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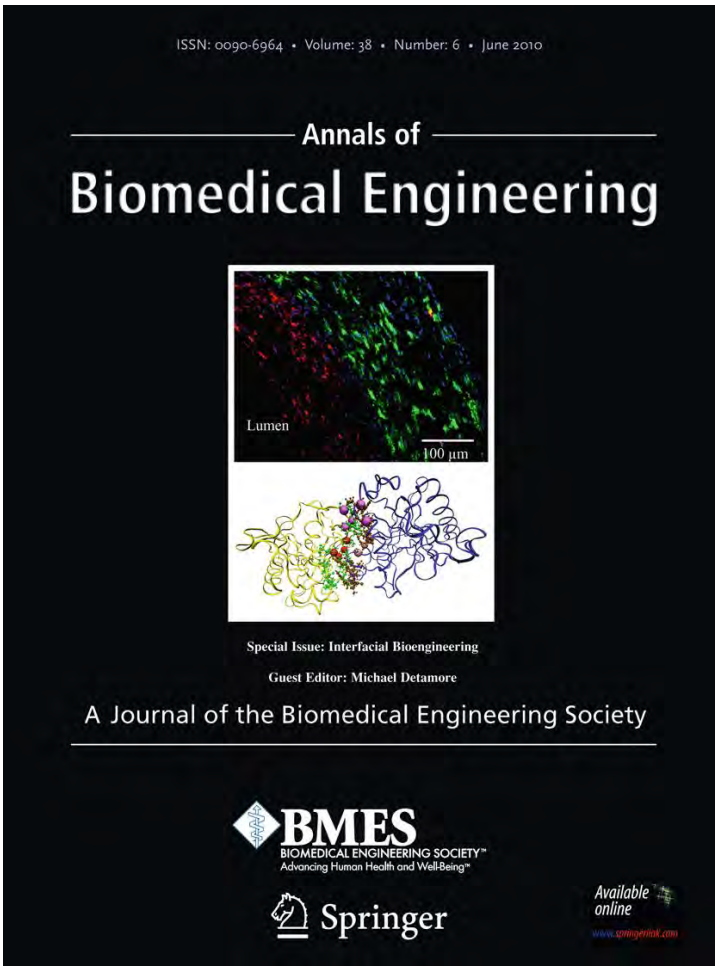

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The Interface Between Generating Renal Tubules and a Polyester Fleece in Comparison to the Interstitium of the Developing Kidney

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Abstract—An increasing number of investigations is dealing with the repair of acute and chronic renal failure by the application of stem/progenitor cells. However, accurate data concerning the cell biological mechanisms controlling the process of regeneration are scarce. For that reason new implantation techniques, advanced biomaterials and morphogens supporting regeneration of renal parenchyma are under research. Special focus is directed to structural and functional features of the interface between generating tubules and the surrounding interstitial space. The aim of the present experiments was to investigate structural features of the interstitium during generation of tubules. Stem/progenitor cells were isolated from neonatal rabbit kidney and mounted between layers of a polyester fleece to create an artificial interstitium. Perfusion culture was performed for 13 days in chemically defined Iscove's Modified Dulbecco's Medium containing aldosterone (1×10^{-7} M) as tubulogenic factor. Recordings of the artificial interstitium in comparison to the developing kidney were performed by morphometric analysis, scanning and transmission electron microscopy. The degree of differentiation was registered by immunohistochemistry. The data reveal that generated tubules are embedded in a complex network of fibers consisting of newly synthesized extracellular matrix proteins. Morphometric analysis further shows that the majority of tubules within the artificial interstitium develops in a surprisingly close distance between 5 and 25 μm to each other. The abundance of synthesized extracellular matrix acts obviously as a spacer keeping generated tubules in distance. For comparison, the same principle of construction is found in the developing parenchyma of the neonatal kidney. Most astonishingly, scanning electron microscopy reveals that the composition of interstitial matrix is not homogeneous but differs along a cortico-medullary axis of proceeding tubule development.

Keywords—Tissue engineering, Perfusion culture, Kidney, Tubule, Artificial interstitium, Collagen type III.

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INTRODUCTION

Recovery from renal failure requires the replacement of injured tissue with new cells that restore epithelial integrity and functionality within tubules. An increasing number of papers is therefore dealing with strategies for repair of parenchyma by the help of stem/progenitor cells.[5,12] However, recent data show that an effective therapy is still far away from a widespread clinic application. Unsolved issues in renal tissue engineering are the concentration of stem/progenitor cells at the site of damage, their integration in a diseased environment, the process of differentiation into nephron-specific cells, and the spatial development of tubules within the kidney.[32]

Part of actual research is focusing on cell biological mechanisms involved in the formation of tubules during regeneration.[4] Due to the spatial microarchitecture of the kidney experiments are frequently performed applying three-dimensional culture experiments in combination with primary cells or cell lines.[2,30] Normally the cells are coated by extracellular matrix proteins such as collagen or Matrigel®. Applying serum-containing medium the cells migrate within the coat of extracellular matrix proteins, proliferate and aggregate to form cysts and tubules.[23] However, during long-term culture the coat of extracellular matrix proteins hinders exchange of nutrition and respiratory gas. Up-to-date typical nephron-specific differentiation and the synthesis of an intact basal lamina in generated tubules are lacking. Since the cells do not exhibit an fetal origin, it remains questionable to what extent these models reflect a stem/progenitor cell-derived process of tubule regeneration.

Pioneering experiments related to regeneration with stem/progenitor cells were performed by culturing isolated nephrogenic mesenchyme from mouse fetus on one side and spinal cord on the other side of a filter.[9,24]

In these transfilter experiments both tissues were coated by agarose. During culture in medium containing serum the interaction between both tissues through the pores results in the development of tubules within the mesenchyme. Most impressive of this method is the high degree of cellular differentiation within generated tubules. However, the need of an inducer tissue secreting morphogens, the necessary application of serum containing undefined factors, the limited time for maintenance, and the minimal amount of tissue available for cell biological analysis pertains to the disadvantages.

Development of tubules was further investigated on intact metanephric organ anlage cultured within a filter insert.[8] To facilitate exchange of respiratory gas the tissue is kept near the gas–fluid interface. The integrity of the growing organ supports at the begin of culture tubule development, but the continuously increasing mass of parenchyma hinders the provision with fresh nutrition, which in turn limits further growth. For that reason a capillary for perfusion of medium is placed inside the hilus. During a 10-day period of culture in medium containing serum the explants process through the early stages of nephrogenesis. Using this type of protocol the onset of necrosis is delayed, while morphology of the growing organ is well preserved.

To avoid coating by extracellular matrix proteins and to raise renal tubules in chemically defined medium, an advanced culture technique was developed. Renal stem/progenitor cells are placed between two layers of a polyester fleece to simulate an artificial interstitium.[19] The space between the fibers facilitates exchange of nutrition and respiratory gas. Transport of always fresh and chemically defined medium in a perfusion container guarantees a constant provision with nutrition and respiratory gas during long-term culture. Experiments demonstrate that the generation of tubules at the interface of this artificial interstitium is a powerful model to investigate processes involved in renal regeneration. For example, development of renal stem/progenitor cells can be induced by aldosterone.[11] The signal is mediated via the mineralocorticoid receptor (MR), since antagonists such as spironolactone or canrenoate prevent tubulogenic development.[18] Disturbing the molecular interaction between MR and heat shock protein 90 by geldanamycin or radicicol results in a lack of tubule formation.[17]

Thus, the interface between two layers of polyester fleece promotes the spatial development of numerous tubules in culture under controlled conditions. Since there are no proteins derived from a coating process, it became possible for the first time to investigate synthesis of interstitial matrix proteins surrounding generated tubules. To obtain first insights in the arrangement of these compounds morphometry, electron microscopy and immunohistochemistry was performed. Most astonishingly, the presented data reveal that generating tubules avoid a direct contact but keep a minimal distance to each other. This spatial separation is caused by linking the basal lamina of tubules with synthesized fibers of the extracellular matrix and with fibers of the polyester fleece. Finally, for comparison the interface between tubules and extracellular matrix was investigated in the developing kidney.

MATERIALS AND METHODS

Isolation of Tissue Containing Renal Stem/Progenitor Cells

For the culture experiments 1-day-old New Zealand rabbits were anesthetized with ether and killed by cervical dislocation. Both kidneys were removed and dissected from pole to pole into a ventral and dorsal part as described earlier.[11] By stripping off the capsula fibrosa with fine forceps a thin tissue layer containing numerous epithelial stem/progenitor cells within collecting duct ampullae and nephrogenic mesenchymal stem/progenitor cells can be harvested (Fig. 1a).

Mounting Stem/Progenitor Cells in a Tissue Carrier for Perfusion Culture

For generation of tubules isolated renal tissue is placed in plane position between layers of polyester fleece (I-7, Walraf, Grevenbroich, Germany) measuring 5 mm in diameter during culture. This sandwich-like configuration creates an artificial interstitium with the freshly isolated tissue in the middle and the polyester fleece covering the upper and lower side (Fig. 1b).[11,19]

The sandwich set-up containing renal stem/progenitor cells was mounted then in a base ring of a Minusheet® tissue carrier. First, a polyester fleece measuring 13 mm in diameter was placed in the tissue carrier. Then, the sandwich set-up was inserted and covered by a further polyester fleece with 13 mm in diameter. The tissue carrier was used in a perfusion culture container with horizontal flow characteristics (Minucells and Minutissue, Bad Abbach, Germany). After closing the lid of the perfusion culture container, the layers of fleece keep the isolated tissue in central and flat position (Fig. 1c). As shown earlier the interface between the fleece layers produces an artificial interstitium promoting the spatial development of tubules during the culture period over 13 days. The area for tubule formation was 5 mm in diameter and up to 250 μm in height.

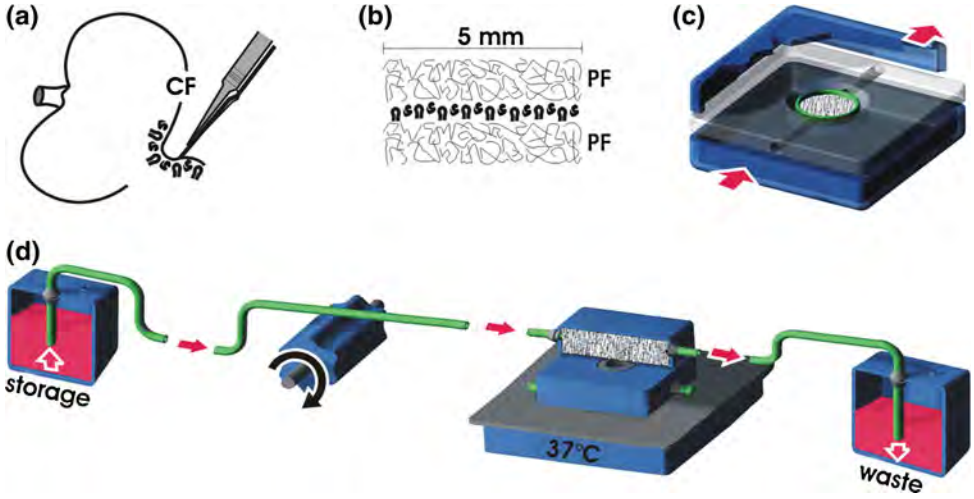

FIGURE 1. Schematic illustration of isolating renal stem/progenitor cells and generation of tubules at the interface of an artificial interstitium (a–d). (a) By stripping off the capsula fibrosa (CF) from neonatal rabbit kidney by forceps renal stem/progenitor cells within mesenchyme and collecting duct ampullae can be isolated. (b) Isolated tissue is placed between two layers of polyester fleece (PF). (c) For stabilization the tissue is mounted in a tissue carrier and inserted in a culture container with horizontal flow (*arrow*). (d) During perfusion culture fresh medium is transported (*arrow*) for 13 days at a rate of 1.25 mL/h by a peristaltic pump. To maintain a constant temperature of 37 °C, the culture container is placed on a thermoplate and covered with a lid.

Perfusion Culture

To generate tubules perfusion culture was performed as described earlier (Fig. 1d).[11,18] During a period of 13 days always fresh medium was transported with 1.25 mL/h by an IPC N8 peristaltic pump (Ismatec, Wertheim, Germany). To maintain a constant temperature of 37 °C the perfusion culture container was placed on a thermoplate (Medax, Kiel, Germany) and covered with a transparent lid.

For the culture chemically defined IMDM (Iscove's Modified Dulbecco's Medium including Phenolred, GIBCO/Invitrogen, Karlsruhe, Germany) with 50 mmol/l HEPES (GIBCO) for maintenance of a constant pH of 7.4 under atmospheric air was used. To prevent infections an antibiotic-antimycotic cocktail (1%, GIBCO) was present in all culture media. As tubulogenic factor aldosterone (1×10^{-7} M, Fluka, Taufkirchen, Germany) was added.

Histochemical Labeling

To analyze cell biological features, cryosections of 20 μm thickness were prepared and fixed in ice-cold ethanol. After washing 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-labeling (SBA, Vector, Burlingame, USA) the samples were exposed to fluorescein-isothiocyanate (FITC)-conjugated lectin diluted 1:2000 in blocking solution for 45 min as earlier described.[18] For immunohistochemical label monoclonal antibodies such as mab anti-laminin γ1 (kindly provided by Dr. L. Sorokin, Lund, Sweden),[19] mab anti-P_{CD} Amp 1,[27] mab anti-Na/K-ATPase alpha 5 (Developmental Studies Hybridoma Bank, Iowa City, USA) and mab anti-collagen type III (III-53, Calbiochem, Schwalbach, Germany) was applied in blocking solution. After washing with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA), the specimens were then incubated for 45 min with donkey-anti-mouse-IgG-fluorescein-isothiocyanate (FITC) or goat-anti-rat-IgG-rhodamine (Jackson Immunoresearch Laboratories, West Grove, USA) diluted 1:50 in this solution. Following several washes with PBS, the sections were embedded with Slow Fade Light Antifade Kit (Molecular Probes, Eugene, USA) and then analyzed using an Axioskop 2 plus microscope (Zeiss, Oberkochen, Germany). Fluorescence images were taken with a digital camera at a standard exposure time of 1.3 s and thereafter processed with Corel DRAW 11 (Corel Corporation, Ottawa, Canada).

Confocal Fluorescence Microscopy

To investigate spatial arrangement of extracellular matrix proteins in generated tubules, confocal fluorescence microscopy with a LSM 710 (Zeiss, Oberkochen, Germany) was performed. Specimens

were fixed in 70% ethanol and labeled by Soybean agglutinin (SBA), mab anti-collagen type III and anti-laminin γ1.

Scanning Electron Microscopy

To analyze the growth pattern of generated tubules within the polyester interstitium, scanning electron microscopy (SEM) was performed. Specimens were fixed in 70% ethanol, dehydrated in a graded series of ethanols, transferred in acetone, critical point dried with CO_2 and sputter-coated with gold (Polaron E 5100, Watford, GB). Then, the samples were examined in a scanning electron microscope DSM 940 A (Zeiss, Oberkochen, Germany) as described earlier.[29]

Transmission Electron Microscopy

For transmission electron microscopy, specimens were fixed in 2% glutaraldehyde containing 0.1 M sucrose and 0.1 M cacodylate buffer for 5 h at room temperature. After several washes with PBS, the tissue was postfixed in 1% osmium tetroxide in 1 M PBS, rinsed with PBS, and then dehydrated in graded series of ethanols and embedded in Epon polymerized at 60 °C for 48 h. Ultrathin sections were performed with a diamond knife on an ultramicrotome EM UC6 (Leica GmbH, Wetzlar, Germany). Sections were collected onto grids (200 mesh) and contrasted using 2% uranyl acetate and lead citrate.

Morphometry

To determine the amount of developed tubules, whole mount SBA-labeled specimens were used. The distance between the basal lamina of neighboring tubules was measured on magnified DIN A4 illustrations. To register the number of generated tubules, WCIF ImageJ (Bethesda, MD, USA) was used as morphometric program (Fig. 2b). Independently from their length the individual distance between SBA-labeled tubules within a microscopic opening of 500 × 850 μm was registered.

Amount of Cultured Constructs

A total of 48 specimens was isolated and kept 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 three independent experiments. All experiments are in accordance with the animal ethics committee, University of Regensburg, Regensburg, Germany.

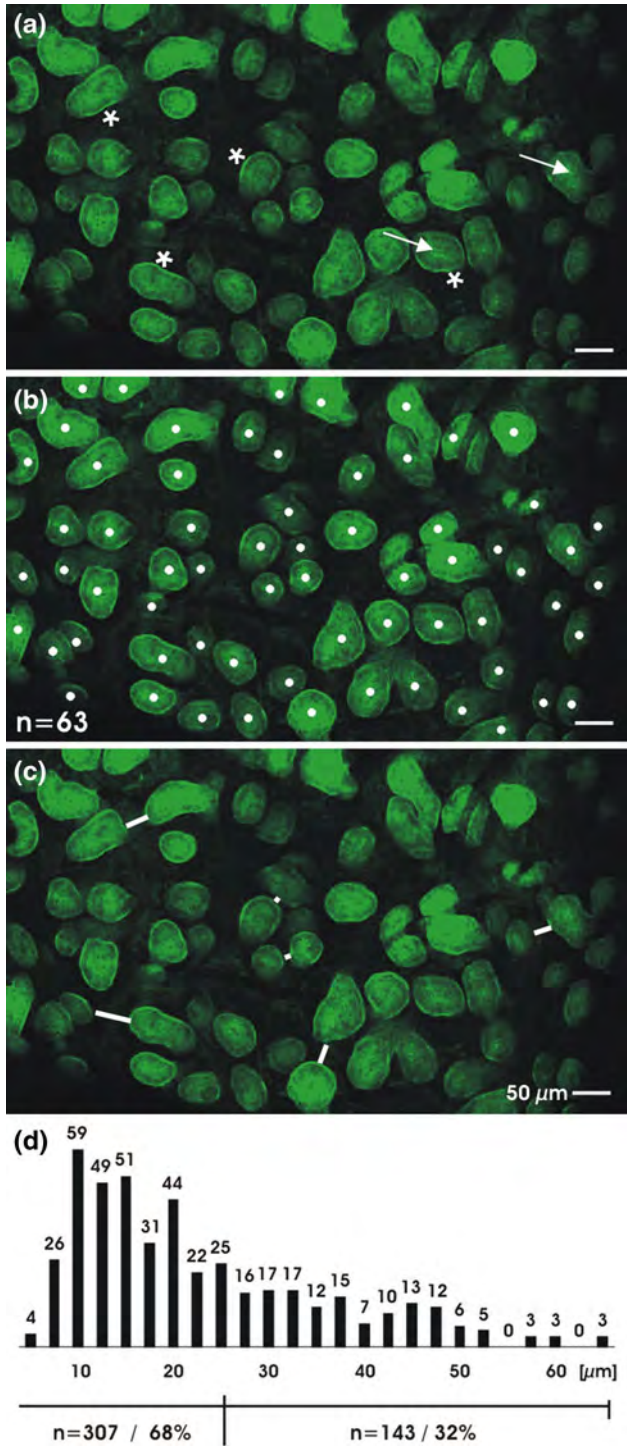

FIGURE 2. Confocal fluorescence microscopy on SBA-labeled tubules generated over 13 days (a–d). (a) Tubules exhibit a basal lamina (*asterisk*) and a lumen (*arrow*). (b) Morphometry on a microscopic opening of 500 × 850 μm shows in this individual experiment 63 (*white spot*) developed tubules. (c) Example demonstrates that generated tubules grow in small-, medium- and big-sized interstitial distances (*white lines*). (d) Morphometry of 450 recordings shows that 68% (n = 307) of tubules exhibit a distance between 5 and 25 μm, while 32% (n = 143) are separated by a distance between 26 and 65 μm.

RESULTS

Arrangement of Tubules

The renal stem/progenitor cells were isolated from the cortex of neonatal rabbit kidney and cultured between layers of polyester fleece. For 13 days the tissue was kept in perfusion culture with chemically defined IMDM containing aldosterone (Fig. 1).

To analyze the spatial development of generated tubules, the artificial interstitium was opened at the end of culture by separating the polyester fleece layers with fine forceps. Specimens were labeled then by SBA to visualize the spatial distribution of generated tubules. Confocal fluorescence microscopy demonstrates that numerous labeled tubules can be seen in a longitudinal, transversal and oblique course (Fig. 2a). All of them exhibit a continuously developed basal lamina, lining epithelial cells and a visible lumen.

To determine the number of SBA-labeled tubules, cross sections were analyzed by morphometry. In the presented case, 63 tubules are detected (Fig. 2b). Surprisingly, whole mount label further demonstrates that generated tubules do not contact each other (Fig. 2c). Small, medium, and wide distances can be registered. To obtain exact information about the gap between generated tubules, 450 recordings were made (Fig. 2d). The data show that a gap smaller than 2.5 μm does not occur. A distance between 5 and 25 μm was found in 307 cases (68%). It is obvious that the most frequently found space is 10 μm ($n = 59$). In contrast, gaps between 26 and 65 μm are less frequently detected ($n = 143$). The accumulation of gaps in a range between 5 and 25 μm is a clear hint that the distance between generated tubules does not occur accidentally but appears to be organized and tightly packed.

Extracellular Matrix Between Generated Tubules

For more detailed analysis the interstitial space between generated tubules was investigated after immunohistochemical label (Fig. 3). Immuno-positive label for collagen type III is found in both along the basal lamina and in the gap between generated tubules (Fig. 3a). This observation shows that at the basal aspect of generated tubules interstitial matrix is synthesized. The experiments further demonstrate that laminin γ1 is localized together with collagen type III in the basal lamina of generated tubules, but also in the space between (Fig. 3b). In consequence, collagen type III and laminin γ1 appear as candidates creating the gap between generating tubules (Fig. 3c).

Structural Features of the Interstitial Space Between Generated Tubules

To obtain detailed insight in the ultrastructure of the interstitial space between generated tubules, transmission electron microscopy was performed (Fig. 4). The surface view shows that generated tubules exhibit a polarized epithelium (Fig. 4a). The luminal plasma membrane of epithelial cells borders a clearly visible lumen. The luminal and lateral plasma membranes are separated by a tight junctional complex. The basal plasma membrane is in contact with a basal lamina. Higher magnification depicts that the basal lamina is composed of several layers as it is known from the kidney (Fig. 4b). A lamina rara interna faces the basal plasma membrane of epithelial cells, while a lamina densa and a lamina rara externa cover the tubules at the outer surface. The lamina fibroreticularis acts as connecting element to the interstitial space containing numerous collagen fibers. In some cases,

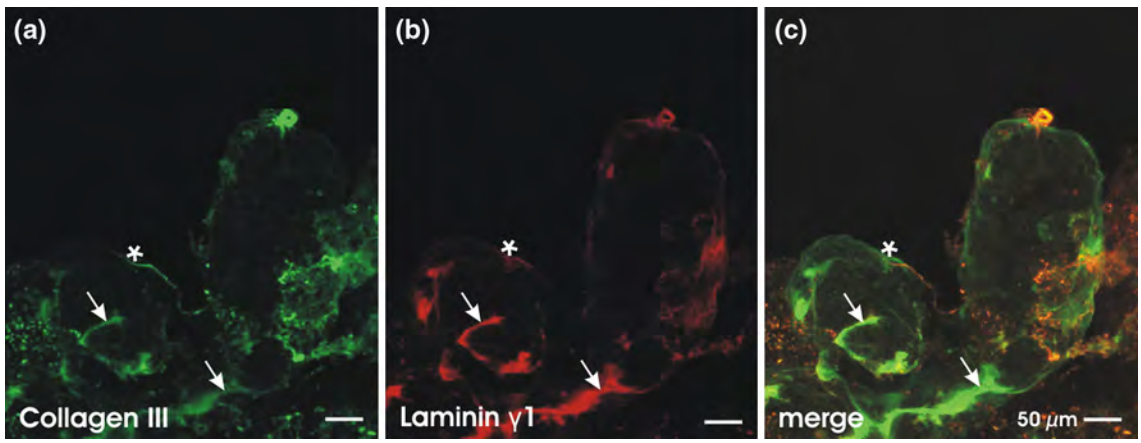

FIGURE 3. Confocal analysis of whole mount-labeled tubules generated over 13 day at the interface of an artificial interstitium (a–c). (a) Label for collagen type III is found at the basal lamina (*asterisk*) and within the gap (*arrow*) between the tubules. (b) Label for laminin γ1 is found within the basal lamina (*asterisk*) and within the gap (*arrow*) between the tubules. (c) Merge illuminates co-localization of both molecules within the basal lamina (*asterisk*) and within the intertubular gap (*arrow*).

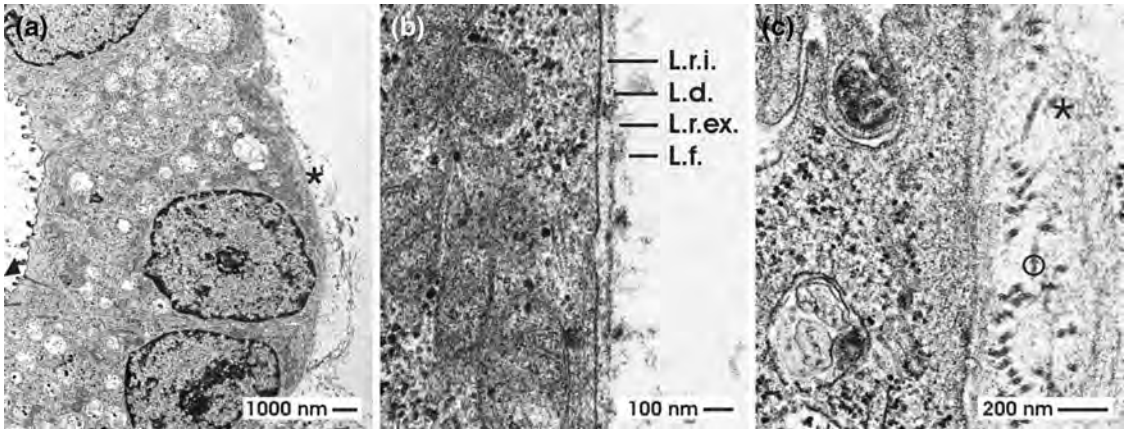

FIGURE 4. Transmission electron microscopy on generated tubules after 13 days of culture (a–c). (a) Surface view demonstrates that tubules contain a polarized epithelium. At the border between the luminal and lateral plasma membrane a tight junctional complex is developed (*arrow head*). The basal side of the epithelium rests on a basal lamina (*asterisk*). (b) Higher magnification shows the basal aspect of the epithelium and the basal lamina consisting of a lamina rara interna (l.r.i.), lamina densa (l.d.), lamina rara externa (l.r.ex.) and a lamina fibroreticularis (l.f.). (c) Higher magnification depicts that the lamina fibroreticularis (*asterisk*) reveals occasionally an increased thickness containing numerous collagenous fibrils (O).

the lamina rara externa and the lamina fibroreticularis show an increased thickness (Fig. 4c). The inconstant thickness of these layers may explain the varying distances between generated tubules as shown in Fig. 2.

SEM was accomplished to receive three-dimensional information about molecules spacing generated tubules (Figs. 5a, 5d, and 5g). A surface view demonstrates tubules contacting the polyester fleece. The polyester fibers exhibit an average diameter of 10 μm and a smooth surface without recognizable roughness. Development of tubules occurs in the vicinity of fibers (Fig. 5a). Micrographs further show that a basal lamina is covering the complete outer surface of tubules. A part of tubules grows in a parallel fashion (Fig. 5d), while others exhibit a dichotomous branching (Fig. 5g). Numerous of the tubules have no contact, while others have only a loose contact to the fibers of the polyester fleece. Higher magnification illustrates that the basal lamina of generated tubules is covered by a network of extracellular matrix proteins obviously synthesized by interstitial cells (Figs. 5b, 5e, and 5h). Collagen fibers are lining as well between the basal lamina of tubules as to neighboring fibers of the polyester fleece.

On the basal lamina of generated tubules dispersed interstitial cells are found (Fig. 5b, 5e, and 5h). They exhibit a more or less round shape. In some cases their surface appears smooth (Fig. 5b), while in other cases a network of filopodia or extracellular matrix fibers is protruding from the interstitial cells (Figs. 5e and 5h).

However, looking by SEM to filopodia of interstitial cells it cannot be decided, where a cell is ending and at which point the extracellular matrix starts (Figs. 5b, 5e, and 5h). For that reason TEM was carried out to analyze the transition from cellular protrusions to the extracellular matrix (Figs. 5c, 5f, and 5i). Ultrathin sections reveal that always a close contact exists between protrusions of interstitial cells and attached fibers consisting of synthesized extracellular matrix. Surprisingly, at the contact site the plasma membrane appears solubilized and the microstructure is barely recognizable. Instead, amorphous material is protruding through the plasma membrane into the cytoplasm.

Interstitium of the Developing Renal Parenchyma

Features of the interstitium found in generated tubules may be influenced by the culture environment (Figs. 3–5). For comparison the interstitium of developing parenchyma in neonatal kidney was investigated (Figs. 6–9). Semithin sections through the outer cortex of neonatal rabbit kidney show in vertical (Figs. 6a and 6b) and longitudinal (Fig. 6c) direction tubules embedded in the interstitium. It can be recognized at this early stage of development that tubules do not exhibit a close contact to each other but are separated by an astonishingly wide interstitial space.

To obtain further detailed data concerning the interstitium of developing parenchyma in neonatal kidney, histochemical and ultrastructural analysis were performed. In the developing cortex four different zones can be distinguished (Fig. 7a). Underneath the organ capsule both the population of mesenchymal (mes) nephrogenic stem/progenitor cells and within the tip of the collecting duct ampulla (A) epithelial stem/progenitor cells are localized. At the neck of the

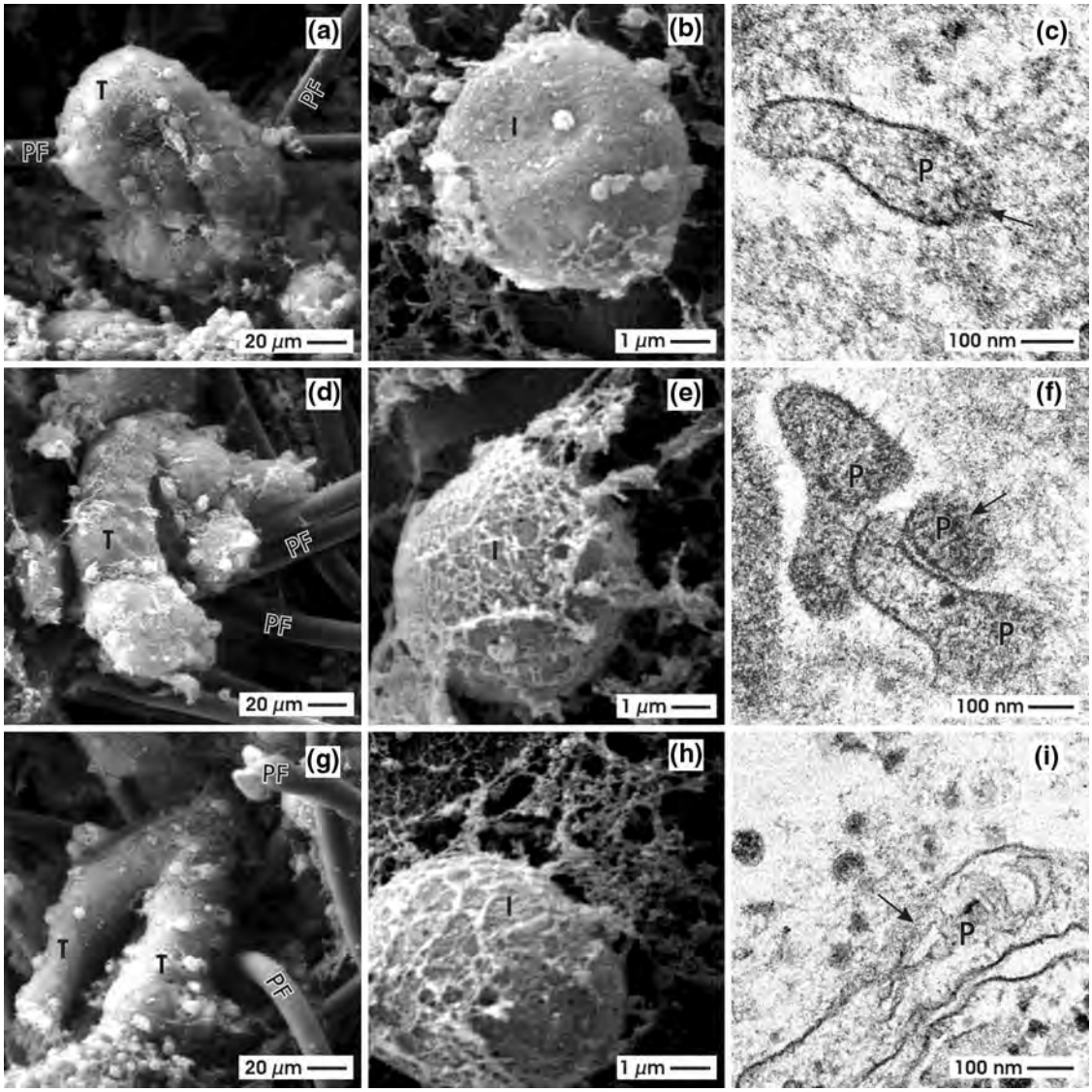

FIGURE 5. Scanning (a, b, d, e, g, h) and transmission (c, f, i) electron microscopy of tubules generated for 13 days at the interface of an artificial polyester interstitium. (a, d, g) SEM shows that fibers of the polyester fleece (PF) are detected in a longitudinal, transversal and oblique course. They exhibit a homogeneous composition and a smooth surface without recognizable protrusions or roughness. The diameter of fibers is 10 μm in average. Development of tubules (T) occurs in the space between the fibers. (a) A basal lamina is covering completely the outer surface of tubules. (d, g) A part of tubules grows in a parallel fashion, while others exhibit a dichotomous branching. (b) On the outer surface of generated tubules dispersed interstitial cells (I) are found exhibiting a round shape. (e, h) On the surface of interstitial cells a network of filopodia and extracellular matrix is recognized. (c, f, i) TEM demonstrates that a close contact exists between protrusions (P) of interstitial cells and attached fibers consisting of synthesized extracellular matrix. At the contact site (*arrow*) the plasma membrane appears solubilized. Amorphous material is protruding through the plasma membrane into the cytoplasm.

collecting duct ampulla comma-shaped bodies as first visible signs of nephron formation are detected. Down in the neighborhood of the ampulla shaft further matured nephrons are present. Thus, the axis of a developmental gradient is lining vertically from the organ capsule through the cortex corticis, the tip, the neck and finally to the shaft of the collecting duct ampulla. Along this gradient the specific features of the interstitium were investigated.

A clearly orientated semithin section shows that the mesenchymal nephrogenic stem/progenitor cells beyond the organ capsule and the epithelial stem/progenitor cells in the tip of the collecting duct ampulla are separated by a wide interstitium (arrows) (Fig. 7b). A wide interstitial cleft is also recognizable at the lateral side of the tip, the neck down to the shaft of the collecting duct ampulla. Here, matured Principal (P) and Intercalated (IC) cells become visible.

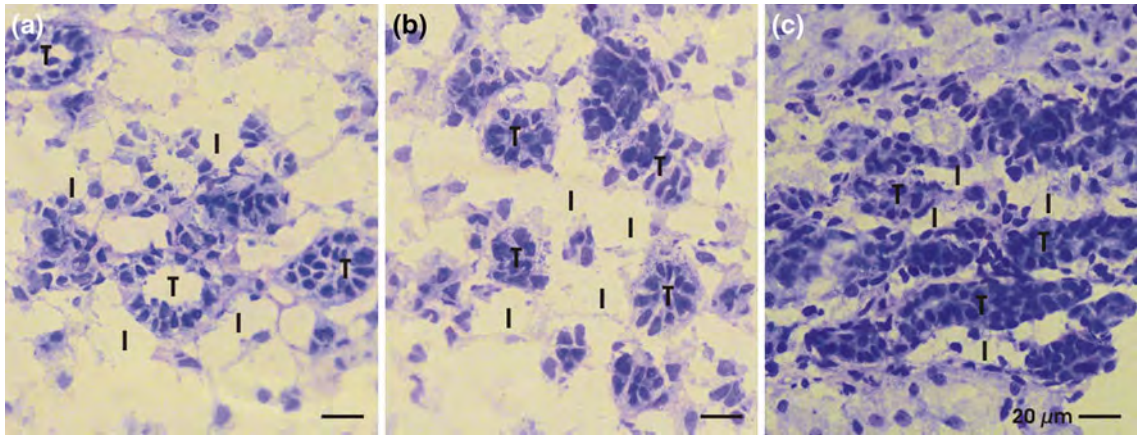

FIGURE 6. Light microscopy of renal parenchyma within the developing zone of neonatal rabbit kidney (a–c). (a, b) Cross and (c) in longitudinal sections depict tubules (T) within the interstitium (I).

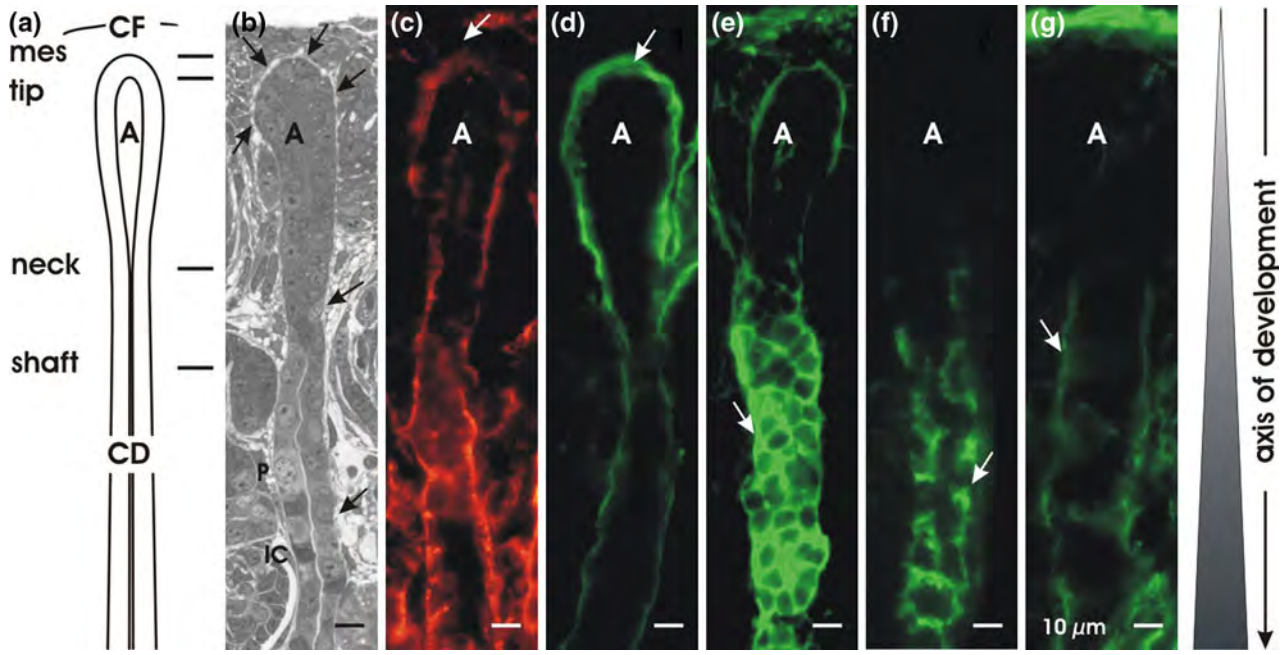

FIGURE 7. Features of the interstitium in the developing renal parenchyma. (a) Beyond the capsula fibrosa (CF) the mesenchyme (mes), the tip, neck and shaft of the collecting duct (CD) ampulla (A) of neonatal kidney are seen. (b) Semithin section shows that the mesenchyme and the tip of the collecting duct ampulla are separated by a distinct interstitial space (*arrows*) lining down to the lateral side of the tip, neck and shaft of the collecting duct ampulla. Matured Principal (P, *light*) and Intercalated (IC, *dark*) cells become visible in the shaft of the collecting duct ampulla. (c) Immuno-label for laminin $\gamma 1$ is lacking in the mesenchyme beneath the capsule (*arrow*), while distinct label is found in the basal lamina of all zones of the collecting duct ampulla. (d) Intensive label for P_{CD} Amp1 is found at the basal lamina of the ampulla tip (*arrow*), while the reaction is continuously decreasing towards the neck and shaft. (e) An inverse reaction is found for SBA label. Only faint reaction is detected in the basal lamina of the tip, while within the neck and shaft of the collecting duct ampulla strong cellular label is present (*arrow*). (f) Label for Na/K ATPase alpha 5 is lacking within the tip of the collecting duct ampulla, while intensive reaction is found in the neck and shaft (*arrow*). (g) Collagen type III is lacking in the mesenchyme and around the tip of the collecting duct ampulla, while primary arise is found around the neck (*arrow*).

To obtain more information about the developmental processes, histochemistry was performed (Figs. 7c–7g). Label for laminin $\gamma 1$ is lacking in the mesenchyme underneath the organ capsule (arrow), but it is found in the basal lamina of all zones of the collecting duct ampulla (Fig. 7c). Intensive reaction for P_{CD} Amp1 is found at the basal lamina of the ampulla tip (arrow), but the label is decreasing towards the neck and shaft of the ampulla (Fig. 7d). An inverse result is found for SBA label (Fig. 7e). Faint reaction is detected in the basal lamina of the tip, while within the neck and shaft of the collecting duct

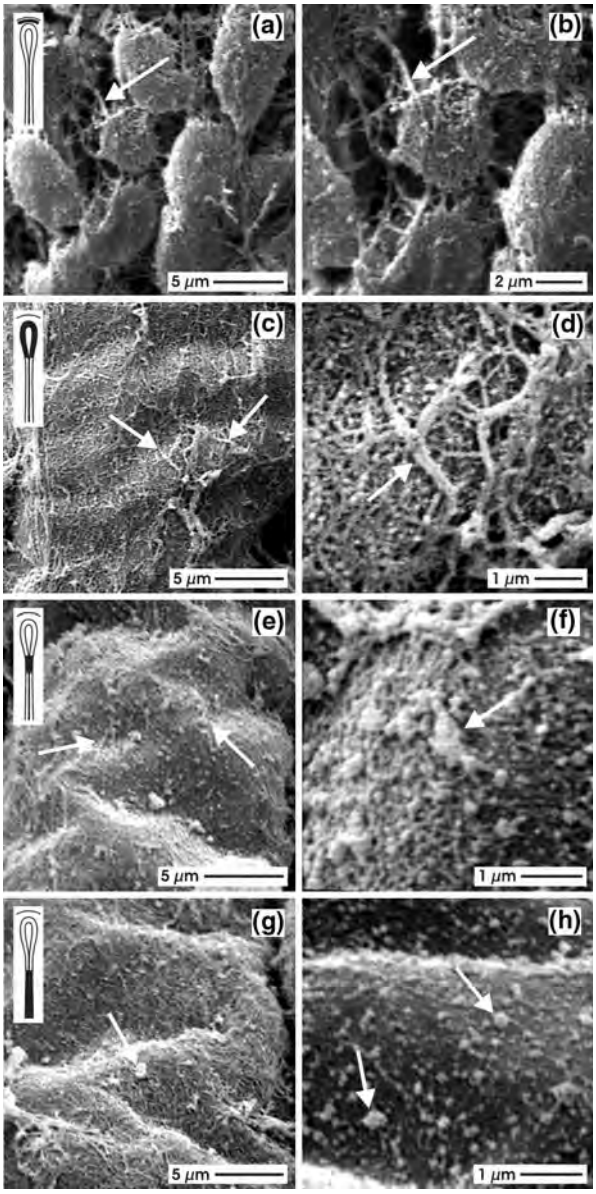

FIGURE 8. Scanning electron microscopy (SEM) in the cortex of neonatal rabbit kidney demonstrates structural elements of the interstitium in the mesenchyme (a, b) beyond the capsule, (c, d) around the tip, (e, f) the neck and (g, h) the shaft of the collecting duct ampulla. (a, b) The surface of mesenchymal cells exhibits short microvilli and numerous protrusions (*arrow*) searching contact to neighboring cells. It cannot be recognized if the protrusions consist exclusively of cellular material or if also extracellular matrix is included. (c, d) View to the tip of the collecting duct ampulla reveals a rough surface including arborising fibers (*arrow*) consisting of extracellular matrix. (e, f) Around the neck of the collecting duct ampulla the number and size of fibers decreases (*arrow*), so that the surface becomes smooth. Here numerous round particles adherent to fibers can be recognized. (g, h) Along the shaft of the collecting duct ampulla the number of round particles (*arrows*) within the lamina fibroreticularis decreases and strong fibers are lacking.

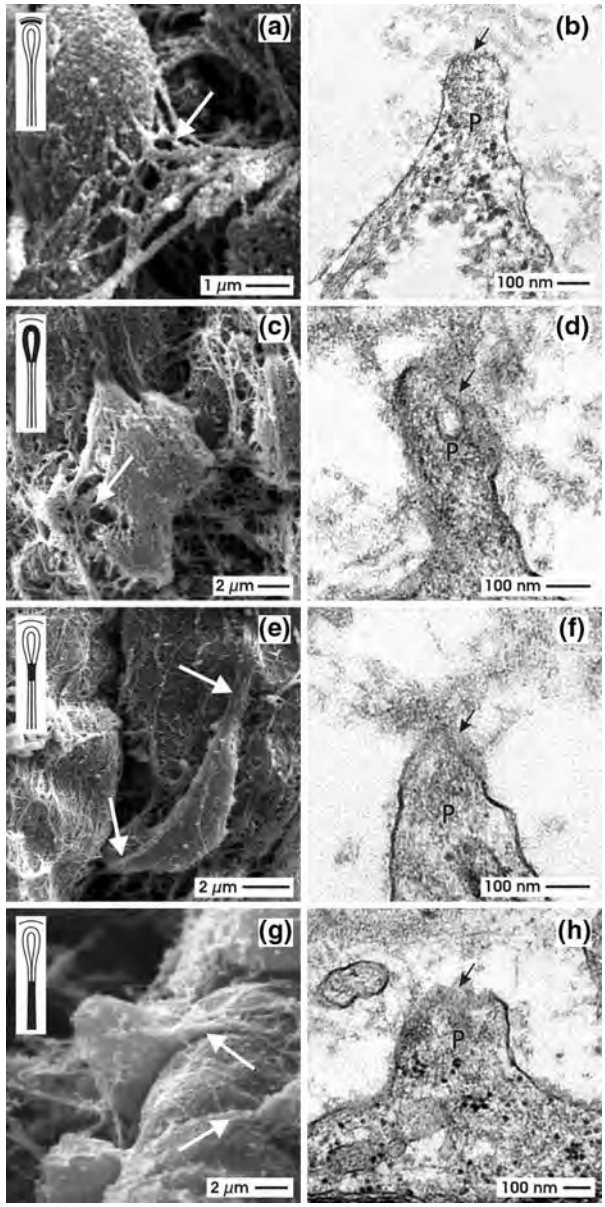

FIGURE 9. Scanning (SEM) (a, c, e, g) and transmission (TEM) (b, d, f, h) electron microscopy of the cortex in neonatal rabbit kidney. (a) SEM depicts that cells in the mesenchyme are connected over a loose network of thin fibers (*arrow*). (c) Around the tip of the collecting duct ampulla flat interstitial cells with numerous filopodia and a dense network of extracellular matrix fibers are seen (*arrow*). (e) At the neck of the ampulla interstitial cells are flat showing two or three foot-like protrusions (*arrows*). At the contact site with the extracellular matrix numerous filopodia are present. (g) Along the shaft of the ampulla interstitial cells exhibit a cuboidal shape. At the cell side facing the extracellular matrix remarkable food-like protrusions and filopodia keeping contact with extracellular matrix (*arrows*). (b, d, f, h) TEM illuminates that a close contact exists between protrusions (P) of interstitial cells and attached fibers consisting of extracellular matrix. At the contact site the plasma membrane appears solubilized. The amorphous material protrudes through the plasma membrane into the cytoplasm (*arrow*).

ampulla strong cellular label is present. Within the collecting duct ampulla label for Na/K ATPase alpha 5 is lacking (Fig. 7f). In contrast, intensive reaction for Na/K ATPase alpha 5 is found in the ampulla shaft (arrow) and the further matured collecting duct. Surprisingly, collagen type III is lacking in the mesenchyme and around the tip of the collecting duct ampulla, while primary appearance of label is found around the neck (arrow) and the shaft of the ampulla (Fig. 7g). Thus, collagen type III is not synthesized as a primary structural element in the developing renal interstitium.

Structural Elements Within the Interstitium

To gain insights in structural features of the developing interstitium, scanning electron microscopy (SEM) was performed (Fig. 8). SEM demonstrates underneath the organ capsule mesenchymal nephrogenic stem/progenitor cells. It is obvious that they are keeping an astonishingly wide distance to each other (Fig. 8a). The surface of mesenchymal cells exhibits short microvilli and numerous protrusions searching contact to neighboring cells (Fig. 8b). It cannot be recognized if the protrusions consist exclusively of cellular material or if also extracellular matrix is included. In contrast, view to the tip of the collecting duct ampulla reveals a rough surface including numerous arborising fibers (Figs. 8c and 8d). In the neck of the collecting duct ampulla, the number and size of fibers decrease, so that the surface becomes smooth (Figs. 8e and 8f). Yet, numerous more or less round particles adherent to fibers can be recognized (Fig. 8f). In the shaft of the collecting duct ampulla, the number of round particles within the lamina fibroreticularis decreases and strong fibers are lacking (Figs. 8g and 8h). Thus, following the cortico-medullary axis structural elements within the developing interstitium are heterogeneously composed. This kind of segmentation appears in parallel to histochemical changes occurring along the axis between the mesenchymal stem/progenitor cells, the ampulla tip, ampulla neck and ampulla shaft of the collecting duct (Fig. 7).

Cells in the Different Zones of the Developing Interstitium

To obtain information about cells communicating with structural elements in the developing interstitium, SEM was performed. Thus, the cortex corticis (Fig. 9a), the ampulla tip (Fig. 9c), the ampulla neck (Fig. 9e) and the shaft of the collecting duct ampulla (Fig. 9g) were analyzed. The zone of nephrogenic mesenchyme beyond the organ capsule reveals that numerous cells are embedded in a loose network consisting of thin fibers (arrow) (Fig. 9a). It cannot be decided if cellular protrusions or extracellular matrix or both are seen. Further down, around the tip of the collecting duct ampulla flat interstitial cells with numerous protrusions are localized (Fig. 9c). They are embedded within a dense network of extracellular matrix fibers. At the neck of the ampulla interstitial cells are flat showing two or three foot-like protrusions (arrow) (Fig. 9e). The surface of the cells is smooth. At the contact site with extracellular matrix numerous cellular protrusions are present. In contrast, in the shaft of the ampulla, interstitial cells exhibit a more or less cuboidal shape (Fig. 9g). At the cell side facing the extracellular matrix, remarkable food-like protrusions (arrow) are detected.

SEM illuminates three-dimensional features of cell and extracellular matrix within the developing interstitium (Figs. 9a, 9c, 9e, and 9g). However, following the protrusion of cells it is impossible to recognize a clear demarcation line between the end of the cell and the beginning of extracellular matrix. To investigate this special interface, transmission electron microscopy (TEM) was performed first in the mesenchymal stem/progenitor cell group within the cortex corticis (Fig. 9b), around the ampulla tip (Fig. 9d), the ampulla neck (Fig. 9f) and the collecting duct ampulla shaft (Fig. 9h). Surprisingly, in all series it was found that both protrusions and extracellular matrix have a close contact to each other. However, this interface is special, since the plasma membrane appears to be solubilized to a more or less degree as it was earlier observed in generated tubules (Figs. 5c, 5f, and 5i). Most interestingly, in all cases the amorphous material of extracellular matrix is protruding through the plasma membrane into the cytoplasm (arrow in Figs. 9b, 9d, 9f, and 9h).

DISCUSSION

The interstitium is an important functional space occurring in all parenchymal organs. In the kidney it can be recognized as a narrow slit between the basal lamina of tubules and the capillaries. Although barely visible in light microscopy (arrow, Fig. 7b), the interstitial space is of great importance in the healthy kidney.[14] The interstitium consists of extracellular matrix, the surrounding fluid and the interstitial cells. The skeletonal portion contains mainly collagen type III sustaining the three-dimensional structure of the organ.[7] All processes of tubule reabsorption and secretion have a transit across the fluid portion localized between the collagen fibers. In the diseased kidney, inflammatory cells infiltrate the interstitium. The resulting increase of interstitial cells and extracel-

lular matrix causes renal fibrosis.[6] During this process tubule cells loose epithelial features and convert to fibroblast-like cells.[3] This epithelial-mesenchymal transition (EMT) is paralleled for example by the expression of fibroblast-specific protein-1(FSP1),[13] heat shock protein 47 (HSP 47)[10,16,21] and α-smooth muscle actin (SMA).[31]

In contrast, mesenchymal-epithelial transition (MET) plays an important role, when stem/progenitor cells are applied to support regeneration in acute or chronic renal failure.[1,33] The key for a successful therapeutic concept is to learn about involved cell biological processes promoting epithelial differentiation during tubule formation. The process of regeneration includes mechanisms organizing the spatial distribution of tubules. In the kidney, this separation is caused by extracellular matrix proteins synthesized during organ development. To obtain insights in the arise of extracellular matrix elements developing renal parenchyma in the kidney and tubules generating at the interface of an artificial polyester interstitium was analyzed.

Interstitium of the Developing Renal Parenchyma

Insights in the structure of the interstitium can be obtained on an exactly orientated section through the cortex of neonatal rabbit kidney (Figs. 7a and 7b). Developing, maturing, and matured tissue can be found here lining along a developmental axis from the organ capsule through the cortex down to the medulla.[15] In the cortex, four different zones of maturation can be distinguished. It comprises the mesenchyme (including nephrogenic stem/progenitor cells), the tip (including epithelial stem/progenitor cells), the neck (maturing), and the shaft (matured) of the collecting duct ampulla (Fig. 7a).

Light microscopy further reveals that the interstitium of developing renal parenchyma is extending as well between the mesenchymal cells as around the basal aspect of the collecting duct ampulla (arrow, Fig. 7b). The interstitial space can be further recognized as a slit running from the tip to the neck and down to the shaft of the collecting duct ampulla. Here, the arise of matured P and IC cells within the collecting duct tubule can be seen.

SEM illuminates better that the mesenchyme containing nephrogenic stem/progenitor cells exhibits astonishingly wide intercellular spaces reflecting an extended interstitium (Figs. 8a and 8b). In addition, numerous anastomizing fibers connect the sides of neighboring cells. Performing SEM it cannot be decided yet if the contacting structures consist exclusively of cellular protrusions or if also extracellular matrix is involved (Fig. 9a). However, TEM clearly elucidates that this interface is special. It consists of both synthesized extracellular matrix fibers in close contact with finger-like cellular protrusions (Figs. 9b, 9d, 9f, and 9h). Most conspicuous is that amorphous extracellular matrix material is protruding through the plasma membrane into the cytoplasm.

Heterogeneity of Structural Elements in the Developing Interstitium

The tip of the collecting duct ampulla is the specific site, where mesenchymal nephrogenic stem/progenitor cells interact reciprocally with epithelial stem/progenitor cells during nephrogenesis.[20,25] This molecular process results in the formation of a comma-shaped body as a first morphological sign of a developing nephron. Thus, the interstitium at the ampulla tip was analyzed by SEM (Figs. 8c and 8d). A dense network of extracellular matrix is found here. It consists of numerous fibers with varying diameter as it was shown earlier.[26,29] In contrast, in the ampulla neck the roughness, the diameter and the length of the skeletonal elements decrease (Figs. 8e and 8f). Instead, more or less round and bone-shaped particles are found to be attached to the fibers of the extracellular matrix. At the shaft of the collecting duct ampulla, the surface of extracellular matrix becomes smooth and fibers become rare (Figs. 8g and 8h). In consequence, the portions of the interstitium along the four zones of the collecting duct ampulla are heterogeneously composed. Up to date it is speculative if the different shape is due to different protein composition. The heterogeneity of the extracellular matrix could coincidence with particular functions occurring during nephrogenesis. However, more information about individual structural composition is up to date not available and has to be investigated in future.

Arise of Structural Elements in the Interstitium

The main structural element within the renal interstitium is collagen type III or earlier described as reticulin.[7,22] One could assume that the primary expression of collagen type III is a driving force forming the structural portion of the interstitium and featuring thereby the distance between generating tubules. To obtain exact information, the primary appearance of collagen type III was investigated (Fig. 7g). Most interestingly, immuno-label demonstrates that collagen type III is lacking as well in the nephrogenic mesenchyme as along the tip and neck of the collecting duct ampulla. However, further down in the shaft of the collecting duct ampulla primary appearance of collagen type III is found. In contrast, label for laminin γ1 is present not only in the shaft, but

also in the neck and tip of the collecting duct ampulla, while in the mesenchyme it could not be detected (Fig. 7c).[19] Thus, label for collagen type III is missing in the zone of renal stem/progenitor cells, but it is co-localized with laminin γ1 in the maturing zone along the shaft of the collecting duct ampulla. In consequence, the experiments clearly show that collagen type III is not the primary skeletonal element of the interstitium in developing renal parenchyma. More appropriate candidates are earlier described structural elements such as collagen type IV,[29] SBA-labeled molecules within the basal lamina (Fig. 7e), microfibers[26] or P_{CD} Amp1[28] (Fig. 7d).

Interface Between Generating Tubules

Experiments were further made to investigate the structured portion of the interstitium keeping generating tubules in distance (Figs. 2a– 2c). The surface view on whole mount SBA-labeled specimens demonstrates that generated tubules do not fuse, but are separated by each other. Morphometric analysis elucidates that a majority of the tubules generated at an artificial interstitium exhibits a distance in the range between 5 and 25 μm (Fig. 2d). Up to date a reason for the discrete distance between generated tubules is not known. Moreover, generated tubules exhibit a co-localization of collagen type III and laminin γ1 along the basal lamina (Fig. 3), as it is found in the developing part of the kidney (Figs. 7c and 7g). The reaction of collagen type III is also found in the interstitium of generated tubules. This result illuminates that collagen type III appears as an important molecule linking the artificial polyester interstitium with the basal lamina of generated tubules. SEM and TEM support this finding, since the basal lamina of generated tubules is surrounded by bundles of synthesized extracellular matrix (Figs. 4 and 5a, 5d, 5g). Regarding morphometric data (Fig. 2d), it appears that the linking of polyester fibers and synthesized extracellular matrix is determining the distance between generated tubules. Experiments are under work to illuminate further the interface influencing the development at an artificial interstitium.

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