www.jpnim.com Open Access eISSN: 2281-0692
Journal of Pediatric and Neonatal Individualized Medicine 2017;6(1):e060111
doi: 10.7363/060111
Received: 2016 Jul 05; accepted: 2016 Jul 15; published online: 2016 Dec 07

Invited review

The rabbit nephrogenic zone in culture: past, present and future as a model to investigate causes of impaired nephrogenesis

Will W. Minuth

Institute of Anatomy, University of Regensburg, Regensburg, Germany

Abstract

In preterm infants, intrauterine as well as extrauterine influences are held responsible for causing prematurity of renal parenchyma and impaired nephrogenesis, leading to a high incidence of severe kidney diseases later in life. Although involved noxae and resulting molecular effects are quite different, all of them converge to the nephrogenic zone which is restricted to the outer cortex of a developing kidney. Covered by the organ capsule, it consists of aligned ureteric bud-derived collecting duct (CD) ampullae containing epithelial stem cells, nephrogenic mesenchymal stem cells, renal vesicles and S-shaped bodies.

Owing to the complex composition of the nephrogenic zone and the different noxae, it is appropriate to investigate impaired nephrogenesis with an adequate *in vitro* system. In this case, isolation and culture of the nephrogenic zone from neonatal rabbit kidney is particularly well-suited. As compared to human specimens, it exhibits to a large extend a comparable microarchitecture. However, a decisive advantage is that it can be easily and quickly isolated in original composition with microsurgical techniques. Thus, pieces of the explant are available to a variety of advanced culture experiments. Formation of renal spheroids can be used for drug toxicity testing. Mounting in a tissue carrier makes it possible to register functional differentiation of the CD epithelium. Perfusion culture within an artificial interstitium enables investigation of spatial development of tubules. The present article has been written to inform about past and present results, recognized risks and future challenges.

Keywords

Preterm infants, impaired nephrogenesis, neonatal rabbit kidney, nephrogenic zone, isolation, tissue culture model.

Corresponding author

Will W. Minuth, Institute of Anatomy, University of Regensburg, University Street 31, D-93053 Regensburg, Germany; e-mail: will.minuth@vkl.uni-regensburg.de.

How to cite

Minuth WW. The rabbit nephrogenic zone in culture: past, present and future as a model to investigate causes of impaired nephrogenesis. J Pediatr Neonat Individual Med. 2017;6(1):e060111. doi: 10.7363/060111.

Introduction

Although the majority of preterm infants survive infancy, it is becoming increasingly clear that they exhibit an elevated risk for the development of hypertension and renal impairment later in life [1]. There are several reasons for this: when infants are born preterm, nephrogenesis is still active so that confrontation with intrauterine and/or extrauterine growth restrictions, exposure to certain medication or hypoxia/hyperoxia hamper development, finally resulting in a decreased nephron endowment and a lowered renal function [2-4]. From a pathological view this process is recognized by an altered width of the nephrogenic zone and a greater percentage of morphologically abnormal glomeruli with dilated Bowman's space and shrunken tuft as compared to controls [5].

A sustainable solution of the problem could be to stimulate the period of nephrogenesis therapeutically and thereby prolong postnatal kidney development in preterm infants for 4 to 6 weeks [6]. Consequently, such a therapeutic approach focuses on the nephrogenic zone, which is restricted to the outer cortex in the kidney of a preterm infant. Optical microscopy demonstrates that the entire machinery leading to nephron formation is concentrated in this area [7, 8].

To understand the special topology of the nephrogenic zone, a short look at the development of the kidney is given. The organ anlage in a human kidney starts around the 6th week of gestation by molecular interactions between the invading ureteric bud containing epithelial stem cells and surrounding nephrogenic mesenchymal stem cells [9, 10]. Driven by a spatiotemporal program including branching morphogenesis [11], the parenchyma develops up to birth (36th week of gestation) in a strict radial extension [12]. As a result, first the medulla, then the inner, mid and finally the outer cortex are formed [13]. Corresponding to that, a developmental gradient exists with matured nephrons in the inner and mid cortex, maturing nephrons in the outer cortex and developing nephrons in the nephrogenic zone [14]. Covered by the organ capsule, all of the renal stem cell niches are aligned within the nephrogenic zone. Each of them consists of a ureteric bud-derived collecting duct (CD) ampulla containing epithelial stem cells in its tip. They are facing stem cells of the metanephric mesenchyme. At the lateral aspect of a CD ampulla, renal vesicles, the S-shaped bodies as first visible signs of active nephrogenesis are recognized (**Fig. 1**).

Regarding the cell biological level, it is unknown whether impairment of nephrogenesis is caused by a misleading of morphogenic signals, by metabolites interfering with the process of nephron induction,

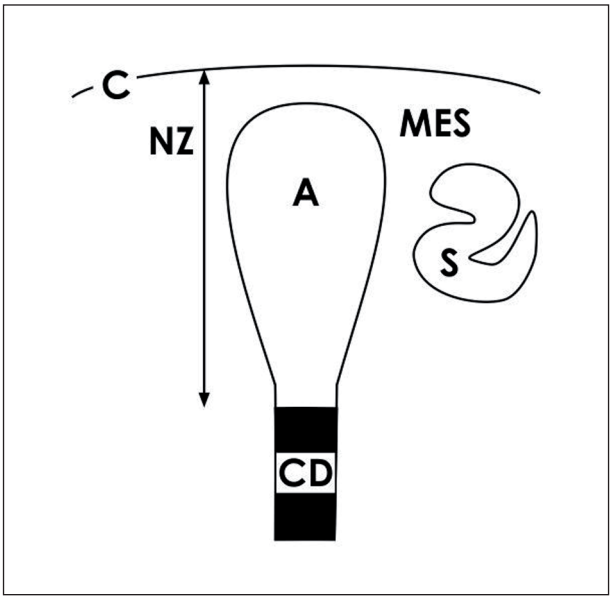

Figure 1. Schematic illustration depicts spatial expansion of the nephrogenic zone (NZ) in the developing mammalian kidney. Its outer side is covered by the organ capsule (C), while the inner side is limited by maturing collecting duct (CD) tubules at the neck of a CD ampulla (A). The stem cell niches are aligned within the nephrogenic zone. Each of them consists of a ureteric bud-derived CD ampulla containing epithelial stem cells at the tip, while competent mesenchymal stem cells (MES) are concentrated between the tip of each ampulla and the capsule. At the lateral side of a CD ampulla, renal vesicles, the S-shaped bodies (S) are recognized thus indicating active formation of nephrons.

by a loss of competence in mesenchymal stem cells or by a disturbed communication between mesenchymal and epithelial stem cells. As a consequence, a successful therapeutic concept that compensates for impaired nephrogenesis in preterm infants must consider the unique position of the nephrogenic zone, the special microarchitecture of contained niches and the specific morphogenetic signalling in this area. In this coherence it is entirely unknown whether original morphogens or less potent analogues can be administered to prolong nephrogenesis therapeutically. All of these basic issues need to be addressed experimentally, before development of a safe therapy can start.

It is plausible that basic research cannot be performed on the kidneys of preterm infants. Instead, this complex project requires an adequate *in vitro* system that resembles natural conditions and provides precise data about causes leading to impaired nephrogenesis on a cell biological level. In this case, it is logical to isolate the nephrogenic zone of neonatal rabbit kidney in original composition and use it for sophisticated culture experiments.

Isolation of the nephrogenic zone

The nephrogenic zone of neonatal rabbit kidney can be isolated using a simple microsurgical technique [15-17]. In a schematic illustration it is seen that the nephrogenic zone is restricted to the outer cortex and that it is covered by the organ capsule (**Fig. 2a**). Only in this area occur CD ampullae containing epithelial stem cells, mesenchymal stem cells, renal vesicles and S-shaped bodies (**Fig. 1**). Although it has been tried several times, no animal species other than neonatal rabbit admits isolation of the nephrogenic zone in its original composition by stripping it off with fine forceps.

For isolation, one-day old New Zealand rabbits are narcotised and then sacrificed. After opening the abdomen, a kidney is held on its hilum for isolation with a fine forceps. Owing to the special position of the nephrogenic zone, touching of the capsule is avoided. After isolation, a kidney is transferred to sterile balanced saline solution to remove adipose tissue cautiously with fine forceps. In a next step the kidney is divided along the poles into an anterior and posterior half and in the middle between the poles with a small scissor. For isolation of the nephrogenic zone, pieces of the kidney are transferred to a dish filled with balanced saline solution.

Under a surgical microscope it can be seen, after a while, that the edges of the organ capsule are

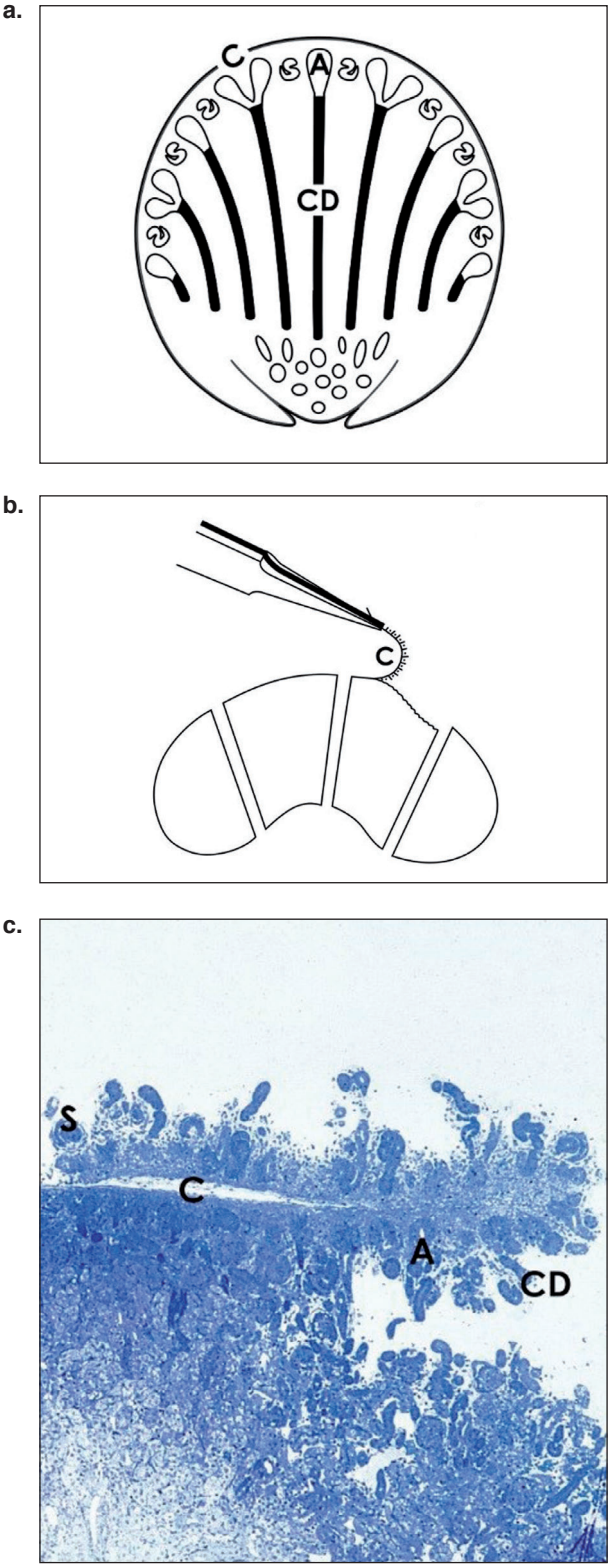

Figure 2. Illustration informs about the position of the nephrogenic zone within the neonatal rabbit kidney and its isolation. **a.** Just like in humans, the rabbit nephrogenic zone is restricted to the outer cortex and bordered by the organ capsule (C). On the inner side maturing collecting duct (CD) tubules occur. In-between CD ampullae (A), renal vesicles and S-shaped bodies (S) are present. **b.** With the use of a fine forceps the nephrogenic zone can be stripped off in its original composition. **c.** Optical microscopy shows a nephrogenic zone that is partly isolated.

curling up. Now the capsule is further stripped off carefully with fine forceps (**Fig. 2b**). A semithin section under optical microscopy illustrates that the inner side of the stripped off capsule is not blank. In a specific pattern, demolished endings of CD tubules, ampullae, renal vesicles and S-shaped bodies adhere to the capsule (**Fig. 2c**). By applying this simple isolation method, multiple pieces of the nephrogenic zone can be harvested for subsequent culture experiments and cell biological analysis within a few minutes. Finally, with an iridectomy scissor the explant is cut into pieces of desired size.

Each piece of an explant represents the nephrogenic zone adherent to the inner side of the organ capsule in original composition (**Fig. 3**). Analysis by scanning electron microscopy depicts that stumps of developing CD tubules are connected with the neck of an ampulla. Renal stem cell niches are represented by epithelial stem cells contained in the tip of a CD ampulla (in this perspective not visible) and neighbouring mesenchymal stem cells. Between CD ampullae occur renal vesicles and S-shaped bodies that are enclosed by a basal lamina. Current literature informs that this well-defined microarchitecture is maintained by microfibers of extracellular matrix spanning between each of the renal stem cell niches and the inner side of the organ capsule [18].

After isolation, pieces of the rabbit nephrogenic zone are available for a variety of culture experiments. In the past, formation of renal spheroids and functional development of the CD epithelium were analysed, while in recent years spatial generation of tubules has been investigated.

Formation of renal spheroids

For beginning experiments, pieces of the isolated nephrogenic zone were transferred to a conventional culture medium, such as Dulbecco's Modified Eagle Medium (DMEM), containing 10% foetal bovine serum (**Fig. 4a**). Unexpectedly, curling up of each explant is observed (**Fig. 4b**). This reaction is obviously caused by constriction of smooth muscle cells occurring within the organ capsule [18]. Histology further exhibits that, within 24 hours,

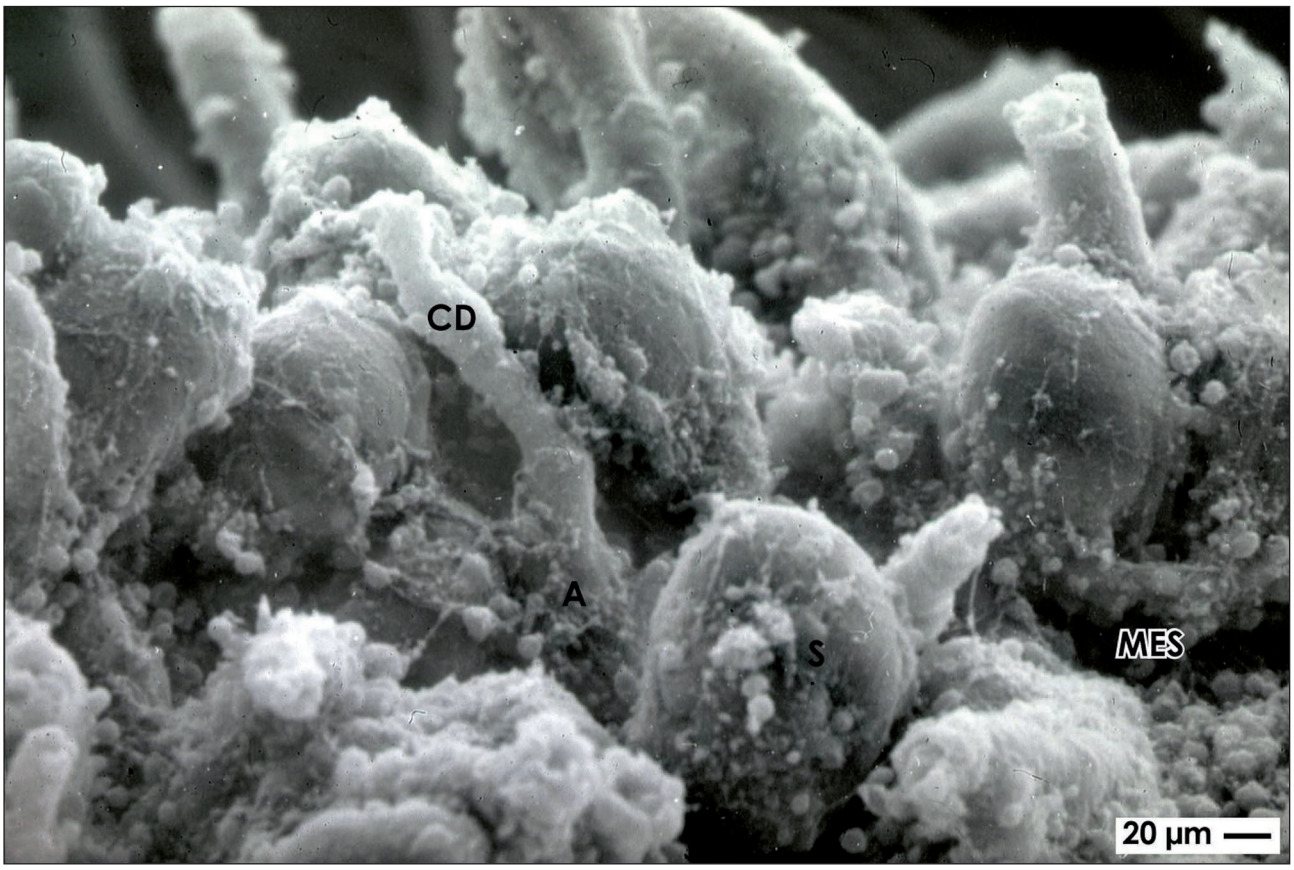

Figure 3. View to the interior side of isolated nephrogenic zone by scanning electron microscopy (SEM). Demolished collecting duct tubules (CD) protrude like spines of a hedgehog. Each of them is connected with a CD ampulla (A). In-between renal vesicles, S-shaped bodies (S) and mesenchymal stem cells (MES) are recognized. In this perspective the capsule is at the bottom and therefore not visible.

formerly called "globular bodies" now termed renal spheroids are forming. They are completely covered by a monolayer of epithelial cells (**Fig. 4c**).

Established epithelial cells on the surface of a spheroid develop polar differentiation. Their luminal side faces the culture medium, while its basal side rests on a basal lamina, which is in contact with the capsule support. Microscopic analysis informs that the established epithelium resembles the renal CD tubule. It can be recognized that the outgrowth of cells during epithelium formation occurs at the neck of each CD ampulla (**Fig. 4b**). The outgrowth depends on applied serum concentration and is arrested by various inhibitors of protein and glycoprotein synthesis such as cycloheximide, actinomycin C1, tunicamycin, 6-diazo-5-oxo-norleucine, vinblastine, colchicine or cytochalasin B. Development of the epithelium is paralleled by a specific glycoprotein synthesis [19, 20]. Epithelium development is not inhibited by inhibitors of DNA synthesis such as cytosine arabinoside, mitomycine and hydroxyurea [21]. Exposure of spheroids to culture medium including basement membrane synthesis inhibitors such as hydroxyl-D-proline, L-azetidine-2-carboxylic acid, and o- and p-nitrophenyl-xylopyranoside eliminates fibroblasts mesenchymal stem cells beyond the CD epithelium [22].

Further on, a special glycoprotein (renal gpCD I) was isolated from the CD epithelium covering spheroids for use in producing an antiserum [23]. Immunohistochemistry reveals label on the CD epithelium of cultured spheroids, on medullary as well as cortical CD tubules but not on the CD ampullae in the nephrogenic zone of neonatal rabbit kidney. With this strategy it was possible to analyse for the first time the primary expression of a glycoprotein specifically involved in CD tubule differentiation.

As long renal spheroids are small in size, they are an excellent experimental tool in culture for screening of drug toxicity. When the diameter of a spheroid increases, cells on their surface are further provided with culture medium. However, in its interior, a lack of nutrition and oxygen propagates leading to uncontrolled destruction of tissue. For that reason spheroids were regarded as being less suitable for ongoing analysis of renal CD epithelium development.

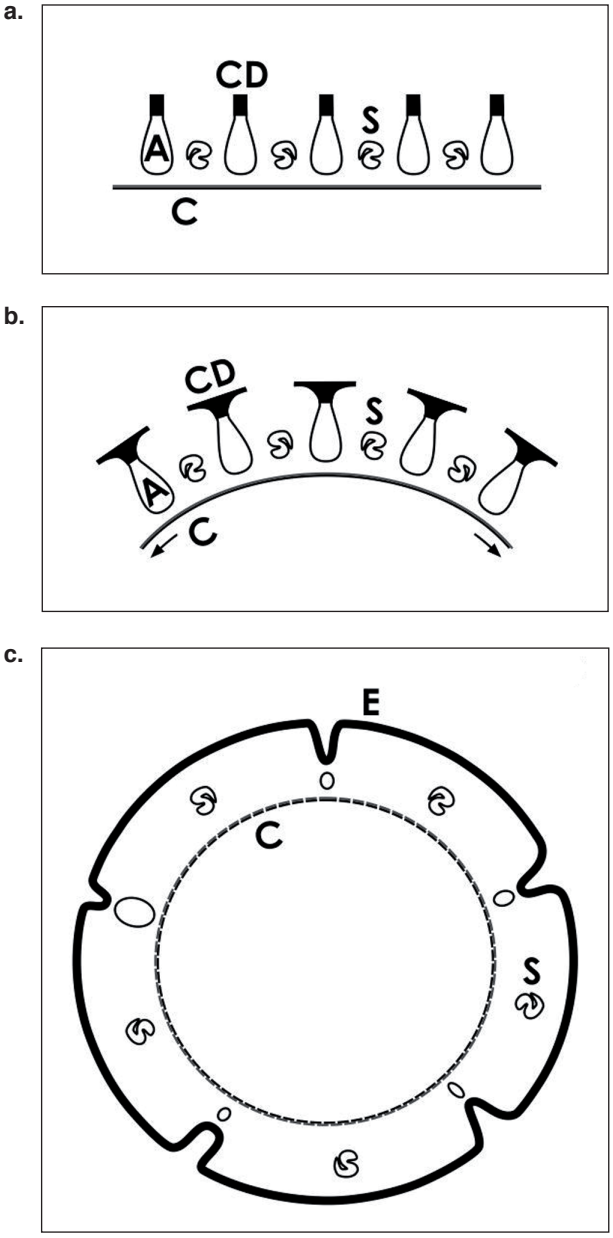

Figure 4. Schematic illustration informs about development of the isolated nephrogenic zone into a spheroid. **a.** For culture a piece of the isolated nephrogenic zone consisting of the capsule (C), adherent ampullae (A), demolished CD tubules (CD) and S-shaped bodies (S) is placed in medium containing fetal bovine serum. **b.** During the first 24 hours a curling (arrows) of the explant is observed. In parallel, cells at the ampulla neck grow out to cover the outer surface of the explant. **c.** During ongoing culture a single layer of a polarized CD epithelium (E) is established on the surface.

Mounting the nephrogenic zone in a tissue carrier

The aim in further experiments was to analyse differentiation of epithelial stem/progenitor cells into a functional CD epithelium. To prevent the earlier mentioned curling (**Fig. 4b**), the nephrogenic zone was mounted in a tissue carrier so that the CD epithelium could develop on the entire surface of the explant in a planar fashion (**Fig. 5**). Mounting of the isolated nephrogenic zone was started under a surgical microscope by placing

the explant on the base part of a tissue carrier that exhibits an inner cylindrical opening of 6 mm (**Fig. 5a**). Then the nephrogenic zone was fixed by a tension ring like the skin of a drum. Henceforth, the tissue carrier guaranteed mechanical stability and prevented damage of the mounted nephrogenic zone.

Generation of a CD epithelium to register differentiation

At the start of experiments little information was available about growth factors and electrolyte environment that stimulate stem/progenitor cells to develop into a differentiated CD epithelium. To learn about this, a piece of the isolated nephrogenic zone mounted in a tissue carrier (**Fig. 5a**) was then exposed to culture medium containing 10% foetal bovine serum. This treatment caused ampulla cells to grow out at the neck of each CD (**Fig. 5b**). Within 24 hours a monolayer of epithelial cells on the entire surface of the explant was established (**Fig. 5c**). During ongoing culture, differentiation into a polarized CD epithelium took place (**Fig. 6**). Its glossy luminal surface faced the culture medium, while its basal side rested on the fibrous capsule.

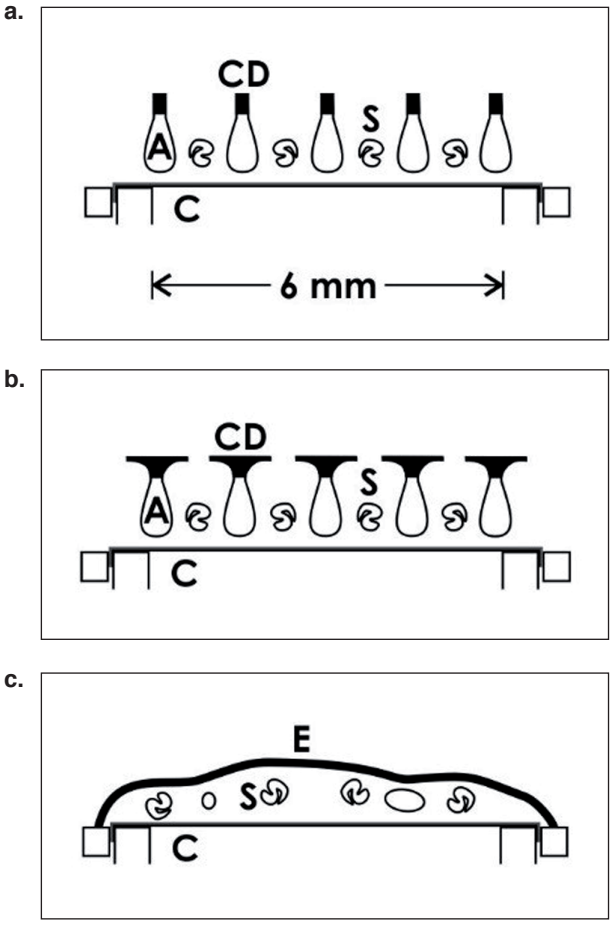

Figure 5. Schematic illustration explains mounting of the nephrogenic zone in a tissue carrier and development of a flat CD epithelium on the surface. **a.** For culture, a piece of the isolated nephrogenic zone consisting of the capsule (C), demolished CD tubules, ampullae (A) and S-shaped bodies (S) is mounted on a tissue carrier and then placed in medium containing fetal bovine serum. **b.** During culture, cells at the ampulla neck grow out to cover the surface of the explant. **c.** During ongoing culture, a single layer of a polarized CD epithelium (E) is established. Its luminal side faces the culture medium, while its basal side rests on the capsule support.

Recording of physiological parameters

A basic question was whether the epithelium established on the surface of the explant exhibited typical functional features (**Figures 5c** and **6**). To perform physiological recordings, a tissue carrier including the mounted CD epithelium was transferred to a double-chamber with integrated electrodes [24]. Control epithelia generated in culture medium without additives revealed leaky physiological features. However, when epithelia were pre-incubated in medium containing aldosterone (1×10^{-6} M), a transepithelial resistance of 0.83 ± 0.2 kΩcm^2 and a transepithelial potential difference of -59.5 ± 4.4 mV was registered, thus indicating tight physiological characteristics.

Addition of the Na^+ channel blocker amiloride (1×10^{-6} M) to the luminal perfusion fluid largely abolished the transepithelial potential difference but increased the resistance. This result showed that a polarized principal cell CD epithelium with an active sodium transport was developing. Using the patch clamp technique, the presence of a high-conductance K^+ channel in apical membranes of the cultured CD epithelium was demonstrated [25]. This channel is highly selective for K^+, has a conductance of 180 and can be completely blocked by Ba^{2+} and partially by Na^+. Finally, luminal application of the chloride channel blocker 5-nitro-2-(3-phenylprophlaamino)-benzoic acid (NPPAB, 1×10^{-7} M) produced a significant hyperpolarization indicating the presence of a Cl^- conductance in the basolateral plasma membranes [26]. Thus, recording of physiological parameters revealed that stem/progenitor cells derived from the CD ampulla neck develop under *in vitro* conditions into a CD epithelium with functional characteristics as recognized in the adult rabbit kidney.

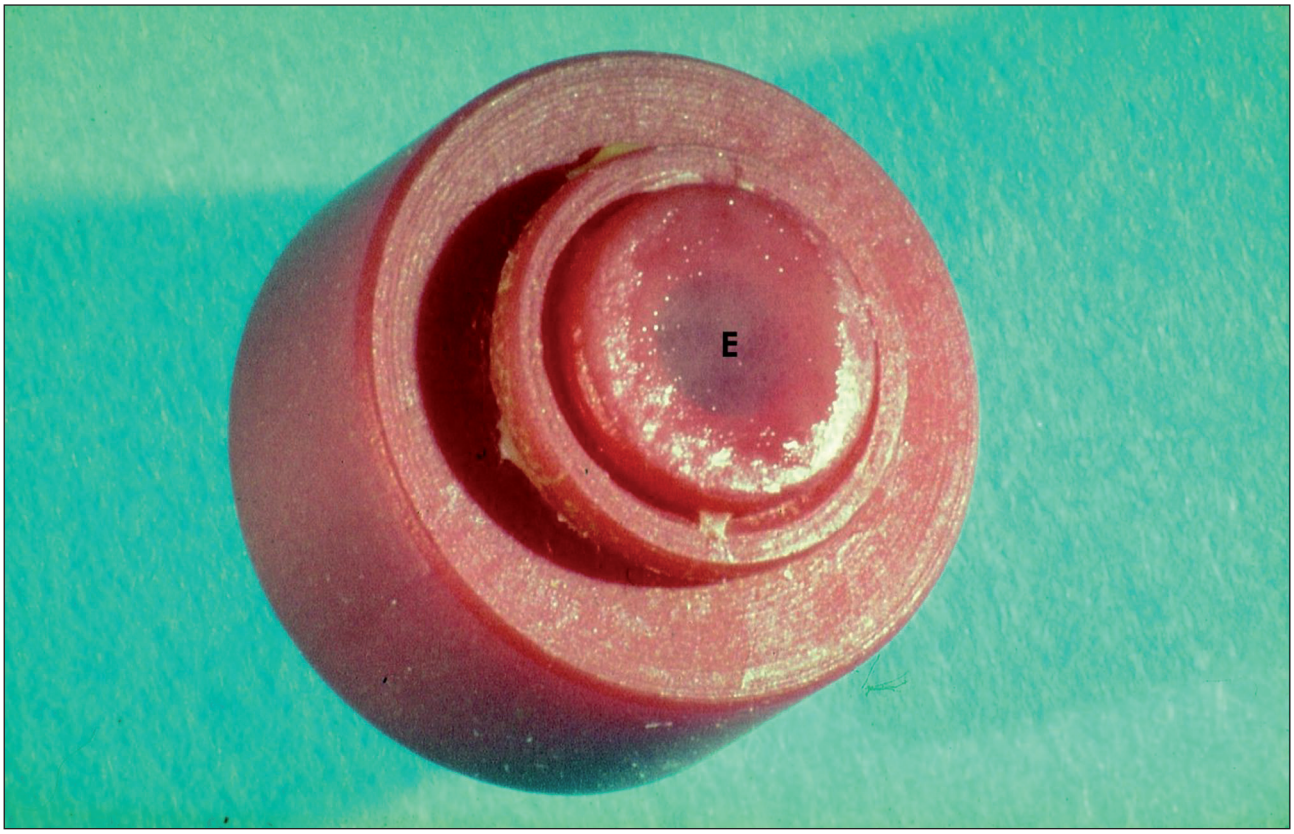

Figure 6. Photographic illustration demonstrates a tissue carrier (6 mm inner diameter) with a mounted CD epithelium (E). Its luminal (glossy) side faces the culture medium, while its basal side rests on the capsule support.

Registration of cell biological features

The following series of experiments was performed to register the cell biological profile of the cultured CD epithelia and to compare it with CD ampullae in the nephrogenic zone, maturing and matured CD tubules of the neonatal kidney. The electron microscope showed that cells of generated CD epithelia exhibited explicit features of polar differentiation, such as a cilium and microvilli at the luminal cell poles, formation of a tight junction complex between the lateral and apical plasma membranes and development of a basal lamina exhibiting a lamina rara, lamina densa and lamina fibroreticularis. Immunohistochemistry showed the expression of epithelium-specific cytokeratins [27]. Administration of aldosterone during culture of the CD epithelium led to an upregulation of the α-subunit of Na/K-ATPase in parallel to a significant magnification of the basolateral plasma membrane in the form of foldings. This phenomenon reflects a functional hypertrophy as observed in the adult kidney [28].

To detect more differences between renal epithelial stem/progenitor cells and a differentiated CD epithelium, monoclonal antibodies (mab) were raised owing to the lack of commercially available markers at that time. Immunohistochemistry revealed that generated mab renal CD1 was unreactive in control epithelia, whereas mabs renal CD2 and renal CD3 showed an intense label on cultured CD epithelia after chronic administration of aldosterone [29, 30]. It was further observed that exposure of cultured CD epithelia to a luminal-basal fluid gradient for 10 minutes altered cell geometry, evoked internalization of the luminal plasma membrane and provoked transcytosis [31]. Moreover, administration of aldosterone for a few hours led to a change in the methylation pattern of cytosolic proteins [32]. Although an increase in protein synthesis was observed, surprisingly no typical ‘aldosterone induced proteins’ were detected [33]. Instead, within 3 hours of aldosterone application a distinct increase on citrate synthase activity was registered [34]. During the course of experiments it became more and more obvious that aldosterone had an important influence not only on Na^+ transport in the matured CD tubule system but also a stimulating effect on its functional maturation [35].

Modulation of environment by perfusion culture

Experiences show that static culture in a dish does not meet the physiological requirements of a CD epithelium during the phase of functional differentiation. To offer cells an optimal adhesion on a support and a constant provision with always fresh culture medium, the construction of the MINUSHEET® system was started [36, 37]. This technique is based on individually selected biomaterials that are mounted in a tissue carrier. For cell seeding a tissue carrier is first placed in a 24 well plate for static culture. Then, for the phase of differentiation, it is transferred to a series of perfusion culture containers (Minucells and Minutissue, Bad Abbach, Germany) [38].

In principle, during perfusion culture CD epithelia are exposed to a continuous flow of always fresh medium so that the metabolized medium is not re-circulated but collected in a separate waste bottle. The transport of culture medium is best accomplished by a peristaltic pump (ISMATEC, IPC N8, Wertheim, Germany). Optimal results are obtained when medium is transported with 1.25 ml/h for a period of at least 10 days. Experiments are performed under atmospheric air with a pH (7.2-7.4) stabilized culture medium. For maintaining a defined temperature of 37°C within a perfusion culture container, a heating plate (MEDAX-Nagel, Kiel, Germany) is used.

Exposure of developing epithelia to always fresh medium

To investigate development of epithelial stem/progenitor cells into principal and intercalated cell types of a CD epithelium, perfusion culture was applied. Owing to the lack of suitable markers further monoclonal antibodies had to be raised. With this experimental combination it became possible to analyze expression of the renal CD 7 antigen in cultured specimens and in the developing kidney [39, 40]. For example, CD ampullae in the nephrogenic zone did not reveal label for renal CD 7, while generated CD epithelia and matured CD tubules in the rabbit kidney exhibited intense label on all of the cells. Application of Peanut Agglutinin (PNA) as a marker for intercalated cells in the matured CD tubule revealed that only 8% of cells were positive when CD epithelia were cultured without hormone administration. In contrast, CD epithelia treated with aldosterone and arginine vasopressin produced 72% positive cells. Application of aldosterone in combination with insulin increased the number of PNA-positive cells up to 90%. Using this approach it was further demonstrated that differentiation into principal and intercalated cells proceeded through a transitional precursor cell type at the neck of a CD ampulla [41].

Confrontation of developing CD epithelia with a fluid gradient

Due to leaky physiological barrier characteristics, a renal CD epithelium was exposed at the beginning of its functional development to the same fluids on the luminal and basal sides. However, when polar differentiation proceeded, a tight junction complex sealed the intercellular space so that a functional barrier could arise. In parallel transport, features were upregulated so that the CD epithelium was still exposed to different fluids on its luminal and basal sides.

To analyze the influence of electrolytes on maturation of the CD epithelium, the nephrogenic zone was isolated, mounted in a tissue carrier and incubated for the initial 24 hours in a culture medium containing serum so that a planar CD epithelium could form on the entire surface of the explant (**Fig. 5**). A tissue carrier with an established epithelium was then transferred to a gradient culture container that enabled transport of fluid with different composition to the luminal and basal sides of the CD epithelium [42]. At the end, CD epithelia were harvested and investigated with immunohistochemistry. When CD epithelia were exposed on both sides to standard Iscove's modified Dulbecco's Medium (IMDM) containing 112 mmol/l Na^+ and 85 mmol/l Cl^-, less than 10% immunopositive cells were registered in the CD epithelium by means of an epithelium-specific monoclonal antibody 703. In contrast, when the medium at the basal side of the epithelium was adapted to serum of neonatal rabbits (137 mmol/l Na^+ and 99 mmol/l Cl^-), more than 80% positive cells were detected [43]. During the run of the experiments it was further shown that immunoreactivity started to develop after an unexpected long lag period of 4 days, then increased during the next 5 days to reach a maximum at day 14 [44]. After switching back from gradient to isotonic culture conditions on both sides, immunoreaction for facultative proteins on the CD epithelium decreased within 5 days, while constitutively expressed proteins remained stable [45].

Cell differentiation by fluid environment

Further experiments illustrated that for the maintenance of a CD epithelia various culture media, such as IMDM, Medium 199, Basal Medium Eagle, Williams' Medium E, McCoys 5A Medium and DMEM without administration of serum, can be used principally [46]. In those series constitutively expressed proteins such as cytokeratin 19, renal CD9, Na/K ATPase and laminin were present in all cells of the generated CD epithelia. In contrast, a group of facultative proteins detected by monoclonal antibodies 703, 503 and PNA, respectively, was found only in individual series, so that each culture medium produced CD epithelia with the same morphological characteristics, but each with an individual immunohistochemical pattern. Finally, it was demonstrated that astonishingly low doses of 3-6 mmol/l NaCl added to the luminal perfusion culture medium were able to evoke changes in the profile of differentiation [47]. Equally remarkable was that an embryonic CD epithelium was able to tolerate a NaCl load linearly between 3 to 24 mmol/l and up to 100 mmol/l, however, by adapting individual cell features including expression of cyclooxygenase [48, 49].

A crucial point during development of the CD tubule epithelium in the kidney was the first contact with urea. To obtain concrete information, generated CD epithelia were exposed to urea during perfusion culture. Interestingly, addition of urea did not damage the cells and did not influence expression of principal cell specific proteins such as Troma 1, cytokeratine 19, occludine, aquaporin 2 or 3. However, administration of urea influenced binding of PNA known as a marker for β-type intercalated cells within the adult cortical CD tubule of the rabbit kidney. Control epithelia exhibited 90% of PNA binding cells, while urea treated specimens showed only 10% of positive cells [50]. It remains to be explored whether functional features, such as acid-base transport, were developed.

Further on, maturation of the CD tubule occured at the CD ampulla neck in the nephrogenic zone. In this area also endothelial cells form cords, but a perfused capillary system including erythrocytes was not observed at that time. Of special interest was to find out whether the oxygen concentration had a special influence on differentiation. Surprisingly, generated CD epithelia survived and maintained good morphology for 14 days under all tested oxygen conditions [51]. However, while expression of cytokeratin 19 remained unchanged, Na/K ATPase was downregulated under low oxygen, whereas COX-2 expression increased drastically.

In sum, the illustrated data exhibit that, besides aldosterone, electrolytes and the local oxygen concentration had a significant influence on the functional maturation of a CD epithelium. Finally, the data presented could be increased, since the gradient perfusion culture technique was stepwise improved technically [52-55].

Development of microvasculature

CD ampullae, renal vesicles, S-shaped bodies and mesenchymal stem cells that adhered to the capsule in original composition were contained in the isolated nephrogenic zone (**Fig. 3**). At the start of the experiments it was not known whether also endothelial cells that are able to survive in culture in the explants occur. Consequently, distribution of endothelial cells and development of microvasculature were investigated. Owing to the lack of suitable markers reacting on rabbit kidney, monoclonal antibodies EnPo1 and EC1 were raised [56, 57]. Immunohistochemistry with these antibodies delivered new information about specific development of renal capillaries. For example, when the nephrogenic zone was kept in culture under permanent transport of serum-free culture medium, only few and tiny strands of endothelial cells were recorded [58]. However, with administration of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), aldosterone and 1,25-hydroxyvitamin D3, development of a dense microvascular network took place [59]. Histochemistry with mab EnPo1 further illustrated that S-shaped bodies developed during perfusion culture into podocytes and parietal cells of Bowman's capsule [60]. Surprisingly, with short term administration, bFGF, 9-cis retinoic acid and VEGF for only 3 hours and subsequent perfusion culture for 13 days were found to be potent morphogenic pulses for development of an intense microvasculature network within the isolated nephrogenic zone [61].

Generation of renal tubules within an artificial interstitium

In previous experimental set-ups an embryonic CD epithelium was cultured as a planar (two-dimensional; 2D) layer on a renal capsule support to perform physiological recordings or to analyze

influences of the fluid environment on functional differentiation (**Figures 5** and **6**). In contrast, in the following series of experiments pieces of the isolated nephrogenic zone were cultured to investigate spatial formation of tubules (three-dimensional; 3D) (**Fig. 7**). To avoid contact with xenogenic molecules and uncertain growth factors during generation of tubules, addition of serum or serum substitutes to the culture medium was not performed. Although frequently used, coating with extracellular matrix proteins was not done. Instead, chemically defined culture media were applied including an antibiotic-antimycotic solution (Gibco, Invitrogen, USA).

Creation of an artificial interstitium

Generation of renal tubules requires guidance by a scaffold. Thus, pieces of the isolated nephrogenic zone were mounted between layers of a I-7 polyester fleece (Walraf, Grevenbroich, Germany) and transferred to a MINUSHEET® tissue carrier [62] (**Fig. 7a**). The applied fleece created an artificial interstitium that performed essential tasks. First of all, it is an excellent substitute for microfibers naturally occurring in the extracellular matrix of the interstitial space within the kidney. Furthermore, the fibers of the fleece serve as a scaffold that provides excellent mechanical stability during perfusion culture (**Fig. 7b**). In addition, the space between the polyester fibers is optimal for even distribution of culture medium so that a constant provision with nutrition and respiratory gas takes place. Applying this experimental set-up, it became possible for the first time to register development of renal tubules over weeks [63] (**Fig. 7c**).

Tubulogenic action of aldosterone

Unexpectedly, the starting series of experiments with the isolated nephrogenic zone in an artificial interstitium was disappointing, since administration of a conventional culture medium, such as IMDM, for 14 days led to complete disintegration of tissue [64]. Moreover, addition of stimulators known from literature for embryonic renal parenchyma development, such as epidermal growth factor (EGF), a cocktail of insulin, transferrin and selenium (ITS), retinoic acid (RA), cholecalciferol (VitD3) or bovine pituitary extract (BPT) did not stimulate formation of tubules.

However, it was detected that a continuous administration of aldosterone was able to induce

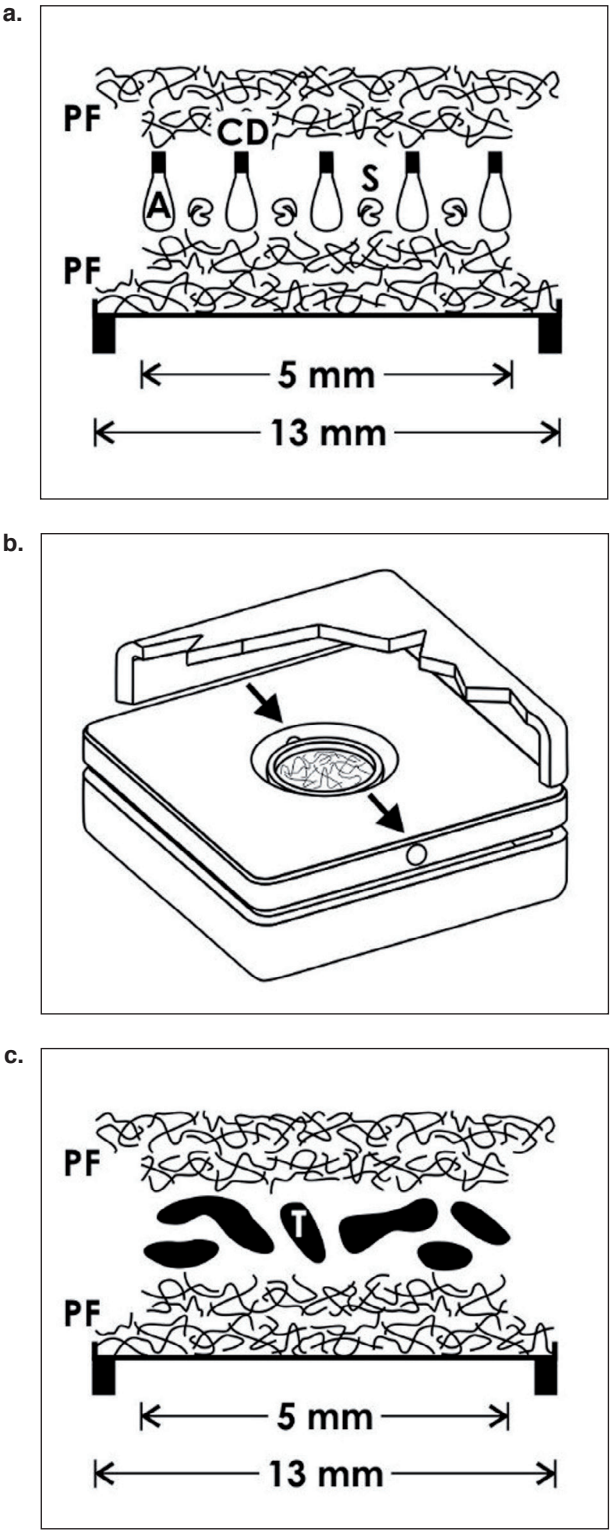

Figure 7. Schematic illustration informs about culture of the isolated nephrogenic zone within an artificial interstitium to generate tubules. **a.** At the start, a piece of the isolated nephrogenic zone consisting of the capsule, demolished CD tubules, ampullae (A) and S-shaped bodies (S) is placed between layers of polyester fleeces (PF) and then mounted in a MINUSHEET® tissue carrier. **b.** Perfusion culture is performed with always fresh and chemically defined medium (arrows) in a container with horizontal flow characteristics. **c.** After 14 days, generated tubules (T) can be analyzed by cell biological methods.

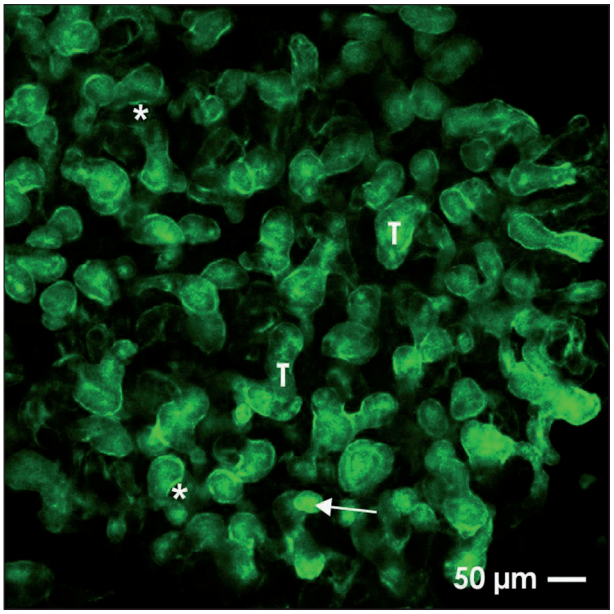

Figure 8. Perfusion culture of the nephrogenic zone within an I-7 polyester fleece after 13 days in IMDM containing aldosterone. Confocal microscopy and label by fluorescent Soybean Agglutinin (SBA) shows that numerous tubules (T) exhibiting a lumen (arrow) and a basal lamina (asterisk) have developed.

tubulogenesis (**Fig. 8**). The tubulogenic action of aldosterone depends on its applied concentration. Presence of 1 x 10^{-6} M or 1 x 10^{-7} M aldosterone in the culture medium for 13 days produced numerous tubules, while application of 1 x 10^{-8} M to 1 x 10^{-10} M led to a continuous decrease and finally to a loss of tubule formation [65]. Surprisingly, the tubulogenic action depended exclusively on aldosterone and not on its molecular precursors such as cholesterole, pregnenolone, progesterone, 11-deoxycorticosterone (DOCA) or progesterone [66]. The effect of aldosterone is mediated via binding on the mineralocorticoid receptor, since application of aldosterone, together with antagonists such as 1 x 10^{-4} M spironolactone or canrenoate, completely inhibited development of tubules. Moreover, western blotting experiments with isolated proteins of the nephrogenic zone demonstrated that the mineralocorticoid receptor was present so that an unspecific tubulogenic effect of aldosterone was excluded [67]. Finally, molecular interference by geldanamycin (3.6 x 10^{-6} M), radicicol (1 x 10^{-6} M), quercetin (2 x 10^{-4} M) or KNK 437 (1 x 10^{-4} M) illustrated that successful signaling of aldosterone is based on an intact interaction between the mineralocorticoid receptor and heat shock proteins (hsp) 90 and 70 [68].

The interface between developing tubules and the artificial interstitium

Extension of generated tubules can be increased by piling and paving of the isolated nephrogenic zone between multiple layers of a polyester fleece [69]. Since coating by extracellular matrix proteins does not impede the view inside the polyester fleece, an examination of the polyester fibers and the basal lamina of generated tubules with scanning electron microscopy is possible [70]. In this coherence it was detected that an intact basal lamina covered generated tubules. Surprisingly, it contained three categories of evenly distributed pores. Although their physiological function is unknown, the pores also occur in CD tubules of the neonatal kidney, so that they do not arise due to culture artifacts.

Immunohistochemistry further reveals that the generated tubules do not dangle but are fastened by tiny strands of collagen type III. The fibers span between the basal lamina and the polyester fibers of the fleece [71]. Transmission electron microscopy demonstrates that the basal lamina of generated tubules consists of a lamina rara, lamina densa and lamina fibroreticularis, as is found in the basal lamina of matured tubules within the kidney [72].

Search of an environment caching cell stress

An unanswered question is whether the nephrogenic zone develops, during culture, other cell biological features as observed within a developing kidney. To obtain some insights, in the following experiments the focus was directed at cache cell stress. First of all, for minimizing osmotic shock the nephrogenic zone was isolated not in buffered saline solution but in the same medium that was used for subsequent culture. To recognize intact and misdirected development, specimens were screened routinely by a set of markers such as SBA and antibodies reacting with laminin γ1, cytokeratin 19, TROMA-I, Na/K ATPase α5, E-cadherin, cingulin and occludin.

To optimize the spatial extension and density of generated tubules, besides the earlier used I-7 fleece, also Posi-4, -5, -6 and -7 polyester fleeces (Positech, Hallwil, Switzerland) were tested [73, 74]. It was recognized that the kind of polyester fleece and especially the geometry between the fibers strongly influenced the spatial distribution and density of generated tubules [75]. In optimal cases the majority of tubules within an artificial

interstitium developed in a close distance between 5 and 25 μm, which is comparable to the situation within the developing kidney. Also I-7, Posi-4 as Posi-5 fleeces were principally suitable in promoting development of renal tubules. Since changes in the geometry of polyester fibers leads to altered spatial growth patterns, the influence of extracellular matrix synthesis during growth of tubules has to be considered [76].

Detection of atypical development

In numerous experiments the isolated nephrogenic zone was mounted between layers of polyester fleece for perfusion culture in chemically defined IMDM [77]. To stimulate formation of tubules, aldosterone was administered as described earlier. Of particular interest was to know whether other steroid hormones also exhibit a tubulogenic effect. Surprisingly, when progesterone, pregnenolone, 11-desoxycorticosterone or dihydrotestosterone were applied, microscopy elucidated that intense formation of tubules was lacking, but an atypical aggregation of SBA-labeled cells in the form of cell islets was present. Especially noticeable was that numerous SBA-labeled cells within extended clusters were observed after application of testosterone, 17-β-estradiol, corticosterone and dexamethasone [78].

Registration of atypical cell islets and clusters as well as only minimal tubule development was the reason for analyzing specimens using transmission electron microscopy. In a standard protocol, fixation was first performed in a conventional glutaraldehyde solution. However, tubules generated in the presence of aldosterone showed no peculiarities. Then, special fixation for transmission electron microscopy was used to unveil masked details. For example, when specimens were fixed in glutaraldehyde solution containing ruthenium red or tannic acid, abundant and earlier unrecognized extracellular matrix at the basal lamina and within the interstitium of generated tubules was visible [79].

Atypical development during generation of tubules might be caused by the fluid environment. To exclude a harming effect of previously used IMDM, in further series of experiments Williams' E Medium, Basal Medium Eagle, McCoy's 5A Modified Medium and Medium 199 were applied. However, microscopic screening revealed that these media were less suitable owing to poor morphological preservation and presence of numerous vacuoles in epithelial cells of generated tubules [80].

In contrast, promising morphological results were obtained with Leibovitz's L-15 Medium and CO_2 Independent Medium [81]. When the nephrogenic zone was cultured in these media in combination with an I-7 polyester fleece and fixed by conventional glutaraldehyde for transmission electron microscopy, generated tubules showed an excellent preservation of morphological features and a homogenous cell population in developed tubules. However, fixation by glutaraldehyde solution, including tannic acid, revealed that, besides normal bright cells, also numerous atypical dark cells of unknown function were present in the tubule epithelium [82] (**Fig. 9**). Moreover, abundant extracellular matrix in the interstititium of generated tubules became visible, when fixation was performed with glutaraldehyde solution including cupromeronic blue or ruthenium red [83]. Finally, replacement of I-7 against Posi-4 polyester fleece did not prevent development of an atypical bright-dark cell mosaic within the tubule epithelium [84].

These unexpected results must be seen against the background that optical microscopy and immunohistochemistry did not inform about the occurrence of a pathologic bright-dark cell mosaic

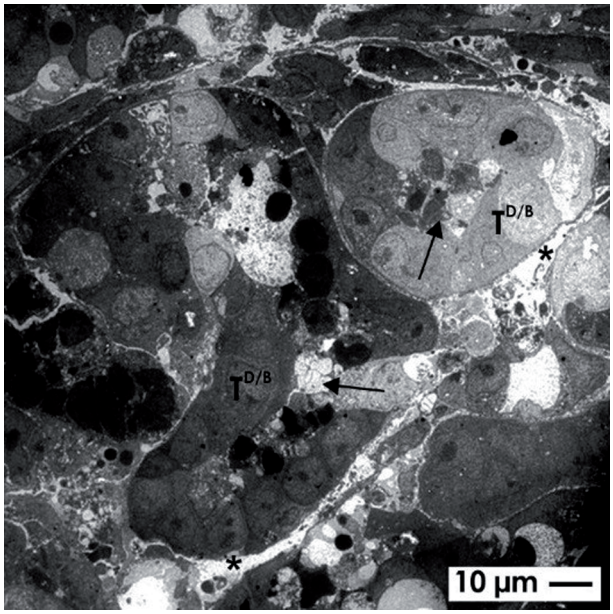

Figure 9. Atypical development of tubules in the nephrogenic zone cultured within an artificial interstitium for 13 days. Fixation of specimens in glutaraldehyde solution containing tannic acid for transmission electron microscopy demonstrates that in generated tubules ($T^{D/B}$) a mosaic of dark (D) and bright (B) cells is contained. Such a heterogeneous cell population is not known in the kidney. Lumen (arrow), basal lamina (asterisk).

in the tubule epithelium. Thus, for recognition of cell biological risks hidden within the nephrogenic zone, pathologists must take a closer look at generated specimens by means of transmission electron microscopy in combination with innovative tissue fixation.

Perspectives for the future

When the nephrogenic zone of neonatal rabbit kidney is isolated, it consists of CD ampullae including epithelial stem cells, nephrogenic mesenchymal stems, condensed vesicles and S-shaped bodies that adhere to the capsule in original composition (**Figures 1** and **2b,c**). Seen in this way, in the explant the complete machinery is present to maintain stemness, to synthesize morphogens and to develop new nephrons. To preserve this constellation and support development, the isolated nephrogenic zone was mounted in an artificial interstitium and exposed to chemically defined media during perfusion culture [69]. Despite these favorable and controlled culture conditions, in the course of experiments it was recognized that formation of complete nephrons was lacking for reasons not yet known. Instead, development of numerous CD tubules was observed. It remained unclear as to whether some proximal nephron segments occur in the cultures since explicit markers for rabbit species were not available.

Although a highly sophisticated culture environment was applied, generation of CD tubules was paralleled by the rise of pathologic features in the form of cell islets, cluster formation, a bright-dark cell mosaic in the tubule epithelium and abundant extracellular matrix in the interstitial space [78, 82, 83] (**Fig. 9**). However, this disappointing result was seen as a signal to inform about atypical development, contained cell biological risks and find a strategy for avoiding it in future. Since illustrated abnormal development *in vitro* was not observed during development of the kidney, this must have been caused by stress during isolation and by still not adequately adjusted culture conditions. This again is a clear indicator that the environment within the nephrogenic zone is particularly protecting contained structures and that the involved interstitial fluid is more complexly composed than was previously assumed.

Search for suitable culture media

Initially, media were developed for isolated but proliferating cells in culture but not for the different highly specialized stem/progenitor cells and arising nephrons of the nephrogenic zone. To find a compromise, in our performed experiments we used a series of commercially available culture media that were earlier applied for isolated renal cells in another context. Despite an intense search, an optimal medium for the culture of the isolated nephrogenic zone, considering the aforementioned aspects, was not found. This can be recognized by the fact that numerous CD tubules in culture developed, while formation of nephron segments was lacking, although renal vesicles and S-shaped bodies were contained in the explant.

Thus, when the isolated rabbit nephrogenic zone reacts so sensitively to isolation and exposure to a culture fluid environment, special emphasis must be directed in future to work on its fine-tuning. That must take into account site-specific needs such as an adjusted electrolyte composition and an individual offer of nutrition. Focus must be also directed at the respiratory gas requirement, cell stress reactions and peculiar metabolic processes. Without a sound experimental knowledge of a 'good' and a 'bad' fluid environment, the isolated rabbit nephrogenic zone will not give adequate responses that provide knowledge about impaired nephrogenesis in preterm infants.

Antibiotics and antimycotics without potential nephrotoxicity

As shown in numerous experiments, pieces of the nephrogenic zone were isolated and cultured for prolonged periods of time. For primary cultures in long term experiments it is necessary to add an antibiotic-antimycotic solution (100x; Gibco, Invitrogen, USA) to prevent infections. During an experiment this was administered according to the manufacturer's recommendations. Although generally used, it was not systematically investigated, whether the contained penicillin, streptomycin and amphotericin B have a toxic influence on cells of the isolated nephrogenic zone, causing in turn impaired nephrogenesis *in vitro*. Thus, the challenge for the future is also to identify improved culture media antibiotics and antimycotics that do not exhibit a toxic effect on embryonic, differentiating and matured renal cells.

Maintaining stemness and competence for induction

In the isolated nephrogenic zone of neonatal rabbit kidney, epithelial stem cells contained in

the tip of each CD ampulla were juxtaposed with mesenchymal stem cells in their original stem cell niche arrangement. An up-to-date unanswered question is whether after isolation and culture the stemness in both types of stem cells is maintained. Furthermore, it is unknown whether the competence to respond to nephron induction is preserved in mesenchymal stem cells. It is also not known whether synthesis, secretion and transport of site-specific morphogens stay intact after the process of isolation. However, indirect information is obtained from the fact that a significant formation of new nephrons was not registered in the culture experiments performed. So far, the missing *de novo* formation of nephrons in culture might reflect faults in morphogenic signaling that can be seen as a mirror causing impaired nephrogenesis in preterm infants.

Disturbed morphogen signaling within the niche must be identified

Many data exist that deal with renal stem cell features at the stage of kidney anlage, morphogen signaling during initial ureteric bud branching and subsequent nephron formation [10, 14]. Surprisingly however, little information is available about the mechanisms that stimulate or inhibit synthesis, secretion and transport of involved morphogens during formation of renal parenchyma in the terminal phase of gestation. In this context it is unknown whether each of the niches acts autonomously or whether all niches aligned beyond the organ capsule are regulated by a supervisory system. In particular, one has to ponder the fact that all of the renal niches vanish in infants shortly before a normal birth regulated by an unknown mechanism.

To find some of the answers, pieces of the isolated rabbit nephrogenic zone in culture can be exposed to single or a cocktail of involved morphogens such as Glial cell line-derived neurotrophic factor (GDNF), Wingless/Int-1 signal proteins (Wnts) or Fibroblast growth factors (FGFs). Such experiments can test whether stem cell niche activity is maintained for a prolonged period of time and if it is capable of producing new nephrons. In this connection, one has to consider that an absent *de novo* formation of nephrons might be caused either by a down-regulation of morphogen synthesis or depend on disturbed signaling, hampered cell to cell communication via tunneling nanotubes, or by an altered synthesis and degradation of extracellular matrix within the renal stem cell niche [85-87].

Metabolites may hamper nephron formation

Capillaries are under construction within the nephrogenic zone, but an intact blood circulation is not available. For this reason nephron formation occurs in a bradytrophic district, where nutrition and respiratory gas is slowly provided over an astonishingly wide distance by diffusion. In turn, noxious agents need a relatively long time span to reach the nephrogenic zone. Consequently, the same time is necessary to eliminate harmful metabolites. This constellation makes it difficult to estimate the time span when harming by metabolites starts, how long it takes and how it can be stopped by a therapeutic intervention so that repair might have a chance. As a consequence, isolated pieces of the rabbit nephrogenic zone can be used to investigate their individual metabolism. Then, by exposure of the nephrogenic zone to a harmful environment alterations of its metabolome can be recognized. There is so much to do – so let us get started!

Remarks

The nomenclature in the text is used according to previously published literature [13, 85-87].

Acknowledgements

The author thanks the Institute of Anatomy, University of Regensburg, D-93053 Regensburg, Germany for financial support and technical assistance.

Declaration of interest

The Author declares that there is no conflict of interest.

References

1. Kandasamy Y, Smith R, Writh IM. Oligonephropathy of prematurity. Am J Perinatol. 2012;29(2):115-20.
2. Gubhaju L, Sutherland MR, Black MJ. Preterm birth and the kidney: implications for long-term renal health. Reprod Sci. 2011;18(4):322-33.
3. Saint-Faust M, Boubred F, Simeoni U. Renal development and neonatal adaption. Am J Perinatol. 2014;31(9):773-80.
4. Abitbol CL, DeFreitas MJ, Strauss J. Assessment of kidney function in preterm infants: lifelong implications. Pediatr Nephrol. 2016;31(12):2213-22.
5. Sutherland M, Gubhaju L, Moore L, Kent AL, Dahlstrom JE, Horne RSC, Hoy WE, Bertram JF, Black MJ. Accelerated maturation and abnormal morphology in the preterm neonatal kidney. J Am Soc Nephrology. 2011;22:1365-74.

6. Fanos V, Castagnola M, Faa G. Prolonging nephrogenesis in preterm infants: a new approach for prevention of kidney disease in adulthood. Iran J Kidney Diseases. 2015;9(3):180-5.
7. Faa G, Sanna A, Gerosa C, Fanni D, Puddu M, Ottonello G, Van Eyken P, Fanos V. Renal physiological regenerative medicine to prevent chronic renal failure: should we start at birth? Clin Chim Acta. 2015;444:156-62.
8. Fanni D, Sanna A, Gerosa C, Puddu M, Faa G, Fanos V. Each niche has an actor: multiple stem cell niches in the preterm kidney. Ital J Pediatr. 2015;41:78.
9. Nagalakshmi VK, Yu J. the ureteric bud epithelium: morphogenesis and roles in metanephric kidney patterning. Mol Reprod Dev. 82(3):151-66.
10. Krause M, Rak-Raszewska A, Pietilä I, Quaggin SE, Vainio S. Signaling during kidney development Cells. 4(2):112-32.
11. Meyer TN, Schwesinger C, Bush KT, Stuart RO, Rose DW, Shah MM, Vaughn DA, Steer DL, Nigam SK. Spatiotemporal regulation of morphogenic molecules during in vitro branching oft he isolated ureteric bud: toward a model of branching through budding in the developing kidney. Dev Biol. 2004;275(1):44-67.
12. Fanos V, Loddo C, Puddu M, Gerosa C, Fanni D, Ottonello G, Faa G. From ureteric bud to the first glomeruli: genes, mediators, kidney alterations. Int Urol Nephrol. 2015;47(1):109-16.
13. al-Awqati Q, Goldberg MR. Architectural patterns in branching morphogenesis in the kidney. Kidney Int. 1998;54(6):1832-42.
14. Sanna A, Fanos V, Gerosa C, Vinci L, Puddu M, Loddo C, Faa G. Immunohistochemical markers of stem/progenitor cells in the developing human kidney. Acta Histochem. 2015;117(4-5):437-43.
15. Minuth WW, Kriz W. Culturing of renal collecting duct epithelium as globular bodies. Cell Tissue Res. 1982;224(2):335-48.
16. Minuth WW, Kriz W. Renal Collecting Duct Cells Cultured as Globular Bodies and as Monolayers. J Tissue Cult Meth 1982;7(3):93-6.
17. 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.
18. Minuth WW, Denk L. Structural links between the renal stem/progenitor cell niche and the organ capsule. Histochem Cell Biol. 2014;141(5):459-71.
19. Minuth WW. Cell associated glycoproteins synthesized by cultured renal tubular cells. Histochemistry. 1982;76(1):89-106.
20. Minuth WW. Glycoprotein synthesis of renal collecting duct epithelium cultured as Globular Bodies. Renal Physiol. 1983;6(2):87-102.
21. Minuth WW. Induction and inhibition of outgrowth and development of renal collecting duct epithelium. Lab Invest. 1983;48(5):543-8.
22. Minuth WW, Essig E. Effects of glycoprotein and basement membrane synthesis inhibitors on the growth of cultured renal collecting duct epithelium. Histochemistry. 1984;80(5):475-82.
23. Minuth WW, Lauer G, Bachmann S, Kriz W. Immunocytochemical localization of a renal glycoprotein (gpCDI) synthesized by cultured collecting duct cells. Histochemistry. 1984;80(2):171-82.
24. Gross P, Minuth WW, Kriz W, Frömter E. Electrical properties of renal collecting duct principal cell epithelium in tissue culture. Pflügers Arch. 1986;406(4):380-6.
25. Gitter AH, Beyenbach KW, Christine CW, Gross P, Minuth WW, Frömter E. High-conductance K+-channel in apical membranes of principal cells cultured from rabbit renal cortical collecting duct anlagen. Pflügers Arch. 1987;408:282-90.
26. Gross P, Minuth WW, Ketteler M, Frömter E. Ionic conductances of cultured principal cell epithelium of renal collecting duct. Pflügers Arch. 1988;412:434-41.
27. Minuth WW, Gilbert P, Lauer G, Aktories K, Gross P. Differentiation properties of renal collecting duct cells in culture. Differentiation. 1986;33(2):156-67.
28. Minuth WW, Gross P, Gilbert P, Kashgarian M Expression of the α-subunit of Na/K-ATPase in renal collecting duct epithelium during development. Kidney Int. 1987;31(5):1104-12.
29. Minuth WW, Gilbert P. The expression of specific proteins in cultured renal collecting duct cells. Histochemistry. 1988;88(3-6):435-41.
30. Minuth WW, Gilbert P, Gross P. Appearance of specific proteins in the apical plasma membrane of cultured renal collecting duct principal cell epithelium after chronic administration of aldosterone and arginine vasopressin. Differentiation. 1988;38(3):194-202.
31. Lauer G, Minuth WW. Apico-basal osmotic gradient induces transcytosis in cultured renal collecting duct epithelium. J Membrane Biol. 1988;101(2):93-101.
32. Minuth WW, Steckelings U, Gross P. Methylation of cytosolic proteins may be a possible biochemical pathway of early aldosterone action in cultured renal collecting duct cells. Differentiation. 1987;36(1):23-34.
33. Minuth WW, Zwanzig M, Gross P. Action of aldosterone on protein expression in cultured collecting duct cells from neonatal rabbit kidney. Ren Physiol Biochem. 1989;12(4):213-27.
34. Minuth WW, Struck M, Zwanzig M, Gross P. Action of aldosterone on citrate synthase in cultured renal collecting duct cells. Ren Physiol Biochem. 1989;12(2):85-95.
35. Zwanzig M, Minuth WW, Gross P. Action of aldosterone on cultured renal collecting duct cells during the latent period. Ren Physiol Biochem. 1990;13(6):285-94.
36. Minuth WW, Rudolph U. A compatible support system for cell culture in biomedical research. Cytotechnology. 1990;4(2):181-9.
37. Minuth WW, Dermietzel R, Kloth S, Hennerkes B. A new method culturing renal cells under permanent superfusion and producing a luminal-basal medium gradient. Kidney Int. 1992;41(1):215-9.
38. Minuth WW, Stöckel G, Kloth D, Dermietzel R. Construction of an apparatus for perfusion cell cultures which enables in vitro experiments under organotypic conditions. Eur J Cell Biol. 1992;57(1):132-7.
39. Minuth WW, Fietzek W, Kloth S, Aigner J, Herter P, Röckl W, Kubitza M, Stöckl G, Dermietzel R. Aldosterone modulates PNA binding cell isoforms within renal collecting duct epithelium. Kidney Int. 1993;44(3):537-44.

40. Aigner J, Kloth S, Kubitza M, Kashgarian M, Dermietzel R, Minuth WW. Maturation of renal collecting duct cells in vivo and under perifusion culture. Epithelial Cell Biol. 1994;3(2):70-8.
41. Aigner J, Kloth S, Jennings ML, Minuth WW. Transitional differentiation patterns of Principal and Intercalated Cells during renal collecting duct development. Epithelial Cell Biol. 1995;4(3):121-30.
42. Minuth WW, Aigner J, Kloth S, Steiner P, Tauc M, Jennings ML. Culture of embryonic renal collecting duct epithelia in a gradient container. Pediatr Nephrol. 1997;11(2):140-7.
43. Minuth WW, Steiner P, Strehl R, Kloth S, Tauc M. Electrolyte environment modulates differentiation in embryonic renal collecting duct epithelia. Exp. Nephrol. 1997;5(5):414-22.
44. Steiner P, Strehl R, Kloth S, Tauc M, Minuth WW. In vitro development and preservation of specific features of collecting duct epithelial cells from embryonic rabbit kidney are regulated by the electrolyte environment. Differentiation. 1997;62(4):193-202.
45. Minuth WW, Steiner P, Strehl R, Schumacher K, de Vries U, Kloth S. Modulation of cell differentiation in perfusion culture. Exp Nephrol. 1999;7(5-6):394-406.
46. Schumacher K, Strehl R, Kloth S, Tauc M, Minuth WW. The influence of culture media on embryonic renal collecting duct cell differentiation. In Vitro Cell Dev Biol Anim. 1999;35(8):465-71.
47. Schumacher K, Klotz-Vangerow S, Tauc M, Minuth WW. Embryonic renal collecting duct cell differentiation is influenced in a concentration-dependent manner by the electrolyte environment. Am J Nephrol. 2001;21(2):165-75.
48. Schumacher K, Strehl R, de Vries U, Minuth WW. Advanced technique for long term culture of epithelia in a continuous luminal - basal medium gradient. Biomaterials. 2002;23(3):805-15.
49. Schumacher K, Castrop H, Strehl R, de Vries U, Minuth WW. Cyclooxygenases in the collecting duct of neonatal rabbit kidney. Cell Physiol Biochem. 2002;12(2-3):63-74.
50. Schumacher K, Strehl R, Minuth WW. Urea restrains aldosterone-induced development of peanut agglutinin-binding on embryonic renal collecting duct epithelia. J Am Soc Nephrol. 2003;14(11):2758-66.
51. Strehl R, Schumacher K, Minuth WW. Controlled respiratory gas delivery to embryonic renal epithelial explants in perfusion culture. Tissue Eng. 2004;10(7-8):1196-203.
52. Minuth WW, Strehl R, Schumacher K, de Vries U. Long term culture of epithelia in a continuous fluid gradient for biomaterial testing and tissue engineering. J Biomater Sci Polym Ed. 2001;12(3):353-65.
53. Minuth WW, Schumacher K, Strehl R. Renal epithelia in long term gradient culture for biomaterial testing and tissue engineering. Biomed Mater Eng. 2005;15(1-2):51-63.
54. Minuth WW, Strehl R. Technical and theoretical considerations about gradient perfusion culture for epithelia used in tissue engineering, biomaterial testing and pharmaceutical research. Biomed Mater. 2007;2(2):R1-11.
55. Minuth WW, Denk L, Roessger A. Gradient perfusion culture – simulating a tissue specific environment for epithelia in biomedicine. J Epithel Biol Pharmacol. 2009;2:1-13.
56. Kloth S, Meyer D, Röckl W, Miettinen A, Aigner J, Schmidbauer A, Minuth WW. Characterization of an endothelial protein in the developing rabbit kidney. Differentiation. 1992;52(1):79-88.
57. Kloth S, Aigner J, Schmidbauer A, Minuth WW. Interrelationship of renal vascular development and nephrogenesis. Cell Tissue Res. 1994;277(2):247-57.
58. Kloth S, Schmidbauer A, Kubitza M, Weich HA, Minuth WW. Developing renal microvasculature can be maintained under perfusion culture conditions. Eur J Cell Biol. 1994;63(1):84-95.
59. Kloth S, Ebenbeck C, Kubitza M, Schmidbauer A, Röckl W, Minuth WW. Stimulation of renal microvasculatur development under organotypical culture conditions. FASEB J. 1995:9(10): 963-7.
60. Kloth S, Aigner J, Kubitza M, Schmidbauer A, Gerdes J, Moll R, Minuth WW. Development of renal podocytes cultured under medium perifusion. Lab Invest. 1995;73(2):294-301.
61. Kloth S, Gerdes J, Wanke C, Minuth WW. Basic fibroblast growth factor is a morphogenic modulator in kidney vessel development. Kidney Int. 1998;53(4):970-8.
62. Minuth WW, Strehl R, Schumacher K. Tissue Factory: conceptual design of a modular system for the in-vitro generation of functional tissues. Tissue Eng. 2004;10(1-2):285-94.
63. Minuth WW, Sorokin L, Schumacher K. Generation of renal tubules at the interface of an artificial interstitium. Cell Physiol Biochem. 2004;14(4-6):387-94.
64. Minuth WW, Denk L, Heber S. Growth of embryonic renal parenchyme at the interphase of a polyester artificial interstitium. Biomaterials. 2005;26(33):6588-98.
65. Heber S, Denk L, Hu K, Minuth WW. Modulating the development of renal tubules growing in serum-free culture medium at an artificial interstitium. Tissue Eng. 2007;13(2): 281-92.
66. 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):4418-28.
67. Minuth WW, Denk L, Hu K, Castrop H, Gomez-Sanchez C. The tubulogenic effect of aldosterone is attributed to intact binding and intracellular response of the mineralocorticoid receptor. Central Eur J Biol. 2007;2(3):307-325.
68. Minuth WW, Blattmann A, Denk L, Castrop H. Mineralocorticoid receptor, heat shock proteins and immunophilins participate in the transmission of the tubulogenic signal of aldosterone. J Epithel Biol Pharmacol. 2008;1:24-34.
69. 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.
70. Blattmann A, Denk L, Strehl R, Castrop H, Minuth WW. The formation of pores in the basal lamina of regenerated renal tubules. Biomaterials. 2008;29(18):2749-56.
71. Minuth WW, Denk L, Blattmann A, Castrop H. Collagen type III is an important linking molecule between generated renal tubules and an artificial interstitium. J Clin Rehabilitative Tissue Eng Res. 2008;12(32):6201-8.

72. Minuth WW, Denk L, Meese C, Rachel R, Roessger A. Ultrastructural insights in the interface between generated renal tubules and a polyester interstitium. Langmuir. 2009;25(8): 4621-7.
73. Roessger A, Denk L, Minuth WW. Potential of stem/progenitor cell cultures within polyester fleeces to regenerate renal tubules. Biomaterials. 2009;30:3723-32.
74. Minuth WW, Denk L, Roessger A. Towards a guided regeneration of renal tubules at a polyester interstitium. Materials. 2010;3: 2369-92.
75. Miess C, Roessger A, Denk L, deVries U, Minuth WW. The interface between generating renal tubules and a polyester fleece in comparison to the interstitium of the developing kidney. Ann Biomedical Eng. 2010;38(6):2197-209.
76. Glashauser A, Denk L, Minuth WW. Polyester fleeces used as an artificial interstitium influence the spatial growth of regenerating tubules. J Tissue Sci Eng. 2011;2(2):105.
77. Minuth WW, Denk L, Roessger A. Cell and drug-delivery therapeutics for controlled renal parenchyma regeneration. Adv Drug Deliv Rev. 2010;62(7-8):841-54.
78. Minuth WW, Denk L, Glashauser A. Promoting and harmful effects of steroid hormones on renal stem/progenitor cell development. J Tissue Sci Eng. 2010;1:101.
79. Minuth WW, Denk L. Interstitial interfaces show marked differences in regenerating tubules, matured tubules and the renal stem/progenitor cell niche. J Biomedical Materials Research Part A. 2012;100A:1115-25.
80. Minuth WW, Denk L, Gruber M. Search for chemically defined culture medium to assist initial regeneration of diseased renal parenchyma after stem/progenitor cell implantation. Int J Stem Cell Res Transplantation. 2013;1:202.
81. Minuth WW, Denk L. Initial steps to stabilize of microenvironment for future implantation of stem/progenitor cells in diseased renal parenchyma. Transplantation Technology. 2013;1:2.
82. Minuth WW, Denk L. Tannic acid label indicates abnormal cell development coinciding with regeneration of renal tubules. BMC Clinical Pathology. 2014;14:34.
83. Minuth WW, Denk L. Detection of abnormal extracellular matrix in the interstitium of regenerating renal tubules. Int J Mol Sci. 2014;15(12): 23240-54.
84. Minuth WW, Denk L. Atypical features in regenerating tubules point to a risk for implantation of renal stem/progenitor cells. Int J Stem Cell Res Transplantation. 2015;03(2):101-8.
85. Minuth WW, Denk L. When morphogenic proteins encounter special extracellular matrix and cell-cell connections at the interface of the renal stem/progenitor cell niche. Anat Cell Biol. 2015;48(1):1-9.
86. Minuth WW, Denk L. What is the functional background of filigree extracellular matrix and cell-cell connections at the interface of the renal stem/progenitor cell niche? J Pediatr Neonatal Individualized Medicine. 2016;5(1):e050115.
87. Minuth WW, Denk L. Special morphological features at the interface of the renal stem/progenitor cell niche force to reinvestigate transport of morphogens during nephron induction. Biores Open Access. 2016;5(1):49-60.