pH 6.0. After pretreatment in 5% normal goat serum in PBS, the cryostat sections were incubated overnight in a humidified chamber at 4°C with the primary antibodies, diluted in PBS-1% BSA.

Binding sites of the primary antibodies were revealed with Cy3-conjugated goat antirabbit IgG and with a fluorescein isothyocyanate (FITC)-conjugated goat antimouse IgG (both Jackson ImmunoResearch Laboratories, West Grove, PA). For nuclear staining, 4′,6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MO, USA) was added to the working dilution of the secondary antibodies. Double labelings were performed using cocktails of mouse and rabbit primary antibodies and of the respective secondary antibodies.

For control of unspecific binding of secondary antibodies we made control incubations by omitting the primary antibody. These control experiments were negative. The sections were coverslipped, using DAKO-Glycergel (Dakopatts), to which 2.5% 1,4-diazabicyclo[2,2,2]octane (DABCO; Sigma, St. Louis, MO, USA) was added as fading retardant.

Sections were studied by epifluorescence with a Polyvar microscope (Reichert Jung, Vienna, Austria). The fluorescence for DAPI, Cy3 and FITC were recorded separately using U1, G1 and B2 filter modules, respectively. Overlays of the three channels were performed with the Image-Pro software. Images were acquired with a charge-coupled device camera (Visicam 1280, Visitron Systems, Puching, Germany) and processed by Image-Pro and Photoshop software.

Results

Light microscopy and electron microscopy of cells in mitosis

The kidneys of six healthy young adult rats were studied with the light microscope. For each rat a series of 50 semithin sections including the cortex and the outer medulla was examined. A total of 65 mitotic cells were documented in the proximal tubule and of 19 cells in the collecting duct and the distal tubule. The latter includes the thick ascending limb, the distal convoluted tubule, and the connecting tubule. For the morphology of mitotic cells at the level of electron microscopy we rely on micrographs of cells, which have been seen by chance in the course of various studies with 5–6-week-old Wistar rats. Sixteen are proximal tubular cells, 19 distal tubular cells.

Apart from the arrangement of chromatin the mitotic cells did not differ much from other tubular epithelial cells (Figs. 1, 2). However, they mostly appeared larger and frequently their apex bulged into the tubular lumen. The area of the mitotic spindle did not contain organelles. The density of mitochondria appeared somewhat decreased in many mitotic cells (Fig. 1), probably reflecting the increased cellular volume rather than a lower absolute content of the organelle. This notwithstanding the density of mitochondria remained high in mitotic cells. All mitotic cells in the proximal tubule displayed microvilli (Figs. 1, 2). However, their density was often lower than in neighboring cells. Like for mitochondria this decrease is probably due to the increase of cell volume without production of additional microvilli. Tight junctions of the mitotic cell with the neighboring quiescent cells were mostly detectable (Fig. 2).

Immunofluorescence of cells in mitosis

We examined whether mitotic cells display proteins that are characteristic of tubular epithelial cells in the kidney. The Na-K-ATPase is abundant in the basolateral membranes of most cell types of the nephron. NaPi-IIa and megalin are found exclusively in the luminal (brush-border) membrane of proximal tubules. We also examined a protein of peroxisomes, PMP70. In the kidney peroxisomes are particularly large and abundant in proximal tubular cells (Baumgart 1997). Antibodies against those four proteins strongly labeled tubular cells but there was none detectable in cells of the interstitium, vessels or glomeruli. The mitotic figures were revealed by the DNA-binding fluorochrome DAPI.

In cells of the distal nephron as well as the cortical and outer medullary-collecting duct Na-K-ATPase is densely packed in the infolded basolateral membrane. The nucleus is situated above the infoldings. All of the 14 observed distal tubular cells in mitosis indicated a dense basolateral labeling for Na-K-ATPase (Fig. 3). In sections labeled with anti-Na-K-ATPase we examined the segment S3 of the proximal tubule in addition to the distal nephron. Due to the simple shape of the cells in S3, in contrast to S1 and S2, immunolabeling of the basolateral membrane provides a clear image of the outlines of each single cell. The 27 mitotic cells found in this segment displayed Na-K-ATPase in their basolateral membranes at levels comparable to neighboring cells (Fig. 3).

The proximal tubule was further examined for NaPi-IIa, megalin and PMP70. All of the 108 observed dividing cells displayed either NaPi-IIa (7 cells) (not shown), megalin (47 cells) or PMP70 (54 cells) (Fig. 4). Frequently, the intensity of immunolabeling for brush border proteins was distinctly lower in the mitotic cells than in neighboring cells.

Vimentin was expressed in glomeruli, vessels and in some interstitial cells, but it was never detected in tubular cells, including cells in mitosis (Fig. 5).

Differentiation markers in PCNA-expressing cells

We next compared the abundance of the above mentioned differentiation markers in cells in the late G1 and S-phase of the cell cycle, as detected with PCNA. In

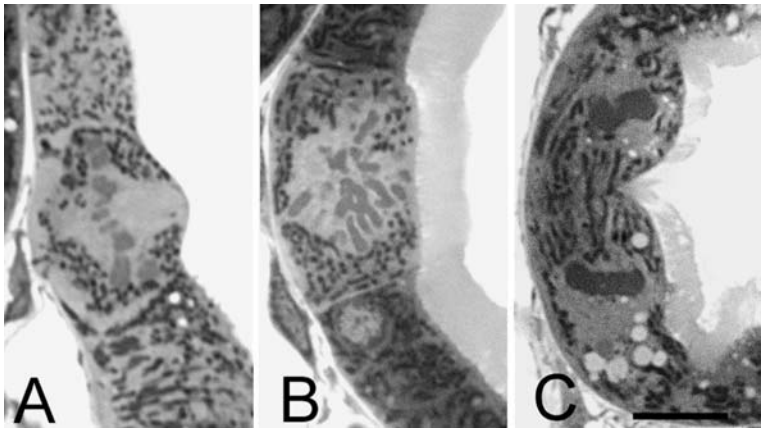

Fig. 1 Light microscopy of mitotic tubular cells. **a** Late distal tubule; the obvious cellular delimitation, the nearly mitochondria-free rim at the base of the cell representing infoldings of the basal plasma membrane, and the high amount of mitochondria are typical features of an intercalated cell. **b** Proximal tubule S3; the mitotic cell is clearlydelimited and enlarged. **c** Proximal tubule S1, telophase. Epon sections, 1 μm thick, AzurII/methylene blue. *Bar*: 10 μm

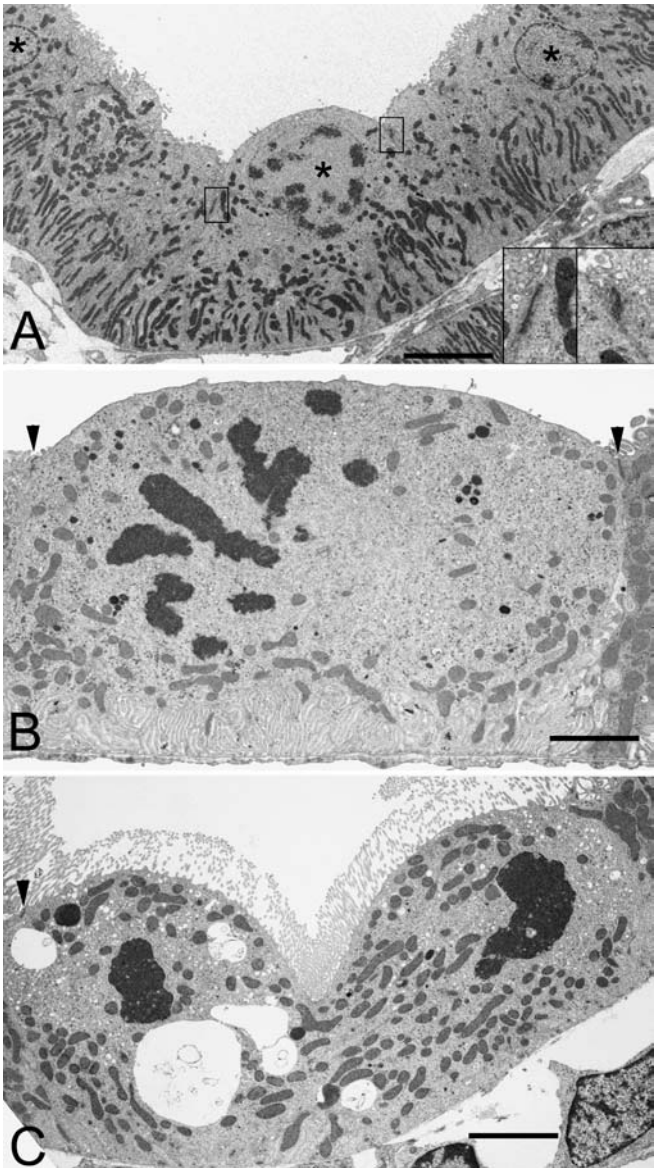

Fig. 2 Electron microscopy of mitotic cells. **a** Early distal tubule, the cell in the center is in the prophase; tight junctions are enlarged in the inserts, nuclei are labeled with asterisks. **b** Connecting tubule cell, displaying the characteristic basal rim of plasma membrane infoldings; *arrowheads* show tight junctions.**c** Proximal tubule, telophase. *Bar*—4 μm in **a** and **c**, 2 μm in **b**

sections double-labeled for Na-K-ATPase and PCNA we found 53 and 56 PCNA-positive cells in the distal tubule and in the proximal tubule, respectively. In sections labeled with NaPi-IIa, megalin and PMP70 we found 108, 43 and 90 PCNA-positive proximal tubular cells, respectively.

The PCNA-labeled proximal and distal tubular cells (total of 350) showed without any exception the same intensity of immunolabeling as neighboring cells for each of the four markers (Figs. 6, 7). Thus, cells in the S-phase of the mitotic cycle are polarized with respect to the basolateral and luminal cell membrane domains. The abundance of characteristic proteins of the basolateral membrane, of the brush border and of peroxisomes qualify them as fully differentiated cells.

Characterization of label-retaining cells by immunofluorescence

When applied in vivo BrdU, a synthetic analog of thymidine, is incorporated in the DNA of cycling cells in which it can be then detected by specific antibodies. Since repeated cycles dilute the label, only slow cycling cells, which are per definition the stem cells, but not the rapidly cycling transit amplifying cells, will remain detectable for weeks after application of BrdU. So far the capacity to retain BrdU, or an equivalent label, has been the only way to identify putative stem cells in the kidney (Maeshima et al. 2003; Oliver et al. 2004).

The BrdU was injected in newborn rats. The BrdU-retaining cells were examined in the proximal tubule of rats sacrificed 8, 14 or 35 weeks later. Two rats were examined at each time point. The BrdU was detected by immunofluorescence. In addition the sections were immunolabeled for either Na-K-ATPase and NaPi-IIa or Na-K-ATPase and PMP70. Whereas the incidence of label-retaining cells decreased with time qualitative differences were not found between the three time points. Therefore, all observations were pooled. Three hundred and forty-eight and 83 label-retaining cells were found in Na-K-ATPase/NaPi-IIa—and Na-K-ATPase/PMP70-labeled sections, respectively. These cells were in the cortex. None were found in the outer stripe of the medulla, reflecting the fact that this zone was not yet developed when BrdU was injected.

The vast majority of label-retaining cells displayed similar abundance of the markers as the neighboring unlabeled cells (Fig. 8). However, six of 348 cells did not show NaPi-IIa. Out of the six, three distinctly expressed Na-K-ATPase. Thus, only three out of 431 label-retaining cells did not show any of the used differentiation markers.

Discussion

The main objective of the present study was to examine whether cycling cells in healthy rat kidneys display a lower level of differentiation than quiescent cells. Cell turnover is very low in kidneys of adult rats (Witzgall et al. 1994). Therefore we used juvenile rats in which the kidney is fully developed but the nephrons still grow in length.

In light microscopy and electron microscopy mitotic cells displayed high amounts of mitochondria, characteristic for transporting epithelia. In the proximal tubule they displayed a brush border. Tight junctions were visible in the electron microscope. Thus the mitotic cells found in this study could not be qualified as undifferentiated precursors. This conclusion was supported by immunofluorescence. Mitotic cells expressed high levels

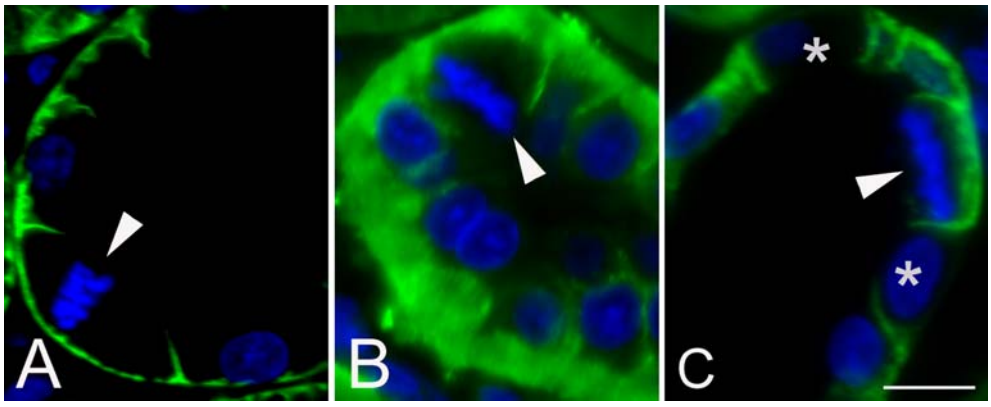

Fig. 3 Na, K-ATPase in mitotic cells (*arrowheads*). The Na, K-ATPase is detected by immunofluorescence (*green*). The DNA is labeled with DAPI (*blue*). **a** Proximal tubule S3. **b** Early distal convoluted tubule. **c** Connecting tubule; intercalated cells (*asterisks*) do not show Na, K-ATPase. *Bar*: 10 μm

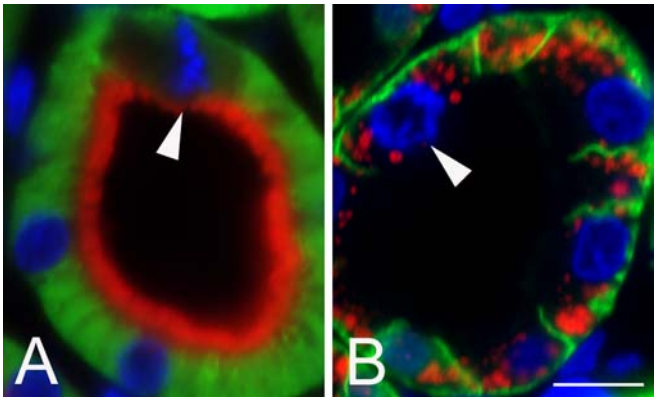

Fig. 4 Proximal tubular marker proteins in mitotic cells (*arrowheads*). Immunofluorescence for Na, K-ATPase (*green*), megalin (*red* in **a**) and PMP70 (*red* in **b**). The DNA is labeled with DAPI (*blue*). **a** Proximal tubule S1. **b** Proximal tubule S3. *Bar*: 10 μm

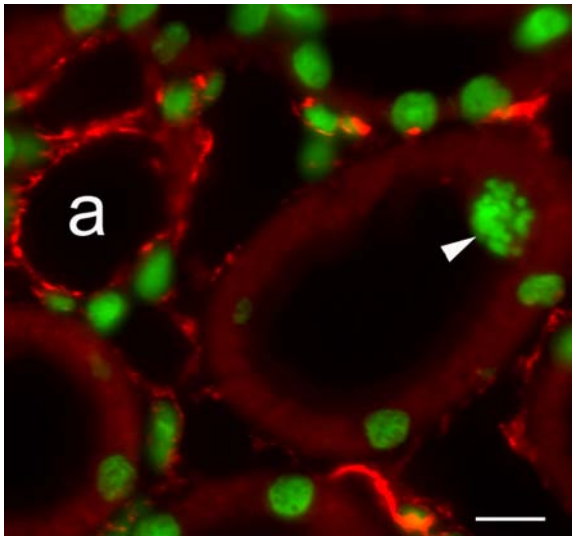

Fig. 5 Absence of vimentin in a mitotic cell (*arrowhead*) in a proximal tubule. Immunofluorescence for vimentin (*red*). The DNA is labeled with DAPI (*green,false color*). **a** Arteriole. *Bar*: 10 μms

of proteins, which are characteristically abundant in renal tubules, namely Na-K-ATPase, NaPi-IIa, megalin and PMP70. The exclusive localization of Na-K-ATPase in the basolateral membrane and of NaPi-IIa and megalin in the luminal membrane show that the cells remain polarized during mitosis.

The results summarized above indicate that mitotic cells display characteristic features of the epithelium where they are found. However, a comparison of the density of cell organelles or of the abundance of a given antigen between mitotic cells and quiescent cells is difficult due to the increased cell volume and the profound alteration of the cell architecture which is brought about by the formation of the mitotic spindle. Thus, in order to be able to detect quantitative differences between cycling cells and quiescent cells we labeled the former before the formation of the spindle with anti-PCNA. The PCNA is found in the nuclei of cells in the late G1 as well as in the S phase. The expression of Na-K-ATPase and of PMP70 as well as of brush border proteins was identical in PCNA-positive cells as in neighboring tubular cells. Thus, most if not all cycling cells are differentiated and cannot be distinguished from neighboring cells on account of criteria used in this study.

In earlier studies, which analyzed the recovery from acute tubular necrosis, it has been proposed that tubular cells dedifferentiate before cycling. Indeed, the strong proliferation which follows massive cell death is preceded by the expression of proteins, that are present in the metanephric mesenchyme, but not in the mature nephron, including vimentin and Pax-2 (Bonventre 2003; Ichimura et al. 2004; Imgrund et al. 1999). The absence of vimentin in cycling cells in the present study suggests that such a pattern of phenotype regression is not required for entry into the cycle in the healthy kidney.

Besides the description of dividing cells a second objective of the present study was to evaluate the level of differentiation of label-retaining cells, the putative stem cells (Maeshima et al. 2003; Oliver et al. 2004). Application of BrdU shortly after birth during a period of rapid renal growth guarantees the labeling of a large amount of tubular cells. Our observations on the distribution of BrdU were similar to those made in a previous study (Oliver et al. 2004). Indeed, immediately

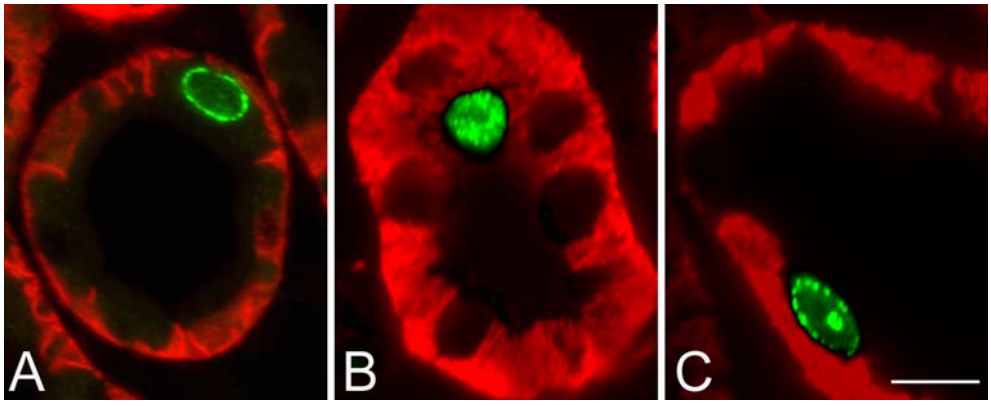

Fig. 6 Double-labeling for PCNA (*green*) and Na, K-ATPase (*red*). **a** Proximal tubule S3. **b** Early distal convoluted tubule. **c** Late distal convoluted tubule. *Bar*: 10 μm

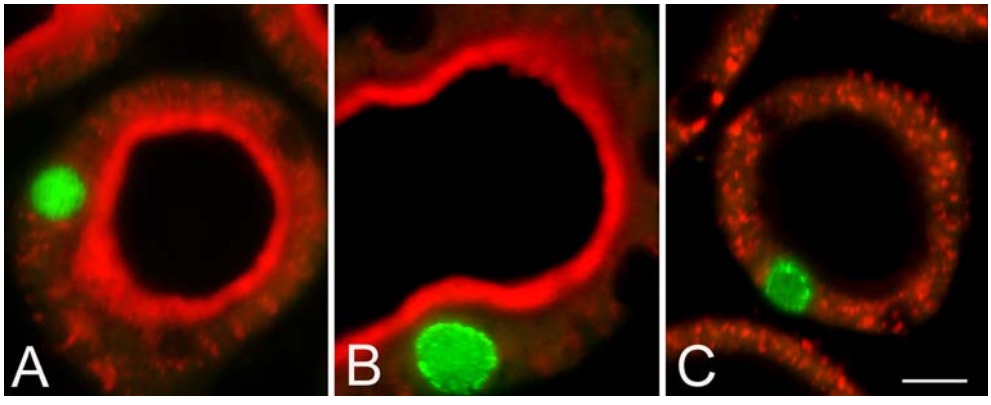

Fig. 7 Double-labeling for PCNA (*green*) and NaPi-IIa (*red* in **a**), Megalin (*red* in **b**) or PMP70 (*red* in **c**). *Bar*: 10 μm

after the end of the application of BrdU, on day 7, a high fraction of tubular cells were labeled. Already after 4 weeks of washout the incidence of BrdU-positive cells was low in the cortex and very low in the outer medulla. Compared to these two zones it remained higher in the papilla, where, besides tubular cells, interstitial cells were frequently labeled (not shown). The decrease of tubular BrdU-positive cells is readily explained by the high mitotic activity in the elongating tubules, and hence the strong dilution of BrdU in the chromatin. However, a small number of cells in the cortex still displayed moderate to strong immunofluorescence after 35 weeks. This shows that some cells have undergone at most a few cycles following the period of labeling. Label-retaining

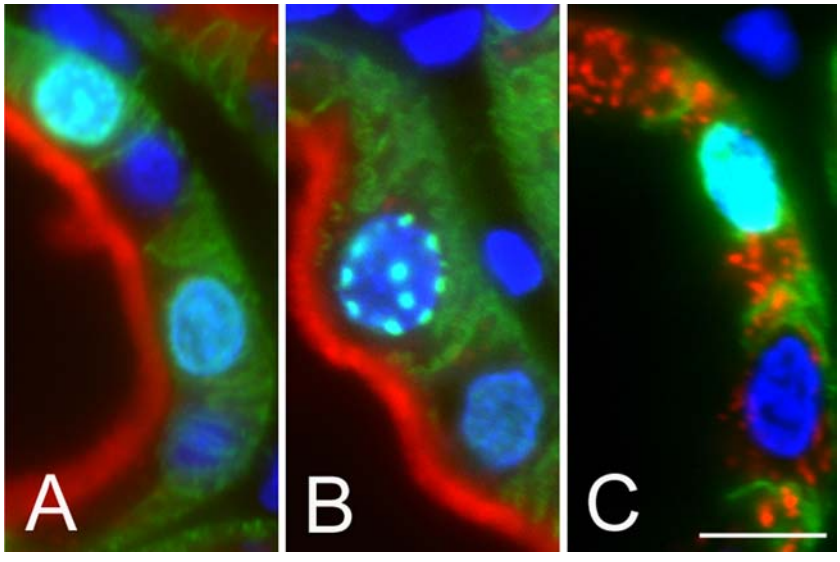

Fig. 8 Triple-labeling for BrdU (*green* in nuclei), Na, K-ATPase (*green*) and NaPi-IIa (*red* in **a** and **b**) or PMP70 (*red* in **c**) in proximal tubule. Eight weeks (**a**, **c**) or 35 weeks (**b**) after BrdU application. *Bar*: 10 μm. Although anti-BrdU and anti-Na-K-ATPase were both mouse antibodies, the unique locations of the two antigens (nuclei and basolateral membranes, respectively) allowed a specific detection

cells expressed high amounts of proteins that are specific for differentiated tubular cells. Actually with respect to those markers the vast majority of label-retaining cells were identical to the neighboring cells and therefore they can hardly be stem cells. Thus, label retention is probably not an adequate criterion to detect stem cells in renal tubules. This can be well explained on the basis of the present knowledge on stem cells in solid tissues (Alison et al. 2002; Lehrer et al. 1998). The division of a stem cell yields a daughter stem cell and a fast cycling cell. The latter undergoes between three and five successive divisions. The last division produces differentiated cells. Thus, according to that model, during application of BrdU, for each BrdU-labeled daughter stem cell, between eight and 32 labeled terminally differentiated cells will be generated. When the very low rate of tubular cell proliferation in the adult is considered (Witzgall et al. 1994), it becomes obvious that differentiated tubular cells live many weeks. Therefore, a large proportion of BrdU-retaining cells are necessarily differentiated cells rather than stem cells.

It has been established that a stem cell/TA cell system is responsible for homeostasis in the healthy epithelium in the epidermis, the cornea and the intestine (Brittan and Wright 2004; Fuchs et al. 2004; Jensen et al. 1999; Lehrer et al. 1998). In these epithelia morphologically and functionally different cell types are found in a close vicinity, due to stratification or, for the intestine, to intermingling of different cell types. In addition, in those epithelia the microenvironment varies across short distances. For instance, in the epidermis, the cells of the basal layer and those of the bulge of the hair follicle are situated in environmental niches. The same is true for cells in the limbus of the cornea and cells in the crypts of the intestine. Such niches appear to play a crucial role in the biology of stem cells (Fuchs et al. 2004; Lehrer et al. 1998). When attempting to apply the stem cell concept to the nephron one is confronted with several problems. First, in contrast with the epithelia mentioned above the nephron is made of serially connected, histologically and functionally distinct segments. If each segment had an own stem cell system, small populations of stem cells and TA cells should be intermingled among the highly differentiated cells all along the nephron. This could hardly have remained undetected by the many investigators who examined the nephron in the electron microscope during more than half a century. In contrast, if stem cells were restricted to a niche their progeny would need to migrate extraordinary long distances across different types of epithelia in order to supply new cells to the various segments. A further challenge to the hypothesis of tubular stem cells lies in the need for every cell in a simple epithelium to cope with the composition of the luminal fluid. It is difficult to imagine how a precursor lacking the specific transport proteins of the neighboring cells could regulate its intracellular ionic milieu and thus its volume.

In conclusion, most if not all cycling cells in the cortex and the outer medulla of the rat kidney display a similar level of differentiation as the neighboring cells. In addition, tubular label-retaining cells in these parts of the kidney are differentiated cells. These data are compatible with the hypothesis that differentiated tubular cells are self-renewing in the healthy kidney. It is also conceivable that tubular stem cells might be highly differentiated. However that would seem to be at odds with the broadly accepted notion of stem cells as progenitors with the potential to produce more than one differentiated progeny.

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