Collagen type Ⅲ is an important linking molecule between generated renal tubules and an artificial polyester interstitium

Reticulin as a linker between generated tubules and the interstitium

W.W. Minuth[1], L. Denk[1], A. Blattmann[1], H. Castrop[2]

Abstract: In the field of regenerative medicine, much consideration has been given to stem/progenitor cells for the future treatment of acute and chronic renal failure. For this strategy to be effective, however, cell biological information about tubule development within the diseased organ is needed. Unresolved cell-biological issues relating to this kind of treatment include ① the integration of stem/progenitor cells, ② their differentiation into site-specific cell types, and ③ the spatial formation of new tubules. To better understand the mechanisms related to this technology, renal tubules were generated at the interphase of an artificial interstitium by using advanced culture techniques. Stem/progenitor cells derived from neonatal rabbit kidney were covered with layers of polyester fleece, placed in a perfusion culture container, and superfused for 13 days with fresh and chemically defined Iscove's Modified Dulbeccos Medium (IMDM) containing aldosterone (1×10^{-7} mol/L). The spatial growth of tubules was registered by scanning electron microscopy (SEM) and on whole mounts or cryosections labeled with soybean agglutinin, silver stain and monoclonal antibodies reacting with collagen type Ⅲ or laminin γ1. SEM revealed that the generated tubules were completely covered by a basal lamina. The lamina fibroreticularis exhibited numerous fibers connecting the basal aspect of generated tubules with the surrounding polyester fibers. Cryosections labeled with monoclonal antibodies anti-collagen type Ⅲ and silver stain demonstrated the formation of numerous fibers spanning between the basal lamina of generated tubules and neighboring polyester fibers. In matured kidney tubules the same arrangement of collagen type Ⅲ fibers is observed as that for generated tubules. This work shows that collagen type Ⅲ is a relevant molecular linker between the basal aspect of generated renal tubules and the polyester fibers of the artificial interstitium.

Key Words: tissue engineering; perfusion culture; stem/progenitor cells; kidney; tubule; artificial interstitium; basal lamina; collagen type Ⅲ; polyester

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INTRODUCTION

A challenge in future regenerative medicine is the treatment of acute and chronic renal failure with the application of stem/progenitor cells [1-4]. However, up-to-date necessary knowledge is lacking regarding stem cell implantation in the kidney, their integration within a diseased environment and their capability to function in the repair of renal tubules.

During fetal kidney development, the transition of renal stem/progenitor cells into tubules is a rather complex process [5]. It proceeds continuously during kidney development in the outer cortex beyond the organ capsule [6-8]. At this specific site renal tubules originate from both mesenchymal nephrogenic stem cells and collecting duct ampulla-derived epithelial stem cells. Multiple growth factors mediate the primary steps to develop into the nephron anlage [9]. The ensuing cell-biological process comprises epithelial cell spreading in combination with control of spatial tubule formation [10-11]. The progress of functional development of an individual tubule segment is recognized by the appearance of a polarized epithelium exhibiting a lumen and basal aspect including the synthesis of a basal lamina. The final steps of development include the spatial extension of the tubule resulting in a defined length, diameter, and determination of a straight or convoluted course. At present, it is unknown which factors trigger all of these cellular communications, the spatial growth and the segmentation into defined tubule portions.

The complexity of kidney micro-architecture hinders investigations into the basic mechanisms of tubule development. Consequently, a powerful *in-vitro* system is essential, making it feasible to investigate the spatial formation of tubules. In the last few years, stem/progenitor cells derived from the outer cortex of neonatal rabbit kidneys were successfully used for the generation of tubules [12]. Dynamic culture of isolated embryonic renal tissue at the interface of an artificial interstitium results in the formation of numerous soybean agglutinin-labeled tubules. The process of development is initiated by the administration of aldosterone [13], is dependent on the applied hormone concentration [14] and can be antagonized by addition of spironolactone or canrenoate [15]. Generated tubules are maintained for more than two weeks in a highly differentiated state. To perform the experiments under controlled conditions, addition of serum is avoided, and, instead, fresh and chemically defined Iscove's Modified Dulbeccos Medium (IMDM) is constantly applied [16].

It is currently unknown, which molecular interactions support the spatial development of renal tubules at the interface of an artificial interstitium. Thus, the present study assessed the molecular linking between generated tubules and the surrounding artificial interstitium. For these experiments, the generated tubules were not coated with extracellular matrix proteins. Avoiding coating the view to the interface between tubules and artificial interstitium is not stacked by proteins derived

Author information: Will W. Minuth has been Professor for Anatomy at the University of Regensburg since 1989; Philip Morris Research Prize "Challenge of the Future" in 1992 for his development of an innovative cell culture system; co-founded Minucells and Minutissue GmbH in 1993; member of the German Academy for Transplantation Medicine; developed new strategies for the manufacture of tissue constructs; and expert advisor to international research projects in the field of tissue engineering. More than 100 publications on proceedings in perfusion culture technique developed by Will W. Minuth have been published. Advantages of the perfusion culture system are:
① The selection of a variety of cell supports.
② Individual influence on the differentiation of cells.
③ Perfusion of the cultures with always fresh media.
④ Microscopical observation.
⑤ Long-term culture.
⑥ Tissue engineering for surgical implantation.
⑦ Testing of biomaterials.

from the coating process. As a result, this new technique makes it possible for the first time to investigate the basal aspect of generated tubules and the surrounding fleece fibers using scanning electron microscopy. The actual experiments demonstrate that generated tubules are surrounded by a network of synthesized collagen type III fibers linking the basal lamina with the artificial polyester interstitium. Comparing fetal kidney development to these *in-vitro* experiments highlights the constructional necessity of collagen type III fibers which appears to be essential for the generation of tubules in a spatial environment.

MATERIALS AND METHODS

Isolation of embryonic explants containing renal stem/progenitor cells

One-day-old New Zealand rabbits were anesthetized with ether and sacrificed by cervical dislocation as described earlier [12]. Both kidneys were removed immediately. Each kidney was dissected in two parts. By stripping off the capsula fibrosa with fine forceps, a thin tissue layer containing numerous epithelial stem cells within the collecting duct ampullae and nephrogenic stem cells within the mesenchyme were harvested.

Basic 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 sides of embryonic tissue (Figure 1a) [16-17].
During culture the basic sandwich set-up was held in this specific position to prevent damage to the growing tissue. Then a base ring of a tissue carrier 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 carrier. 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. After closing the lid of the perfusion culture container, the complete tissue-fleece construction is fixed in an exact position. The interphase between the fleece layers used as an artificial interstitium provides an optimal microenvironment for the development of tubules throughout the entire culture period.

Dynamic culture for the generation of renal tubules

To generate renal tubules chemically defined IMDM (including Phenolred, GIBCO/Invitrogen, Karlsruhe, Germany) was used [16]. To maintain a constant pH of 7.4 under atmospheric air containing 0.3% CO_2 HEPES (50 mmol/L, GIBCO, USA) is added to the medium. To evoke tubulogenic development aldosterone (1×10^{-7} mol/L, Fluka, Taufkirchen, Germany) was present in the culture medium. To prevent infection an antibiotic-antimycotic cocktail (1%, GIBCO, USA) was added to all culture media. Throughout the entire experimental phase of 13 days fresh medium was constantly perfused at a rate of 1.25 mL/h using an IPC N8 peristaltic pump (Ismatec, Wertheim, Germany). To maintain a constant temperature of 37 ℃, the culture container was placed on a thermoplate (Medax - Nagel, Kiel, Germany) and covered with a transparent lid.

Scanning electron microscopy

For scanning electron microscopy, 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, UK). Generated tubules were fixed in 70% ethanol and processed as described and examined in a scanning electron microscope DSM 940 A (Zeiss, Oberkochen, Germany) [18].

Lectin- and antibody-labeling

Whole mount specimens or cryo-sections of 20 μm thickness were fixed in ice-cold ethanol. After being washed with phosphate buffered saline (PBS), the specimens were blocked with PBS containing 1% bovine serum albumin and 10% horse serum for 30 minutes. For soybean agglutinin (Vector, Burlingame, USA) -labeling the samples were exposed to fluorescein-isothiocyanate (FITC) -conjugated lectin diluted 1:2 000 in blocking solution for 45 minutes as described. For immunohistochemistry monoclonal antibodies anti-collagen type III (III-53, Calbiochem, Schwalbach, Germany) and monoclonal antibodies anti-laminin γ1 (kindly provided by Prof. Dr. L. Sorokin, Lund, Sweden) were applied as primary antibodies for 1 hour in blocking solution after washing with PBS. The specimens were then incubated for 45 minutes with donkey-anti-mouse-IgG-fluorescein-isothiocyanate or goat-anti-rat-IgG-rhodamine (Jackson Immuno- research Laboratories, West Grove, USA) diluted 1:50 in PBS containing 1% bovine serum albumin. 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 seconds and thereafter processed with Corel DRAW 11 (Corel Corporation, Otawa, Canada). The counting of tubules was performed with a WCIF Image J

morphometric program (Wayne Rasband, National Institute of Health, USA).

Silver contrasting for reticulin label

Silver stain procedure for interstitial fibers (reticulin) was performed according the method described previously [19].

Amount of cultured specimens

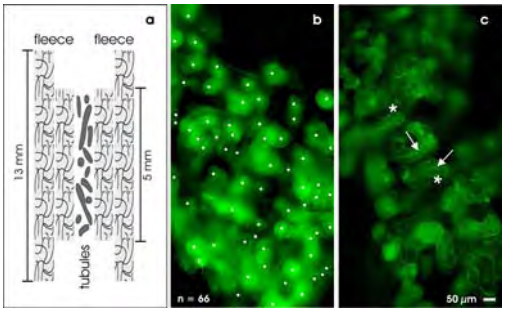
A total of 40 embryonic tissues were isolated and maintained in culture for the present study. All of the experiments were performed at least in triplicate. The data provided in the text are the mean of at least three independent experiments.

RESULTS

Generation of tubules in IMDM containing aldosterone

For the present culture experiments, the capsula fibrosa from neonatal rabbit kidney was stripped off together with a thin adherent tissue layer containing numerous stem/progenitor cells. To generate renal tubules at the interface of an artificial interstitium the isolated embryonic tissue is enclosed by layers of polyester fleece (Figure 1a) in a perfusion culture container. A smaller fleece with 5 mm in diameter is in direct contact with the developing tissue, while a larger 13 mm fleece is covering the sandwich configuration. A peristaltic pump constantly transports always fresh IMDM containing aldosterone into the perfusion container for 13 days.

The first series of experiments shows the interphase of the artificial interstitium after separation of the fleece layers. The area for tubule formation was 5 mm in diameter and the spatial environment was up to 250 μm in height. Labeling whole-mount specimens with soybean agglutinin reveals numerous and densely packed tubules (Figure 1b). In the case shown, 66 tubules were detected within a microscopic opening of 425 μm×230 μm. As shown in another example with individual focus, the generated tubules exhibit a basal lamina, lining with epithelial cells and a visible lumen (Figure 1c).



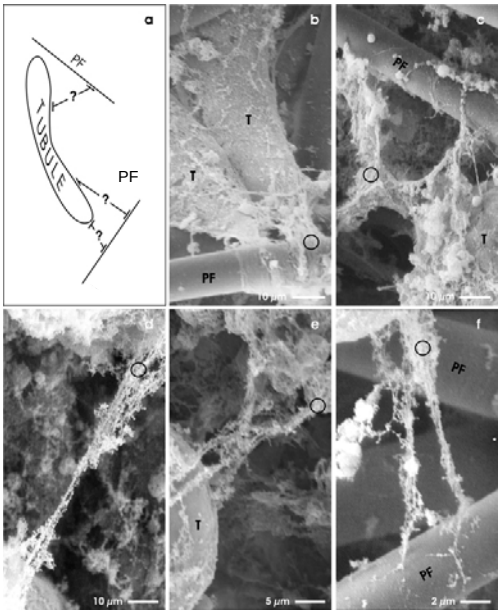
During dynamic culture the growing tissue is in contact with a polyester fleece measuring 5 mm in diameter and covered by fleeces with 13 mm in diameter on the upper and lower sides (a).
Opening the artificial interstitium after culture reveals an area with tubule growth measuring 5 mm in diameter. In this case 66 soybean agglutinin-labeled tubules are seen (b).
Generated tubules exhibit a lumen (arrow) and a basal lamina (asterisk, c).

Figure 1 Schematic illustration of renal tubules generated at the interphase of an artificial interstitium

Scanning the interphase of an artificial interstitium

In a second set of experiments the interface of an artificial interstitium was analyzed by scanning electron microscopy to investigate connecting elements between the basal lamina of generated tubules and the surrounding polyester fibers (Figure 2a). Since the cultures were not coated by extracellular matrix proteins, the view is not stacked by external proteins derived from the coating process. This fact makes it possible to analyze renal tubules generated at the interface of an artificial interstitium. It appears that the generated tubules grow either fully separated from the polyester fleece fibers or that they had only a loose neighboring contact to them. An integration of polyester fibers into the tubules is not obvious.

The scanning electron microscopy surface view further demonstrates that the basal aspect of the tubules was completely covered by a basal lamina. An important observation is that thin fibers of extracellular matrix spanned between the basal lamina of generated tubules and the polyester fibers of the artificial interstitium (Figure 2b). In the typical case, an extracellular matrix fiber originated from the lamina fibroreticularis of generated tubules and stretched towards the surface of neighboring polyester fibers belonging to the artificial interstitium (Figures 2c,d). Before reaching the surface of the polyester, the extracellular matrix fiber showed one or more dichotomous branchings (Figures 2c-f). The surface of the extracellular matrix fibers exhibited a more or less fuzzy coat (Figure 2f). In none of the cases, however, did the SEM analysis reveal if the extracellular matrix fibers were synthesized by tubular or interstitial fibroblastic cells.



Schematic illustration of tubular growth between polyester fleeces (a). Generated tubules (T) are growing in vicinity of polyester fibers (PF, b, c). Between the basal lamina of generated tubules and the polyester fibers newly synthesized extracellular matrix (○) is detected (d,e).
On the surface of extracellular matrix single interstitial cells can be recognized (c, d)

Figure 2 Scanning electron microscopy at the interface of an artificial interstitium

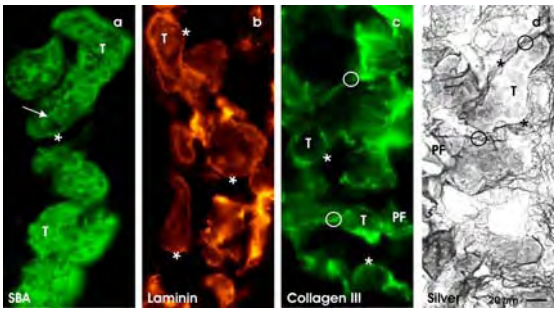
Linking molecules between generated tubules and the artificial interstitium

In the third series of experiments, cryostat sections of generated tubules were made to investigate histochemical features of fibers spanning between the basal lamina of generated tubules and the polyester fibers of the artificial interstitium (Figure 3). For control, tubules were labeled with fluorescent soybean agglutinin (Figure 3a).

The epithelial cells within the tubules were apparent. In contrast, interstitial compounds, such as extracellular matrix proteins, were not stained. Immunohistochemical labeling with monoclonal antibodies anti-laminin γ1 demonstrated the presence of a basal lamina on generated tubules (Figure 3b). Also in this case the interstitial space between the tubules was not labeled with monoclonal antibodies anti-laminin γ1.

Staining of the tissue with anti-collagen type III, however, revealed that the basal aspect of generated tubules exhibited intense labeling. In addition, a series of fibers is spanning between generated tubules and polyester fibers of the artificial interstitium (Figure 3c).

Silver staining, according to Gomori, further exhibits intense labeling at the basal lamina and within a network spanning between generated tubules and polyester fibers (Figure 3d).



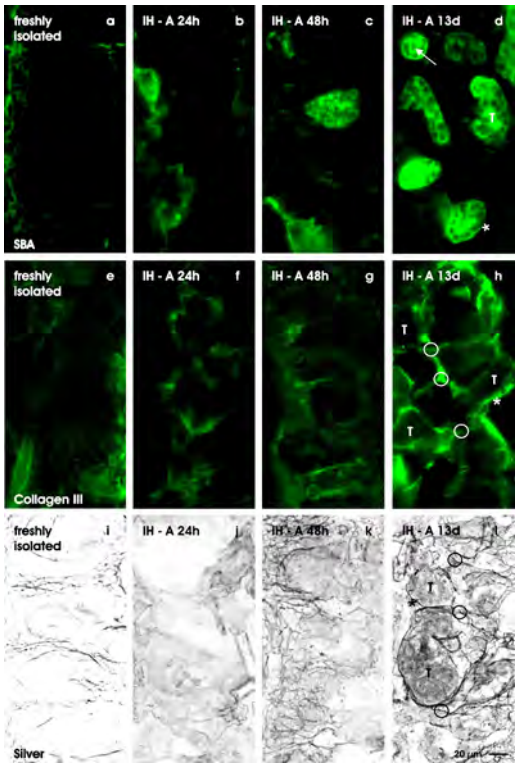
Cryosections reveal after soybean agglutinin-label numerous tubules (T) containing a basal lamina (asterisk) and a lumen (arrow, a).
The basal lamina of generated tubules is positive for laminin γ1 (b).
Anti-collagen type III(c) and silver staining (d) intensively label the basal lamina and interstitial fibers. Newly synthesized extracellular matrix (○). Polyester fibers (PF).

Figure 3 Histochemical labeling of generated tubules at the interface of an artificial interstitium after 13 days

To obtain further information about extracellular matrix fibers spanning between the basal aspect of generated tubules and the artificial interstitium, specimens were investigated at the start (Figures 4a, e, i), after 24 hours (Figures 4b, f, j), 48 hours (Figures 4c, g, k) and 13 days (Figures 4d, h, l) of culture. As a control, cellular soybean agglutinin-label was lacking at the beginning of culture (Figure 4a) and was barely found after 24 hours (Figure 4b) and 48 hours (Figure 4c), while strong expression was found after 13 days (Figure 4d) of culture within generated tubules. Labeling for collagen type III was absent at the start (Figure 4e) and after 24 hours of culture (Figure 4f). After 48 hours of culture, a faint labeling appeared (Figure 4g), which was recognized as a strong reaction after 13 days (Figure 4h) of culture. Silver staining was detected only as a very faint label after isolation (Figure 4i) of renal stem/progenitor cells and after 24 hours (Figure 4j) of culture. After 48 hours (Figure 4k) of culture, the silver staining was enhanced and reached a maximum at day 13 of culture (Figure 4l). Thus, soybean agglutinin- (Figure 4a), collagen type III- (Figure 4e) and (to a lesser degree) silver staining (Figure 4i) -label were lacking after isolation of tissue, while after 13 days of culture intensive labeling of both stainings was detected. It demonstrates that soybean agglutinin-, collagen type III- and silver staining appeared to be dependent on the time of culture.

Interstitium within the developing kidney

The previous experiments with generated tubules demonstrate that soybean agglutinin- (Figure 4a) and collagen type III- label (Figure 4e) were not found after isolation of renal stem/progenitor cell containing tissue, but that they develop within 13 days of culture (Figures 4d, h). This result has to be compared with embryonic, maturing and matured structures of the developing kidney (Figure 5). Using exactly orientated cortico-medullary tissue sections, this developmental gradient can be analyzed by histochemical methods on the neonatal kidney.



Tissue was analyzed at the begin (a, e, i), after 24 hours (b, f, j), after 48 hours (c, g, k) and after 13 days (d, h, l) of culture.
Label for soybean agglutinin is not found after isolation of renal stem/progenitor cells (a) and barely expressed after 24 (b) and 48 hours (c), while intensively developed after 13 days of culture (d).
Label for collagen type III is not found after isolation of renal stem/progenitor cells (e) and barely expressed after 24 (f) and 48 hours (g), while intensively detected after 13 days of culture (h).
Label for silver stain is not found after isolation of renal stem/progenitor cells (i) and barely developed after 24 (j) and 48 hours (k), while intensively marked after 13 days of culture (l).
Tubules (T) with lumen (arrow), basal lamina (asterisk) and newly synthesized extracellular matrix (○)

Figure 4 Histochemical label of embryonic renal tissue generated at the interface of an artificial interstitium for soybean agglutinin (a-d), anti-collagen type III (e-h) and siver stain (i-l)

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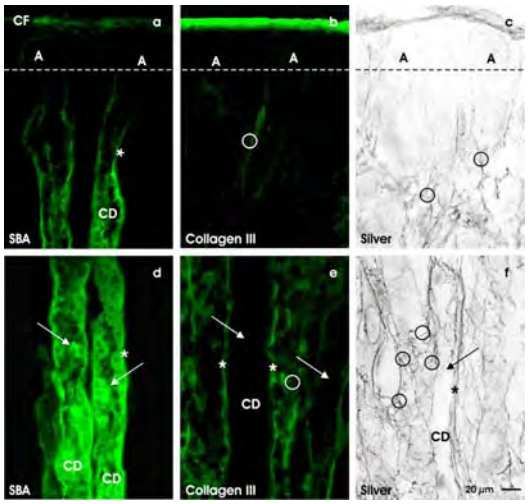
In the last set of experiments, the primary appearance of collagen type Ⅲ- and silver stain-positive fibers was investigated. The thin tissue layer between the organ capsule and the dotted line contained epithelial stem cells within the collecting duct ampullae, while the nephrogenic stem cells are located around the ampullae. Thus, the embryonic tissue beyond the capsule (CF) and above the dotted line did not show labeling for soybean agglutinin (Figure 5a), or collagen type Ⅲ (Figure 5b), and only a faint label for silver stain (Figure 5c). The layer of tissue containing renal stem/progenitor cells above the dotted line was used after isolation for the current culture experiments (Figures 4a,e,i, 5a,b,c).

In contrast, the tissue below the dotted line contained maturing tissue of the neonatal kidney. Here, in the ampulla shaft area and further down in the more matured collecting duct tubule, increasing soybean agglutinin-label was found (Figure 5a). While collagen type Ⅲ-label was not detected in the embryonic outer cortex, it showed a slight label in the maturing region of neonatal kidney (Figure 5b).

Also, silver staining exhibited a faint label in the maturing area of the cortex beyond the dotted line (Figure 5c).

Finally, in the matured inner cortex of the neonatal kidney, intense cellular soybean agglutinin-labeling was found within the collecting duct tubule (Figure 5d). Strong collagen type Ⅲ - (Figure 5e) and silver stain-label (Figure 5f) was detected at the basal aspect of the matured collecting duct tubule and within the renal interstitium.



Histochemical label in the embryonic outer cortex of neonatal rabbit kidney (a-c) and in the matured deep cortex (d-f) after soybean agglutinin-label (a, d), anti-collagen type Ⅲ label (b, e) and silver stain (c, f).

Beyond the renal capsule (CF) within the collecting duct ampullae (A) and the nephrogenic mesenchyme label for soybean agglutinin (a), collagen type Ⅲ (b) and silver stain (c) is lacking.

However, below the dotted line in the maturing part all three marks start to be expressed.

In the deep cortex intensive label for soybean agglutinin (d) in the collecting duct (CD), collagen type Ⅲ (e) and silver stain (f) in the interstitium is detected. Basal lamina (asterisk), lumen (arrow) and newly synthesized extracellular matrix (○).

Figure 5 Gradient of development in the neonatal rabbit kidney

Thus, the soybean agglutinin-, collagen type Ⅲ-, and silver stain-label in the embryonic (above dotted line, Figures 5a-c), maturing (below dotted line, Figures 5a-c) and matured zone (Figures 5d-f) of neonatal kidney demonstrated an unambiguously recognizable developmental gradient within the organ. Comparison of the histochemical pattern between generated tubules (Figures 3, 4) with the developmental gradient in the neonatal kidney (Figure 5) is an important tool in determining the actual state of tissue development reached in culture. This gradient clearly demonstrates that collagen type Ⅲ is an essential molecule expressed in kidney development and in renal tubules generated at the interface of an artificial interstitium.

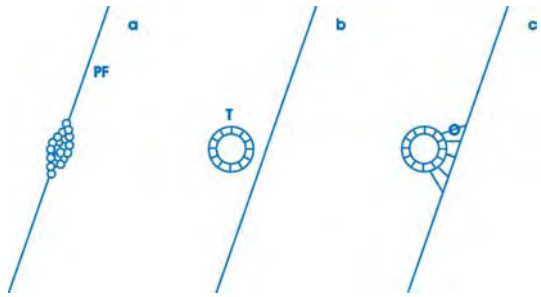
DISCUSSION

Renal tubules have previously been raised by coating cell lines, primary cells or isolated embryonic tissue with extracellular matrix compounds [20-23]. In all of these cases, the culture was performed in serum-containing medium and in the stagnant environment of a culture dish. To offer better controlled conditions, the protocol presented here generates tubules in a chemically defined medium using a perfusion container without coating with extracellular matrix proteins. Instead, the isolated renal stem/progenitor cells were covered with layers of polyester fleece on the upper and lower sides [16-17]. Since the interstitial space between the developing tubules was not stacked by proteins from a coating process, this technique makes it possible to investigate the primary derivation of extracellular matrix proteins using scanning electron microscopy and histochemical analysis.

The interface of the artificial interstitium used for renal stem/progenitor cells resulted in the spatial formation of numerous tubules under the influence of aldosterone as tubulogenic factor. The progress of development from an embryonic into a mature state can be marked by the formation of a lumen, the primary expression of tight junctions, the up-regulation of Na/K-ATPase and the development of a basal lamina containing laminin γ1 as previously described [13,17].

Since the environmental factors supporting the spatial development of tubules are unknown, the present experiments were performed to obtain first information about the molecular linkage between the generated renal tubules and the surrounding artificial interstitium. Scanning electron microscopy analysis illuminated that the basal lamina of generated tubules was found in close proximity to the tubules but that it apparently avoids direct interaction with fibers of the polyester fleece. It appears, that the polyester fibers do not exhibit a guiding influence on the spatial development of the tubules. Thus, the artificial interstitium is functionally not comparable to a scaffold. Instead the tubules develop at the interface between the two layers of polyester fleece used as artificial interstitium. The growth of tubules inside the fleece and between the fibers does not seem to be supported. In the case the polyester fibers exhibit an attractive influence on embryonic renal cells, they would spread on the surface of the polyester fibers (Figure 6a). However, a spreading of cells on the fibers is

not observed. In consequence, the fibers exhibit a repulsive effect, since they prevent spreading on their surface and support development of tubules (Figure 6b) and in more or less vicinity of the polyester fibers (Figure 6c).



A positive effect of fibers would attract the cells to grow on their surface (a).
A repulsive effect influences the tubules (T) to grow fully separated from the PF (b).
A beneficial effect stimulates the tubules to grow in the vicinity of PF and stimulates to form extracellular matrix (○, c).

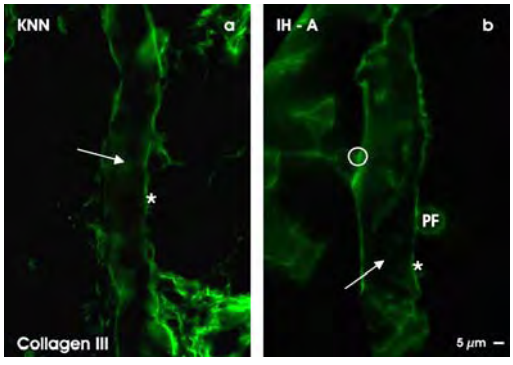
Figure 6 Schematic illustration of attractive and repulsive growth of renal stem/progenitor cells in relation to a polyester fibers (PF)

Scanning electron microscopy (Figure 2) and histochemistry (Figure 3) further revealed that numerous newly synthesized fibers consisting of extracellular matrix proteins were spanning between the basal lamina of generated tubules and the surface of polyester fibers of the artificial interstitium. For the first time, it has been shown that generated tubules are fastened on the polyester fibers. These spanning fibers consisting of extracellular matrix were not present after isolation of renal stem/progenitor cells, but they were synthesized during a 13 days culture period (Figure 4). Not all, but at least part of the extracellular matrix fibers were immuno-positive for collagen type Ⅲ and could be labeled with silver stain. All these features indicate that a reticulin fiber network is synthesized as it is present within the kidney.

The collagen type Ⅲ/silver stain-positive fibers spanning between generated tubules and the polyester fibers of the artificial interstitium may be due to culture artifacts. Consequently, histochemical experiments were carried out with embryonic, maturing and matured kidney in comparison to generated tubule specimens (Figure 5). On tubules generated at the interface of an artificial interstitium (Figures 4h,l) and within the kidney (Figures 5e,f) the same type of extracellular matrix fibers is found [24-26]. The primary appearance of this type of extracellular matrix fibers is occurring in dependence on the state of organ development. Beyond the organ capsule in the embryonic outer cortex of the neonatal kidney, this type of fiber does not surround stem/progenitor cells or developing tubules as demonstrated by soybean agglutinin (Figures 4a, 5a), anti-collagen type III- (Figures 4e, 5b) and silver stain-label (Figures 4i, 5c). In contrast, in deeper areas of the maturing cortex first signs of extracellular matrix fibers surrounding the tubules were detected. Most impressive was the interstitial fiber network established around the collecting duct tubules. As demonstrated previously, the same type of tubule is generated in the presented experiments. So far the demonstrated collagen type Ⅲ- (Figures 5e, 7a) and silver stain- (Figure 5f) positive fibers belong to the naturally occurring extracellular matrix compounds surrounding a renal tubule.

These extracellular matrix componds appear to serve as a mechanical fastener and as a means to keep the tubules at a discrete distance from the polyester fibers. Obviously this mechanism promotes the growth of epithelial cells within the tubule and hinders the growth of cells on the surface of the polyester fleece fibers. Additionally, it may be a hint that development of tubules can be improved by offering a collagen type Ⅲ fiber network [27].

Future culture experiments will clarify whether or not the development of collagen type Ⅲ-positive fibers in the kidney (Figure 7a) and on generated tubules (Figure 7b) are related to an epiphenomenon or if they actively support development. Consequently, experiments are under way to stimulate collagen type Ⅲ synthesis for example by the addition of ascorbic acid to the culture medium. An alternative experimental strategy involves coating the polyester fibers with collagen type Ⅲ to support molecular linking of tubules during development. A further approach could be the generation of tubules using Small Intestine Submucosa as an artificial interstitium. This matrix consists of a natural collagen type Ⅲ fiber network. Independent from the outcome of these experiments in the present paper first information about molecular fastening between generated tubules and the interface of an artificial interstitium is demonstrated. All of this information is crucial for improving strategies to treat renal diseases with the help of stem cells [28-29]. In conclusion, fibers consisting of collagen type Ⅲ are found to be natural fastening elements between the basal lamina of generated renal tubules and the polyester fibers of an artificial interstitium.



In both kidney and generated tubules it is demonstrated that collagen type Ⅲ is localized as well in the basal lamina as on interstitial fibers.
Basal lamina (asterisk), lumen (arrow), newly synthesized extracellular matrix (○), polyester fiber (PF)

Figure 7 Immunohistochemical label of collecting duct tubules for collagen type Ⅲ in the deep cortex of neonatal kidney (a) and on a generated tubule (b) after 13 days of culture

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REFERENCES

1 Maeshima A, Sakurai H, Nigam SK. Adult kidney tubular cell population showing phenotypic plasticity, tubulogenic capacity, and integration capability into developing kidney. J Am Soc Nephrol 2006;17(1):188-198
2 Hammerman MR. Cellular therapies for kidney failure. Expert Opin Biol Ther 2006;6(2):87-97
3 Vigneau C, Zheng F, Polgar K, et al. Stem cells and kidney injury. Curr Opin Nephrol Hypertens 2006;15(3):238-244
4 McTaggart SJ, Atkinson K. Mesenchymal stem cells: immunobiology and therpeutic potential in kidney disease. Nephrology 2007;12(1):44-52
5 Dressler G. Tubulogenesis in the developing mammalian kidney. Trends Cell Biol 2002;12(8):390-395
6 Strehl R, Trautner V, Kloth S, et al. Existence of a dense reticular meshwork surrounding the nephron inducer in neonatal rabbit kidney. Cell Tissue Res 1999;298(3):539-48
7 Schumacher K, Strehl R, De Vries U, et al. SBA-positive fibers between the CD ampulla, parenchyme, and renal capsule. J Am Soc Nephrol 2002;13(10):2444-2453
8 Schumacher K, Strehl R, Minuth WW. Characterization of micro-fibers at the interface between the renal collecting duct ampulla and the cap condensate. Nephron Exp Nephrol 2003;95(2):43-54
9 Karihaloo A, Nickel C, Cantley LG. Signals which build a tubule. Nephron Exp Nephrol 2005;100(1):40-45
10 Hogan BL, Koledziej M. Molecular mechanisms of tubulogenesis. Nature Rev 2002;3(7):513-523
11 Lubarsky B, Krasnow MA. Tube morphogenesis: making and shaping biological tubes. Cell 2003;112(1):19-28
12 Minuth WW. Neonatal rabbit kidney cortex in culture as tool for the study of collecting duct formation and nephron differentiation. Differentiation 1987;36(1):12-22
13 Heber S, Denk L, Hu K, et al. Modulating the development of renal tubules growing in serum-free culture medium at an artificial interstitium. Tissue Eng 2007;13(2):281-292
14 Minuth WW, Denk L, Hu K. The role of polyester interstitium and aldosterone during structural development of renal tubules in serum-free medium. Biomaterials 2007; 28(30):4418-4428
15 Minuth WW, Denk L, Hu K, et al. Tubulogenic effect of aldosterone is attributed to intact binding and intracellular response of the mineralocorticoid receptor. Central Eur J Biol - CEJB 2007; 2(3):307-325
16 Hu K, Denk L, de Vries U, et al. Chemically defined medium environment for the development of renal stem cells into tubules. Biotechnol J 2007;2(8):992-995
17 Minuth WW, Denk L, Castrop H. Generation of tubular superstructures by piling of renal stem/progenitor cells. Tissue Eng Part C Methods 2008;14(1):3-13
18 Blattmann A, Denk L, Strehl R, et al. The formation of pores in the basal lamina of regenerated tubules. Biomaterials 2008; 29(18): 2749-2756
19 Romeis B. Mikroskopische Technik.17th ed. Munich: Urban & Schwarzenberg 1989:510-511
20 Inoue CN., Sunagawa N, Morimoto T, et al. Reconstruction of tubular structures in three-dimensional collagen gel culture using proximal tubular epithelial cells voided in human urine. In vitro Cell Dev Biol Animal 2003;39(8-9):364-367
21 Meyer TN, Schwesinger C, Bush KT, et al. Spatiotemporal regulation of morphogenetic molecules during in vitro branching of the isolated ureteric bud: toward a model of branching through budding in the developing kidney. Dev Biol 2004;275(1):44-67
22 Hellman NE, Greco AJ, Rogers KK, et al. Activated extracellular signal-regulated kinases are necessary and sufficient to initiate tubulogenesis in renal tubular MDCK strain I cell cysts. Am J Physiol Renal Physiol 2005;289(4):F777-F785
23 Han HJ, Sigurdson WJ, Nickerson PA, et al. Both mitogen activated protein kinase and the mammalian target of rapamycin modulate the development of functional renal proximal tubules in matrigel. J Cell Sci 2004;117(Pt9):1821-1833
24 Montes GS. Structural biology of the fibers of the collagenous and elastic systems. Cell Biol Int 1996;20(1):15-27
25 Douglas T, Heinemann S, Bierbaum S, et al. Fibrillogenesis of collagen types I, II, and III with small leucine-rich proteoglycans decorin and biglycan. Biomacromolecules 2006;7(8):2388-2393
26 Fleischmajer R, Jacobs L, Perlish JS, et al. Immunochemical analysis of human kidney reticulin. Am J Pathol 1992;140(5):1225-1235
27 Mondalek FG, Lawrence BJ, Kropp BP, et al. The incorporation of poly(lactic-co-glycoloc) acid nanoparticles into porcine small intestinal submucosa biomaterials. Biomaterials 2008; 29(9):1159-1166
28 Cantley LG. Adult stem cells in the repair of injured renal tubule. Nature Clin Pract Nephrol 2005;1(1):22-32
29 Steenhard BM, Isom KS, Cazcarro P, et al. Integration of embryonic stem cells in metanephric kidney organ culture. J Am Soc Nephrol 2005;16(6):1623-1631

Collagen type Ⅲ is an important linking molecule between generated renal tubules and an artificial polyester interstitium。

Reticulin as a linker between generated tubules and the interstitium

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W.W. Minuth，解剖学教授，1989 年起至今任职于德国雷根斯堡大学，1992 年因成功研制出一种新型细胞培养系统而获得 Philip Morris Research Prize 奖，1993 年还与其同伴共同创建了 Minucells and Minutissue Vertriebs GmbH 公司。W.W. Minuth 教授是德国移植医学学会成员，研发了制造器官移植组织结构的新方法，也是国际组织工程领域研究项目的顾问专家。

W.W. Minuth 已发表 100 多篇灌注培养技术的相关论文。

灌注培养系统的优点：

① 支持多种组织细胞的选择。

② 排除了胞外基质材料中胶原成份对细胞分化的影响。

③ 可对新鲜组织细胞进行连续灌注培养。

④ 可进行细胞的长期体外培养。

⑤ 体外灌注培养条件下可见细胞增长与增殖。

⑥ 可进行生物材料支持下的三维细胞培养。

摘要：在再生医学领域，已经有很多人考虑在将来用干/祖细胞治疗急慢性肾功能衰竭。但是，要使这种治疗方案行之有效，需要了解关于患病肾脏肾小管发育的细胞生物学信息。与干/祖细胞治疗急慢性肾功能衰竭相关尚待明晰的细胞生物学问题包括：①干/祖细胞的整合。②干/祖细胞的定向分化类型。③新形成肾小管的空间构成。为了更好的了解这项技术的相关机制，文章应用了先进的培养技术在人工非细胞外基质材料界面条件下构建肾小管。将新生兔肾来源的干/祖细胞用多层聚酯纤维网覆盖，放置在灌注培养器中，用含 1×10^{-7}mol/L 醛固酮的新鲜合成的在 DMEM 基础上改良的 IMDM 培养基诱导培养 13 d。扫描电镜下，在大豆凝集素、银染和抗 III 型胶原或

层粘连蛋白γ1 单克隆抗体标记的全标本包埋爬片或冰冻切片上观察肾小管的生长情况。扫描电镜观察结果显示，新生成的肾小管完全被基底膜覆盖，纤维网状板有大量的纤维与生成的肾小管基底面和周围人工聚酯材料相互交联。抗 III 型胶原染色和银染结果表明，在新生成的肾小管的基底膜和邻近的人工聚酯材料间有大量的纤维交织，在发育成熟的肾小管中有与新生成肾小管相同排列形式的 III 型胶原纤维。以上实验结果说明，III 胶原是联系新生成的肾小管基底面和人工基质聚酯纤维的分子纽带。

关键词：组织工程学；灌注培养；干/祖细胞；肾；小管；人工间质；基底膜；III型胶原；聚酯

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中国专家评价 1： Will .W. Minuth 是德国 University of Regensburg，Department of Molecular and Cellular Anatomy 的教授，研究领域是 Tissue engineering，查询以他为首的团队的研究成果，可以知道本篇论文是该团队多年来系统研究的最新成果之一，并且已经列入投稿名单(Will.W. Minuth, L. Denk, A. Blattmann, H. Castrop (2008) collagen type Ⅲ is an important linking molecule between generated renal tubules and an artificial interstitium, Submitted.)。

从论文本身的内容看，技术运用全面，实验数据可信，图示清晰，学术价值高。

中国专家评价 2： 文章采用人工聚酯材料夹心法，用含醛固酮的诱导培养基对分离的新生家兔肾组织切片进行连续灌注培养，结果表明在此体系下诱导培养 13 d 后，可分化获得具有肾小管形态的结构，经扫描电镜和免疫荧光检测证实新生的肾小管周围形成了典型的纤维网状的胞外基质成分，其中含有III型胶原，并且新生的III型胶原成为联系肾小管和人工聚酯材料的纽带，但新生的III型胶原在肾组织切片诱导分化为肾小管样结构中所扮演的角色还有待进一步研究确定。

文章立题新意，设计合理，结果有一定的创新性。

中国专家评价 3： 文章的创新之处是首次采用人工非细胞外基质材料支持肾组织切片的体外分化培养，避免了传统方法所采用的细胞外基质材料中胶原成分对实验结果及分析的干扰；其次，文章首次将连续灌注培养引入肾组织切片离体培养研究中，与以往静态培养体系相比，该培养方式更接近于体内情况，研究结果证实了该方法的优越性。

研究设计缜密、图表清晰、结果可信，其意义主要在于上述方法学的改进和应用，并再次证实了肾组织中存在具有分化为肾小管的干细胞。

中国专家评价 4： 文章是三维细胞培养(TDCC)技术所获得的又一项重要成果，立题新颖，设计合理，结果有创新性，反映了在聚酯材料上用人工脉管取代细胞外基质生长支架,在完全可控制的生长条件下，肾干细胞能分化产生结构完整的肾小管,可见腔和纤层，细胞外基质生成明显，III型胶原的作用地位证明明确,在肾的发育生物学研究和组织工程领域做出了新的又一独特贡献。