From the Renal Stem Cell Niche to Functional Parenchyme

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Summary

Our present experiments focus on a future alternative for the therapy of acute or chronic renal failure. Regarding the course of these diseases it is unknown why the kidneys loose their ability for regeneration of parenchyme. Consequently, we investigate mechanisms, which stimulate renal stem cells to develop new tubules. This process forms a three-dimensional tubule with a defined length, diameter and an exact geometrical extension. The construction of suitable microreactors for perfusion culture is a key step in this process. We apply a brandnew technique enabling us to generate tubules at the interphase of an artificial interstitium. Collagen coating is avoided and serum-free IMDM is applied. Aldosterone plays a key role, since it stimulates tubulogenesis in the cultured embryonic tissue.

Introduction

The multitude of patients with chronic or acute renal failure shows that the kidney [1] has in comparison to liver [2,3] a decreased capability for functional regeneration. In view of this severe clinical background the question arises, why the diseased kidney is no more able to regenerate nephron segments. Of special interest is the question why the regeneration is inhibited and how this process is triggered on the cellular basis. An ideal form of therapy in the future would be to evoke a process of regeneration and to use it therapeutically [4]. One could imagine that the renewal of parenchyme occurs via a stimulation of non-diseased parenchymal cells or via an activation of tissue-specific stem cells [5,6]. Independent of the chosen strategy it is apparent that an application or an activation of stem cells alone is not sufficient to trigger the course of therapy. Most important, one has to learn now to optimize the development of stem cells, so that finally three-dimensional and functional tubules arise. However, at present the knowledge about these processes is minimal.

Stem Cells within the Kidney

The tubular system of the kidney arises from two very different stem cell populations [1,7,8]. The different portions of the collecting duct develop from the arborisations of the ampullae, which are derivates of the ureteric bud. Each collecting duct ampulla contains in its tip epithelial stem cells, which induces by reciprocal interaction the neighbouring mesenchymal stem cells to develop into different nephron segments [9,10,11]. Thus, all the segments of the nephron are derivates of nephrogenic mesenchymal stem cells, which develop after induction first into a Comma-shaped and then a S-shaped body. These condensates are the first morphological signs of nephron development. By an unknown mechanism the upper part of the S-shaped body forms all the tubular portions of the nephron. Thus, it results in the development of the proximal, intermediate and distal tubule. In the course of further development the cells of the presumptive distal tubule search contact with the lateral aspect of the collecting duct ampulla to form the connecting tubule. It is assumed that the cells of the connecting tubule are derivates of the collecting duct.

From the Stem Cell to a Structured Tubule

The development of a three-dimensional tubular structure appears at first view very simple, but it is a complex cellbiological process. The stem cells have to form a functional epithelium, which is integrated into a three-dimensional tubule with exact geometrical dimensions [12]. This development happens in steps and starts by the transition of stem cells into precursor cells of an epithelium. First, these cells serach a sociality with close contacts to neighbouring cells. Then they develop a polarisation including a basement membrane and a lumen. Finally a three-dimensional structure of a tubule appears with a defined inner and outer diameter. Further the geometrical properties such as length, formation of a convolute or a straight direction are determined.

A tubular structure does not simply appear after the application of a single growth factor. In contrast, the development depends on various parameters such as morphogens, growth factors, a specific extracellular matrix, special nutritional and oxygen needs and finally physiological environment. These stimuli do not appear at the same time, but are triggered in special periods of competence and are controlled by different molecular pathways [6,12]. At present we elaborate in which way these events can be experimentally influenced (Fig. 1). By the use of innovative culture experiments we gained first insights to generate renal tubules under very controlled conditions.

Use of Renal Tissue Containing Stem Cells

Our aim is to gain insights into the processes involved in the generation of renal tubules derived from renal stem cells. The complex architecture of the kidney hinders experiments in animals. Consequently, embryonic renal tissue has to be isolated and a special tissue culture model has to be applied. Performance of these experiments results in important experiences, indicates frontiers and further challenges.

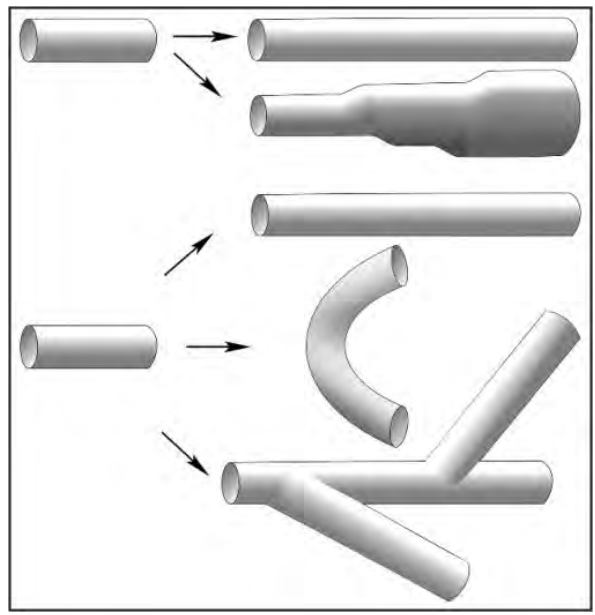

Fig. 1: Schematic illustration of a tubule formation. During this process a precursor (left side) develops a three-dimensional tubule with a defined inner and outer diameter (right side). Furtheron a straight forward development, a convolution and an arborisation are determined.

To broaden our experience in the generation of renal tubules we use the kidneys of newborn rabbits. In contrast to mouse kidney and the related transfilter culture techniques developed by Grobstein [13,14,15] the neonatal kidney of rabbit has the advantage, that at birth embryonic renal tissue is found in sufficient amounts beyond the renal capsule for culture experiments and cellbiological analysis. In addition, the embryonic tissue can be harvested without the loss of the mother rabbit.

Stripping off the capsule from neonatal rabbit kidney with fine forceps a thin layer of embryonic tissue of constant thickness adheres to the explant (Fig. 2). In the explant nephrogenic mesenchymal stem cells, Comma-, S-shaped bodies and collecting duct ampullae with epithelial stem cells are found. By this simple micro-surgical method a thin layer of embryonic renal tissue up to 1 cm^2 in square can be harvested. Up to date no other species is known, which allows to harvest such amount of embryonic renal tissue by this simple preparation technique [16].

Adequate In-Vitro Methods

After isolation of embryonic renal tissue one needs to decide, which in-vitro method is the most suitable one to generate tubules. Giving three examples we like to show that tubules do not automatically arise, but need innovative culture methods. One aim is the application of serum-free culture medium. Further the embedding of tissue in extracellular matrix such as collagen should be avoided. Thus, cultures are not raised in the presence of fetal calf serum (FCS) or by coating f.e. with extracellular matrix proteins such as Matrigel derived from tumor cells.

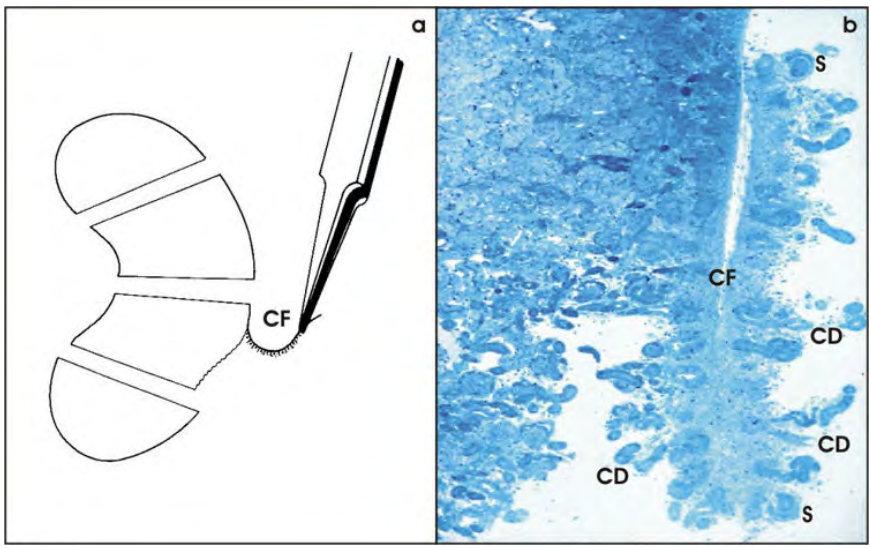

Fig. 2: Isolation of renal stem-/progenitor cells from neonatal rabbit kidney. a) Schematic illustration using fine forceps the renal capsule is stripped off with an adherent layer of nephrogenic blastema. b) The isolated explant consists of renal capsule (CF), collecting duct ampullae (CD), S-shaped bodies (S) and mesenchymal stem cells.

1. Culture in static environment

When embryonic renal tissue is placed at the bottom of a culture dish and incubated in medium, after a few days cells grow out of the explant and emigrate in the periphery (Fig. 3). After 8 to 10 days a more or less confluent monolayer has grown by spreading of cells (Fig. 3a,b). However, tubules do not appear within the explant or in the area of emigrated cells. This means that with classic culture methods a nice cell monolayer (Fig. 3c) can be raised but tubules do not appear.

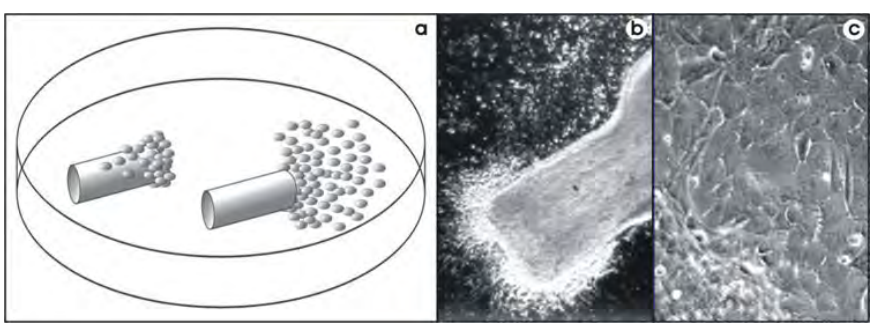

Fig. 3: Culture of embryonic renal tissue at the bottom of a culture dish. a) Schematic illustration of an explant with outgrowing cells. Partly the cells overgrow the explant, partly they migrate at the bottom of the culture dish. b) Microscopical illustration of an explant with emigrating cells. c) Monolayer of cells derived from an embryonic renal explant at the bottom of a culture dish.

2. Perfusion cultures

In comparison with static cultures perfusion of medium improves features of differentiation. For these experiments the isolated embryonic renal tissue is placed on a tissue carrier with 6 mm inner diameter and incubated for 24 hours in serum-containing medium under static culture conditions (Fig. 4a). During this period epithelial stem cells of the collecting duct ampullae grow out and form a polarily differentiated epithelium at the complete surface of the explant. Surprisingly, S-shaped bodies and the nephrogenic mesenchymal stem cells beyond the epithelium degenerate.

After 24 hours of culture the tissue carrier can be placed in a gradient perfusion container (Fig. 4b). In this container the tissue is provided at the luminal and basal side with the same or with different media [17].

The culture system is used on a laboratory table. The used silicone tubes are gaspermeable. Thus, during transportation the medium is equilibrated against atmospheric air. A peristaltic pump transports the culture medium with 1 ml/h and a warming plate with a cover lid maintains the temperature of 37° C. Arising air bubbles are eliminated in a gas expander module before the medium is reaching the culture container. The waste medium is collected in bottles and is not re-circulated.

Numerous publications show that using perfusion culture, tissue can be kept for many weeks in an optimal degree of differentiation. The described experiments demonstrate that a nice but flat epithelium is established (Abb.4c). However, in this kind of experiments no tubular elements are developed. Neither with different media, nor with different gradient culture conditions tubular elements could be generated.

3. Perfusion culture with an artificial interstitium

To generate renal tubules it was necessary to improve the perfusion culture technique. Normally cells or a piece of tissue within a culture dish or in a

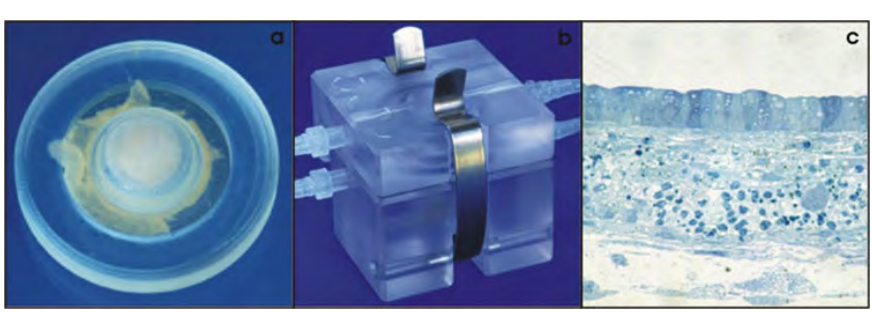

Fig. 4: Embryonic renal tissue in perfusion culture. a) The isolated tissue is placed on a tissue carrier with 6 mm inner diameter. b) The tissue carrier is placed in a gradient culture container and is perfused with medium at the luminal and basal side. c) Microscopical view of an explant cultured for 14 days in a gradient container. At the surface a polarily differentiated collecting duct epithelium is established, while the S-shaped bodies beyond the epithelium are degenerated.

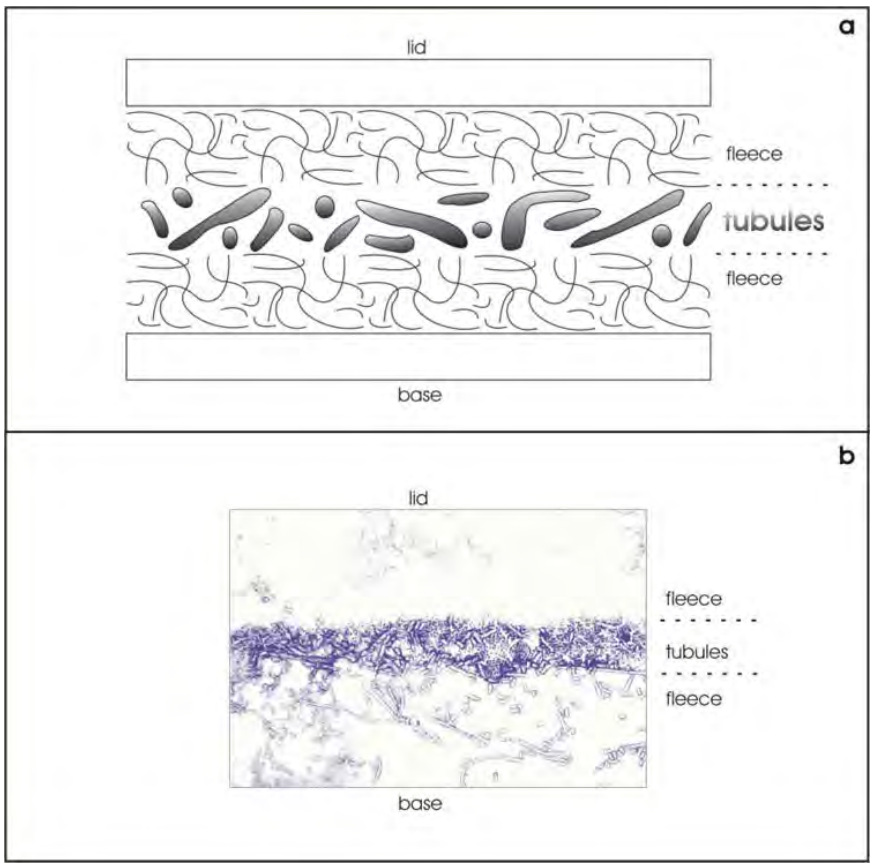

Fig. 5: Culture of an embryonic renal explant at the interphase of an artificial interstitium. a) Schematic illustration of tubules, which developed after 14 days at the interphase of a polyester fleece. b) Microscopical view of a micro-tissue consisting of renal tubules at the interphase of the polyester interstitium after 14 days in culture.

perfusion culture container is surrounded by a big volume of medium. This means that an unnecessarily large dead volume is present. By reduction of the geometry of the culture container the dead volume can be reduced.

A further reduction of the dead volume is reached by a fleece of polyester, which is placed in a perfusion culture container. By this new method the characteristics of medium flow and the growth conditions at the interphase of the polyester fleece are improved (Fig. 5) [19].

Generation of Renal Tubules

For the generation of tubules the isolated embryonic renal tissue is placed in a perfusion culture container between two pieces of a polyester fleece with 6 mm in diameter. With this method an artificial interstitium around the

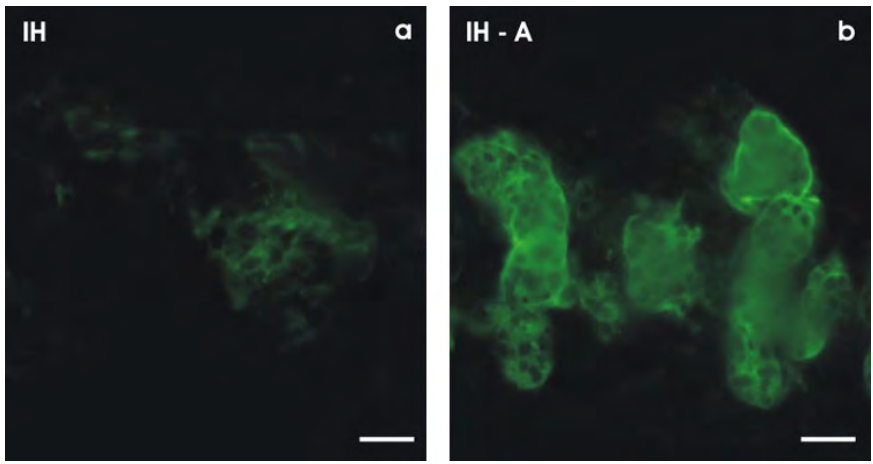

Fig. 6: Labelling of renal tissue cultured for 14 days at the interphase of an artificial interstitium with SBA. a) Culture in IMDM demonstrates only single fluorescent cells. b) Culture in IMDM containing aldosterone (1 x 10^{-7} M) reveals numerous tubules. Bar 20ìm

explant is produced (Fig. 5). Immediately after isolation of the tissue the culture is started in serum-free IMDM (I) containing HEPES (H) as biological buffer for 14 days. Applying this method the generation of renal tubules is possible in serum-free culture medium without coating by extracellular matrix proteins [18,19]. Most importantly, the development of tubules is dependent on the application of aldosterone (A, Fig. 6).

On perfectly orientated sections one can see, that the generated tubules reveal a lumen and a basal lamina. Relatively rarely perfect longitudinal sections can be analysed, since the generated tubules are not placed in a parallel order but are randomly organized. Sections of cultured tissue show tubules with a length of over 1000 µm.

Tubules of the kidney are three-dimensional structures with a certain length and a defined inner and outer diameter, a lumen and a basal lamina, which points out a functional polarisation. Consequently, we investigated by histochemical methods, if such features are developed in generated tubules. Labelling of tissue by Soybean Agglutinin (SBA, Fig. 6b) or with antibodies against cytokeratin 19 oder Troma-1 (cytokeratin Endo-A, no figure) show, that tubules resembling the adult collecting duct are raised. Immunohistochemical label against laminin ã1 shows, that a clearly recognizable basal lamina is produced. The basolateral staining of cells with an antibody against Na/K ATPase is a further indication that a polar differentiation is established.

More New Effects of Aldosterone

An exciting result of the present experiments is that aldosterone induces the formation of renal tubules. Numerous experiments with other hormones demonstrated, that the generation of tubules could only be obtained by the

application of aldosterone (Abb. 6b,7) [20]. Culture of embryonic renal tissue at the interphase of an artificial interstitium in IMDM und without application of aldosterone showed no generation of tubules (Fig. 6a).

Concentration-dependent action: Further experiments revealed that aldosterone induces the generation of tubules in a concentration-dependent manner. A low dosage of aldosterone between 1 x 10^{-10} M und 1 x 10^{-9} M fails to develop tubules. An increasing concentration of 1 x 10^{-8} M reveals only a mediocre action, while concentrations between 1 x 10^{-7} M und 1 x 10^{-6} M generate a maximal number of developed tubules. In contrast, at a concentration of 1 x 10^{-5} M aldosterone shows an inhibiting action.

Needed period of hormone application: Aldosterone has to be contained for a certain period of time in the culture medium to generate renal tubules. The culture of embryonic renal tissue for 14 days shows, that an initial application of 1 x 10^{-7} M aldosterone for 1, 2 or 3 days is too short for the development of tubular elements. In contrast, incubation of aldosterone over initial 4 or more days promotes growth of numerous tubules. Thus, an application over at least initial 4 days is necessary to raise tubules.

Period of tubular development: The development of SBA-positive tubules takes surprisingly long periods of time. Culture of embryonic renal tissue in IMDM for 1 to 5 days containing 1 x 10^{-7} M aldosterone shows that during this culture period only few SBA-positive cells can be recognized. Starting from the 8th day of culture numerous tubules become visible. A maximum of tubules can be recognized after day 10, which can be maintained for at least 8 weeks.

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Information about the culture system www.minucells.de

References

1. Al-Awqati Q, Oliver JA. Stem cells in the kidney. Kidney Int 61:387-395 (2002).
2. Dahlke Mh, Popp FC, Larsen S, Schlitt Hj, Rasko JE. Stem cell therapy of the liver – fusion or friction? Liver Transpl 10(4):471-9 (2004).
3. Ohashi K, Kay MA, Yokoyama T, Kuge H, Kanehiro H, Hisanaga M, Ko S, Nakajima Y. Stability and repeat regeneration of the engineered liver tissue under the kidney capsule in mice. Cell Transplant 14(9):621-27 (2005).
4. Maeshima A, Sakurai H, Nigam SK. Adult kidney tubular cell population showing phenotypic plasticity, and integration capability into developing kidney. J Am Soc Nephrol 17(1):188-98 (2006).
5. Mene P, Polci R, Festuccia F. Mechanisms of repair after kidney injury. J Nephrol 16(2):186-95 (2003).
6. Kim D, Dressler GR. Nephrogenic factors promote differentiation of mouse embryonic stem cells into renal epithelia. J AM Soc Nephrol 16(12):3527-34 (2005).
7. Strehl R, Kloth S, Aigner J, Steiner P, Minuth WW. P_{CD}Amp1, a new antigen at the interface of the embryonic collecting duct epithelium and the nephrogenic mesenchym. Kidney Int 52: 1469-77 (1997).

8. Sajithal G, Zou D, Silvius D, Xu PX. Eya 1 acts as a critical regulator for specifying the metanephric mesenchyme. Dev Biol 284(2):323-36 (2005).
9. Grimm DH, Karihaloo A, Cai Y, Somlo S, Cantley LG, Caplan MJ. Polycystin-2 regulates proliferation and branching morphogenesis in kidney epithelial cells. J Biol Chem 281(1):137-44 (2006).
10. Sariola, H. Nephron induction revisited: from caps to condensate. Curr Opin Nephrol Hypert. 11:17-21 (2002).
11. Schumacher K, Strehl R, de Vries U, Groene HJ, Minuth, WW. SBA-positive fibers between the CD ampulla, mesenchyme, and renal capsule. J Am Soc Nephrol 13: 2446-53 (2002).
12. Eisen R, Walid S, Ratcliff DR, Ojakian GK. Regulation of epithelial tubule formation by Rho family GTPases. Am J Physiol Cell Physiol ;Epub ahead of print (2005).
13. Grobstein C. Trans-filter induction of tubules in mouse metanephrogenic mesenchyme. Exp Cell Res 10(2):424-40 (1956).
14. Sariola H, Ekblom P, Henke-Fahle S Embryonic neurons as in vitro inducers of differentiation of nephrogenic mesenchyme. Dev Biol 132(2):271-81 (1989).
15. Ye P, Habib SL, Ricono JM, Kim NH, Choudhury GG, Barnes JL, Abboud HE, Arara MY. Fibronectin induces ureteric bud cells branching and cellular cord and tubule formation. Kidney Int 66(4):1356-64 (2004).
16. Minuth, WW. Neonatal rabbit kidney cortex in culture as tool for the study of collecting duct formation and nephron differentiation. Differentiation 36:12-22 (1987).
17. 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 23,3:805-15 (2002).
18. Minuth WW, Sorokin L, Schumacher K. Generation of renal tubules at the interface of an artificial interstitium. Cell Physiol Biochem 14(4-6):387-94 (2004).
19. Minuth WW, Denk L, Heber S. Growth of embryonic renal parenchyme at the interphase of a polyester artificial interstitium. Biomaterials 26:6588-98 (2005).
20. Heber S, Denk L, Minuth WW. Influence of aldosterone on the development of renal tubules. Submitted 2006