Regenerating Tubules for Kidney Repair

W.W. Minuth, L. Denk, and A. Roessger

Abstract Stem/progenitor cells are in the focus of regenerative medicine for a future therapy of acute and chronic renal failure. However, broad knowledge about parenchymal regeneration in kidney is lacking. For that reason developmental pathways leading from stem/progenitor cells to newly formed tubules have to be investigated. A new technique promotes renal stem/progenitor cells to form numerous tubules between layers of polyester fleeces. This artificial interstitium replaces coating by extracellular matrix proteins, supports spatial extension of renal tubules, and can be used with chemically defined Iscove's modified Dulbecco's medium (IMDM) during a long-term culture period of 13 days. The development of tubules is stimulated by aldosterone and depends on the applied hormone concentration. The tubulogenic effect cannot be mimicked by precursors of the aldosterone synthesis pathway or by other steroid hormones. Antagonists such as spironolactone or canrenoate prevent the development of tubules, which indicates that the mineralocorticoid receptor (MR) is involved. Administration of geldanamycin, radicicol, quercetin, or KNK 437 in combination with aldosterone blocks development of tubules by disturbing the contact between MR and heat-shock proteins. Transmission electron microscopy (TEM) further demonstrates that generated tubules exhibit a junctional complex between the apical and the lateral plasma membrane. At the basal aspect a continuously developed basal lamina is present. Immuno-label for Troma I (cytokeratin Endo-A) shows isoprismatic cells, while label for laminin $\gamma 1$, occludin, and Na/K-ATPase $\alpha 5$ confirms typical features of a polarized epithelium. Finally, the introduced system makes it possible to pile and pave renal stem/progenitor cells, so that the spatial development of tubules can be systematically investigated.

Keywords Artificial interstitium · Kidney · Stem/progenitor cells · Aldosterone · Perfusion culture

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1 Promoting Regeneration in Kidney

The multitude of patients with chronic or acute renal failure shows that the kidney has in comparison to other organs a decreased capability for functional regeneration. In view of this clinical background the question arises why the diseased kidney is not able to regenerate new nephron segments. Of special experimental interest is the question which cell-biological process inhibits the regeneration of parenchyme.

An ideal form of therapy for the future could be to induce a process of regeneration and to steer it therapeutically [1, 2]. One could imagine that the renewal of parenchyme occurs via a stimulation of non-diseased parenchymal cells or via an activation of tissue-specific stem/progenitor cells. Independent of the chosen strategy it is apparent that an application or an activation of stem/progenitor cells alone is not sufficient to trigger the optimal course of therapy. Most important, one has to learn to promote the spatial development of tubules and their integration in a diseased environment. However, at present the knowledge about these processes is minimal and the complex micro-architecture of the kidney makes it difficult to investigate such processes (Fig. 1).

For that reason controlled in vitro experiments are performed to learn about optimizing stem/progenitor cell development and to steer their spatial growth so that finally three-dimensional structured tubules arise. Only with this basic knowledge it will be possible in future to steer regeneration within a diseased kidney.

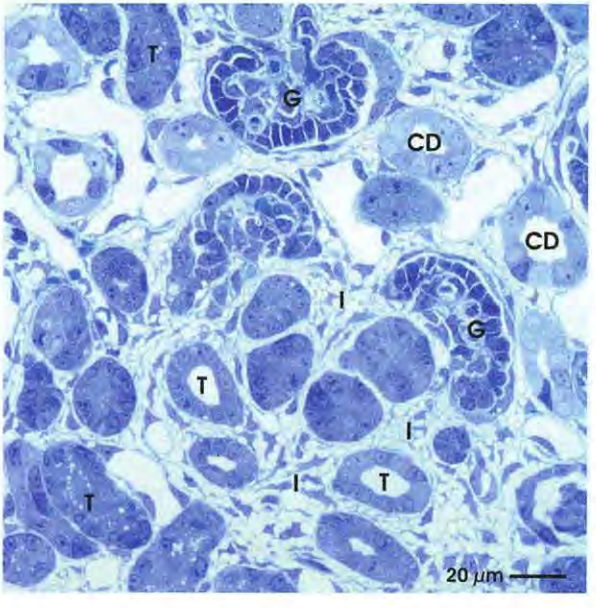

Fig. 1 Horizontal semi-thin section of the cortex from neonatal rabbit kidney. The illustration shows developing glomeruli (*G*), tubules (*T*), and collecting ducts (*CD*) embedded in the interstitium (*I*)

2 Viewing Renal Stem/Progenitor Cell Niches

In our model system the niche for stem/progenitor cells can be demonstrated in the outer cortex of neonatal rabbit kidney (Fig. 2). This region is developing for days still after birth. Beyond the capsule two kinds of very different stem/progenitor cell populations are recognized. The collecting duct tubule (CD) develops from the ampullae (A) located below the renal organ capsule (CF). Each collecting duct ampulla contains in its tip epithelial stem/progenitor cells. At the basal aspect of the ampullar epithelium mesenchymal stem/progenitor cells are found, which develop into the different nephron segments.

After a reciprocal interaction between cells in the tip of the collecting duct ampulla (A) and the nephrogenic mesenchymal stem/progenitor cells, first signs of nephron anlagen as comma-shaped and further matured S-shaped bodies are recognized. By an unknown mechanism the capsule (CF)-orientated wing of the S-shaped body forms all the tubule portions of the nephron, while the medulla-oriented wing is the origin of the glomerulus. The tubular portion develops further into the proximal, intermediate, and distal nephron segments.

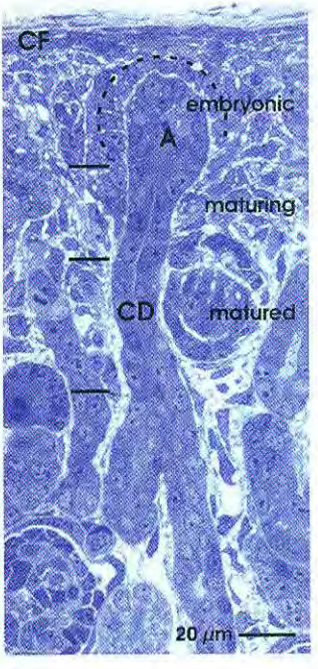

Fig. 2 Vertical section through the cortex of neonatal rabbit kidney. Beyond the capsula fibrosa (*CF*) epithelial stem/progenitor cells are found within the ampulla (*A*) tip of the collecting duct (*CD*). Nephrogenic mesenchymal cells surround the basal aspect of the ampulla (*dotted line*). The outer cortex can be divided into an embryonic, maturing, and matured zone

3 Telling Stem/Progenitor Cells to Form Structured Tubules

At the first view the development of a three-dimensional tubule derived from renal stem/progenitor cells appears simple, but in reality it is a rather complex cell-biological process, which is up to date not understood [3, 4]. As seen from a theoretical sight the intricate development comprises the sprouting of cells for reaching the necessary amount, the formation of a lumen, and the elongation of the tubule segment (Fig. 3) [5–8]. Thus, renal stem/progenitor cells first transdifferentiate, then they are multiplied to form a polarized epithelium. The epithelium is integrated into a structured tubule exhibiting exact geometrical dimensions such as length and inner and outer diameter. For renal tubules it is unknown if the process forming a lumen occurs by wrapping, budding, cavitation, or hollowing. The development continues at the basal side of the tubule by the development of a basal lamina. During the

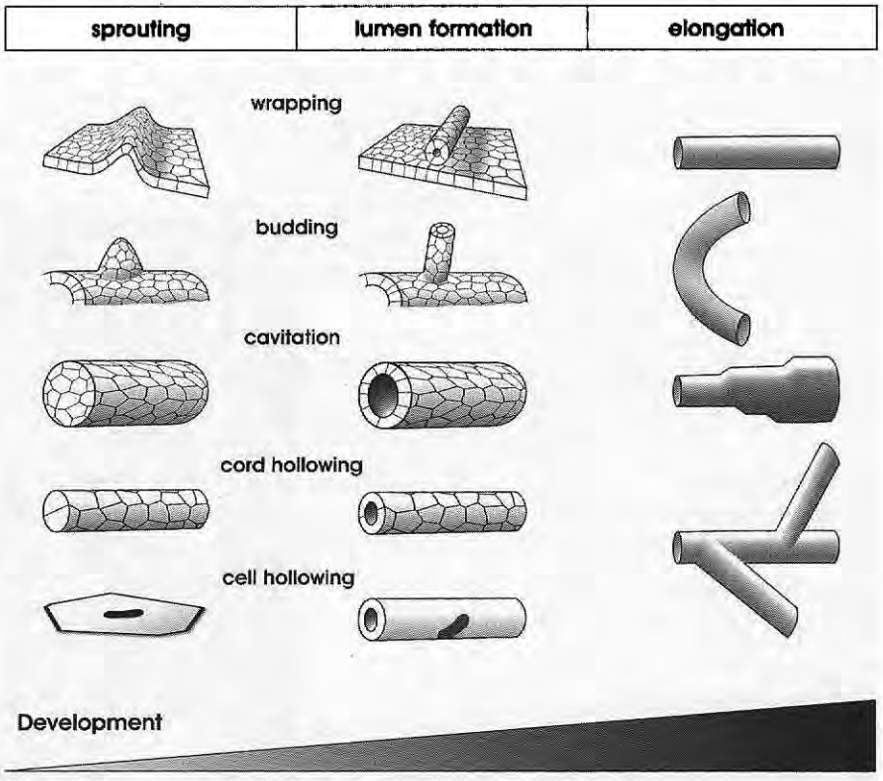

Fig. 3 Schematic development of a polarized tubule comprising sprouting, lumen formation, and elongation. During this process embryonic cells transdifferentiate and develop into a three-dimensional tubule with a defined inner and outer diameter. Further on a straightforward development, a convolution, or an arborization is triggered

following growth phase, geometrical properties such as straight course, length, convolution, or branching of the tubule are realized. Finally a structured tubule with a defined inner and outer diameter arises.

It is obvious that a tubule does not appear automatically after the application of a single growth factor. In contrast, the complex development depends on parameters such as different morphogenic factors, selected adhesion substrates, intercellular communication, and the fluid environment. All these factors have to complement one another to promote histotypical development and to prevent arise of atypical features.

The molecular interactions during the process of reciprocal induction between renal stem/progenitor cells are intensively investigated over a period of decades. However, only little knowledge is available concerning the development of the different tubule segments. For example, the morphogenetic factors are unknown, which trigger the segmentation into the proximal tubule, the loop of Henle, the distal tubule, the connecting tubule, and finally the heterogeneously composed collecting duct. It is further unclear why parts of the proximal and distal tubule develop convolutes, while all the other segments show a straightforward orientated course. In this coherence it is also unknown why some of the nephron segments are homogeneously composed, while the connecting and collecting duct tubules exhibit a heterogeneous cell population.

4 Registering Development of Epithelial Stem/Progenitor Cells

When renal stem/progenitor cells are taken into culture it is important to know about their current embryonic, maturing, and matured states of development. In running experiments the degree of differentiation can be compared between the in vivo and in vitro situation. To register the development potent histochemical markers are required, which make it possible to distinguish between an embryonic, a mature, and an adult state of differentiation. This difference can be visualized, for example, by soybean agglutinin (SBA) labeling. The lectin recognizes terminal *N*-acetylgalactosamine (GalNAcα1) residues on glycoproteins. In comparison to an antibody label the staining with a lectin saves 2 h of incubation time.

Cryosections of the neonatal rabbit kidney reveal that cells within the tip of the collecting duct ampulla (A) lack SBA label. This site of the collecting duct ampulla (A) is known to be the niche for epithelial stem/progenitor cells. It is found below the capsula fibrosa (CF) in the outer cortex of the neonatal rabbit kidney (Fig. 4). In contrast, the maturing cells on the ampulla neck gain SBA label, while the ampulla shaft and further matured collecting duct (CD) tubules reveal an intensive SBA label. Thus, comparison between the ampulla tip, the neck, the shaft, and the functional tubule illustrates a unique developmental gradient showing renal stem/progenitor cells and maturing, respectively, matured epithelial cells within the collecting duct tubule.

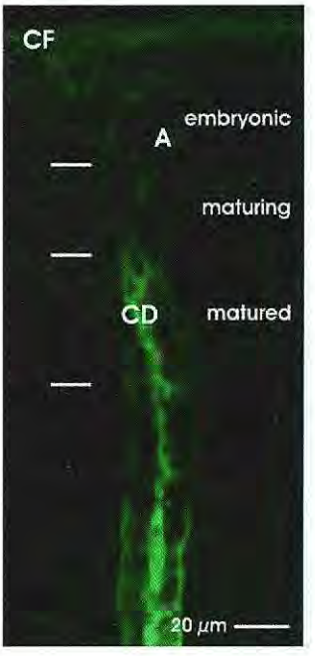

Fig. 4 SBA label demonstrates the developmental gradient between the ampulla (*A*), the neck, and the shaft of the collecting duct (*CD*) tubule. The epithelial stem/progenitor cells within the ampulla are not recognized by the lectin, while the neck shows first signs of reaction. Intensive reaction is found in the matured collecting duct

5 Isolating Renal Stem/Progenitor Cell Containing Tissue

To investigate regeneration of tubules under in vitro conditions a suitable source of renal stem/progenitor cells is needed. Due to the limited size of embryonic mouse or rat specimens, neonatal rabbit kidney is selected as a cell-biological model, since even after birth the embryonic cortex of the organ contains numerous stem cell niches in their original extracellular environment [9]. Further on, the embryonic tissue layer is easily accessible for isolation and can be harvested in sufficient amounts for culture and cell-biological analysis (Fig. 5a).

For the generation of renal tubules embryonic explants from the outer cortex of newborn rabbit kidney can be used. Stripping off the capsula fibrosa (CF) with fine forceps a thin layer of embryonic tissue of constant thickness adheres to the explant (Fig. 5b). Besides the isolated epithelial stem/progenitor cells located in the tip of the collecting duct ampulla, surrounding nephrogenic mesenchymal stem/progenitor cells and S-shaped bodies are found. By this simple micro-surgical method a thin embryonic tissue layer of up to 1 cm^2 can be harvested. Up to date no other species is known, which makes it possible to harvest embryonic tissue containing renal stem/progenitor cells (rS/Pc) in such an amount by this simple preparation technique.

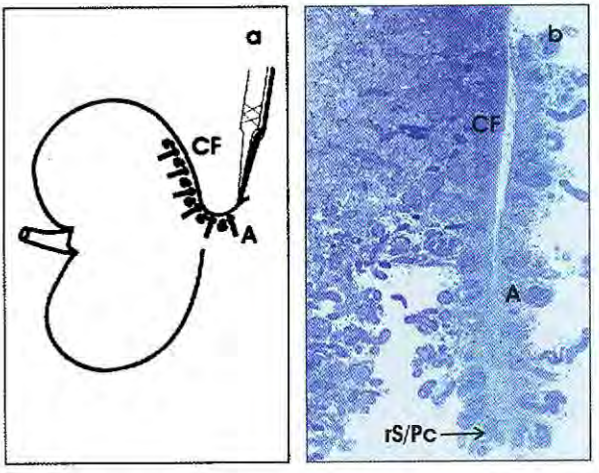

Fig. 5 Isolation of renal stem/progenitor cells from neonatal rabbit kidney. (**a**) Using fine forceps the renal capsula fibrosa (*CF*) is stripped off with an adherent layer of embryonic tissue. (**b**) Semithin section of an isolated explant consisting of renal organ capsule (*CF*), collecting duct ampullae (*A*), renal mesenchymal stem/progenitor cells (*rS/Pc*), and S-shaped bodies

6 Engineering a Micro-environment for Structural Development

When renal stem/progenitor cells are used in future for the repair of parenchyme in acute or chronic renal failure, it is important to investigate their developmental capacity and to learn about formation of tubules. Unsolved issues comprise a suitable implantation technique, the controlled application of growth factors, and the steering of tubule development in a diseased spatial environment of the kidney. However, up to date the growth of stem/progenitor cells is unpredictable. It is, for example, unknown if the complex histoarchitecture of the diseased kidney promotes or even inhibits regeneration of tubules. For that reason sophisticated in vitro experiments have to be performed to obtain exact information about mechanisms involved in the formation of new parenchyme. Such culture experiments have to meet the physiological needs of renal stem/progenitor cells under in vitro conditions. They have to support their development in a spatial environment, to avoid the formation of unstirred layers of medium, and finally to be able to harvest the necessary amount of tissue for cell-biological analysis. Since earlier applied culture methods do not fulfill these needs, an innovative technique for the generation of renal tubules was elaborated [10].

The technical solution is to culture renal stem/progenitor cells between layers of polyester fleece, which replaces the coating by extracellular matrix proteins. For culture the isolated embryonic renal tissue is placed between two punched out layers of polyester fleece (Walraf, Grevenbroich, Germany) measuring 5 mm in diameter

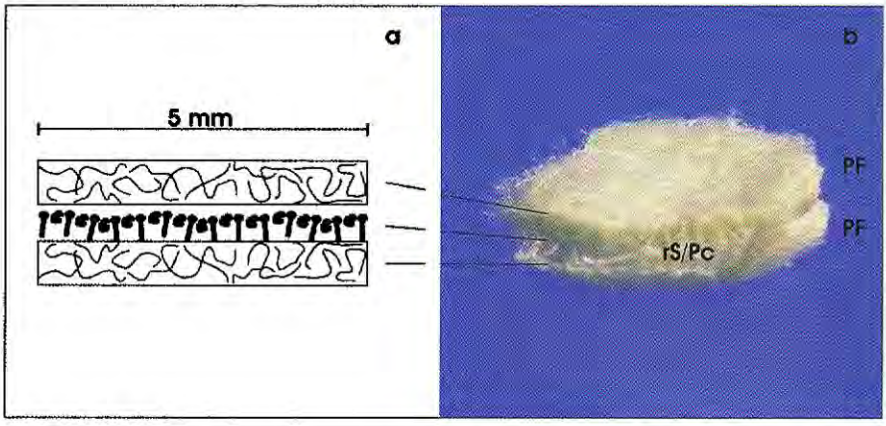

Fig. 6 Creation of an artificial interstitium for the culture of renal stem/progenitor cells. **(a)** Schematic illustration shows that the isolated tissue is placed between two layers of polyester fleece. **(b)** Illustration demonstrates basic sandwich set-up containing isolated embryonic tissue containing renal stem/progenitor cells (*rS/Pc*) between layers of polyester fleece (*PF*)

(Fig. 6a). This arrangement results in a basic sandwich set-up configuration with the freshly isolated embryonic tissue in the middle and layers of polyester fleece covering the outer sides (Fig. 6b).

The interface between the layers of polyester fleece is used as an artificial interstitium, which promotes the spatial development of tubules. Further on, the space between the fleece fibers is an advantage for continuous exchange of culture medium and respiratory gas [11, 12].

7 Offering a Suitable Bioreactor Housing

The basic sandwich set-up containing renal stem/progenitor cells has to be held in its position inside a perfusion culture container to prevent damage to the developing tissue during culture (Fig. 7a). A base ring of a Minusheet® tissue carrier with 13 mm inner diameter is transferred to a perfusion culture container with horizontal flow characteristics (Minucells and Minutissue, Bad Abbach, Germany; www.minucells.de) .

To mount the basic sandwich set-up first a polyester fleece measuring 13 mm in diameter is placed into this tissue carrier (Fig. 7a). Then the basic sandwich set-up containing renal stem/progenitor cells measuring 5 mm in diameter is inserted (Figs. 6, 7a). Finally, a polyester fleece 13 mm in diameter is placed on top of the sandwich as a cover (Fig. 7a, b). After closing the lid of the perfusion container the basic sandwich set-up is fixed in an exact position (Fig. 7c). The spatial area for tubule formation between the polyester fleece layers is 5 mm in diameter and up to 250 μm in height. The specific interface between the fleece layers produces an

Fig. 7 Microreactor design for the culture of a basic sandwich set-up at the interface of an artificial interstitium. (**a**) The basic sandwich set-up containing renal stem/progenitor cells is mounted in a tissue carrier. (**b**) The perfusion container consists of a base plate to hold the tissue carrier with the developing tissue embedded between layers of polyester fleece. (**c**) The container is sealed for culture with a lid

artificial interstitium providing an optimal microenvironment for the development of tubules during the entire culture period.

The principal design of the perfusion culture container consists of a base plate and a lid made of polycarbonate. In the basic version a tissue carrier with developing tissue is placed onto a base plate (Fig. 7b). Then a lid of the container is secured on top. Lid and/or base plate features a medium inlet and outlet on opposing ends. The container is sealed by clamping the lid onto a silicon gasket on the base plate (Fig. 7c). The advantage of such a container is the creation of an artificial interstitium and a decreased overall height that entails a reduction in dead volume and optimizes medium exchange.

8 Providing Always Fresh Culture Medium

To generate renal tubules chemically defined IMDM (Iscove's modified Dulbecco's medium including Phenolred, GIBCO/Invitrogen, Karlsruhe, Germany) is used. In order to maintain a constant pH of 7.4 under atmospheric air containing 0.3% CO_2 HEPES (50 mmol/l) is added to the medium. To induce tubulogenic development of renal stem/progenitor cells aldosterone (1×10^{-7} M, Fluka, Taufkirchen, Germany) is administered and to prevent infections an antibiotic–antimycotic cocktail (1%, GIBCO) is applied in the culture medium.

Dynamic culture is performed throughout the experimental phase of 13 days. Always fresh medium is perfused at a rate of 1.25 ml/h with an IPC N8 peristaltic pump (Ismatec, Wertheim, Germany). To maintain a constant temperature of 37°C, the culture container is placed on a thermoplate (Medax-Nagel, Kiel, Germany) and covered with a transparent lid (Fig. 8).

To avoid unstirred layers of fluid developing tissue at the interphase of an artificial interstitium is supplied with a continuous flow of always fresh culture medium.

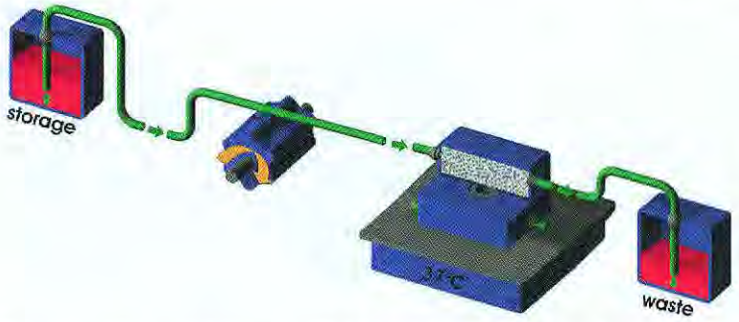

Fig. 8 Dynamic culture containing an artificial interstitium is performed with several modules that are assembled into a working line. A peristaltic pump maintains a flow (1.25 ml/h) of always fresh medium from the storage bottle to the culture container. Used medium that leaves the culture container is not recycled but is collected in a waste bottle

Medium is saturated to 190 mmHg oxygen during transportation. It guarantees an optimal supply for the growing tissue. The high content of oxygen in the medium is reached by a long thin-walled silicone tube. The tubing is highly gas permeable and guarantees optimal diffusion of gases between culture medium and surrounding atmosphere. In this way it is possible to adjust the gas partial pressures within the medium under absolutely sterile conditions because the medium does not get in direct contact with the gases. By maintaining a defined carbon dioxide concentration in the medium this method can be employed to control medium pH via the bicarbonate buffer and irrespective if the system runs in a CO_2 incubator or under atmospheric air. In consequence, the enrichment of oxygen is optimized and the formation of gas bubbles is minimized in this procedure.

9 Visualizing Pattern of Generated Tubules

After a culture period of 13 days at the interface of an artificial interstitium the generated tissue can be analyzed. In order to visualize tubules the artificial interstitium is opened by tearing off the layers of the polyester fleece. Then the specimens are fixed in 70% ethanol and labeled by fluorescent soybean agglutinin (SBA) to analyze the growth pattern (Fig. 9). While the isolated tissue does not exhibit any cellular SBA label, acquisition of SBA binding indicates maturation as it can be recognized during development of the collecting duct within the neonatal kidney (Fig. 4).

Fluorescence microscopy of whole-mount specimens demonstrates that numerous tubules are growing in a spatial arrangement (Fig. 9a, b). When the tubules are developing closely attached to each other the fluorescence is so bright that the details cannot be clearly recognized (Fig. 9a). For that reason areas on the fleece are selected, where the tubules are growing more or less separately from each other (Fig. 9b). Cases are registered, where tubules demonstrate a singular straightforward occurrence, while others reveal a dichotomous branching or curling. When

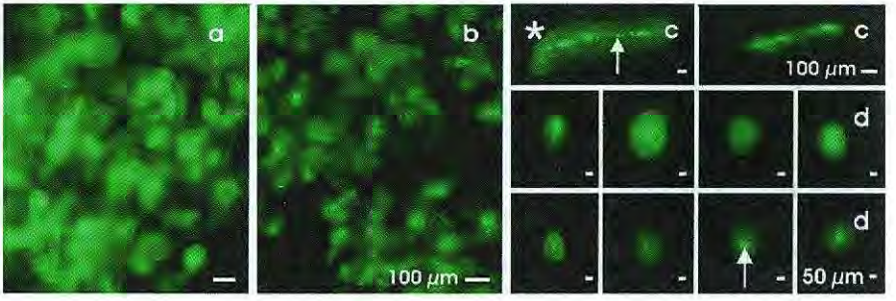

Fig. 9 Fluorescence microscopy of whole-mount specimens labeled by SBA. (**a**, **b**) Opening the artificial interstitium after 13 days of culture reveals a field of densely packed tubules. (**c**) Longitudinal and (**d**) vertical view of generated tubules demonstrates a lumen (*arrow*) and a basal lamina (*asterisk*)

the tubules are not leaving the optical plain, it is possible to follow the longitudinal growth over a distance between 300 and 400 µm. Longitudinal (Fig. 9c) and vertical (Fig. 9d) view depicts that the tubules contain in their center a clearly recognizable lumen, while at the outer surface a smooth basal lamina is present.

10 Scanning the Interface of an Artificial Interstitium

To analyze the covering surface of generated tubules scanning electron microscopy (SEM) was performed (Fig. 10). Special interest is focused on the basal aspect of generated tubules and especially on their contact with surrounding fleece fibers. In contrast to experiments earlier performed by other groups, tubules were generated without coating them by extracellular matrix proteins. This makes it for the first time possible to analyze the basal aspect of generated tubules without the interference of extracellular matrix proteins derived from a coating process [13].

SEM of the fleece without cells demonstrates numerous polyester fibers in a three-dimensional extension (Fig. 10a). The fibers are running in a longitudinal, transversal, and oblique course. They appear to be of homogeneous composition and show a smooth surface without recognizable protrusions or roughness. The average diameter of a polyester fiber is 10 µm. Chemical cross-linking between the polyester fibers cannot be observed.

The area of the polyester fleece used for tissue development exhibits numerous tubules (Fig. 10b). Part of the tubules develop in a parallel fashion in the vicinity of the fleece fibers, some show a curling growth, while others exhibit a dichotomous branching. All of the tubules are covered by a continuously developed basal lamina. The overall view further demonstrates that the tubules have only loose contact with the fibers of the polyester fleece. On the surface of the tubules single interstitial cells and bundles consisting of newly synthesized extracellular matrix proteins are recognized.

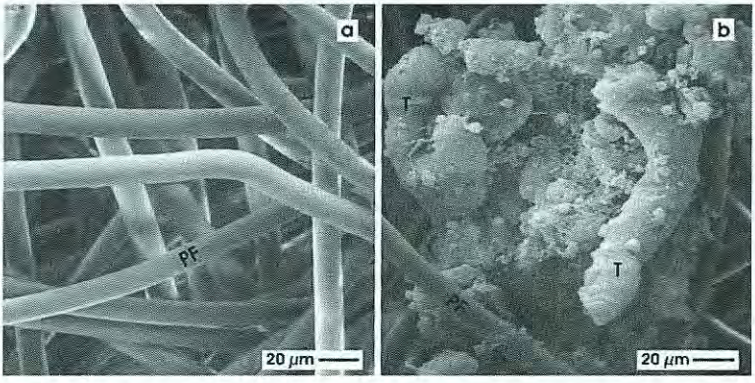

Fig. 10 SEM at the interphase of an artificial interstitium. (**a**) The fibers of the polyester fleece (*PF*) are detected in a longitudinal, transversal, and oblique course. They exhibit a homogeneous composition, a smooth surface without recognizable protrusions or roughness. (**b**) Generated tubules (*T*) grow in close vicinity of the polyester fibers. On the surface of tubules single interstitial cells and thin fibers consisting of extracellular matrix are observed

11 Linking Collagen Between Tubules and Polyester Fibers

In the kidney the tubules are not in contact with others but are separated by the interstitium (Fig. 1). It consists of a network of collagen type III fibers surrounding the tubules. To gain insights in the microenvironment of generated tubules SEM and immunohistochemistry for collagen type III were performed.

SEM shows that bundles of synthesized fibers are spanning between the basal lamina of generated tubules and toward the neighboring polyester fibers (Fig. 11a).

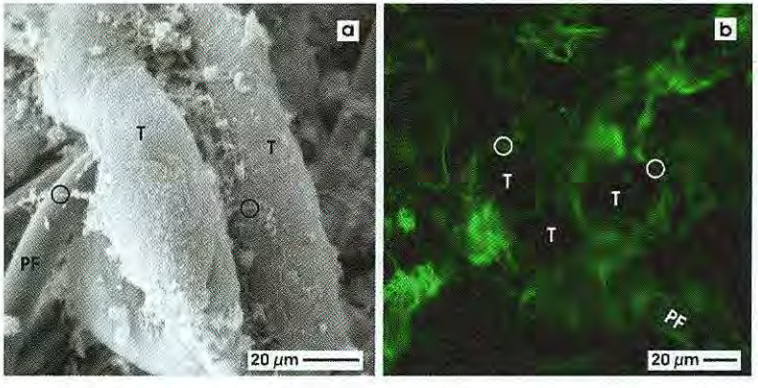

Fig. 11 SEM analysis and immuno-label for collagen type III. (**a**) Synthesized collagen (*O*) is spanning between the basal lamina of both tubules and toward neighboring polyester fibers (*PF*). (**b**) Collagen type III (*O*) is found on the basal lamina of generated tubules (*T*) and on fibers lining toward the polyester fibers (*PF*) of the artificial interstitium

Since the generation of tubules was performed without any coating by extracellular matrix proteins, the basal aspect of generated tubules can also be analyzed by immunohistochemical methods without the interference of proteins derived from a coating process. The label on cryosections demonstrates that collagen type III is contained in the basal lamina of generated tubules and in the surrounding interstitial space (Fig. 11b). This result points out that special attention has to be given to the development of extracellular matrix during generation of tubules, since the cellular differentiation of epithelial cells is strongly correlated with the synthesis of an intact basal lamina and interstitial proteins such as collagen type III.

12 Looking to the Ultrastructure of Generated Tubules

Whole-mount label by soybean agglutinin (SBA, Fig. 9) reveals that generated tubules exhibit a clearly visible lumen in the center and a basal lamina at the outer surface. To obtain further morphological insights transmission electron microscopy (TEM) of the generated tubule epithelium was performed (Fig. 12). Low magnification reveals that tubules generated at the interface of an artificial interstitium show a lining epithelium, a lumen, and a consistently developed basal lamina (Fig. 12a). The tubules occur in close neighborhood to polyester fibers, but it appears that they avoid close contact with them. In the surrounding of a tubule synthesized extra-cellular matrix, single cells and cellular debris are noticed.

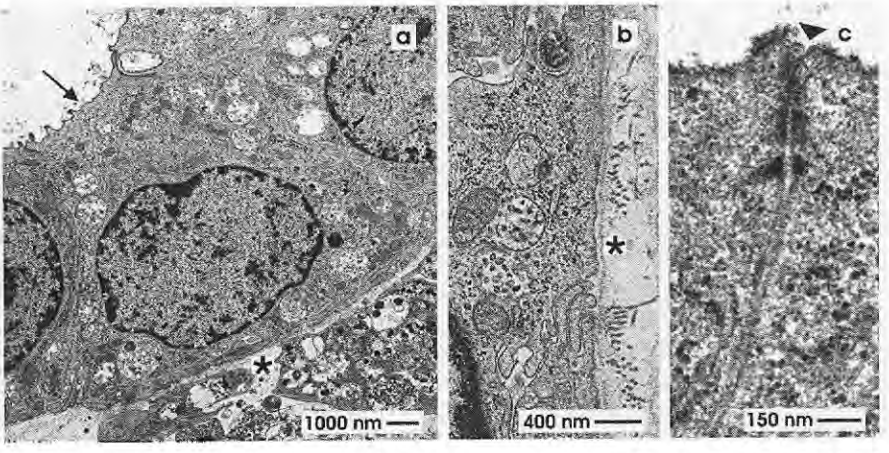

Fig. 12 Transmission electron microscopy of tubules generated at the interface of an artificial interstitium. (**a**) Low magnification reveals that generated tubules contain a polarized epithelium. The apical side borders a lumen (*arrow*), while the basal side contains a basal lamina (*asterisk*). (**b**) The basal lamina consists of a lamina rara interna, a lamina densa, and a lamina fibroreticularis (*asterisk*). (**c**) Between the apical and lateral plasma membrane a junctional complex is developed (*arrow head*). It consists of a zonula occludens, zonula adherens, and a desmosome indicating that a sealing epithelium is established

TEM further demonstrates that an isoprismatic epithelium is established (Fig. 12a). The cells exhibit a large nucleus, which is located in the center of the cytoplasm. In the apical and basal cytoplasm numerous lysosomal elements are found. Small, medium-sized, and large vacuoles are filled to a various degree with electron-dense material. The vacuoles suggest that the containing material has been phagocytosed. At the basal aspect of the epithelium a completely developed basal lamina consists of a lamina rara interna, a lamina densa, and an extended lamina fibroreticularis (Fig. 12b). Neighboring epithelial cells are in close contact with each other. Some microvilli or microplicae of the lateral plasma membrane project into the intercellular space. The basal slits of plasma membranes between neighboring cells are narrow. Higher magnification of TEM illuminates that the luminal and lateral plasma membranes are separated by a typical junctional complex (Fig. 12c). It consists of a zonula occludens, zonula adherens, and a desmosome indicating that a polarized epithelium is established. Thus, all these ultrastructural data show that during culture an obviously sealing epithelium within the generated tubules is developed.

13 Featuring Cell Differentiation

In the presented experiments renal stem/progenitor cells containing tissue was kept in culture for a period of 13 days. Most important is to determine the degree of acquired differentiation in generated tubules. One has to compare the initial state of development during isolation with the harvested tissue at the end of culture. This result further can be compared with the embryonic state, the process of maturation, and finally the adult state in the functional kidney.

To analyze the degree of cell differentiation cryosections were made. For orientation, the surface view of a toluidine blue-stained section demonstrates the distribution of developed tubules at the interface of the artificial interstitium covered by polyester fleeces on both sides (Fig. 13a). Histochemical label for SBA (Fig. 13b) and cyclooxygenase 2 (Cox2, Fig. 13c) reveals an intensive cellular label. Immuno-label for Troma I (Fig. 13d), cytokeratin 19 (Fig. 13e), and E-cadherin (Fig. 13f) exhibits intensive label on tubule cells. Label for occludin demonstrates the development of a junctional belt recognized as faint label in the luminal portion of generated tubules (Fig. 13g). It indicates a functional polarization in the form of a tight junction at the border between the luminal and the lateral plasma membrane of cells within the generated tubules. Immuno-label for Na/K-ATPase $\alpha 5$ reveals an intensive fluorescence at the basolateral aspect of generated tubules (Fig. 13h). All these data show that the generated tubules exhibit a polarized epithelium. Labeling the tissue for laminin $\gamma 1$ finally exhibits an intensive reaction at the basal aspect of generated tubules indicating that a basal lamina is developed during 13 days of culture (Fig. 13i).

SBA, cyclooxygenase 2, Troma I, cytokeratin 19, E-cadherin, occludin, Na/K-ATPase $\alpha 5$, and laminin $\gamma 1$ are naturally found on cells of the adult renal collecting

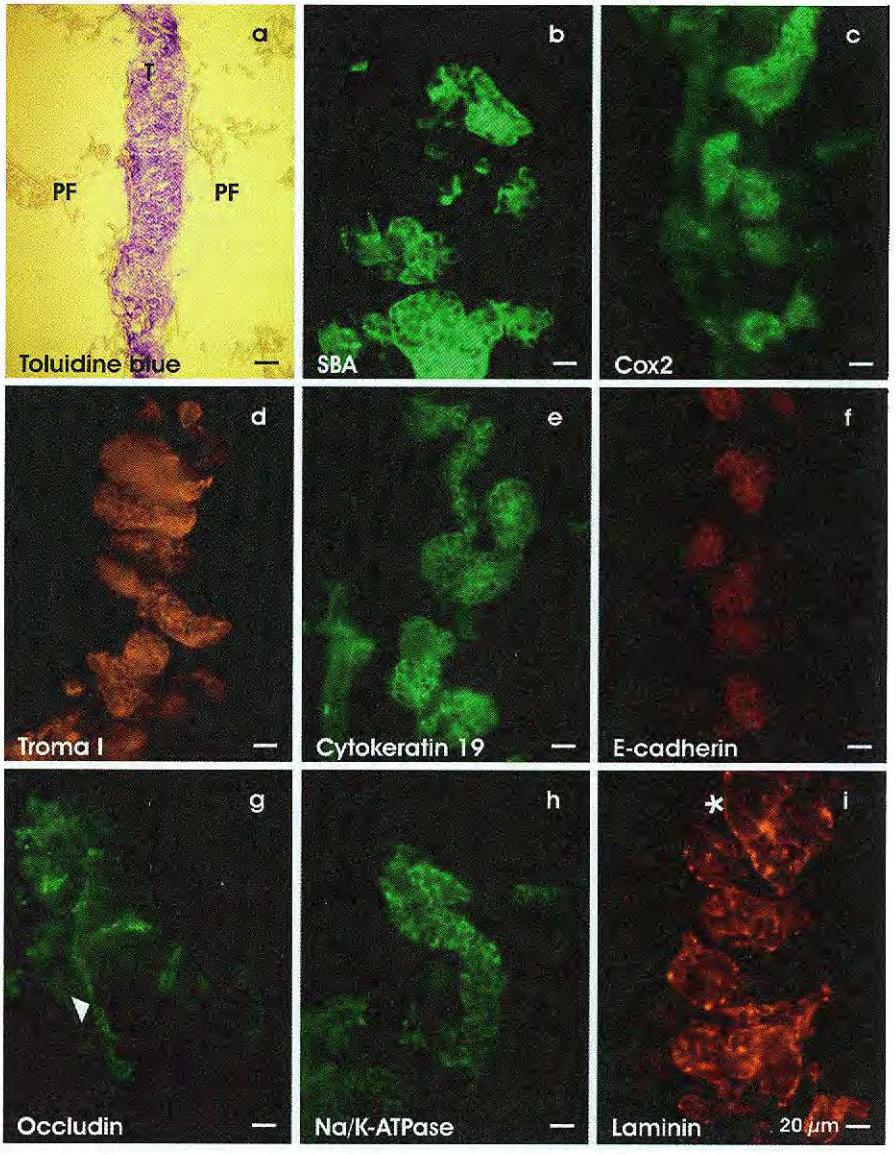

Fig. 13 Histochemical label of tubules generated for 13 days at the interface of an artificial interstitium. (**a**) Toluidine blue stain of a cryosection demonstrates numerous tubules (*T*) grown between fleeces of polyester. Histochemical label for (**b**) SBA and (**c**) cyclooxygenase 2 (Cox2) reveals an intensive cellular label. Label for (**d**) Troma I, (**e**) cytokeratin 19, and (**f**) E-cadherin shows intensive reaction on tubule cells. (**g**) Occludin (*arrow head*) is found in the luminal portion of generated tubules, while (**h**) Na/K-ATPase α5 is detected at the basolateral aspect. (**i**) Laminin γ1 exhibits that a basal lamina (*asterisk*) is formed

duct within the kidney. Since the same markers are detected on tubules generated at the interface of an artificial interstitium (Fig. 13), it is most probable that renal collecting duct-derived tubules are developed.

14 Inducing Tubulogenic Development

Experiments show that SBA-labeled tubules are not developed when the culture of renal stem/progenitor cells is performed without application of aldosterone (Fig. 14a). Cryostat sections show in this case a disintegration of tissue. Only faint rows of cells are developed and in none of the samples structured tubules are observed.

In contrast, administration of aldosterone (1×10^{-7} M) to the standard medium (IMDM) completely changes the developmental pattern of renal stem/progenitor cells generated at the interface of an artificial interstitium (Fig. 14b) [14]. After a culture period of 13 days numerous tubules become visible.

To elaborate a tubulogenic profile of aldosterone the hormone was applied in concentrations ranging from 1×10^{-10} to 1×10^{-5} M. The low dose of 1×10^{-10} M does not stimulate the development of tubules, while concentrations of 1×10^{-9} and 1×10^{-8} M start to induce outgrowth of SBA-labeled cells that form long rows and clusters but not structured tubules. Intact formation of tubules is obtained by the use of 1×10^{-7} and 1×10^{-6} M aldosterone. In contrast, the application of 1×10^{-5} M does not further stimulate a better development of tubules.

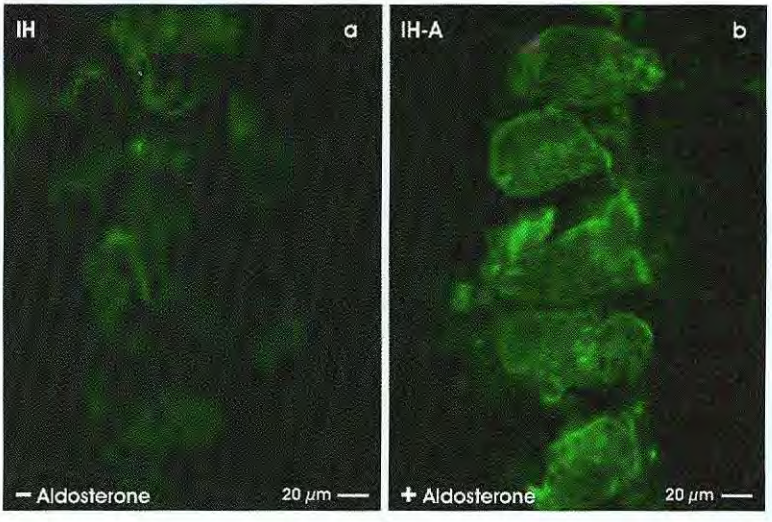

Fig. 14 Culture of embryonic renal tissue at the interphase of an artificial interstitium. **(a)** Generation of tubules cannot be recognized when aldosterone is omitted in IMDM. **(b)** Numerous tubules are observed after application of aldosterone (1×10^{-7} M) in IMDM after 13 days of culture

It was a fully unexpected finding that aldosterone exhibits tubulogenic activity in isolated explants containing renal stem/progenitor cells (Fig. 14b). In the adult kidney the hormone stimulates the Na^+ transport by genomic effects via the mineralocorticoid receptor (MR). A morphogenic effect of aldosterone on renal stem cells sounds on the one hand curiously, but on the other hand, a comparable morphogenic action of the steroid hormone was found on other cells. For example, development of hippocampal neurons is induced by aldosterone via stimulation of the mineralocorticoid receptor (MR), while an activation of the glucocorticoid receptor (GR) is suppressing the development. T37i cells show after application of aldosterone differentiation into cells of brown adipose tissue. This effect can be inhibited by antagonists such as spironolactone or RU-26752. Experiments with cells of the adenohypophysis producing growth hormone demonstrate that their development is triggered by MR and is inhibited by the application of spironolactone. During embryogenesis the blockade of MR with ZK 91587 leads to a reduction of somite number, to reduced growth, and to an alteration of blood vessels in the umbilical cord. In adult cardiac fibroblasts aldosterone increases levels of collagen type I and III synthesis promoting myocardial fibrosis. Finally, for the kidney it was described that aldosterone promotes the synthesis of fibronectin through a Smad2-dependent TGF-β1 pathway in mesangial cells during the development of glomerular sclerosis.

15 Scoring the Development of Tubules

To investigate the influence of hormones or growth factors on the generation of tubules it is most important to determine the degree of differentiation. Depending on the applied substances many or few tubules, cell islets, or even extended cell clusters may arise. For that reason a special score for the development of tubules was elaborated.

The space for tubule development in a basic sandwich set-up is 5 mm in diameter and up to 250 μm in height (Fig. 6). For example, labeling of specimens by SBA reveals numerous tubules exhibiting polarized cells, a visible lumen, and a basal lamina (Fig. 15). The number of generated tubules can be determined with a WCIF ImageJ program (Bethesda, Maryland, USA) by counting each SBA-labeled tubule. Applying this technique on generated specimens, between 41 and 77 tubules are detected within a microscopic opening of 620 × 930 μm.

Besides the overall appearance of tubules, the staining intensity, the formation of a lumen, the development of a basal lamina, their distribution, and their length are registered (Fig. 15). When each of these criteria is scored with 1 point it results in a maximum of 6 points reflecting an ideal development of generated tubules. However, this result will only be obtained when aldosterone is added as tubulogenic factor to the culture medium. When the cultures are properly developed, no cell clusters, no filopodia, and no overgrowth of epithelial cells on the polyester fibers of the artificial interstitium are observed.

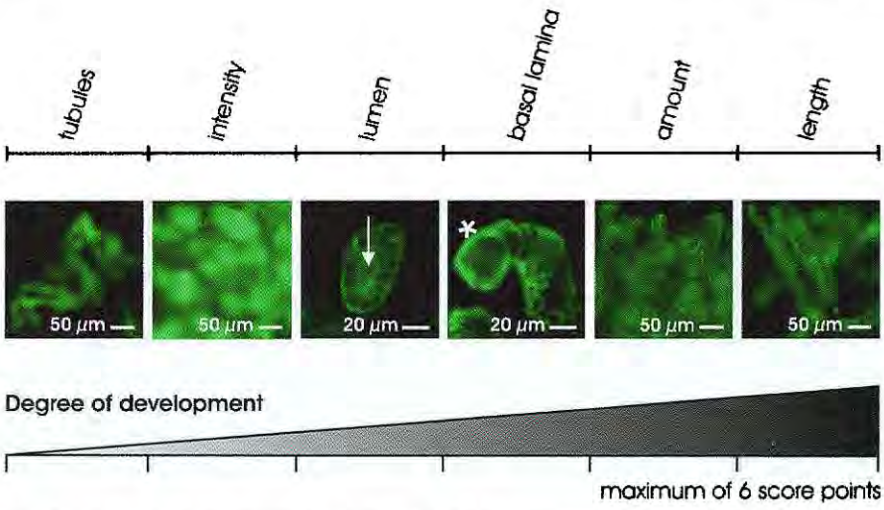

Fig. 15 Scoring the development of tubules. Beside the appearance of tubules, the staining intensity, the formation of a lumen (*arrow*), the development of a basal lamina (*asterisk*), the overall distribution, and the length can be registered

16 Specifying the Morphogenic Action of Aldosterone

From adult kidney it is known that not only aldosterone but also some of its molecular precursors have an affinity to the mineralocorticoid receptor (MR) and influence thereby physiological functions. Consequently it was investigated if also precursors of the aldosterone synthesis pathway show effects on the development of renal stem/progenitor cells [14].

The synthesis of aldosterone starts from cholesterol, which is metabolized over pregnenolone to progesterone, 11-deoxycorticosterone, corticosterone, and 18-hydroxycorticosterone. Physiological experiments with adult kidneys show, for example, that 11-deoxycorticosterone is as effective as aldosterone on the mineralocorticoid receptor, while corticosterone is 100 times less potent.

Culture experiments with precursors of the aldosterone synthesis pathway (Fig. 16, each 1×10^{-7} M) were performed and graded according to the scoring profile (Fig. 15). It is demonstrated that application of cholesterol or pregnenolone does not result in the formation of any SBA-positive tubules (0 point). Treatment with progesterone leads to the development of few tubules but without intensive SBA label (1 point). When 11-deoxycorticosterone is used, only few tubules with a faint SBA label can be detected (1 point). In contrast, administration of corticosterone does not reveal any development of tubules. Instead, numerous SBA-labeled cell clusters are observed in close contact to polyester fibers (0 point). Data for 18-hydroxycorticosterone are missing, since this substance is not commercially available. Administration of aldosterone results in numerous SBA-positive tubules

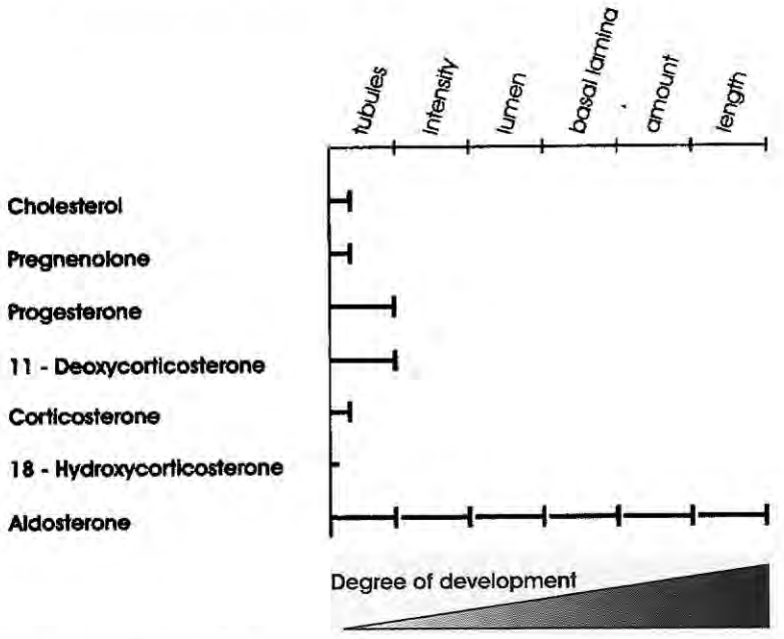

Fig. 16 Tubulogenic effect of aldosterone and its molecular precursors on renal tubule development. Only aldosterone reaches a maximum of 6 points in the score scale. It depicts that only aldosterone but not its precursors lead to the development of SBA-labeled tubules

exhibiting a distinct lumen and a clearly recognizable basal lamina (maximum = 6 points).

The tubulogenic action of aldosterone may be triggered in cooperation via the glucocorticoid receptor (GR). Consequently, the glucocorticoid dexamethasone instead of aldosterone was tested. While the administration of aldosterone (1×10^{-7} M) results in the development of numerous SBA-labeled tubules, the use of dexamethasone (1×10^{-7} M) produces huge clusters of non-polarized cells. Thus, the progress of structured tubule development appears specific for aldosterone by stimulating the mineralocorticoid receptor (MR).

17 Antagonizing the Tubulogenic Signal on MR

It was demonstrated that the generation of renal tubules depends specifically on the presence of aldosterone in the culture medium (Fig. 14). Further it was shown that the tubulogenic effect is dependent on the applied hormone concentration and cannot be mimicked by precursors of the aldosterone synthesis pathway (Fig. 16). In the following experiments it was further investigated if the tubulogenic effect of aldosterone is exclusively mediated via the mineralocorticoid receptor (MR) or if it

is related to an unspecific side effect of the steroid hormone (Fig. 17) [15]. To interfere the binding between aldosterone and MR, antagonists such as spironolactone and canrenoate were used in the culture experiments.

Low dose of spironolactone (1×10^{-7} M) in the presence of aldosterone does not affect the development of SBA-labeled tubules. However, application of a higher concentration of spironolactone (1×10^{-5} M) demonstrates first inhibitory effects and leads to a switch of tubule development. The number of structured tubules is reduced and SBA-labeled cells start to form extended cell clusters. However, presence of 1×10^{-4} M spironolactone in aldosterone-containing medium completely prevents the development of SBA-labeled tubules (Fig. 17b).

Canrenoate has the same inhibitory profile on the tubulogenic action of aldosterone as it is observed with spironolactone. Application of 1×10^{-7} M canrenoate in the aldosterone-containing medium does not affect the development of tubules. However, administration of 1×10^{-6} and 1×10^{-5} M canrenoate drastically reduces SBA-labeled structures. The use of 1×10^{-4} M canrenoate results in a complete lack of SBA-labeled cells and tubules (Fig. 17c).

Thus, the simultaneous administration of aldosterone in combination with spironolactone (Fig. 17b) or canrenoate (Fig. 17c) demonstrates that the tubulogenic effect is inhibited in a dose-dependent manner. The result further shows that the tubulogenic effect of aldosterone is mediated via the mineralocorticoid receptor (MR).

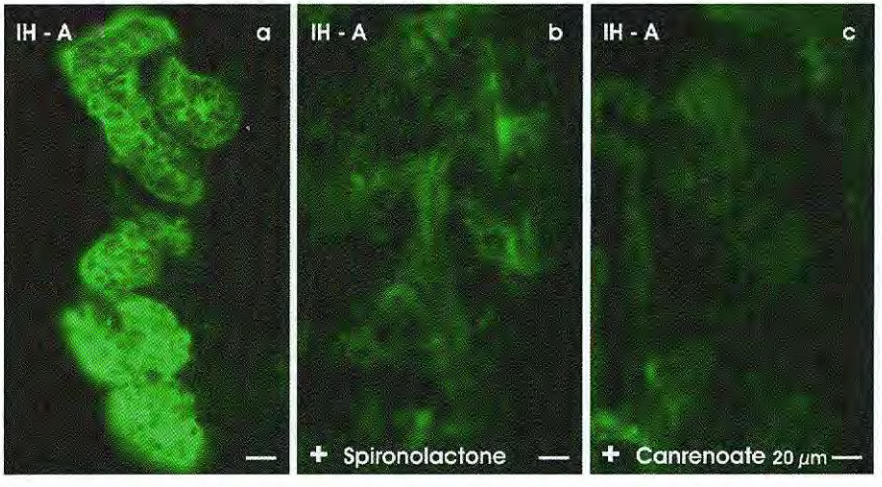

Fig. 17 Antagonizing the tubulogenic action of aldosterone on the receptor level. (**a**) For control, aldosterone generates numerous SBA-labeled tubules. Application of (**b**) 1×10^{-4} M spironolactone or (**c**) 1×10^{-4} M canrenoate in the presence of aldosterone completely inhibits the development of tubules

18 Interfering the Tubulogenic Signaling in the Cytoplasm

Earlier experiments demonstrated that MR is not randomly distributed within the cytoplasm of the target cell but stays in close molecular contact with heat shock proteins (hsp) 90 and 70 (Fig. 18a). Both proteins, in turn, are located in close proximity to immunophilins, especially FKBP 12. To obtain insights in the molecular signaling renal stem/progenitor cells were treated with aldosterone in combination

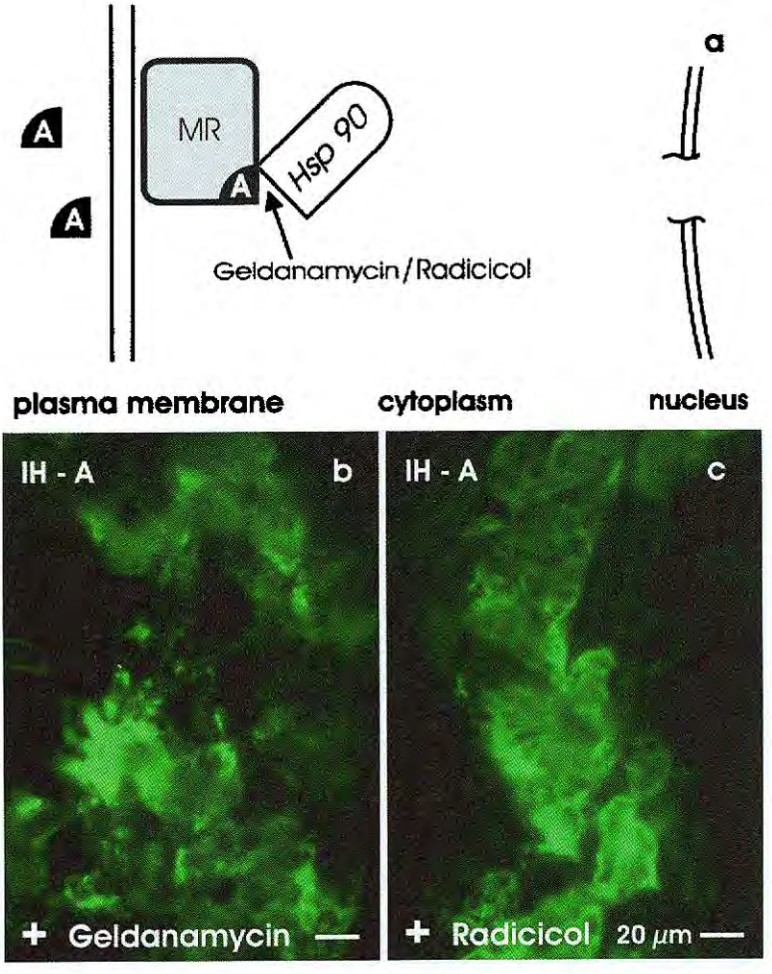

Fig. 18 Cytoplasmic interference of the tubulogenic signal (**a**). Schematic illustration of the aldosterone (*A*)-stimulated mineralocorticoid receptor (*MR*) in relation to *hsp 90*. Fluorescence microscopy on renal tissue generated for 13 days with aldosterone (1×10^{-7} M) in combination with (**b**) 3.6×10^{-6} M geldanamycin or (**c**) 1×10^{-6} M radicicol shows multiple SBA-labeled cells within clusters but solid formation of tubules is lacking, when the binding between *MR* and *hsp 90* is interfered

with substances that disrupt the cytoplasmic interaction between MR, hsp 90, hsp 70, and immunophilins [16].

To disrupt the contact between MR and hsp 90, renal stem/progenitor cells were cultured in IMDM containing geldanamycin (3.6×10^{-6} M) in combination with aldosterone (1×10^{-7} M) for 13 days (Fig. 18b). Geldanamycin specifically binds to hsp 90, thereby blocking the ATP-binding site due to its higher affinity compared to ATP. In this way, it disturbs the contact between hsp 90 and activated MR. The culture experiments show that structured tubules are not found in this series of experiments. Instead numerous SBA-labeled cells are localized in extended clusters.

Radicicol is a macrocyclic antifungal substance that binds in the same way as geldanamycin. It hinders ATP-dependent conformational changes that are required for cytoplasmic interactions with target proteins such as MR. Culture of renal stem/progenitor cells with radicicol (1×10^{-6} M) in combination with aldosterone (1×10^{-7} M) produces only few structured tubules, but numerous SBA-labeled cells in the form of extended clusters (Fig. 18c). In consequence, experiments with geldanamycin (Fig. 18b) and radicicol (Fig. 18c) show that the tubulogenic pathway of aldosterone is blocked by interfering the contact to hsp 90.

19 Producing Renal Superstructures

The presented experiments show the feasibility to generate tubules at the interface of an artificial interstitium (Fig. 9). Further it is demonstrated that aldosterone exhibits a tubulogenic effect on renal stem/progenitor cells (Figs. 14, 15, 16).

The presented new technique opens an experimental way to increase systematically the amount of generated tubules, since the arrangement of the basic sandwich set-up is most advantageous for extending the spatial environment, the supply of medium, and respiratory gas. Since the developing tissue is covered by layers of polyester fleece always fresh medium is transported through the space between the fibers of the polyester fleece separating the tissue layers (Fig. 19). Using this experimental design unstirred layers of fluid are minimized so that for the first time piling and paving of basic sandwich set-ups becomes possible [17].

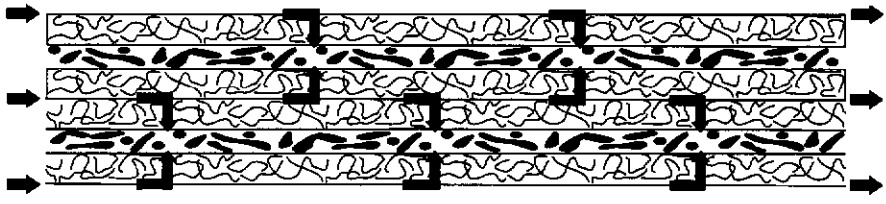

Fig. 19 Piling and paving of basic sandwich set-ups. The supply with culture medium between the tissue layers is optimal, since nutrition and respiratory gases are continuously exchanged through the space between the polyester fibers. *Arrows* indicate the possible flow of medium during dynamic culture

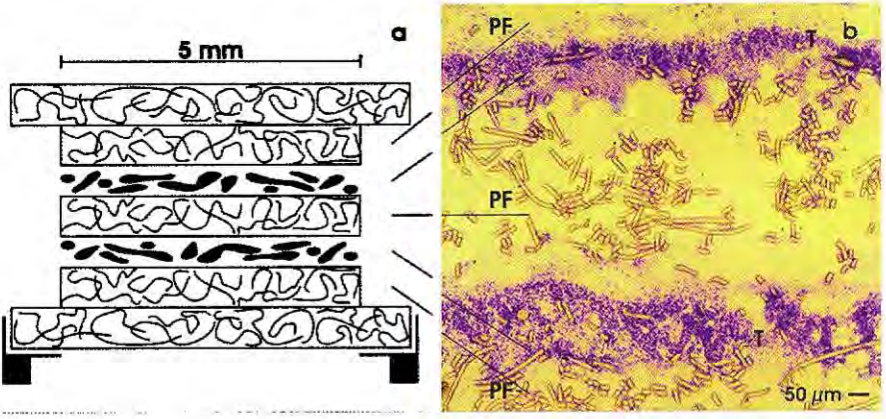

Fig. 20 Schematic illustration of (**a**) piling basic sandwich set-ups. (**b**) Toluidine blue stain of a cryosection shows two piled rows of generated tubules (*T*) cultured for 13 days at the interphase of an artificial interstitium made of polyester fibers (*PF*)

Thus, to raise renal superstructures basic sandwich set-ups containing renal stem/progenitor cells are piled like bricks (Fig. 20). Running experiments demonstrate that tubules in such piled specimens show the same degree of differentiation as it is found in experiments with single rows (Fig. 13).

The advantages of piling and paving basic sandwich set-ups containing renal stem/progenitor cells are numerous (Fig. 20). One could imagine that these superstructures can be used to generate organoid configurations. The use of an artificial interstitium in combination with piling of basic sandwich set-ups may further be advantageous for micro-vascularization. Endothelial cells could be added to the culture medium and infused along the fluid transportation path within the polyester fleeces. The presence of widely distributed endothelial cells will consequently support rapid vascularization of piled and paved basic sandwich set-ups. It is further imaginable that the procedure for piling and paving is not performed by forceps in hand but can be processed in a computerized robotic process. Such superstructures could be raised to investigate under controlled in vitro conditions better the development of organoid structures as it was done in the past. This technique may be also used to generate complex tissue constructs for the minimal invasive implantation of stem/progenitor cells into a diseased kidney.

20 Summing Up

Using renal stem/progenitor cells it was demonstrated that tubules can be generated under controlled perfusion culture at the interphase of an artificial interstitium. The development of tubules formation in a spatial environment is induced by aldosterone. The special experimental configuration makes it possible to pile and pave basic sandwich set-ups containing renal stem/progenitor cells. These new

techniques appear to be a successful step for further investigation of the spatial development of tubules. It appears also as a realistic experimental strategy for a future regeneration of parenchyme within the diseased kidney.

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