
Generation of Renal Tubules at the Interface of an Artificial Interstitium

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Key Words

Renal stem cells • Epithelium • Tubules • Artificial interstitium • Polyester fleece • Perfusion culture • Tissue engineering

Abstract

During kidney development a multitude of tubular portions is formed. Little knowledge is available by which cellbiological mechanism a cluster of embryonic cells is able to generate the threedimensional structure of a tubule. However, this know-how is most important in tissue engineering approaches such as the generation of an artificial kidney module or for the therapy of renal diseases using stem cells. To obtain cellbiological insights in parenchyme development we elaborate a new technique to generate under in vitro conditions renal tubules derived from the embryonic cortex of neonatal rabbits. The aim of the experiments is to establish a specific extracellular environment allowing optimal threedimensional development of renal tubules under serum-free culture conditions. In the present paper we demonstrate features of the renal stem cell niche and show their isolation as intact microcompartments for advanced tissue culture. Perfusion culture in containers exhibiting a big dead fluid volume results

in the development of a flat collecting duct (CD) epithelium at the surface of the tissue explant. In contrast, by fine-tuning the dead fluid volume within a perfusion culture container by an artificial interstitium made of a polyester fleece shows the generation of tubules. It is an up to date unknown morphogenetic information which tells the cells to form tubular structures.

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Introduction

Acute and chronic renal failures show that the regenerative capability of cells within the organ is lost by an unknown mechanism. Thus, neo-formation of parenchyme in the diseased kidney needs a source of cells for regeneration. As earlier shown cells may be stimulated to divide for regeneration by growth factors such as HGF [1], EGF [2] and IGF-1 [3] so that they replace diseased tissue by spreading. An alternative strategy followed the administration of bone marrow derived stem cells (BMSC) or resulting precursor cells [4]. However, the application of hematopoietic stem cells

did not fulfill in the past the expectations set in this therapeutic protocol. Regarding the unique development of the kidney we favor the organ-specific stem cells to learn about regeneration processes. However, as compared to the hematopoietic system and to other tissues like liver, skin or intestine the knowledge about the developmental potential of renal stem cells is up to date inferior [5-7].

One of the peculiarities of the renal stem cell population is that as well an epithelial as a mesenchymal type exists, which develops either into the collecting duct (CD) system or into individual segments of the nephron including the glomerulus [8, 9]. Development of renal parenchyme starts by a reciprocal interaction between the ureter bud-derived epithelial tip of CD ampullae and the competent mesenchymal cells located in the covering cap condensate [10]. By the induction stimulus part of the mesenchymal cells condensates to convert into an epithelium. It includes the presumptive glomerulus and the different premature nephron segments. Over extended periods of time this mechanism frequently repeats, in consequence the developing kidney constantly increases in volume. Although significant advances were made in the last decade to elucidate the molecular mechanisms leading to nephron induction [11, 12], few knowledge is available regarding the threedimensional growth of parenchyme in kidney and in other organs such as lung, liver and pancreas during fetal development [13-16].

Materials and Methods

Isolation of explants containing renal stem cells

One day old New Zealand rabbits were anesthetized with ether and killed by cervical dislocation. Both kidney were removed immediately. Each kidney was dissected in two parts. By stripping off the capsula fibrosa with fine forceps a fully embryonic tissue part is harvested containing numerous CD ampullae, S-shaped bodies and mesenchyme (Fig. 1A-C) [17].

Generation of flat CD epithelia in perfusion culture

The explants were applied on specific tissue holders with 14 mm outer diameter (Fig. 1D). The holders were then placed for 24 hours in a 24-well plate in a CO₂-incubator containing 5% CO₂/95% air atmosphere [17, 18]. During culture of the explants in Iscove's Modified Dulbecco's Medium (IMDM; order # 21980 - 032; Gibco BRL Life Technologies, Eggenstein, Germany) including 10% fetal bovine serum (Boehringer, Mannheim, Germany) an outgrowth of cells from the CD ampullae was observed. Within 24 hours the entire surface of the explant was covered by a monolayer of polarized CD cells. After initiation of culture the tissue holders were then placed

in a perfusion culture container (Fig. 1E,2A; Minucells and Minutissue, Bad Abbach, Germany, www.minucells.de). Fresh medium was continuously perfused for 14 days 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 container was placed on a thermo plate (Medax, Kiel, Germany) and covered by a transparent lid. During perfusion culture IMDM without serum was used as the standard medium. Aldosterone was applied at a concentration of 1 x 10⁻⁷ M (Sigma-Aldrich-Chemie, Deisenhofen, Germany) and 1% antibiotic-antimycotic solution (Gibco BRL-Life Technologies) was added to all culture media. Furthermore, up to 50 mmol/l HEPES (Gibco BRL-Life Technologies) was used in the medium to maintain a constant pH of 7.4 in perfusion culture under atmospheric air containing 0.3% CO₂ [18].

Generation of renal tubules in perfusion culture

For the generation of renal tubules embryonic explants were isolated as described [17] and placed in specific tissue holders (Fig. 1D) [18]. However, the culture period for 24 hours in serum-containing medium was omitted. Consequently, the holders with freshly isolated embryonic renal tissue were immediately placed in a perfusion culture container with a lowered dead fluid volume (Fig. 1E,3A). This was obtained by filling the dead fluid volume of the culture container with a highly porous biocompatible polyester fleece (Walraf, Grevenbroich, Germany) as an artificial interstitium as published earlier [19]. Thus, the embryonic tissue and the polyester material were in close contact (Fig. 3B,C). Fresh serum-free IMDM containing aldosterone was continuously perfused for 14 days at a rate of 1 ml/h with an IPC N8 peristaltic pump (Ismatec, Wertheim, Germany).

Amount of cultured constructs

In total 237 tissue constructs containing tubules were generated for the presented experiments. The mean of generated structure size is given in the text.

Lectin-labeling

Whole mount specimens or cryosections of the generated epithelium (8 µm) respectively of the generated tubules (20 µm) were fixed in ice-cold ethanol and then washed with phosphate buffered saline (PBS). After incubation in blocking solution (PBS + 1% bovine serum albumine (BSA) + 10% horse serum) for 30 minutes the specimens were exposed to fluorescein-isothiocyanate (FITC) -conjugated Soybean agglutinin (SBA) or Dolichos biflorus agglutinin (DBA, Vector Laboratories, Burlingame, USA) diluted 1:2000 in PBS for 45 minutes. Following several washes in PBS tissue was then embedded with Slow Fade Light Antifade Kit (Molecular Probes, Eugene, USA) and analyzed using an Axioskop 2 plus microscope or a laser scanning microscope Zeiss LSM 510 meta (Zeiss, Oberkochen, Germany). Images were made by a digital camera and thereafter processed with Photoshop 5.5 (Adobe Systems, San Jose, CA, USA). For histological control sections were stained with Toluidinblue or Ferrohematoxylin-azophloxin-lightgreen (Goldner).

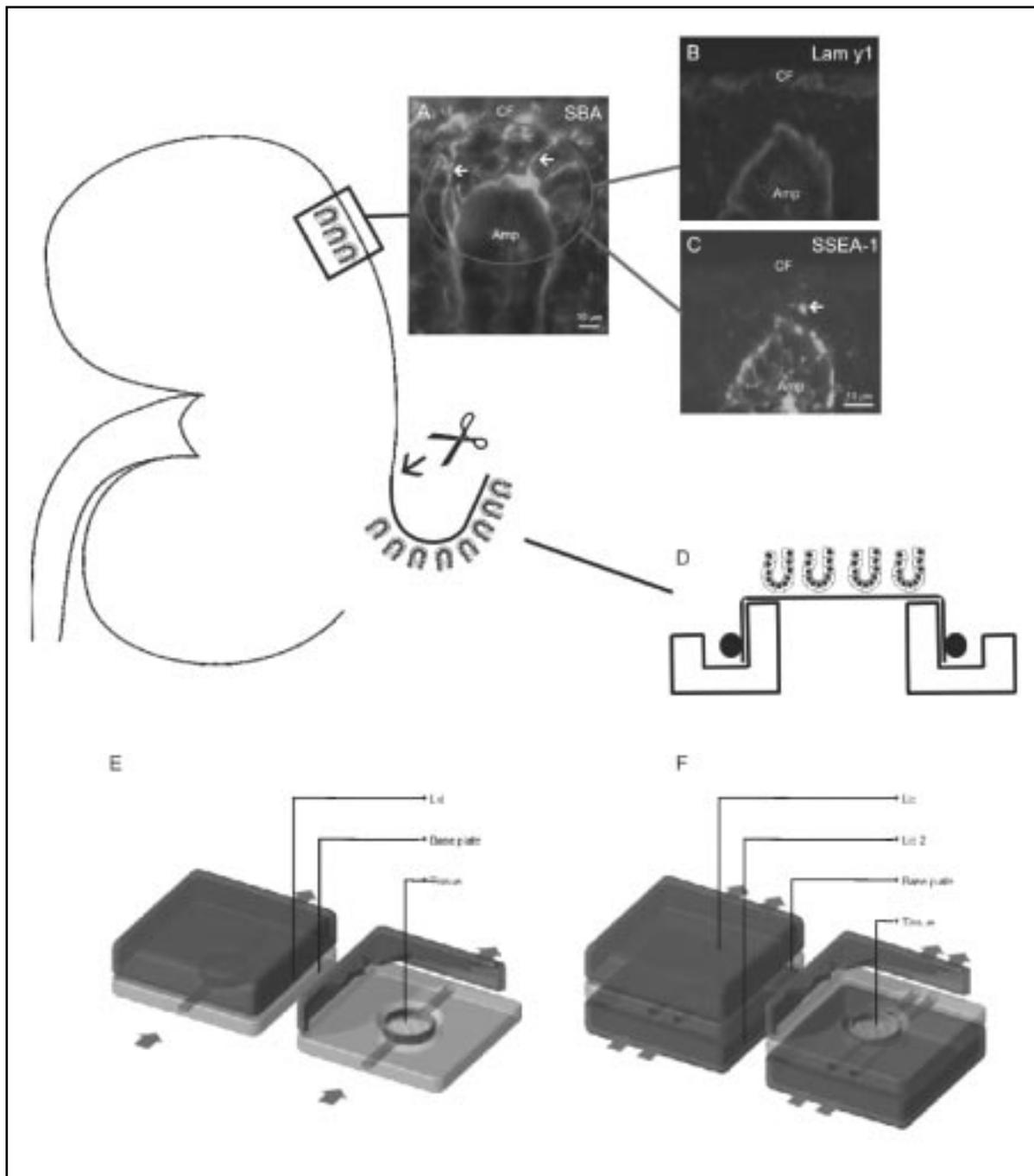


Fig. 1. Localisation and isolation of the renal stem cell compartments. (A) SBA-labeling reveals extending micro-fibers (arrows) between the basal aspect of the CD ampulla (Amp) and the capsula fibrosa (CF). The micro-fibers harbour nephrogenic mesenchymal stem cells. (B) Immunohistochemistry shows laminin $\gamma 1$ in the basement membrane of the CD ampulla. (C) All cells within the ampulla are positive for the stem cell marker SSEA-1. Expression of SSEA-1 is also detected in the surrounding mesenchyme on individual cells (arrow). (D) The embryonic zone of neonatal rabbit kidney can be easily isolated by stripping off the organ capsule. For tissue culture an explant is placed in a specific tissue holder. (E) Illustration of a perfusion culture container containing a tissue holder with 14 mm outer diameter. The container can be used without (Fig. 2A) or with (Fig. 3A) an artificial interstitium. Arrows show medium inlet and outlet. (F) Special construction allows perfusion of medium at the top and base of the tissue constructs (not used in the present experiments).

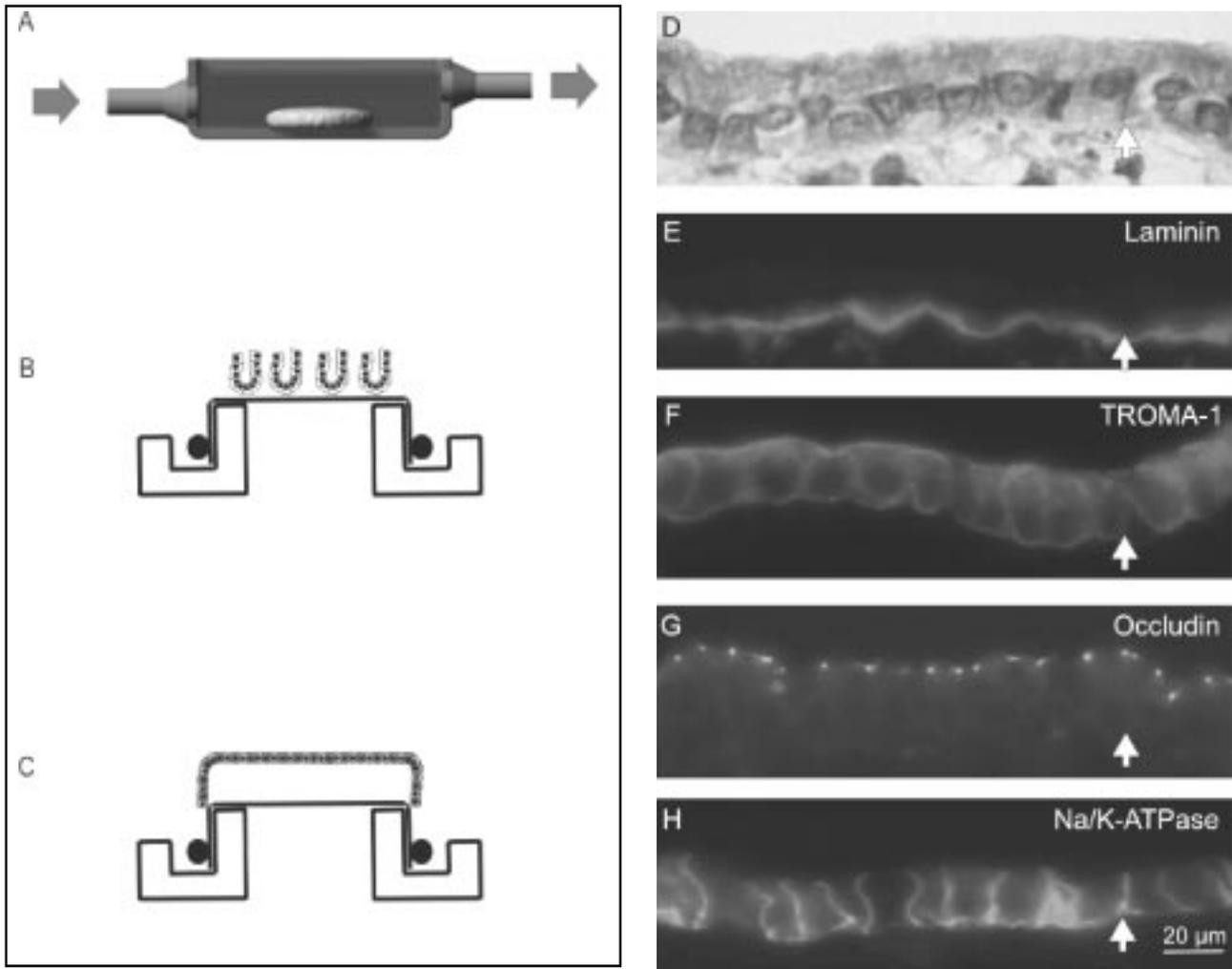


Fig. 2. Generation of a flat CD epithelium in perfusion culture. (A) Perfusion culture container without an artificial interstitium. Arrows indicate the exchange of medium. (B) The embryonic renal tissue is mounted on a holder to prevent curling and damage. (C) After 14 days of perfusion culture a monolayered epithelium outgrown of the CD ampullae covers the explant. Features: (D) Goldner-staining reveals a monolayered epithelium. (E) Immunohistochemistry shows that laminin $\gamma 1$ is found as a typical compound of the basement membrane. (F) Maintenance of typical intermediate-sized filaments as revealed by expression of TROMA-1. (G) Occludin as a typical tight junction protein indicates the primary appearance of polarisation within the epithelium. (H) Basolateral expression of Na/K-ATPase displays the primary upregulation of functional properties. (D-H) Arrows indicate basement membrane.

Immunohistochemistry

The specimens were fixed in ice-cold ethanol. After washing with PBS the sections were blocked with PBS containing 1% BSA and 10% horse serum for 30 minutes. Mab (monoclonal antibody) anti-Na/K-ATPase, mab anti-TROMA-1 and mab anti-SSEA-1 (Development Studies Hybridoma Bank; University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, under contract NO1-HD-7-3263 from the NICHD), mab anti-occludin (Zymed, San Francisco, USA) and mab anti-laminin $\gamma 1$ (L. Sorokin, Lund, Sweden) were applied as primary antibodies for 1h in blocking solution. The specimens were incubated for 45 minutes with donkey-anti-mouse-IgG-

fluorescein-isothiocyanate (FITC)- or biotin-conjugated secondary antibodies diluted 1:200 in PBS containing 1% BSA (Jackson ImmunoResearch Laboratories, West Grove, USA). In the case of the biotin-conjugated antibody the specimens were further processed with a Vectastain Elite ABC reagent followed by application of Vector NovaRED (Vector Laboratories, Burlingame, USA). The sections were then analyzed using an Axioskop 2 plus microscope or a laser scanning microscope Zeiss LSM 510 meta (Zeiss, Oberkochen, Germany). Images were made by a digital camera and thereafter processed with Photoshop 5.5 (Adobe Systems, San Jose, USA).

Results and Discussion

Site-specific peculiarities of renal stem cells can be recognized in the outer cortex of the developing kidney. Both ureter bud- and mesenchyme-derived stem cells are found in close vicinity of the capsule (Fig. 1A-C). Labeling of embryonic human, mice and rabbit kidney with Soybean Agglutinin (SBA) shows that both stem cell populations are connected by numerous micro-fibers, which originate from the basal aspect of each CD ampulla, line through the mesenchyme and terminate in the organ capsule (Fig. 1A) [20]. Immunohistochemistry with antibodies directed to interstitial proteins demonstrates that the micro-fibers are not identical with known proteins of the extracellular matrix [21]. In present experiments immunohistochemistry shows that a basement membrane containing laminin $\gamma 1$ separates each CD ampulla from the mesenchyme (Fig. 1B). All cells within the CD ampulla are positive for the stem cell marker SSEA-1 (Fig. 1C). In addition, a faint reaction with SSEA-1 is displayed by individual mesenchymal cells. Thus, epithelial and mesenchymal renal stem cells are localized in a unique histoarchitectural niche, where they show an unexpected close structural connection to each other during long phases of kidney development.

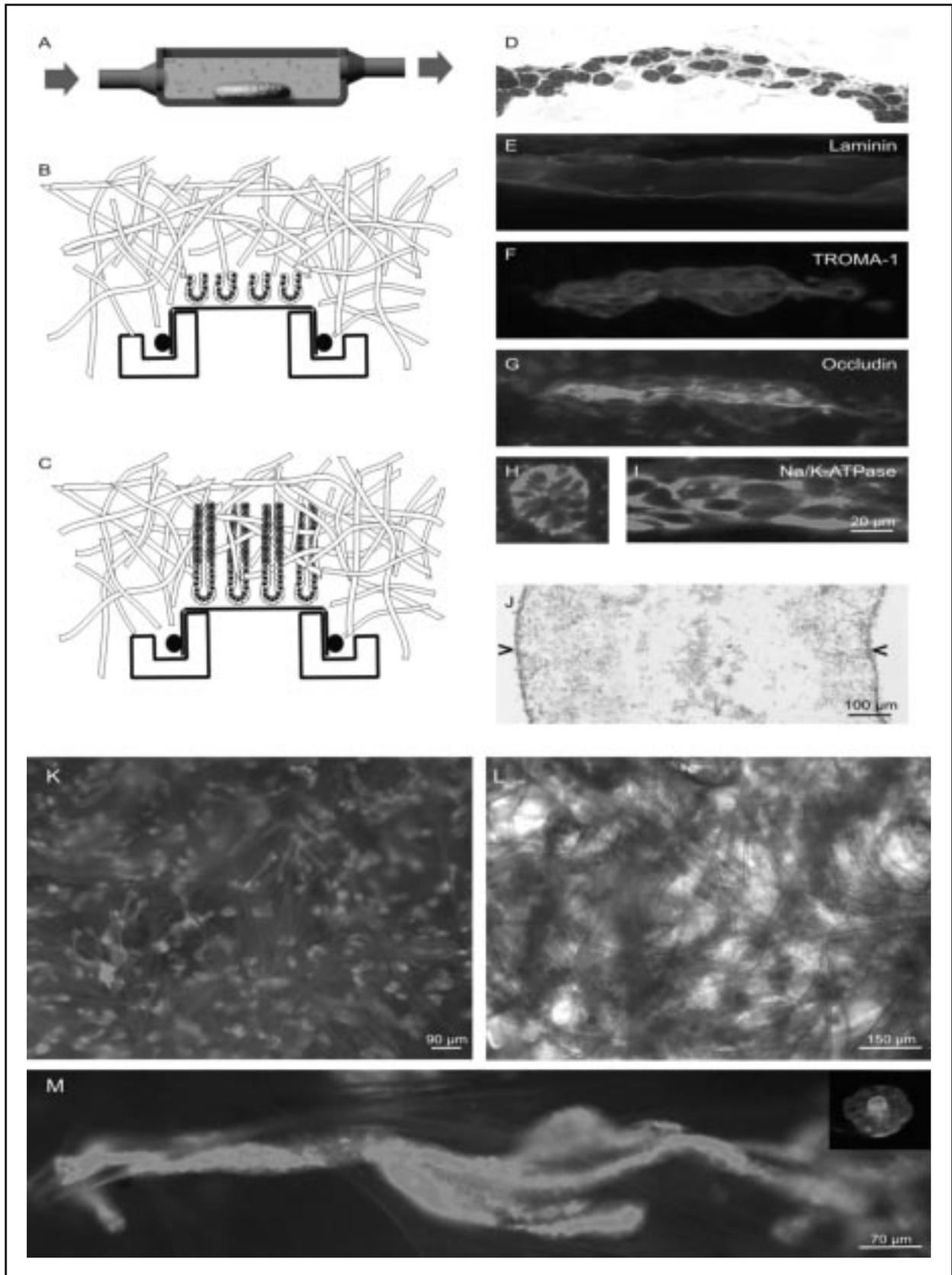
As compared to very small mouse or rat specimens the neonatal rabbit kidney provides an excellent cellbiological model, since the embryonic cortex containing numerous stem cell compartments is easily accessible for isolation. It can be harvested in sufficiently big amounts for tissue culture and cellbiological analysis simply by stripping off the capsula fibrosa with fine forceps [17]. Adherent to the organ capsule the isolated tissue layer contains numerous CD ampullae, S-shaped bodies and nephrogenic mesenchyme. After isolation the tissue is fixed in a specific holder to prevent curling and damage during further experimentation (Fig. 1D,2B,3B). Since the tissue is isolated without enzymatic digestion, numerous stem cell niches can be obtained in their original extracellular environment adherent to the capsula fibrosa.

Culture of the isolated tissue in stagnant environment with serum-containing IMDM for the first 24 hours stimulates the embryonic cells of the CD ampullae to grow out and to form a polarized epithelium on the kidney-specific support (Fig. 2C). Perfusion culture for further two weeks in serum-free IMDM containing aldosterone demonstrates the development of a flat epithelium with 6 mm in diameter containing isoprismatic cells (Fig. 2D-H). Since embryonic tissue is cultured, it is important to analyse the primary appearance of functional features.

Immunohistochemistry shows that the generated epithelium has established a basement membrane containing laminin $\gamma 1$ (Fig. 2E) as found before in the CD ampulla (Fig. 1B). The presence of Troma-1 (Fig. 2F) demonstrates tissue-specific intermediate-sized filaments. Primary appearance of occludin (Fig. 2G) and Na/K-ATPase (Fig. 2H) indicate the upregulation of CD-specific features found in adult tissue. Lack of immunolabel for mab Ki67 reveals in analogy to the adult kidney that the cells within the epithelium stop to divide after day 6 of perfusion culture and remain in the functional interphase (data not shown). As demonstrated in recent investigations the generation of a flat CD epithelium with 6 mm in diameter derived from renal CD ampulla cells appears as a reliable model to investigate basic cellbiological questions such as epithelial polarization, upregulation, modulation and maintenance of CD features [18, 22].

The development of a flat epithelium on a collagenous support is a cellbiological model system, that does not reflect the real situation found within the kidney, since no threedimensional tubular structures are developed (Fig. 2). Thus, for the generation of tubules we had to develop an advanced technique. In a perfusion culture container normally a small piece of growing tissue is surrounded by a big volume of medium, consequently a big dead fluid volume is present (Fig. 1E,2A) [19]. This fluid space can cause substantial hydraulic effects and passes pressure differences directly on the cultured tissue. Further the dead fluid volume is the preferred site for the accumulation of harmful gas bubbles. By reduction of the geometrical sizes of the culture container the dead fluid volume can be decreased but not fully optimised. The technical solution is to fill the culture container with an artificial interstitium consisting of highly porous biocompatible material that distributes and decreases fluid pressure more evenly across the construct by capillary effects (Fig. 3A-C). This material will provide mechanical protection to the tissue and optimises the dead fluid volume within the culture container. Consequently, for the generation of renal tubules we place fleeces made of polyester inside a perfusion culture container to mimik an artificial interstitium (Fig. 3A). The fleece is in direct contact with the growing tissue (Fig. 3B) and its surface is able to support threedimensional growth and differentiation of tubular structures during perfusion culture (Fig. 3C).

Toluidinblue-staining of the generated tissue shows that individual portions of tubuli are established (Fig. 3D). Immunohistochemistry demonstrates that at the basal aspect of the epithelium laminin $\gamma 1$ is expressed, which



◁ **Fig. 3.** Generation of renal tubules. (A) Perfusion culture container with an artificial interstitium shows that the dead fluid volume is filled with a polyester fleece. (B) The fleece surrounds in close vicinity the isolated embryonic tissue. (C) After 14 days of perfusion culture the generation of tubules at the interface of the fleece and the embryonic tissue is obtained. Properties of generated tubules: (D) Toluidinblue staining shows the development of a tubular portion. (E) Laminin $\gamma 1$ expression displays the establishment of a basement membrane. (F) Occurrence of typical intermediate-sized filaments is revealed by expression of TROMA-1. (G) Occludin as a typical tight junction protein indicates correct polarisation and formation of lumina. (H) Cross section, (I) longitudinal section of a tubular portion. Basolateral expression of Na/K-ATPase shows the primary appearance of functional properties in the generated tubules. (J) For control, replacement of the polyester fleece by a piece of capsula fibrosa (arrow head) shows that no tubule formation was obtained. (K) DBA-staining reveals wide distribution of multiple tubules. (L) As revealed by whole mount immunolabel many of the tubules exhibit Na/K-ATPase expression. (M) DBA-staining displays extensive length growth of tubular structures. Inlet shows the establishment of a lumen in a cross section of a tubule.

indicates the formation of a basement membrane (Fig. 3E). Immunolocalisation of TROMA-1 further shows the presence of an intermediate-sized filament typically detected in embryonic and adult renal CD (Fig. 3F). Luminal expression of occludin (Fig. 3G) and basolateral detection of Na/K-ATPase (Fig. 3H,I) shows the primary appearance of important functional proteins. It demonstrates the establishment of a luminal-basolateral polarisation within the generated tubules. All these data speak in favour that within the cultured tissue many tubules are developing, which resemble the adult CD. In contrast, an example for a negative influence of an artificial interstitium is given, when instead of a polyester fleece a capsula fibrosa explant of neonatal rabbit kidney is placed at the opposite side of the developing tubules. In this case the development of native structures is completely inhibited (Fig. 3J).

Experiments with a perfusion culture container containing an artificial interstitium demonstrate that a wide network of numerous renal tubules is formed. After 14 days of culture a surface view on a polyester fleece shows that numerous three-dimensionally randomly distributed tubules are present (Fig. 3K, L, M). The length of individual generated tubular portions ranges up to 800 μm (Fig. 3M), while CD ampullae show after isolation only 130 μm . Whole mount immunolabeling further demonstrates that not all but numerous of generated tubules express Na/K-ATPase to a high degree (Fig. 3L). Cross (Fig. 3H,M) and longitudinal sections of frozen specimens (Fig. 3E-G,I) further show that the generated tubules develop a lumen.

The presented data give first insights in fascinating perspectives using embryonic renal cells for the generation of tubules in the presence of an artificial interstitium during perfusion culture. Experiments to increase the amount, length and differentiation of generated tubules are under work. To date it is unknown, why proximal tubular portions

of the nephron do not seem to develop under the described conditions despite the presence of numerous S-shaped bodies included in the isolated tissue layer. Consequently, we elaborate further improvements of the culture medium and plan the application of suitable growth factors so that S-shaped body-derived tubules will develop.

Handling renal stem cells they learn us that they need a very special environment to develop. Mimicking an interstitium in a perfusion culture container shows at least the feasibility to generate tubular structures by an unknown cellbiological effect (Fig. 3). In contrast, as shown in earlier publications this was not possible in the stagnant environment of a culture dish and it could not be generated in the perfusion culture omitting an artificial interstitium (Fig. 2). It remains to investigate, if the specific arrangement of polyester fibers, the constant elimination of metabolic products or the stabilisation of a morphogenic gradient are responsible for this effect.

Renal stem cells may be applied in future for medical application by approved methods such as infusion, injection or implantation into the diseased organ. However, to enable transplanted cells/tissues to survive in the pathophysiological atmosphere of a diseased organ one has to learn about their needs in an avascular environment [23-26]. Still unknown are important parameters such as optimal extracellular matrix requirements, adequate nutrients and oxygen supply concerning the developmental potential of renal stem cells. To obtain this important information further advanced cultures and tissue engineering experiments are necessary.

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