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The formation of pores in the basal lamina of regenerated renal tubules

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ABSTRACT

Little information is available concerning the generation of renal tubules, but this information is urgently needed in regenerative medicine for the future treatment of acute and chronic renal failures. Of major interests are the integration of stem/progenitor cells, the cellular development and the tubular growth in a spatial environment. In this regard, we investigated the basal aspect of renal tubules generated at the interphase of an artificial interstitium. Stem/progenitor cells derived from neonatal rabbit kidney were mounted inside a specific tissue holder and covered by layers of polyester fleece. The tissue was then kept in a perfusion culture container for 13 days in chemically defined IMDM containing aldosterone (1×10^{-7} M) as a tubulogenic factor. The spatial development of tubules was registered on whole-mount specimens and on cryo-sections labeled with soybean agglutinin (SBA) and tissue-specific antibodies indicating that collecting duct tubules were developed. Scanning electron microscopy (SEM) revealed that the generated tubules were completely covered by a basal lamina. Most interestingly, the matrix was not consistently composed, but exhibited three categories of pores. The most frequently found pore type had an apparent diameter of 133 ± 26 nm followed by a medium-sized pore type of 317 ± 35 nm. Another category of pores with a diameter of 605 ± 101 nm was rather rarely found. All of the pores were evenly distributed and not restricted to particular sites. The newly detected pores are not related to culture artifacts, since they were also detected in collecting duct tubules of the neonatal rabbit kidney. It remains to be evaluated whether these pores support physiological transport functions or if they indicate the site where extracellular matrix proteins are inserted into newly synthesized basal lamina.

1. Introduction

The capability for regeneration varies from tissue to tissue. For example, cells of the hematopoietic system, skin and intestine show rapid rates of turnover, while cells of the kidney exhibit a limited renewal. This cell biological restriction makes it difficult to replace functional parenchyma in the course of acute and chronic renal failures. One of the future therapeutic challenges in kidney regeneration is therefore the renewal of renal cells by the application of stem/progenitor cells [1–3]. However, up-to-date substantiated knowledge is lacking regarding stem cell integration within a diseased environment and functional repair of the renal tubules [4,5].

To investigate basic mechanisms of the spatial development of tubules a powerful tissue culture system is of special importance. We used stem/progenitor cells derived from the outer cortex of neonatal rabbit kidney for the generation of tubules. Applying innovative technology, we found that perfusion culture of renal stem/progenitor cells at the interphase of an artificial interstitium resulted in the formation of numerous SBA-labeled tubules [6]. The development of tubules was initiated by the administration of aldosterone, and was dependent on the applied hormone concentration. The effect could be antagonized by spironolactone or canrenoate [7]. Cultures could be maintained for more than 2 weeks in a highly differentiated state using chemically defined culture medium [8].

The development of renal stem/progenitor cells into renal tubules is a rather complex event consisting of two successive steps. It begins in the embryonic kidney and is mediated by multiple growth factors resulting in both the nephrogenic and the collecting duct-derived tubule anlagen. The ensuing steps comprise cell biological control of tube formation in combination with epithelium development [9,10]. This results in the development of a polarized epithelium exhibiting a lumen and a basal lamina and the extension of tube formation with a defined length, diameter and three-dimensional course. At present, it is unknown which factors trigger the spatial growth and the cellular communications involved in all these processes [11–13].

In the present paper, we analyzed the basal aspect of generated renal tubules derived from renal stem/progenitor cells. In contrast

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to experiments performed by other groups, we generated tubules without coating them with extracellular matrix proteins [14,15]. Because of this, it was possible to analyze the basal aspect of generated tubules by scanning electron microscopy (SEM), since the analytical view was not stacked with extracellular matrix proteins derived from the coating process. In analyzing the basal aspect of generated tubules, we detected that the basal lamina contained up-to-date unknown pores. The same kinds of pores were detected in the basal lamina of tubules within the kidney. Consequently, the pores appear to be common elements of the basal lamina and they do not reflect artifacts due to culture conditions.

2. Materials and methods

2.1. Isolation of embryonic explants containing renal stem/progenitor cells

One-day-old New Zealand rabbits were anesthetized with ether and killed by cervical dislocation as described earlier [16]. Both kidneys were removed immediately. Each kidney was dissected in two parts. By stripping off the capsula fibrosa with fine forceps, a thin embryonic tissue layer containing numerous collecting duct ampullae, S-shaped bodies and nephrogenic mesenchyme was harvested.

2.2. Sandwich set-up in a perfusion culture container

The isolated embryonic renal tissue was placed between two punched out layers of polyester fleece (Walraf, Grevenbroich, Germany) measuring 5 mm in diameter. This arrangement resulted in a sandwich-like configuration with the freshly isolated embryonic tissue in the center and layers of polyester fleece covering the outer side (Fig. 1a). During culture, the basic sandwich set-up was held in this specific position to prevent damage to the growing tissue. Thus, a base ring of a Minusheet® tissue holder with 13 mm inner diameter was transferred to a perfusion culture container with horizontal flow characteristics (Minucells and Minutissue, Bad Abbach, Germany). A polyester fleece measuring 13 mm in diameter was mounted into the tissue holder. Then the basic sandwich set-up containing renal stem/progenitor cells measuring 5 mm in diameter was inserted. Finally, a polyester fleece 13 mm in diameter was placed on top of the sandwich as a cover (Fig. 1a). After closing the lid of the perfusion culture container, the complete tissue–fleece construction was fixed in an exact position. The spatial area for tubule formation was 5 mm in diameter and up to 250 μm in height. The specific interphase between the fleece layers produced an artificial interstitium providing an optimal microenvironment for the development of tubules during the entire culture period [5,17].

2.3. Perfusion culture to generate renal tubules

Perfusion culture was carried out as described earlier [6] (Fig. 1b). Throughout the whole experimental phase of 13 days, fresh medium was perfused at a rate of 1 ml/h with an IPC N8 peristaltic pump (Ismatec, Wertheim, Germany). To maintain a constant temperature of 37 °C, the culture container was placed on a thermoplate (Medax, Kiel, Germany) and covered with a transparent lid.

To generate renal tubules chemically defined IMDM (Iscove's Modified Dulbecco's Medium including Phenolred, GIBCO/Invitrogen, Karlsruhe, Germany) was used [8]. In order to maintain a constant pH of 7.4 under atmospheric air containing 0.3% CO_2, 50 mmol/l HEPES (GIBCO) was added to the medium. To evoke tubulogenic development aldosterone (1×10^{-7} M, Fluka, Taufkirchen, Germany) was supplied to the culture medium. To prevent infection an antibiotic–antimycotic cocktail (1%, GIBCO) was added to all culture media.

2.4. Lectin- and antibody-labeling

Whole-mount specimens or cryo-sections of 20 μm thickness were 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 (SBA, Vector, Burlingame, USA)-labeling the samples were exposed to fluorescein-isothiocyanate (FITC)-conjugated lectin diluted 1:2000 in blocking solution for 45 min as described

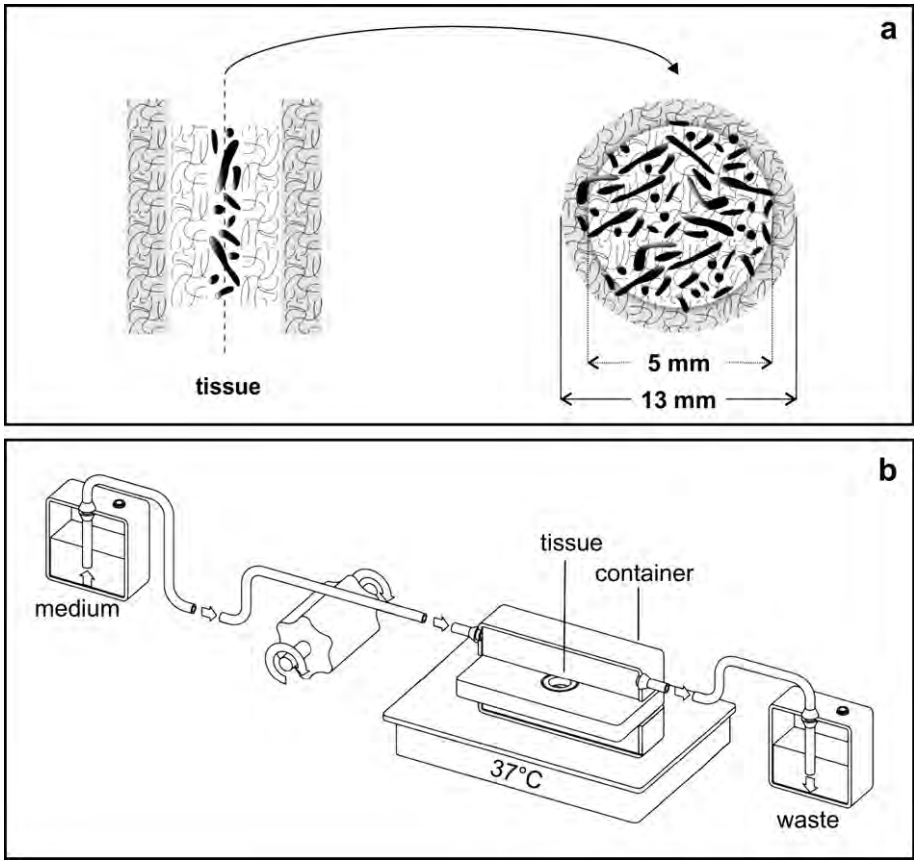

Fig. 1. Schematic illustration of generated tubules at the interphase of an artificial interstitium. The growing tissue was in contact with a polyester fleece measuring 5 mm in diameter (a). Opening the artificial interstitium after culture revealed an area with tubule growth measuring 5 mm in diameter. Perfusion culture was performed with all the time fresh medium for 13 days at a rate of 1 ml/h with a peristaltic pump (b). To maintain a constant temperature of 37 °C the culture container was placed on a thermoplate and covered with a transparent lid.

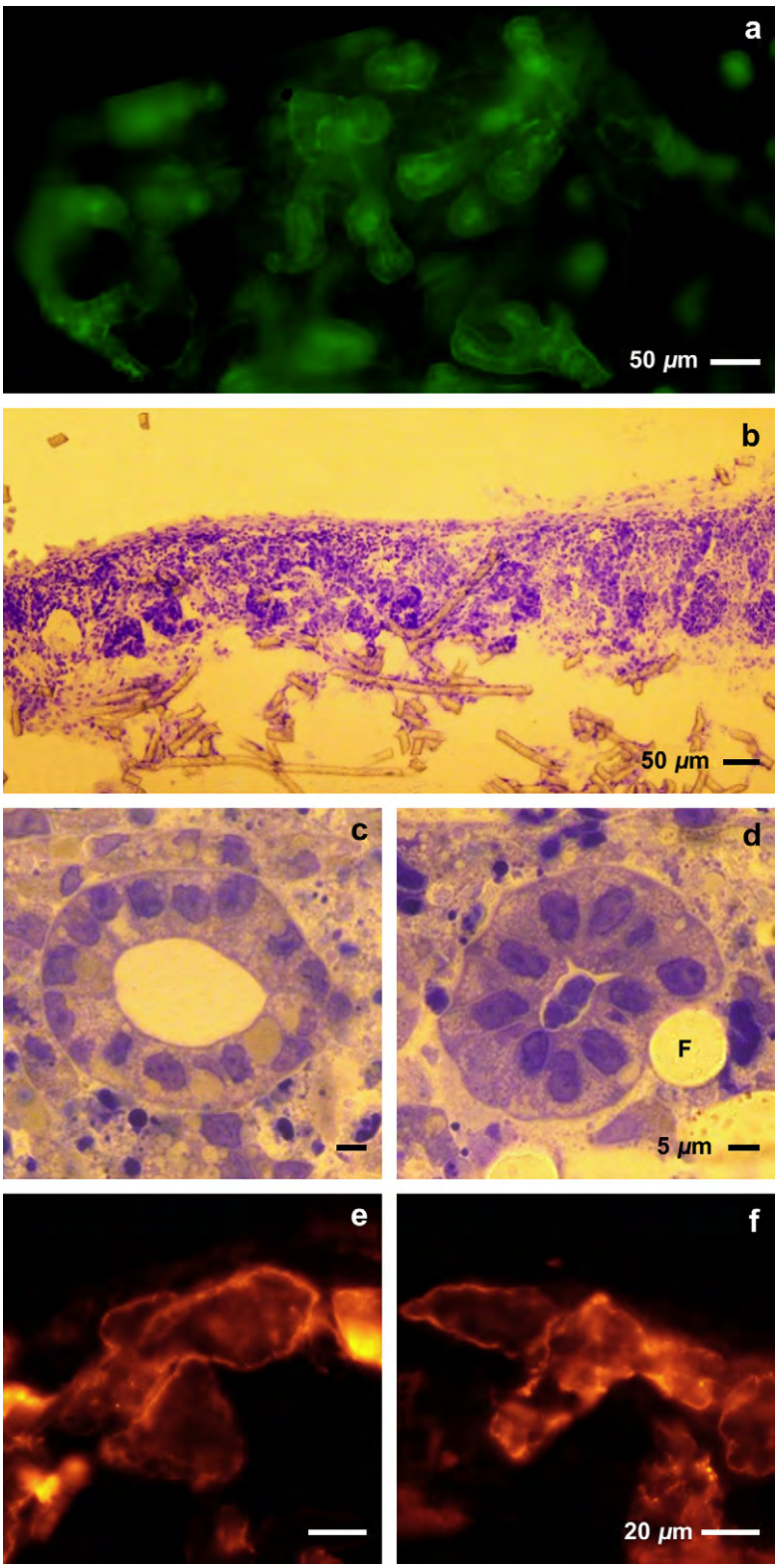

Fig. 2. Morphological view of tubules generated for 13 days at the interphase of an artificial interstitium. Whole mount label with SBA reveals numerous tubules exhibiting a lumen and a basal lamina (a). Cryo-section of cultures after toluidine-staining demonstrates numerous tubules embedded loose connective tissue (b). Semi-thin section after Richardson-staining reveals tubules with a lumen and a basal lamina embedded in loose connective tissue (c and e). Immunohistochemical label by mab anti-laminin γ1 demonstrates that a basal lamina is present (e and f).

[6,7]. For antibody labeling, mab anti-laminin γ1 (kindly provided by Dr. L. Sorokin, Lund, Sweden) was applied as a primary antibody for 1 h in blocking solution after washing with PBS. The specimens were then incubated for 45 min with goat-anti-rat-IgG-rhodamine (Jackson Immunoresearch Laboratories, West Grove, USA) diluted 1:50 in PBS containing 1% BSA. Following several washes with PBS, the sections were embedded with Slow Fade Light Antifade kit (Molecular Probes, Eugene, USA) and then analyzed using an Axioskop 2 plus microscope (Zeiss, Oberkochen, Germany). Fluorescence images were taken with

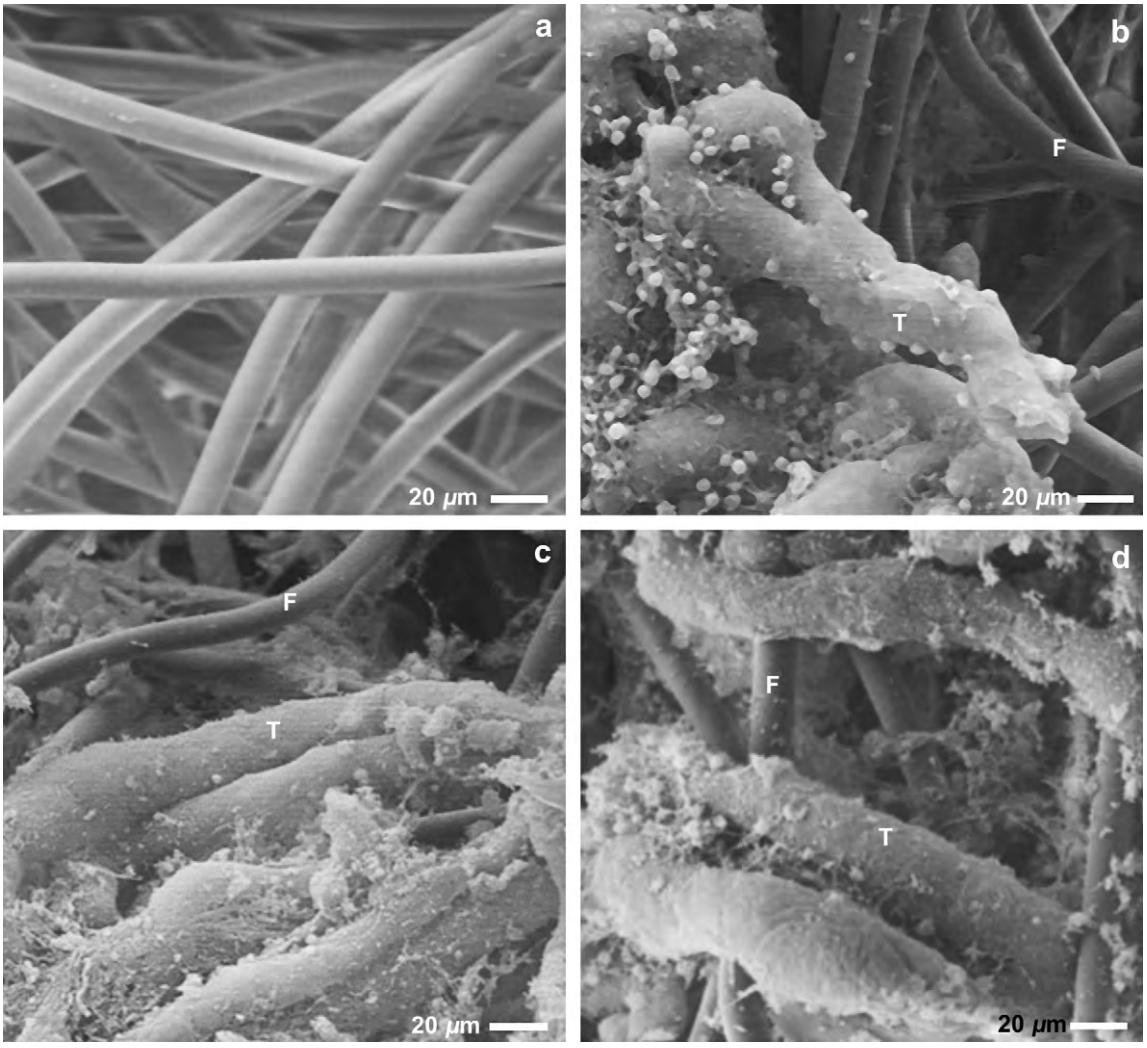

Fig. 3. SEM at the interphase of an artificial interstitium. The fibers of the polyester fleece were detected in a longitudinal, transversal and oblique course (a). They exhibited a homogeneous composition and a smooth surface without recognizable protrusions or roughness. Their diameter was 10 μm in average. The overall view further demonstrated the generated tubules with dichotomous branching (b), while other tubules were found to grow in parallel fashion in close vicinity of the polyester fibers (c and d). On the surface of tubules single interstitial cells and thin fibers consisting of extracellular matrix were observed.

a digital camera at a standard exposure time of 1.3 s and thereafter processed with Corel DRAW 11 (Corel Corporation, Otawa, Canada).

2.5. Embedding of tissue in Epon for semi-thin sections

To analyze semi-thin sections, generated tissue was fixed in 3% glutaraldehyde, dehydrated in a graded series of ethanols, and embedded in Epon. Semi-thin sections were made with a diamond knife on an Ultracut microtome (Leica, Bensheim, Germany) and stained with Richardson solution.

2.6. Scanning electron microscopy

For scanning electron microscopy (SEM) pieces of neonatal rabbit kidney cortex were fixed in 3% glutaraldehyde, dehydrated in a graded series of ethanols, critical point dried with CO_2 and sputter-coated with gold (Polaron E 5100, Watford, GB). The generated tissue was fixed in 70% ethanol. The specimens were examined in a scanning electron microscope DSM 940 A (Zeiss, Oberkochen, Germany) as described earlier [18,19].

2.7. Morphometry and statistics

Magnified SEM micrographs were used to determine the diameter of pores. The diameter of a pore was measured and calculated with a WCIF ImageJ program (Bethesda, Maryland, USA). Statistical analysis was performed by analysis of variance (ANOVA) followed by Bonferroni's test. All data are expressed as mean ± SD. Significant differences were defined as $p < 0.05$.

2.8. Amount of cultured constructs

A total of 37 embryonic tissues were isolated and maintained in culture for the present study. All of the experiments were performed at least in triplicates. The data provided in the text are the mean of at least three independent experiments. All experiments are in accordance with the animal ethics committee, University of Regensburg, Regensburg, Germany.

3. Results

3.1. Culture at the interphase of an artificial interstitium

After stripping off the capsula fibrosa from neonatal rabbit kidney, a thin tissue layer containing numerous stem/progenitor cells was isolated and mounted in a specific holder [19,20]. To generate renal tubules, the isolated embryonic tissue was enclosed by layers of polyester fleece (Fig. 1a). A smaller fleece, 5 mm in diameter, was in direct contact with the tissue, while a larger one, 13 mm in diameter, covered the sandwich configuration. A peristaltic pump constantly transported fresh medium into the perfusion container for a period of 13 days (Fig. 1b). The medium was not re-circulated but was collected in a waste bottle. This method

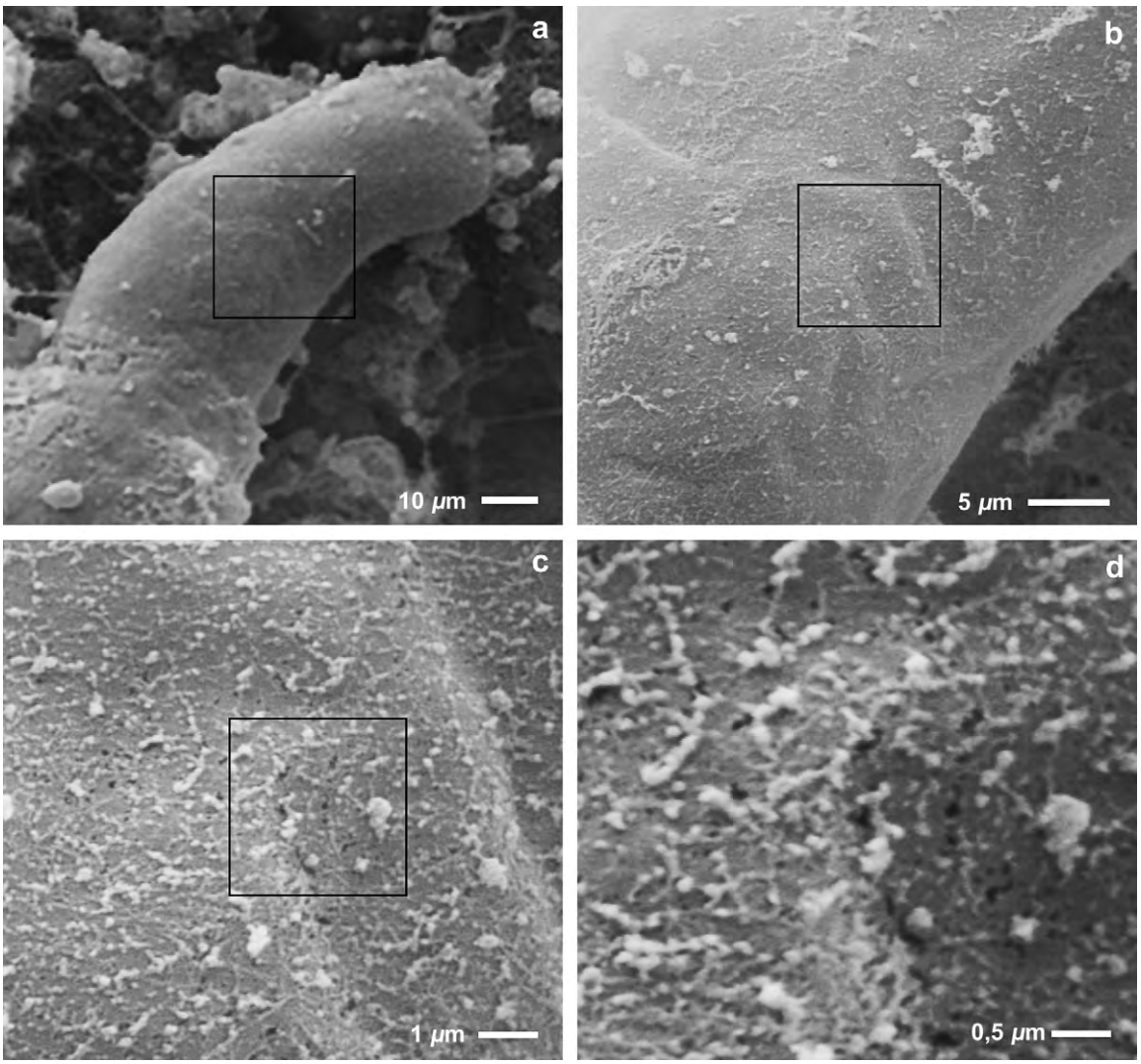

Fig. 4. SEM on the basal aspect of generated tubules at the interphase of an artificial interstitium. Surface view of a tubule (a). Low magnification of the basal lamina (b). Higher magnification (c and d) revealed that the basal lamina was covered by numerous bright particles and numerous pores of different sizes.

enables a constant provision with nutrition and oxygen. The interphase between the fleece layers produced an artificial interstitium, which was beneficial for an optimal development of tubules during a 13 day culture period [7,8].

3.2. Generation of tubules in IMDM containing aldosterone

After culture, the artificial interstitium was opened by separating the fleece layers. The area for tubule growth was 5 mm in diameter at the surface of the fleece. Fluorescence microscopy of whole-mount specimens demonstrated the generation of numerous SBA-labeled tubules (Fig. 2a). Low magnification of tubules exhibited a basal lamina, lining epithelial cells, and a visible lumen. Frozen sections of tissue demonstrated that tubules were embedded in loose connective tissue (Fig. 2b). Semi-thin sections revealed that the generated tubules had a distinct lumen and a basal lamina (Fig. 2c and d). In some cases, the basal lamina of the tubules was in contact with the fibers, but an integration of the fibers into the epithelium was not observed (Fig. 2d). Immunohistochemical labeling with mab anti-laminin γ1 demonstrated that the generated tubules contained laminin at the basal aspect of the tubule (Fig. 2e and f) which indicates the synthesis of a basal lamina.

3.3. Scanning electron microscopy at the interphase of an artificial interstitium

Light microscopical techniques such as whole mount labeling (Fig. 2a), cryo-sections (Fig. 2b), semi-thin sections (Fig. 2c and d), and immunostaining with mab anti-laminin γ1 (Fig. 2e and f) revealed that tubules exhibited features of cellular differentiation when they were generated at the interphase of an artificial interstitium. However, data concerning the ultrastructure of the basal aspect of the generated tubules have been missing. Consequently, we investigated the basal lamina by scanning electron microscopy. For control, we analyzed a polyester fleece free of tissue (Fig. 3a) and compared it with fleeces exhibiting developed tubules (Fig. 3b–d).

Scanning electron microscopy of the polyester fleece without tissue demonstrated numerous fibers in a three-dimensional extension (Fig. 3a). The fibers of the polyester fleece were detected in a longitudinal, transversal and oblique course. They were of homogeneous composition and showed a smooth surface without recognizable protrusions or roughness. Their average diameter was 10 μm. Chemical cross-linking between the fibers was not observed.

In contrast, the area of the polyester fleece used for tissue development exhibited numerous tubules. The overall view

demonstrated that the tubules had only loose contact with the fibers of the polyester fleece (Fig. 3b–d). Some of the tubules exhibited a dichotomous branching (Fig. 3b), while other tubules were found to grow in a parallel fashion in the vicinity of the polyester fibers (Fig. 3c and d). Looking on the surface of the tubules, one could further detect single interstitial cells and numerous thin fibers obviously consisting of synthesized extracellular matrix proteins.

Culture of renal tubules was performed at the interphase of an artificial interstitium without coating with extracellular matrix proteins. For this reason it was possible to investigate further special features of the basal aspect of the generated tubules by scanning electron microscopy (Fig. 4). Low magnification exhibited a smooth surface of the basal lamina of the generated tubules (Fig. 4a). However, higher magnification revealed that the basal lamina was covered by numerous bright particles exhibiting either a round, fibrillar, or crossed shape (Fig. 4b–d). It could not be determined, if these particles were incidentally attached to the basal lamina of developed tubules or if they were regular components of the lamina fibroreticularis of generated tubules. Further, it is currently unknown, if these particles were synthesized by tubular or interstitial cells.

The most impressive result was that the basal lamina of generated tubules exhibited numerous pores of different sizes (Fig. 4d). First, we assumed that the pores may be artifacts due to culture environment or fixation procedure. However, we then detected that the complete surface of the generated tubules was covered by these pores in distinct distance to each other. The pores were evenly distributed in the basal lamina and were not restricted to particular areas.

Analyzing the sizes of the pores in the basal lamina of generated tubules we found that three different categories were present (Fig. 5). Large-sized pores were less common than small-sized pores. An area of 100 μm^2 contained an average of 138 small pores, 17 medium-sized pores and three large ones. The apparent diameter of pores in the biggest category was 605 ± 101 nm, the second 317 ± 35 nm and the third 133 ± 26 nm (Fig. 6a).

Finally, we investigated, whether the newly detected pores were the components only of *in vitro*-generated tubules or if they are also the constituents of the basal lamina of tubules within the kidney. Consequently, the cortex of neonatal rabbit kidney was analyzed by SEM (Fig. 7). We found pores in the basal lamina of the collecting duct (CD) tubule of the neonatal rabbit kidney. However, it appeared that they were less frequent than in the generated tubules. The diameter of pores in the renal CD was slightly smaller compared to those found in the generated tubules (Fig. 7c). In contrast to generated tubules, only two categories of pores were found in the basal lamina of CD tubules within the neonatal rabbit kidney (Fig. 6b). Category type two pores had a diameter of 250 ± 43 nm and category type three a diameter of 114 ± 39 nm. In most of the cases, the opening of the pores was free of any recognizable material, while in some cases the border of the pores was condensed and seemed to protrude into the lumen.

4. Discussion

In previous and present experiments, it was demonstrated that generated renal tubules exhibit a polarized epithelium resembling the renal collecting duct (CD) tubule [21]. The cellular differentiation from an embryonic to a mature state, for example, could be registered by the formation of a lumen (Fig. 2a,c and d), the primary expression of tight junctions, and the development of a basal lamina (Fig. 2c and d) including the presence of laminin $\gamma 1$ (Fig. 2e and f). However, the up-regulation of an individual protein alone did not indicate the formation of a structured basal lamina during generation of tubules, as it is found during development of the kidney.

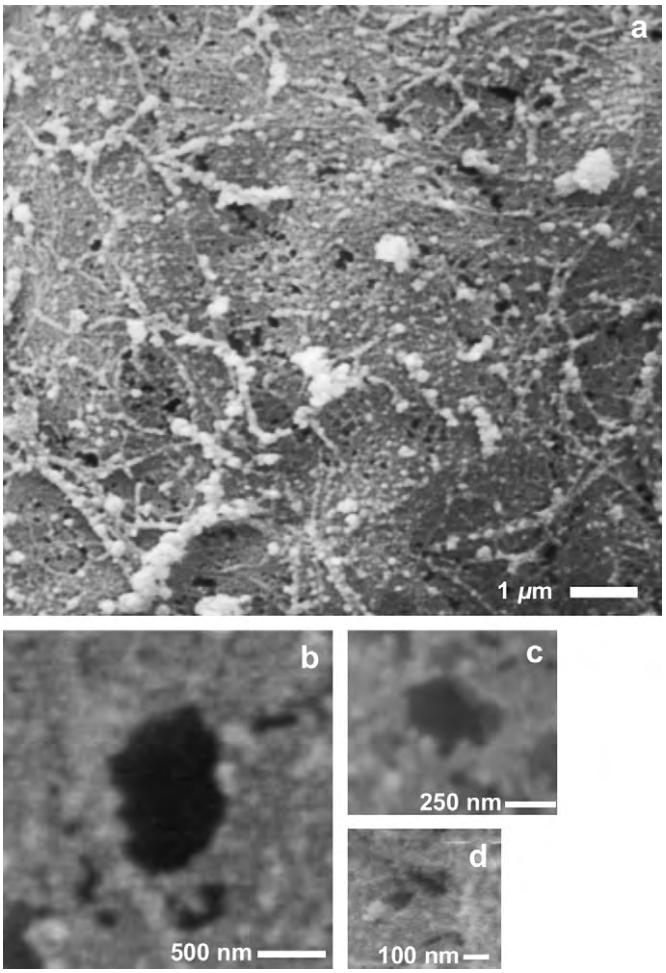

Fig. 5. SEM on the basal lamina of generated tubules (a). Pores with different diameters are shown (b–d).

Consequently, we investigated morphological features of the basal lamina in generated tubules (Figs. 3–5) and in the kidney (Fig. 7) by scanning electron microscopy. Innovative technology

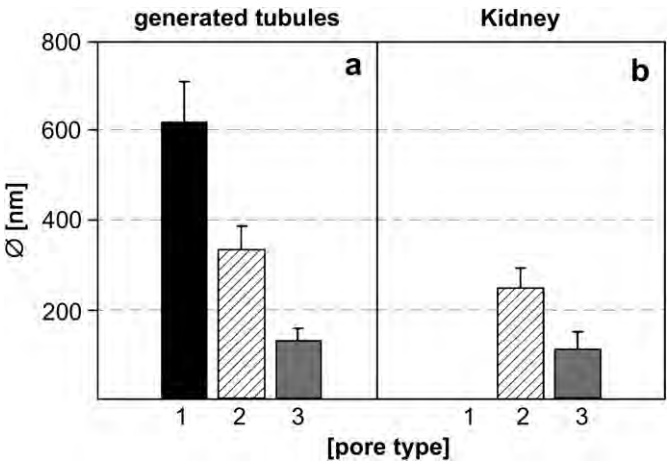

Fig. 6. Illustration of pore categories in the basal lamina of generated tubules (a) and in the collecting duct of the neonatal rabbit kidney (b).

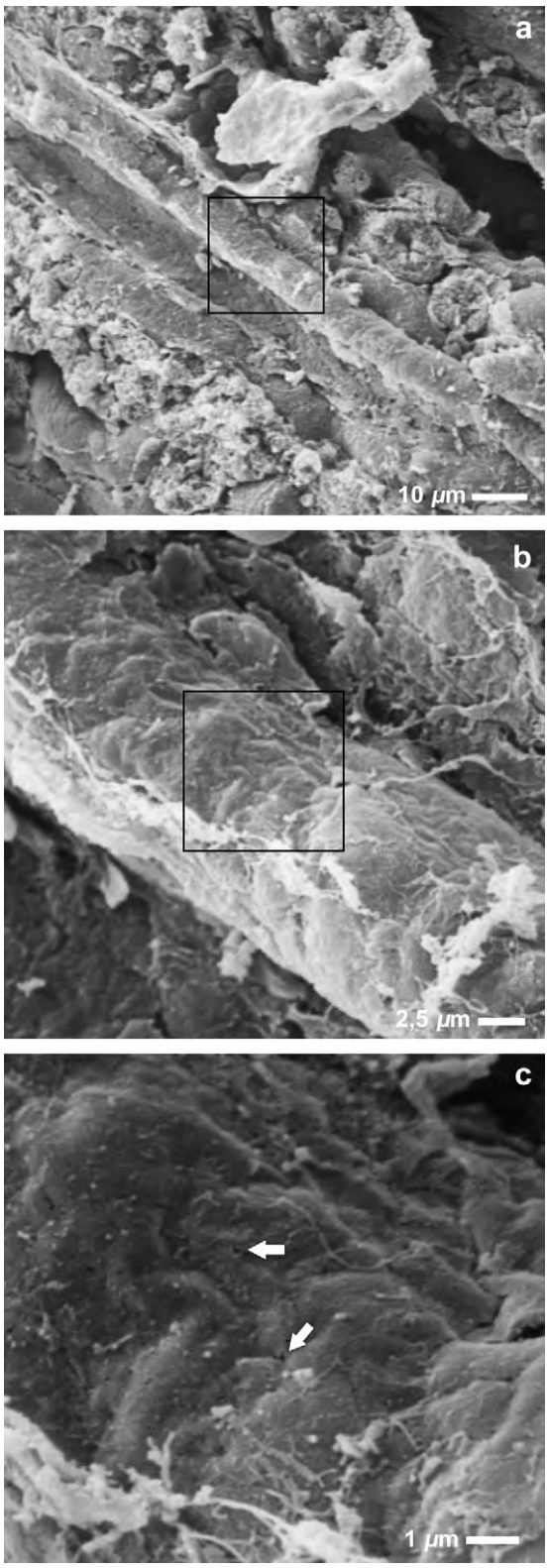

Fig. 7. SEM on the basal lamina of the collecting duct (CD) in the neonatal rabbit kidney. Low magnifaction of the CD (a). Pores with different diameters are shown (b).

such as the culture at the interphase of an artificial interstitium made this feasible. The opening of the artificial interstitium after culture by the separation of the inner fleece layers made the surface of the generated tubules accessible for SEM analysis (Fig. 1a). Since the cultures were not coated by extracellular matrix proteins, the introduced technique allowed undisturbed views of the basal aspect of the generated tubules.

Histochemical (Fig. 2a,e and f) and morphological (Fig. 2b–d) data showed that numerous generated tubules were growing at the interphase of an artificial interstitium. SEM analysis further demonstrated on an ultrastructural level that free access was possible to the basal aspect of generated tubules including the lamina fibroreticularis and lamina rara (Fig. 3b–d). The generated tubules exhibited a constantly developed basal lamina. Epithelial cells were not visible, since they were completely covered by the basal lamina. Low magnification of generated tubules exhibited a smooth surface of the basal lamina (Fig. 4a). However, higher magnification revealed that the lamina fibroreticularis contained numerous bright particles of different shapes (Fig. 4b–d). Until now, the nature of these particles remains to be elaborated.

Further, we detected that the basal lamina of the generated tubules exhibited multiple pores (Fig. 4d). They were evenly distributed in a compact matrix and were not restricted to particular areas. In generated tubules, three different sizes of pores were detected (Figs. 4d and 5). The apparent diameter of the pores in the biggest category was 605 ± 101 nm, the medium ones 317 ± 35 nm, and the smallest 133 ± 26 nm (Fig. 6a). One may argue that the demonstrated pores in generated tubules were artifacts due to culture conditions or preparation technology. However, similar pores were also present in the basal lamina of tubules found in neonatal rabbit kidney (Fig. 7). Category two of the pores exhibited a diameter of 250 ± 43 nm, and category three a diameter of 114 ± 39 nm (Fig. 6b). The occurrence of pores in both the generated tubules and in the neonatal rabbit kidney was a clear hint that pores are regular components in the basal lamina of renal CD tubules.

Reviewing the literature, we found that pores in the basal lamina of *in vitro*-generated tubules were not described earlier. However, pores in the basal lamina of renal tubules were found by other groups [22–24]. The function of these pores is unknown. In these cases, the diameter of pores was between 3 and 13 nm (Table 1). However, the pore diameter found in the generated tubules and in the CD tubules of the neonatal rabbit kidney was between 114 and 605 nm (Figs. 6 and 7). Thus, the pores found in generated tubules and in the neonatal CD tubules are much bigger in diameter compared to previously described pores in renal tubules.

The functions of the newly detected pores in generated tubules remain unknown (Figs. 4–6). They may facilitate the permeation of macromolecules through the basal lamina. They may also be the site, where extracellular matrix proteins are inserted in newly synthesized basal lamina structures.

5. Conclusions

The SEM data presented in this study showed that generated tubules and collecting duct tubules within the neonatal rabbit

Table 1
Overview of pores detected in generated tubules and in the CD of neonatal rabbit kidney in comparison to pores found in the tubular basal lamina of rat and bovine kidneys

	Pore diameter [nm]			Species	Literature
Pore type	III	II	I		
Generated tubules	133 ± 26	335 ± 51	618 ± 89	Rabbit	
Neonatal kidney	114 ± 39	250 ± 43		Rabbit	
Tubular basal lamina	2.8 ± 0.6			Rat	[22]
	4.9 ± 1.5				
	12.6 ± 5.2			Bovine	[23]

kidney exhibit a surprisingly highly structured basal lamina. The homogenously appearing matrix of generated tubules contained three different sizes of pores. In most cases the opening of the pores was free of any recognizable material. However, in some cases we found the border of the pores to protrude into the lumen. The source of the material found at the border of the pores could not be defined, though a kind of extracellular matrix appears most likely.

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