Ultrastructural Insights in the Interface between Generated Renal Tubules and a Polyester Interstitium

Will W. Minuth,* Lucia Denk, Christine Meese, Reinhard Rachel, and Anne Roessger

Department of Molecular and Cellular Anatomy, University of Regensburg, University Street 31, D-93053 Regensburg, Germany

Received November 21, 2008. Revised Manuscript Received January 27, 2009

In regenerative medicine, stem/progenitor cells are emerging as potential candidates for the treatment of renal failure. However, the mechanism of regeneration of renal tubules from stem/progenitor cells is not well-elucidated. In this study, a new method was developed for the generation of tubules replacing coating by extracellular matrix proteins. Renal stem/progenitor cells are mounted between layers of polyester fleece. This artificial interstitium supports spatial development of tubules within 13 days of perfusion culture in chemically defined Iscove's modified Dulbecco's medium (IMDM) containing aldosterone as the tubulogenic factor. Whole mount label by soybean agglutinin (SBA) showed that generated tubules exhibited a lumen and a continuously developed basal lamina. Immuno-labeling for cytokeratin Endo-A demonstrated the presence of isoprismatic epithelial cells, and laminin $\gamma 1$, occludin, and Na/K-ATPase $\alpha 5$ labeling revealed typical features of a polarized epithelium. To get first insight in the interface between tubules and polyester interstitium, transmission electron microscopy (TEM) was performed. The results showed that the generated tubules exhibited polar differentiation with a continuously developed basal lamina consisting of a lamina rara interna, lamina densa, and lamina rara externa. Collagen type III was found to be the linking molecule between the basal lamina and the surrounding polyester fibers by immuno labeling studies. Thus, the findings demonstrate that the spatial development involves the interface between the tubular basal lamina and the polyester interstitium of tubules and is not restricted to the epithelial portion.

1. Introduction

It is an unresolved question, whether stem/progenitor cells can be used for the repair of parenchyma in acute or chronic renal failure in future.[1-4] To address this, it is important to investigate suitable model systems that can lead to renal tubular regeneration from stem/progenitor cells.[5,6] Construction of such systems demands knowledge of the suitable growth factors to be administered in the medium and the structural formation of tubules in a spatial environment. This is further challenged by the complex histoarchitecture of the diseased kidney that promotes or hinders the regeneration of tubules. Hence, effective in vitro experiments are required to elucidate the mechanisms involved in the formation of tubules in a spatial environment and to learn about the controlled steering of this development.[7]

Despite considerable research over five decades, an established method for the controlled formation of structured tubules under defined in vitro conditions is not available. In order to culture renal cells, tubular segments can be isolated and placed at the bottom of a culture dish.[8,9] One would expect that feeding with serum-containing medium will result in the elongation of isolated tubules, although the tubular formation could be absent. In contrast, the cells emigrate out of the tubular segment, and the outer surface of the isolated tubule or the bottom of the culture dish is used as growth substrate (Figure 1a).

To support the formation of spatially organized tubules, more sophisticated techniques are required (Figure 1b). For this, cells collected from urine or Madin-Darby canine kidney (MDCK) cells are coated by extracellular matrix proteins,[10,11] which stimulates the cells to form tubules in a specific three-dimensional environment. However, the relatively thick layer of extracellular matrix coating does not support an optimal exchange of nutrition and respiratory gas, leading to the formation of unstirred layers of medium, causing deleterious accumulation of metabolites.

The earliest culture model with renal stem/progenitor cells showing a spatial development of tubules was pioneered by Grobstein, Saxen, and others.[12-15] Nephrogenic mesenchyma was isolated from a mouse fetus, placed on a nitrocellulose filter, and coated by a layer of agarose. After that the filter was turned, and the spinal cord was mounted (Figure 1c).

*Corresponding author. E-mail: will.minuth@vkl.uni-regensburg.de.
(1) Loo, D.; Beltejar, C.; Hooley, J.; Xu, X. *Methods Cell Biol* **2008**, *86*, 241–255.
(2) Perin, L.; Giuliani, S.; Sedrakyan, S.; DaSacco, S.; De Filippo, R. E. *Pediatr. Res.* **2008**, *63*, 467–471.
(3) Maeshima, A.; Sakurai, H.; Nigam, S. K. *J. Am. Soc. Nephrol.* **2006**, *17* (1), 188–198.
(4) Hammerman, M. R. *Expert Opin. Biol. Ther.* **2006**, *6*, 87–97.
(5) Atala, A. *Pediatr. Res.* **2008**, *63*, 569–575.
(6) McTaggart, S. J.; Atkinson, K. *Nephrology* **2007**, *12*, 44–52.
(7) Giuliani, S.; Perin, L.; Sadrakyan, S.; Kokorowski, P.; Jin, D.; De Filippo, R. *J. Urol.* **2008**, *179*, 365–370.
(8) Ash, S. R.; Cuppage, F. E.; Hoses, M. E.; Selkurt, E. E. *Kidney Int.* **1975**, *7*, 55–60.
(9) Wilson, P. D.; Horster, M. F. *Am. J. Physiol.* **1983**, *244*, C166–174.

(10) Inoue, C. N.; Sunagawa, N.; Morimoto, T.; Ohnuma, S.; Katsushima, F.; Nishio, T.; Kondo, Y.; Linuma, K. *In Vitro Cell. Dev. Biol.: Anim.* **2003**, *39*, 364–367.
(11) Hellman, N. E.; Greco, A. J.; Rogers, K. K.; Kanchagar, C; Balkovetz, D. F.; Lipschutz, J. H. *Am. J. Physiol. Renal Physiol.* **2005**, *289*, F777–785.
(12) Grobstein, C. *Exp. Cell Res.* **1956**, *10*, 424–440.
(13) Saxen, L.; Lehtonen, E. *Differentiation* **1987**, *36*, 2–11.
(14) Hardman, P.; Klement, B. J.; Spooner, B. S. *Dev. Growth Differ.* **1983**, *35*, 683–690.
(15) Avner, E. D.; Ellis, D.; Temple, T.; Jaffe, R. *In Vitro Cell. Dev. Biol.: Anim.* **1982**, *18*, 675–682.
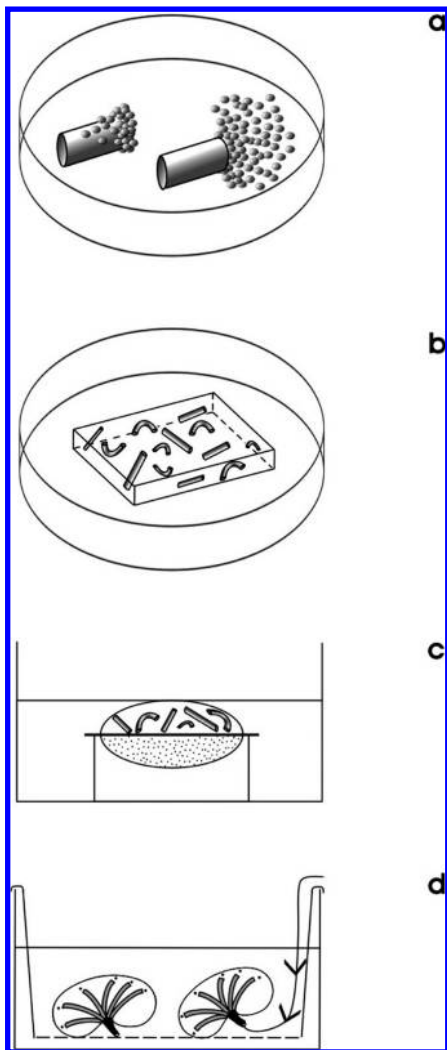
Figure 1. Schematic illustration of renal cell culture and tubule formation. (a) Cells of isolated tubules emigrate to grow on the surface of the tubule segment or on the bottom of the culture dish.[8,9] (b) Isolated cells can be coated by extracellular matrix proteins to form tubules during culture.[10,11] (c) Embryonic renal mesenchyma can be cocultured with spinal cord to develop tubules at the liquid–air interface.[12−15] (d) An anlage from an embryonic kidney can be placed on a filter insert to investigate organ growth. To supply the nutrition, the hilus is perfused (arrowhead) with medium.[7]

The interaction between both the tissues resulted in the development of structured tubules within the coated mesenchymal layer.

Development of tubules was further investigated on an isolated metanephric organ anlage cultured on a filter insert.[7] Here, to facilitate the exchange of respiratory gas, the tissue is kept near the gas–fluid interface (Figure 1d). The integrity of the growing organ supports tubule development, but the continuously increasing mass of tissue hinders provision with fresh nutrition, which in turn limits growth. Hence, a capillary is placed inside the hilus for the perfusion of medium. In culture, the explants process the early stages of nephrogenesis. This protocol delays the onset of necrosis, while preserving the morphology of the growing organ.

Nevertheless, there is an increasing need for the development of better biomaterials for renal tubule regeneration that meet the physiological needs of stem/progenitor cells, supporting development in a spatial environment and avoiding the formation of unstirred layers of medium.

In this study, a new technique for the structural growth of tubules is demonstrated. Renal stem/progenitor cells were cultured between layers of polyester fleece, using the interface as an artificial interstitium for the spatial development of tubules.[16−18] This approach, for the first time, allows investigation of the development of tubules by ultrastructural methods without any interference of stacking proteins derived from a coating process containing undefined extracellular matrix. Moreover, to date, biophysical and biochemical features promoting the spatial development of tubules are unknown. Hence, cell biological and ultrastructural methods were employed to get first insights into the interface between generated tubules and the polyester interstitium.

2. Materials and Methods

Isolation and Culture of Embryonic Explants Containing Renal Stem/Progenitor Cells. One-day-old New Zealand rabbits were anesthetized with ether and killed by cervical dislocation as described earlier.[19] Both kidneys were removed immediately, and each kidney was dissected in two parts. By stripping off the capsula fibrosa with fine forceps, a thin tissue layer containing numerous epithelial stem cells within the collecting duct ampullae and nephrogenic stem cells within the mesenchyme, were harvested (Figure 2a).

For dynamic culture, the isolated embryonic renal tissue was placed within a perfusion container, between two punched-out layers of polyester fleece (Figure 2b; Walraf, Grevenbroich, Germany) measuring 5 mm in diameter. Thus the freshly isolated embryonic tissue was sandwiched between the layers of polyester fleece. The basic setup was held in provided position to prevent damage within a base ring of a tissue carrier with 13 mm inner diameter inside a perfusion culture container (Minucells and Minutissue, Bad Abbach, Germany) (Figure 2c). A polyester fleece measuring 13 mm in diameter was mounted in this tissue carrier. Then the basic sandwich setup containing renal stem/progenitor cells measuring 5 mm in diameter was inserted. Finally, a polyester fleece 13 mm in diameter was placed on top of the sandwich as a cover.

Generation of Renal Tubules under Dynamic Culture. To generate renal tubules, chemically defined Iscove's modified Dulbecco's medium (IMDM with phenolred, GIBCO/Invitrogen, Karlsruhe, Germany) with antibiotic−antimycotic cocktail (1%, GIBCO, Nr. 15240−062) was used. To maintain a constant pH of 7.4, HEPES (50 mmol/L, GIBCO) was added to the medium. To evoke tubulogenic development, aldosterone (1×10^{-7} M, Fluka, Taufkirchen, Germany) was added to the culture medium, and the cells were cultured at 37 °C with 0.3% CO_2.[16] Throughout the entire experimental phase of 13 days, fresh medium was constantly perfused at a rate of 1.25 mL/h using an IPC N8 peristaltic pump (Ismatec, Wertheim, Germany). To maintain a constant temperature of 37 °C, the culture container was placed on a thermoplate (Medax - Nagel, Kiel, Germany) and covered with a transparent lid (Figure 2d).

Lectin- and Antibody-Labeling. Whole mount specimens, cryo-sections of generated tubules (20 μm thickness), and neonatal rabbit kidney (8 μm thickness) were fixed in ice-cold ethanol. After being washed with phosphate buffered saline (PBS), the specimens were blocked with PBS containing 1% bovine serum albumin (BSA) and 10% horse serum for 30 min. For soybean agglutinin (SBA, Vector, Burlingame, CA) labeling, the samples were exposed to fluorescein-isothiocyanate-conjugated lectin (1:1000 in blocking solution) for 45 min. For

(16) Minuth, W. W.; Denk, L.; Hu, K *Biomaterials* **2007**, *28*, 8–28.
(17) Minuth, W. W.; Denk, L.; Castrop, H. *Tissue Eng. C: Methods* **2008**, *14*, 3–13.
(18) Blattmann, A.; Denk, L.; Strehl, R.; Castrop, H.; Minuth, W. W. *Biomaterials* **2008**, *29*, 2749–2756.
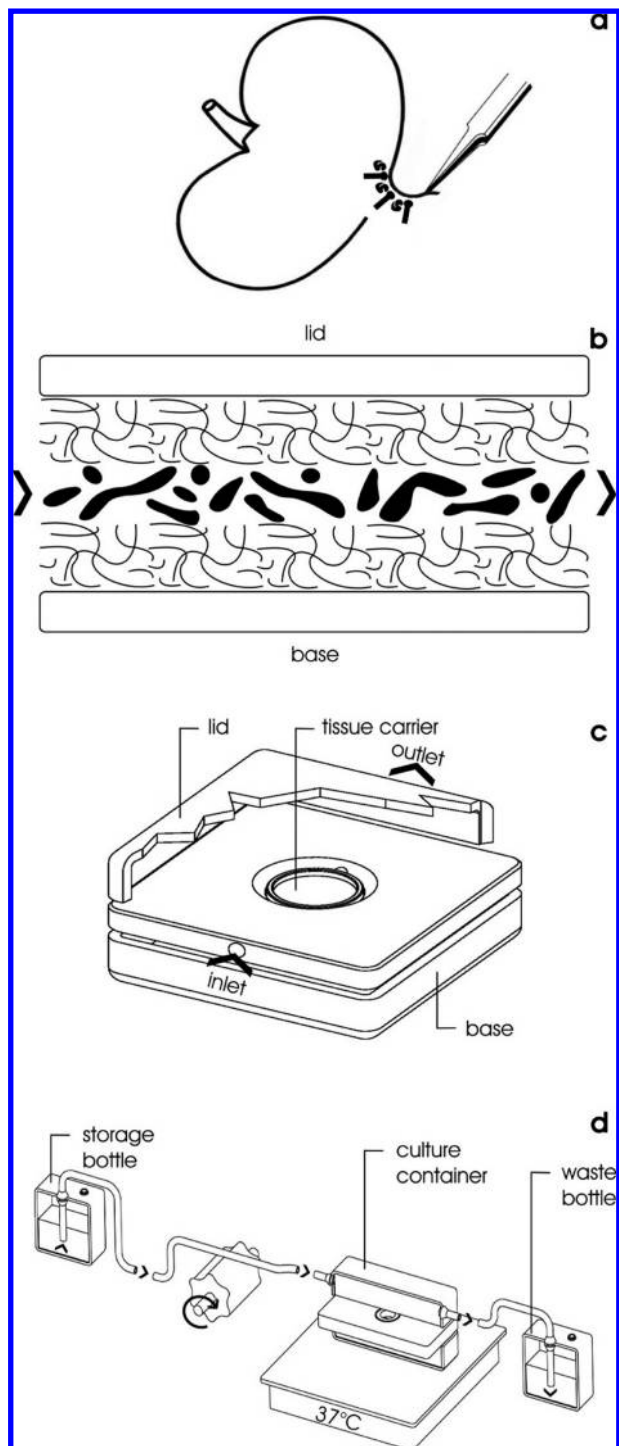(19) Minuth, W. W. *Differentiation* **1987**, *36*, 12–22.

Figure 2. Generation of renal tubules at the interphase of an artificial interstitium. (a) Tissue is isolated by stripping off the capsula fibrosa of neonatal rabbit kidney containing epithelial and mesenchymal stem/progenitor cells.[19] (b) Tubules are generated in contact with polyester fleeces.[16−18] (c) The fleeces are held in a Minusheet tissue carrier placed in the center of a perfusion culture container. (d) During culture, the medium is perfused by a peristaltic pump (1, 25 mL/h) from a storage bottle to the culture container and then to a waste bottle.

immunohistochemistry, monoclonal antibodies against collagen type III (III-53, Calbiochem, Schwalbach, Germany), Na/K ATPase $\alpha5$, cytokeratin Endo-A (both obtained from Developmental Studies Hybridoma Bank, University of Iowa City, IA), occludin (Zymed, San Fransisco, CA), and laminin $\gamma1$ (kindly provided by Prof. Dr. L. Sorokin, Lund, Sweden) were used. The specimens were treated with these primary antibodies for 1 h followed by incubation for 45 min with donkey-anti-mouse-IgG-fluorescein-isothiocyanate or goat-antirat-IgG-rhodamine (Jackson Immuno- research Laboratories, West Grove, PA) (1:50 in PBS containing 1% BSA). Following several washes with PBS, the sections were embedded with Slow Fade Light Antifade Kit (Molecular Probes, Eugene, OR) and then analyzed using an Axioskop 2 plus microscope (Zeiss, Oberkochen, Germany). Fluorescence images were taken with a digital camera with a standard exposure time of 1.3 s. The counting of tubules was done with a WCIF Image J morphometric program (Wayne Rasband, National Institute of Health, Gaithersburg, MD).

Transmission Electron Microscopy (TEM). For TEM, the specimens were fixed in 2% glutaraldehyde containing 0.1 M sucrose in 0.1 M cacodylate buffer (5 h at room temperature) and after several washes with PBS postfixed in 1% osmium tetroxide in 1 M PBS. The tissue was washed with PBS and then dehydrated in graded series of ethanols and embedded in Epon, which was polymerized at 60 °C for 48 h. Ultrathin sections were performed with a diamond knife on a ultramicrotome Ultracut E (Leica GmbH, Wetzlar, Germany). Sections were collected onto 200 mesh copper grids and contrasted using 4% uranyl acetate and lead citrate.

For TEM studies, grids were scanned for suitable specimen areas using a wide-angle video-rate CCD-camera (TV 673 Mk III; Gatan, Munich, Germany) mounted to a TEM CM12 (FEI Co, Eindhoven, The Netherlands) and operating at 120 keV. Electron micrographs were recorded digitally using a slow-scan CCD camera (1024 × 1024 pixels; TVIPS GmbH, Gauting, Germany) at a primary magnification between 3000 and 13 000 (absolute pixel size: 5.2−1.2 nm). Because of the limited field of view of a single image at these magnifications, up to 36 over-lapping images of specimen areas were recorded. A single montage was generated using "tiling software" (EM-MENU 4; TVIPS).

Amount of Cultured Specimen. A total of 96 embryonic tissues were isolated and maintained in culture for the present study. All of the experiments were performed at least in triplicates. The data provided in the text are the mean of at least three independent experiments.

3. Results

Growth Pattern of Tubules. The renal stem/progenitor cells were isolated from the embryonic cortex of neonatal rabbit kidney (Figure 2a) and cultured between layers of polyester fleece (Figure 2b,c). For 13 days the tissue was kept in perfusion culture with chemically defined IMDM, containing aldosterone (Figure 2d). To visualize generated tubules, the interface of the artificial interstitium was opened by tearing off both layers of polyester fleece and labeled by fluorescent SBA (Figure 3a). Fluoresence microscopy demonstrates that numerous tubules were growing in a spatial arrangement. To obtain information about the number of developed tubules, SBA-labeled tubules were registered (Figure 3b). In the presented series, 62 tubules within the defined area of the fleece were registered. Cases were registered, where tubules demonstrated a singular occurrence, while others reveal a dichotomous branching or curling. When the tubules were not leaving the optical plane, there was a longitudinal growth over a distance between 300 and 400 μm. The whole mount specimens further revealed that each tubule was covered by a smooth basal lamina (Figure 3a,c,d). Longitudinal (Figure 3c) and vertical (Figure 3d) growth of tubules further depicts that the tubules contain a clearly recognizable lumen in their center.
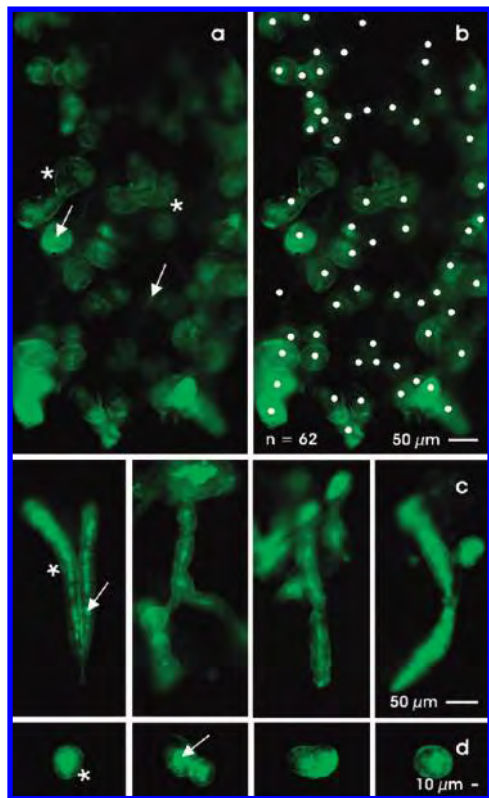
Figure 3. Whole mount label of generated tubules by SBA. (a) The generated tubules present in the artificial interstitium after 13 days of culture. (b) In this individual case, 62 SBA-labeled tubules are seen. Longitudinal (c) and vertical view (d) of generated tubules showing a lumen (arrow) and a basal lamina (asterisk).

Cellular Features of Differentiation. The cryosections of generated tubules were performed to study the features of differentiation. The toluidine blue-stained section showed that the tubules were growing at the interface of the artificial interstitium (Figure 4a). It was clear that the developing tissue was covered by a layer of fleece at the upper and lower side. Immuno-labeling for cytokeratin Endo-A on generated tubules, showed intensive cytoplasmic staining (Figure 4b). Labeling the tissue with antilaminin $\gamma1$ revealed an intensive reaction at the basal region of generated tubules, indicating that a basal lamina is developed during 13 days of culture (Figure 4c). Labeling for occludin demonstrated a junction line as a faint label in the luminal portion of generated tubules (Figure 4d), which suggests a functional polarization between the luminal and lateral plasma membrane of tubule cells. Immuno-labeling for Na/K-ATPase $\alpha5$ revealed intense fluorescence on the basolateral side of the generated tubules (Figure 4e). All these data show that the generated tubules exhibit a polarized epithelium.

Ultrastructural Elements of Tubular Development. A further set of experiments was carried out to obtain insights in the ultrastructure of generated tubules by TEM. The semithin section of a specimen exhibited that tubules generated at the interface of an artificial interstitium have a lumen, a lining epithelium, and a basal lamina (Figure 5a). The tubule occurred in proximity to polyester fibers, but appeared to avoid close contact with them. In the microenvironment of the extracellular matrix, single cells and cellular debris were noticed.

TEM demonstrated an isoprismatic epithelium (Figure 5b). The cells had a large nucleus, which was located in the center of the cytoplasm. In the apical and basal cytoplasm, numerous lysosomal elements were found. Small, medium-sized, and large vacuoles with electron-dense material were found in various degrees. The apical plasma membrane of the tubular epithelium bore some microvilli and often with an electron-dense cell coat. The luminal cell pole slightly protruded toward the lumen (Figure 5b). The basal plasma membrane was smooth and exhibited some infolding. Epithelial cells were in close contact with each other. Some microvilli or microplicae of the lateral plasma membrane projected into the intracellular space. The basal slits of the adjacent plasma membranes were narrow.

The sectional overview (Figure 5b) and higher magnification (Figure 5c) further demonstrated a separation of the luminal and lateral plasma membranes by a typical junctional complex consisting of a zonula occludens, zonula adhaerens, and a desmosome indicating a polarized and physiologically sealing epithelium. At the basal part, a consistently developed basal lamina was covering the tubule (Figure 5b, d). The basal lamina was composed of a lamina rara interna, a lamina densa, and a lamina fibroreticularis (Figure 5d).

To further characterize the basal part of generated tubules, a comparison was made with the collecting duct tubules of the neonatal rabbit kidney. High-magnification studies of TEM showed that the collecting duct tubule of neonatal rabbit was surrounded by a consistently developed basal lamina (Figure 6a), which comprised a lamina rara interna, a lamina densa, and a lamina fibroreticularis. The most interesting aspect was the arrangement of interstitial cells opposing the basal lamina. Numerous broad finger-like protrusions of interstitial cells border the lamina fibroreticularis within the kidney. The distance between the lamina densa and the protrusions of the presented individual specimen was between 66 and 77 nm. Opposing interstitial cells were also found in specimens of generated tubules (Figure 6b). However, the interstitial cells were found to be separated more from the basal lamina, and their protrusions occur less frequently as compared to the kidney. Both generated tubules and opposing interstitial cells were separated over more than 600 nm by an extended lamina fibroreticularis filled with numerous bundles of synthesized collagen. Thus, these structural data show that the basal part of renal tubules and the surrounding interstitial cells are in proximity, and the functional implications of this association are yet to be unravelled.

Collagen Type III and Laminin $\gamma1$ Staining. The last set of experiments was performed to gain first insights into the molecular composition of extracelullar matrix proteins such as collagen type III in comparison to laminin $\gamma1$ surrounding the tubules within the maturing kidney (Figure 7a−d), and on tubules generated at the interface of an artificial interstitium (Figure 7e−g). Double-labeling immuno-histochemical experiments were performed for the proper distinction of the molecules.

In the embryonic zone of neonatal rabbit kidney, immuno-histochemical label for collagen type III showed that this molecule was absent in the interstitium and at the tip of the collecting duct (CD) ampulla (A, Figure 7a). However, major expression of collagen type III was found at the neck of the collecting duct ampulla (dotted line) and was restricted to the basal lamina of the tubule epithelium (Figure 7a′). The interstitial space in this area was free of collagen type III. In contrast, labeling for laminin $\gamma1$ showed its presence at the
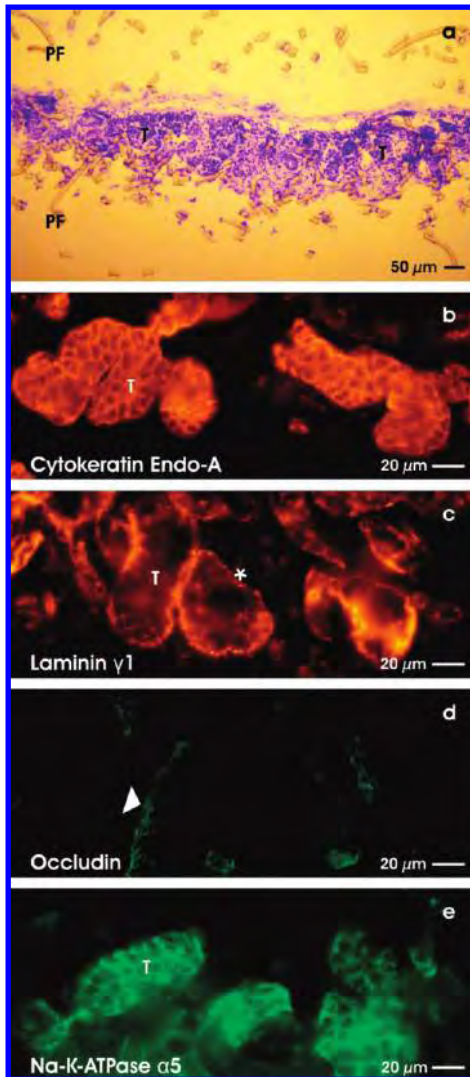
Figure 4. Labeling cryo-sections of generated tubules. (a) Toluidine-stain demonstrates numerous tubules (T) growing between fleeces made of polyester fibers (PF). (b) Immuno-label for cytokeratin Endo-A reveals intensively labeled epithelial cells. (c) Immuno-label for laminin $\gamma1$ shows that generated tubules are surrounded by a basal lamina (asterisk). (d) Immuno-label for occludin reveals that generated tubules contain a junctional complex (arrowhead) with a zonula occludens. (e) Immuno-label for Na−K-ATPase $\alpha5$ exhibits the expression of an important transporter at the basolateral plasma membrane.

Figure 5. Ultrastructure of generated tubules. (a, b) Semithin sections reveal that generated tubules contain a polarized epithelium. The apical side borders a lumen, while the basal side contains a basal lamina (asterisk). The generated tubules (T) grow in vicinity to the polyester fibers (PF). (b) TEM shows isoprismatic epithelial cells. Between the apical and lateral plasma membrane, a junctional complex is developed (arrowhead). (c) The tight junction consists of a zonula occludens (Z.o.), zonula adhaerens (Z.a.), and a desmosome: (D). (d) The basal lamina consists of a lamina rara interna (L.r.i.), a lamina densa (L.d.), and a lamina fibroreticularis (L.f.).

tip of the collecting duct ampulla (Figure 7b). An increasing amount of laminin was seen as an interspersed pattern beyond the neck of the collecting duct ampulla (Figure 7b′). The interstitium did not stain for laminin $\gamma1$, indicating its absence. Thus, in the neck of the ampulla, both collagen type III (Figure 7a′) and laminin $\gamma1$ (Figure 7b′) were present at the basal lamina of the collecting duct tubule, showing the first site where they were coexisting. In the matured cortex of the neonatal kidney, dense staining of collagen type III label was observed at the basal part of the collecting duct tubule and within the surrounding interstitium (Figure 7c). In contrast, the label for laminin $\gamma1$ was restricted to the basal lamina of the tubule epithelium (Figure 7d).

Interestingly, immuno-histochemical labeling for collagen type III (Figure 7e) and laminin $\gamma1$ (Figure 7f) showed that both molecules were found to be coexpressed at the basal part of the generated tubule epithelium. The collagen type III
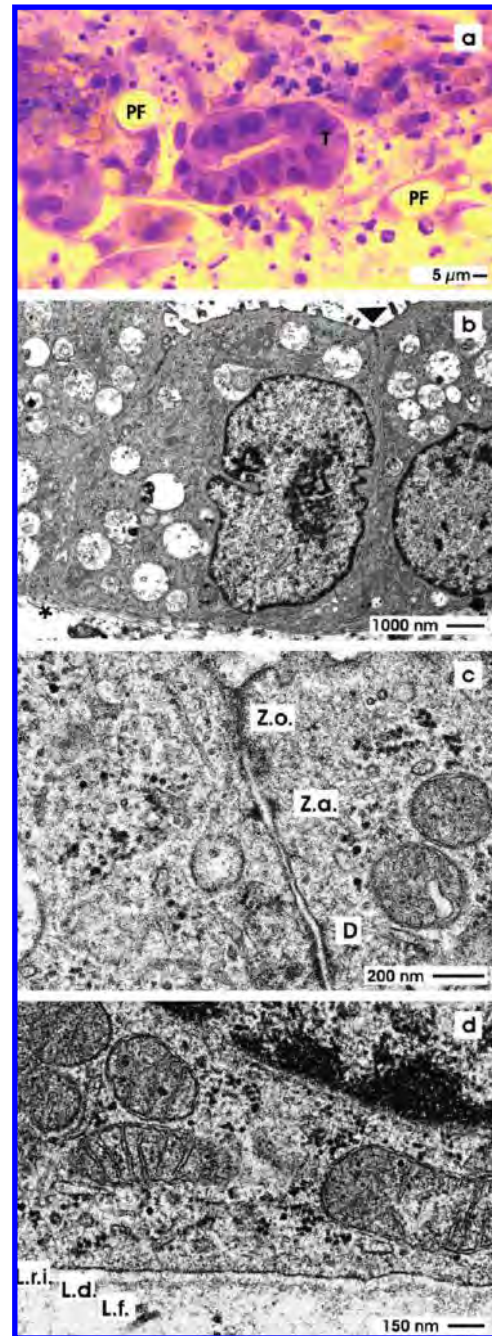
was also detected on the basal part of the epithelium within the interstitial space between the generated tubules (open circles, Figure 7g).

4. Discussion

In regenerative medicine, a special focus is given to stem/progenitor cells as they are emerging as a probable source for the future treatment of acute and chronic renal failure.[3−5]
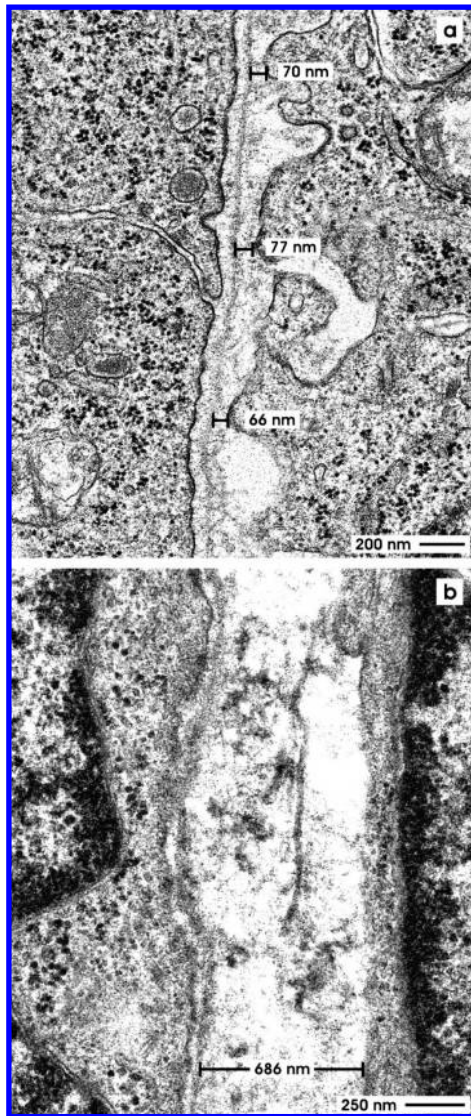
Figure 6. Ultrastructure between collecting duct tubule cells and interstitial cells within the kidney (a) and on generated tubules (b). The collecting duct tubule of neonatal rabbit is surrounded by a consistently developed basal lamina (a). The arrangement of interstitial cells opposing the basal lamina is unusual, since finger-like protrusions of interstitial cells border the lamina fibroreticularis. The distance between the lamina densa and the protrusions is between 66 and 77 nm. Opposing interstitial cells were also found in generated tubules (b). The interstitial cells are found to be more separated and their protrusions occur less frequently as compared to the kidney. The distance between generated tubules and opposing interstitial cells is 686 nm.

Nonetheless, there are unresolved issues, which include the differentiation of stem/progenitor cells into site-specific cell types, the controlled formation of tubules within a spatial environment, and the integration of regenerating tissue within the diseased organ.[20−23] In this study we used a different approach to obtain knowledge about these developmental steps, where renal stem/progenitor cells were kept in perfusion culture between layers of polyester fleece.

(20) Dressler, G. *Trends Cell Biol.* **2002**, *12*, 390–395.
(21) Karihaloo, A.; Nickel, C.; Cantley, L. G. *Nephron Exp. Nephrol.* **2005**, *100*, 40–45.
(22) Hogan, B. L.; Koledziej, M. *Nat. Rev.* **2002**, *3*, 513–523.
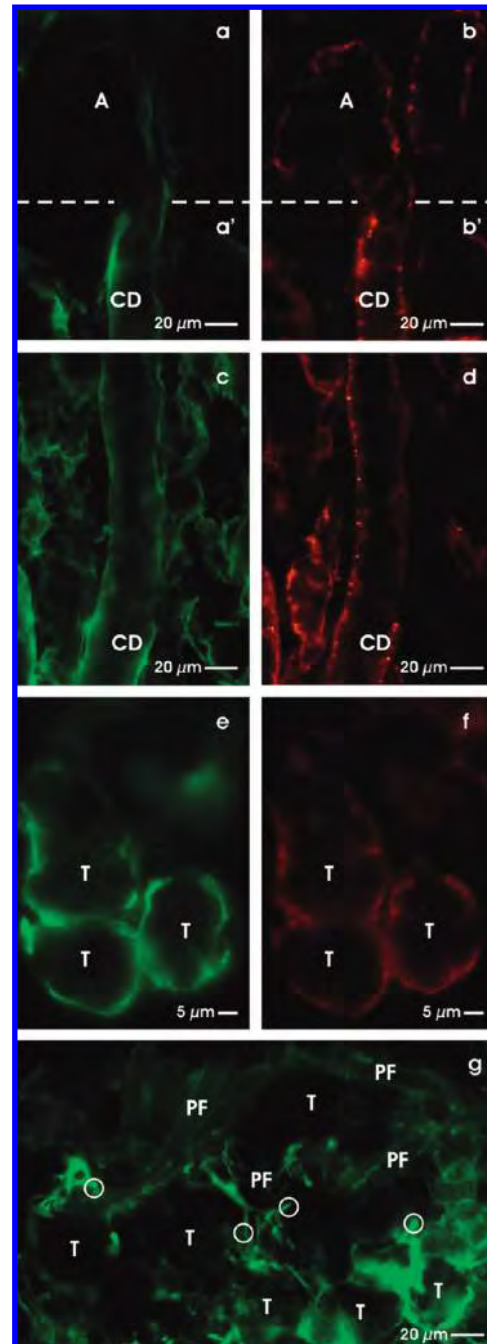(23) Lubarsky, B.; Krasnow, M. A. *Cell* **2003**, *112*, 19–28.

Figure 7. Immuno-label for collagen type III (a,c,e,g) and laminin γ1 (b,d,f) in neonatal rabbit kidney (a−d) and on generated tubules (e−g). Immuno-label for collagen type III (a,c,e,g) and laminin γ1 (b,d,f) in neonatal rabbit kidney (a−d) and on generated tubules (e−g). In the embryonic cortex collagen type III (a) is barely expressed at the collecting duct (CD) ampulla (A), while laminin is found in a punctuate pattern (b). The interstitium is free of label. In the maturing neck of the collecting duct ampulla, the basal lamina of the collecting duct tubule is positive for collagen type III (a′) and laminin γ1 (b′). The dotted line depicts the border between embryonic (a,b) and maturing (a′, b′) renal tissue. In the matured cortex collagen typ III is found in the basal lamina of the collecting duct tubule and in the interstitium, while laminin γ1 is detected exclusively on the basal lamina (d). (e) On generated tubules, collagen type III is found on the basal lamina of tubules (T) and is found to be colocalized with laminin γ1 (f). (g) Collagen type III (open dot) is spanning between generated tubules (T) and polyester fibers (PF).

This artificial interstitium stimulates tubular development in the presence of aldosterone (Figure 2).[16]

Spatial formation of tubules is highly dependent on the substrate, intercellular communication, and the fluid environment.[22-24] All these factors have to complement each another for the formation of structured tubules. To support the spatial development, cells are frequently coated by extracellular matrix proteins (Figure 1b,c). However, the coating mixture contains undefined molecules and produces unstirred layers of fluid. Hence, coating by extracellular matrix proteins was replaced by an artificial interstitium consisting of a polyester fleece, which meets the physiological needs of renal stem/progenitor cells for spatial development of tubules (Figure 2b).

The whole mount labeled with SBA exhibits the overall growth pattern (Figure 3). Immuno-histochemical labeling for cytokeratin endo-A (Figure 4b), laminin $\gamma 1$ (Figure 4c), occludin (Figure 4d), and Na−K-ATPase $\alpha 5$ (Figure 4e) showed the typical cellular features of a polarized tubule epithelium. Structural data showed isoprismatic epithelium (Figure 5b), cells in close contact to each other, and the luminal and lateral plasma membranes separated by a typical junctional complex (Figure 5c). Although we observed a polarized epithelium, its physiological implications are not known.

Since the tubules were generated without coating by extracellular matrix proteins, the analysis of the characteristics of the epithelium is important. Our TEM studies showed a consistently developed basal lamina covering the tubules consisting of lamina rara interna, a lamina densa, and a lamina fibroreticularis, a structure thay is similar to that found in a kidney (Figure 5d). Further, the tubules were surrounded by an extended lamina fibroreticularis (Figure 6b) and the space between generated tubules and opposing interstitial cells was filled with numerous bundles of synthesized collagen.

Semithin (Figure 5a) and ultrathin (Figure 5b−d) sections revealed that the tubules located at the interface of the
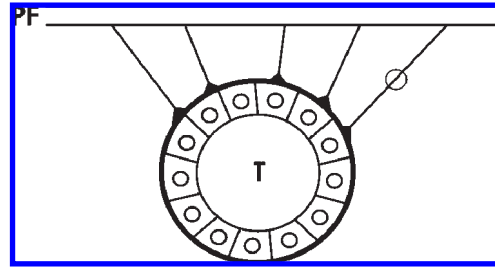

Figure 8. Relation between generated tubules and the artificial interstitium. The basal lamina of generated tubules (T) is connected over collagen type III (open circle) with the surrounding polyester fibers (PF) of the artificial interstitium. Thus, the polyester fibers support molecular linking of synthesized extracellular matrix between tubules and the artificial interstitium.

artificial polyester interstitium were not in a close contact with the surrounding polyester fibers. For further characterization, immuno-histochemical labeling for collagen type III was performed (Figure 7).[25-27] A striking observation was that the collagen type III was present on the basal lamina of generated tubules (Figure 7e) and acted as a linker to the polyester fibers of the artificial interstitium (Figure 7g, 8). One might argue that the newly synthesized collagen type III could be an artifact due to culture conditions. However, in the neonatal kidney, collagen type III is not present in the embryonic zone (Figure 7a), and the primary expression is in the neck of the collecting duct ampulla . In the matured cortex, collagen type III is found on the basal lamina of the collecting duct and within the interstitium (Figure 7c). Thus the appearance of collagen type III in the basal lamina of tubules and in the interstitial space correlated with the observation in generated tubules and in the developing kidney.

(24) Meyer, T. N.; Schwesinger, C.; Bush, K. T.; Stuart, R. O.; Rose, D. W.; Shah, M. M.; Vaughn, D. A.; Steer, D. L.; Nigam, S. K. *Dev. Biol.* **2004**, *275*, 44–67.

(25) Fleischmajer, R.; Jacobs, L.; Perlish, J. S.; Katchen, B.; Schwarzt, E.; Timpl, R. *Am. J. Pathol.* **1992**, *140*, 1225–1235.
(26) Douglas, T; Heinemann, S.; Bierbaum, S.; Scharnweber, D.; Worch, H. *Biomacromolecules* **2006**, *7*, 2388–2399.
(27) Montes, G. S. *Cell Biol. Int.* **1996**, *20*, 15–27.