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# Modulating the Development of Renal Tubules Growing in Serum-Free Culture Medium at an Artificial Interstitium

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## ABSTRACT

**Little information on the structural growth of renal tubules is available. A major problem is the technical limitation of culturing intact differentiated tubules over prolonged periods of time. Consequently, we developed an advanced culture method to follow tubule development. Isolated tissue containing renal progenitor cells was placed in a perfusion culture container at the interphase of an artificial polyester interstitium. Iscove's modified Dulbecco's medium without serum or protein supplementation was used for culture, and the culture period was 13 days. Tissue growth was not supported by addition of extracellular matrix proteins. The development of tubules was registered on cryosections labeled with soybean agglutinin (SBA) and tissue-specific antibodies. Multiple SBA-labeled tubules were found when aldosterone was added to the culture medium. In contrast, culture without aldosterone supplementation displayed completely disintegrated tissue. The development of tubules depended on the applied aldosterone concentration. The use of $1\times10^{-6}$ M and $1\times10^{-7}$ M aldosterone produced numerous tubules, while application of $1\times10^{-8}$ M to $1\times10^{-10}$ M led to a continuous decrease and finally a loss of tubule formation. The development of labeled tubules in aldosterone-treated specimens took an unexpectedly long period of at least 8 days. The morphogenic effect of aldosterone appeared to be mineralocorticoid hormone–specific since spironolactone and canrenoate abolished the development. Finally, dexamethasone induced widely distributed cell clusters instead of tubules.**

## INTRODUCTION

TUBULOGENESIS WITHIN THE MAMMALIAN KIDNEY is a particularly complex process.[1,2] The proximal tubule, the segments of the loop of Henle, and the distal tubule are derivatives of nephrogenic mesenchymal stem cells.[3] In contrast, the collecting duct system arises from epithelial stem cells initially found in the ureter bud and later in the individual collecting duct ampullae.[4] The origin of the connecting tubule is unclear. The nephrogenic tubule is peculiar because each segment is composed of a single cell type while the connecting tubule and the collecting duct exhibit a heterogenous cell population consisting of principal cells and various types of intercalated cells.[5]

The development of renal parenchyme starts with the reciprocal interaction between the epithelial stem cells of an individual collecting duct ampulla and the surrounding nephrogenic mesenchymal cells.[6,7] As a result, the renal vesicle and, later, the S-shaped body become visible. During ongoing development, cellular segmentation of the upper cleft of the S-shaped body becomes apparent, giving rise to maturing cells of the proximal tubule, the loop of Henle, and the distal tubule.

Our aim was to generate renal tubular segments derived from embryonic renal tissue. Such a process starts with embryonic cells, increases the cell mass, defines the dimension of the segment, and develops the shape of a tubule. The developmental process proceeds from an embryonic anlage, maturing into an adult tubule state, which is finally defined by a certain length and a constant inner and outer diameter (Fig. 1A). However, elongation is not the only important factor. For each growing tubular segment appears to exist a

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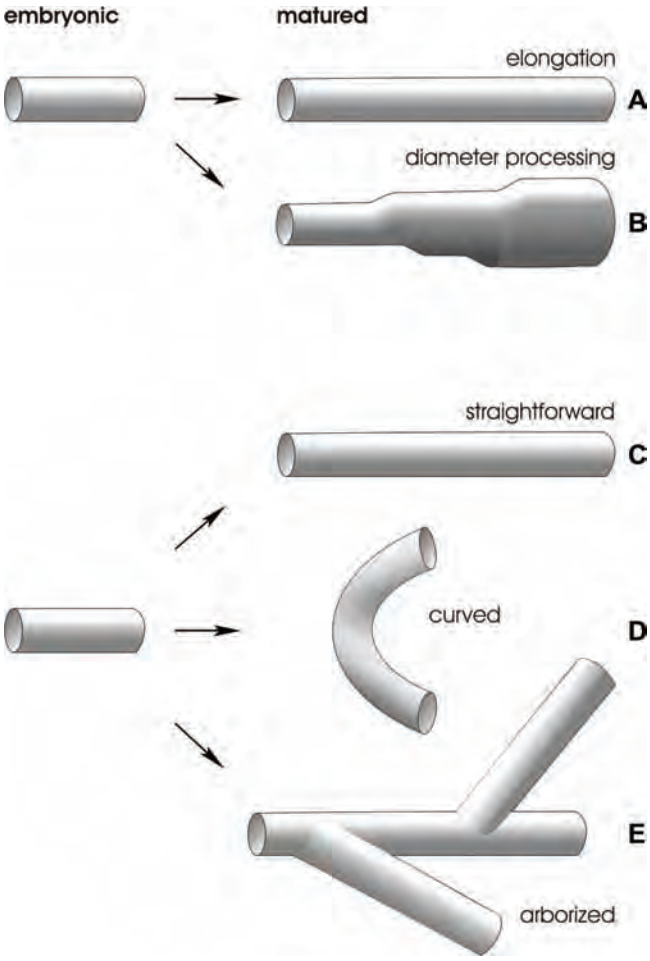


**FIG. 1.** Illustration of tubular growth during development from the embryonic (left side) to the matured state (right side). The illustration shows characteristics for the growth of tubules: elongation with constant diameter (**A**), elongation with discontinuous diameter (**B**), straightforward-directed growth processing (**C**), curved growth (**D**), and discontinuous growth with branching (**E**). Color images available online at www.liebertpub.com/ten

system, which controls the dimensions of the inner and outer diameter (Fig. 1B). Additional morphogenic information must be present for the development of a straightforward-oriented segment (Fig. 1C) or for curved growth within a convolute (Fig. 1D). Finally, the developmental process determines whether an arborization, as seen in the collecting duct ampulla, is prevented or supported (Fig. 1E). Observing the events of tubulogenesis within the growing kidney, it becomes obvious that, to date, little information on the underlying molecular processes is available. However, considering the expanding field of regenerative medicine and the need to optimize the application of stem cells to cure renal failure, exact information on tubule development will be of special interest in the near future.[8–10]

To investigate basic mechanisms of tubulogenesis and to learn about the environmental needs of maturing tubules, the availability of a powerful culture system is of fundamental importance. One of the presuppositions is that embryonic tissue can be maintained over prolonged periods to investigate the progression of tubule development. In a previous paper, we showed the feasibility of culturing renal tubules derived from embryonic tissue at the interphase of an artificial interstitium made of polyester fleece within a perfusion culture container.[11] When this innovative technique is used, the coating of embryonic renal tissue with extracellular matrix proteins is not necessary. Furthermore, culture can be performed in a chemically defined medium that includes hormonal additives.

In the present paper, we present new data that to our knowledge show for the first time the characteristics of the morphogenic action of aldosterone on the growth and long-term maintenance of renal tubules generated under *in vitro* conditions. The progress of tubule development appears to be mineralcorticoid hormone specific since it depends on aldosterone and not on the application of a glucocorticoid such as dexamethasone. It is a new finding, that aldosterone acts in a concentration-dependent manner and requires a long period (8 days) until the first signs of polarized tubules become visible.

## MATERIALS AND METHODS

### *Isolation of embryonic explants containing renal progenitor cells*

One-day-old New Zealand rabbits were anesthetized with ether and sacrificed by cervical dislocation. Both kidneys were removed immediately. Each kidney was dissected into 2 parts. By stripping off the capsula fibrosa with fine forceps, a fully embryonic tissue layer can be harvested; this layer contains numerous collecting duct ampullae, S-shaped bodies, and nephrogenic mesenchyme.[12]

### *Perfusion culture of renal tubules at the interphase of an artificial interstitium*

For a long-term culture, a tissue holder with 14-mm outer diameter was placed in a perfusion culture container (Fig. 2A; Minucells and Minutissue, Bad Abbach, Germany) as described elsewhere.[13] To minimize the dead fluid volume within the culture container, the freshly isolated embryonic renal tissue was placed between a layer of highly porous biocompatible polyester fleece (Walraf, Grevenbroich, Germany) as an artificial interstitium on top of the holder. Thus, the embryonic tissue and the polyester material were in close contact. Always fresh serum-free Iscove's modified Dulbecco's medium (IMDM) that included Phenolred (GIBCO/Invitrogen, Karlsruhe, Germany) was used. Furthermore, up to 50 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; GIBCO) was added to the medium to maintain a constant pH of 7.4 under atmospheric air containing 0.3% carbon dioxide. The medium was continuously perfused for

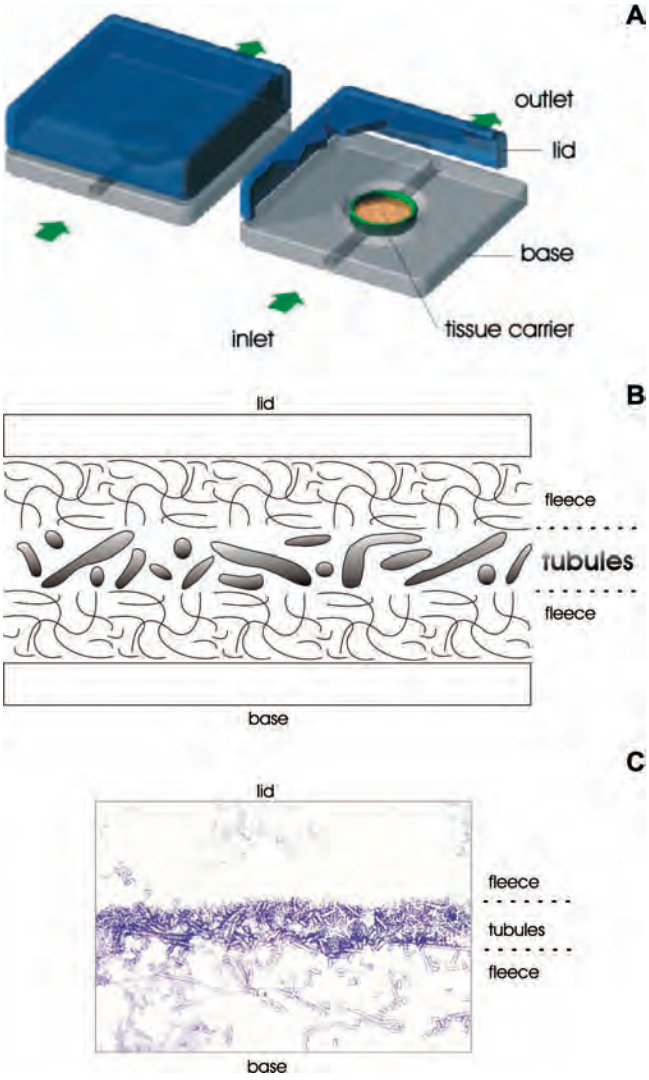


**FIG. 2.** Perfusion culture with an artificial interstitium. Schematic view of a perfusion culture container (**A**). The space between the lid and the base is filled with an artificial interstitium made of a polyester fleece. The tubules develop at the interphase of the artificial interstitium (**B**). The microscopic view (×100) of a cryosection stained with Toluidine blue reveals that polyester fibers restrict the cultured tissue from the upper and lower sides (**C**). Color images available online at www.liebertpub.com/ten

13 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 culture container was placed on a thermoplate (Medax, Kiel, Germany) and covered by a transparent lid. Aldosterone ($1\times10^{-7}$ M; Fluka, Taufkirchen, Germany), dexamethasone ($5\times10^{-6}$ M), triiodothyronine ($1\times10^{-8}$ M), nicotinamide (5 mM), spironolactone ($1\times10^{-4}$ M), and canrenoate ($1\times10^{-4}$ M) were obtained from Sigma (Taufkirchen, Germany). Insulin-transferrin-selenium G supplement (ITS; 1%, GIBCO) were added to individual experimental series. An antibiotic-antimycotic solution (1%, GIBCO) was present in all culture media.

### *Number of cultured constructs*

In total 157 tissue constructs were generated for the presented experiments. The mean number of generated structures is given in the text.

### *Lectin- and antibody labeling*

Cryosections of 20 µm thickness were fixed in ice-cold ethanol. After washing with phosphate-buffered saline (PBS), the sections were blocked with PBS containing 1% bovine serum albumin (BSA) and 10% horse serum for 30 min. For lectin-labeling, the specimens were exposed to fluorescein-isothiocyanate (FITC)–conjugated soybean agglutinin (SBA) (Vector Laboratories, Burlingame, Calif.) diluted 1:2000 in blocking solution for 45 min as described elsewhere.[11] For antibody labeling, monoclonal antibody (mab) anti-sodium potassium adenosine triphosphatase α5 subunit, mab anti-TROMA-1 (cytokeratin Endo-A, both obtained from Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, Iowa City, Iowa, 52242, under contract NO1-HD-7-3263 from the National Institute of Child Health and Human Development), mab anti-occludin (Zymed, San Francisco, Calif.), mab anti-cytokeratin 19 (gift from Dr. R. Moll, Marburg, Germany), and mab anti-laminin γ1 (provided by Dr. L. Sorokin, Lund, Sweden) were applied undiluted as primary antibodies for 1 h. After a washing step with 1% BSA in PBS, the specimens were incubated for 45 min with donkey-anti-mouse IgG FITC or goat-anti-rat IgG rhodamine (Jackson Immunoresearch Laboratories, West Grove, Pa.) diluted 1:50 in PBS containing 1% BSA. Following several washes in PBS, the sections were embedded with Slow Fade Light Antifade Kit (Molecular Probes, Eugene, Ore.) and analyzed by using an Axioskop 2 + micro-microscope (Zeiss, Oberkochen, Germany). Fluorescence images were obtained by a digital camera with a standard exposure time of 3.2 sec and thereafter processed with CorelDRAW 11 (Corel Corporation, Ottawa, Ontario, Canada).

## RESULTS

We sought to isolate embryonic renal tissue derived from a neonatal rabbit kidney for the generation of tubules under advanced culture conditions. Thus, we stripped off the fibrous capsule containing an adherent layer of renal stem cells.[12] The tissue was then placed in a perfusion culture container filled with polyester fleece as an artificial interstitium (Fig. 2). The standard medium was serum-free IMDM that contained HEPES as biological buffer system. To support the development of tubules, frequently applied hormones and growth factors were added. Tissue was cultured under atmospheric air for 13 days. The development of tubules was then screened for cellular SBA labeling. SBA recognizes terminal *N*-acetylgalactosamine (GalNAcα1) residues on glycoproteins.[14] Use of the described titer of fluorescent

SBA labels only matured collecting duct cells. In contrast, in isolated embryonic tissue no cellular label is found. In the present experiments we show that aldosterone plays a key role in the 3-dimensional development of tubules.

### *Definition of developed structures*

*Cell islets:* An islet is defined as a group of few aggregated cells labeled by SBA (Fig. 3A). The cells are frequently in close contact with the polyester fibers (Fig. 3B). Polarized cells, formation of a lumen, or development of a basal lamina are not observed.

*Cell cluster:* A cluster is described as an aggregate of many SBA-labeled cells (Fig. 3C). The diameter of a cluster varies between 30 and 150 µm. Most cells in a cluster do not show polarization; consequently, a lumen cannot be recognized. In some cases, a discontinuous basal lamina is visible. Thus, the surface of a cell cluster is rough. Furthermore, the cells of a cluster show numerous long filopodia that protrude into the medium and contact polyester fibers or neighboring tissue structures (Fig. 3D).

*Tubules:* Developed tubules are described in longitudinal (Fig. 3E) or cross-sectioned (Fig. 3F) view as structures showing polarized cells labeled by SBA. A lumen is visible and a basal lamina borders the smooth outer surface of a tubule. No filopodia and no overgrowth of cells on the polyester fibers are observed.

### *Experimental series without administration of aldosterone*

Dexamethasone, triiodothyronine, nicotinamide, and ITS are frequently applied in renal cell culture protocols. In the first set of experiments we therefore specify whether these substances show a morphogenic effect on developing tubules (Fig. 4A–F, Table 1).

*Basic medium.* For control, embryonic tissue derived from a neonatal rabbit kidney was cultured in the basic medium (which consisted of IMDM that contained HEPES) for 13 days. Supplementation without further substances showed a complete disintegration of tissue. Under these conditions, only thin rows of cells and cell islets develop. In none of the samples were SBA-positive tubular structures observed. The SBA label showed that cells predominantly attach to the surface of the polyester fibers (Fig. 4A).

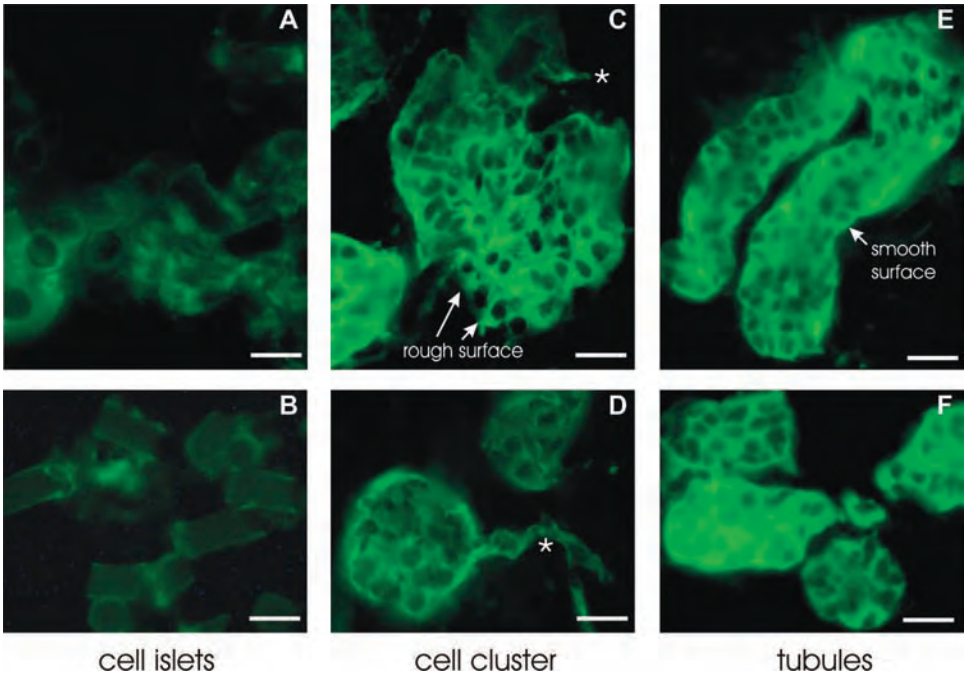


**FIG. 3.** Development of soybean agglutinin–labeled structures in embryonic renal tissue cultured for 13 days. Cell islets: An islet is defined as a group of few aggregated cells (**A**). Frequently the cells are in close contact with the polyester fibers (**B**). Polarized cells and formation of a lumen or a basal lamina are not observed. Cell cluster: A cluster is observed as an aggregate of many cells (**C**). The diameter of a cluster varies between 30 and 150 µm. Most cells in a cluster do not show polarization; consequently, no lumen cannot be seen. In some cases a discontinuous basal lamina is visible. Thus, the surface of a cell cluster is rough. The cells of a cluster show numerous filopodia, which protrude to contact polyester fibers or neighboring tissue structures as joining areas (**D**, asterisk). Tubules: Developed tubules are visible on longitudinal sections (**E**) or cross-sections (**F**) as structures exhibiting polarized cells. A lumen is visible, and a basal lamina borders the smooth outer surface of the tubule. No filopodia and no overgrowth of cells on the polyester fibers are observed. Bar = 20 µm. Color images available online at www.liebertpub.com/ten

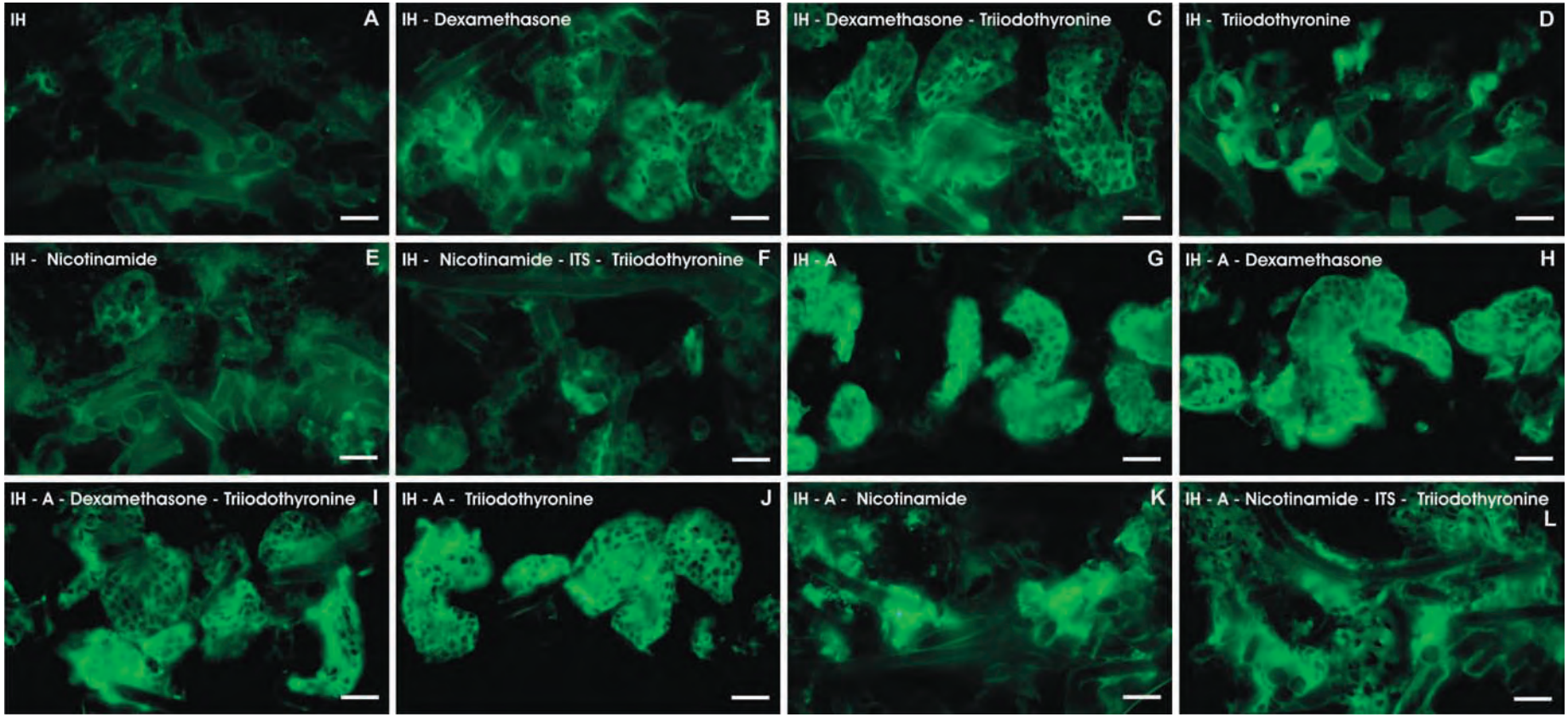


**FIG. 4.** Soybean agglutinin–labeled embryonic tissue cultured for 13 days in serum-free Iscove's modified Dulbecco's medium containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). (**A–F**) Culture without aldosterone administration. Culture without any hormonal supplementation shows few cell islets and leads to tissue disintegration (**A**). Application of dexamethasone frequently produces cell clusters beside few tubules (**B**). Treatment with dexamethasone and triiodothyronine results in big cell clusters and few tubules (**C**). Use of triiodothyronine (**D**) as well as nicotinamide (**E**) shows few cell islets and small cell clusters. Application of nicotinamide, insulin-transferrin-selenium G supplement (ITS) and triiodothyronine demonstrates only a few cell islets (**F**). (**G–L**) Culture containing $1\times10^{-7}$ M aldosterone. Application of aldosterone shows numerous tubules (**G**). The use of dexamethasone in combination with aldosterone leads to numerous cell clusters lined by a discontinuous basal lamina (**H**). Treatment with dexamethasone and triiodothyronine in combination with aldosterone shows the most extended cell clusters and few tubules (**I**). Triiodothyronine in combination with aldosterone shows numerous cell clusters and few tubules (**J**). Nicotinamide in combination with aldosterone demonstrates few small cell clusters (**K**). Application of nicotinamide, ITS, and triiodothyronine results in numerous cell clusters (**L**). Bar = 20 μm. Color images available online at www.liebertpub.com/ten

**Table 1. Perfusion Culture of Embryonic Renal Tissue for 13 days in Iscove's Modified Dulbecco's Medium Containing HEPES without Aldosterone***

| | *IH* | *IH-D* | *IH-D-T* | *IH-T* | *IH-N* | *IH-N-ITS-T* |
|---|---|---|---|---|---|---|
| Cell islets | + | | | + | + | + |
| Cell cluster | | ++ | +++ | + | + | |
| Multiple cells | | ++ | +++ | + | + | |
| Rough surface | | ++ | +++ | + | + | |
| Filopodia | | ++ | +++ | | | |
| Joining areas | | + | +++ | | | |
| Tubules | | + | + | | | |
| Polarized cells | | + | + | | | |
| Smooth surface | | + | + | | | |

*The following substances were added to Iscove's modified Dulbecco's medium (I) with HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (H): dexamethasone (D), triiodothyronine (T), nicotinamide (N), insulin-transferrin-selenium (ITS). The described structures showed differing extents of development: +++ indicates intensive, ++ indicates medium, + indicates low.

*Dexamethasone.* Compared with basic medium, the application of a glucocorticoid (dexamethasone) led to a strong increase of SBA-labeled tissue mass (Fig. 4B). Beside numerous cell clusters, only few tubules became visible. Cell clusters frequently had a diameter greater than 150 µm, contained many cells, and showed a rough surface. Under dexamethasone application, numerous cells developed long filopodia. It appears that they contact polyester fibers and neighboring cell clusters to join both areas.

*Dexamethasone-triiodothyronine.* The highest amount of SBA-labeled tissue was found in cultures treated with dexamethasone in combination with triiodothyronine (Fig. 4C). A typical finding with this hormonal supplementation are extended cell clusters combined with few tubules. The surface of the clusters was rough. Cells of these clusters frequently showed filopodia and joining areas with neighboring clusters.

*Triiodothyronine.* With the application of triiodothyronine, only some cell islets and cells clusters with a rough surface were seen (Fig. 4D).

*Nicotinamide.* The application of nicotinamide resulted in few cell islets and cell clusters showing a rough surface (Fig. 4E). Developed tubules were not observed.

*Nicotinamide-ITS-triiodothyronine.* In this set of experiments, embryonic renal tissue was cultured in IMDM that contained nicotinamide, triiodothyronine, and a cocktail of ITS. Cryostat sections showed a sponge-like tissue formation and only few SBA-labeled cells. The overgrowth of numerous cells on the polyester fibers was conspicuous. No tubules formed, and cell islets developed (Fig. 4F).

## *Experimental series containing aldosterone*

In a second set of experiments, embryonic renal tissue was cultured for 13 days in a medium that contained IMDM with HEPES plus aldosterone ($1\times10^{-7}$ M) alone or in combination with other hormones and growth factors.

*Aldosterone.* Incubation of the generated tissue with SBA showed that numerous tubules were positive for SBA, while some parts of the tissue could not be labeled with lectin (Fig. 4G). The developed tubules could be recognized in cross-sectioned and longitudinal views. Polarized cells lined a basal lamina, and the outer surface of tubules appeared smooth. Overgrowth of cells on polyester fibers, cell islets, or cell clusters was not observed.

Further experiments were performed to determine whether administration of additional hormones or growth factors can enhance the promoting effect on tubule development induced by aldosterone (Fig. 4G–L, Table 2).

*Aldosterone-dexamethasone.* Besides aldosterone, the glucocorticoid dexamethasone was applied to the culture medium in an attempt to increase the number of generated tubules. However, SBA labeling demonstrated that many cells showed only an overgrowth on the polyester fibers (Fig. 4H). In addition, the cells formed numerous cell clusters. Unlike with aldosterone application (Fig. 4G), only a few tubules were detected.

*Aldosterone-dexamethasone-triiodothyronine.* Treatment of cultures with aldosterone, dexamethasone, and triiodothyronine showed the most extended cell clusters in combination with only a few tubules (Fig. 4I).

*Aldosterone-triiodothyronine.* The combination of aldosterone and triiodothyronine led to numerous SBA-labeled cell clusters lined by an inconsistent basal lamina (Fig. 4J).

*Aldosterone-nicotinamide.* SBA labeling revealed an intensive overgrowth of cells on the fibers (Fig. 4K). Some of the cell clusters that developed exhibited a rough surface, with numerous protruding filopodia. No tubules formed.

*Aldosterone-nicotinamide-ITS-triiodothyronine.* The application of a combination of aldosterone, nicotinamide, insulin, transferrin, selenium, and triiodothyronine led to the presence of numerous SBA-labeled cell clusters with a rough surface (Fig. 4L). No tubules developed.

*Summary.* These data show that the administration of aldosterone to the culture medium supports the development of tubules at the interphase of an artifial interstitium (Fig. 4G; Table 2). In contrast, the application of dexamethasone (Fig. 4H) and triiodothyronine (Fig. 4J) alone or in combination (Fig. 4I) does not further improve the development of tubules but rather leads to an intensive growth of cell clusters and a decrease in tubule formation.

**Table 2. Perfusion Culture of Embryonic Renal Tissue for 13 days in Iscove's Modified Dulbecco's Medium Containing HEPES Supplemented with $1\times10^{-7}$ M Aldosterone***

| | *IH-A* | *IH-A-D* | *IH-A-DT* | *IH-A-T* | *IH-A-N* | *IH-A-N-ITS-T* |
|---|---|---|---|---|---|---|
| Cell islets | | | | | | |
| Cell cluster | | ++ | +++ | ++ | + | ++ |
| Multiple cells | | ++ | +++ | ++ | + | ++ |
| Rough surface | | + | +++ | ++ | + | +++ |
| Filopodia | | + | +++ | ++ | | |
| Joining areas | | + | +++ | ++ | | |
| Tubules | +++ | + | + | + | | |
| Polarized cells | +++ | + | + | + | | |
| Smooth surface | +++ | + | + | + | | |

*The following substances were added to Iscove's modified Dulbecco's medium (I) with HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (H): dexamethasone (D), triiodothyronine (T), nicotinamide (N), insulin-transferrin-selenium (ITS). The described structures showed differing extents of development: +++ indicates intensive, ++ indicates medium, + indicates low.

### *Time-dependent development of tubules*

A third set of experiments was designed to determine the time frame in which tubules develop under the presented culture conditions (Fig. 5).

Embryonic renal explants were cultured for 1, 2, 3, 5, 8, and 13 days without (Fig. 5A–F) and with (Fig. 5G–L) administration of aldosterone. In the control series, which did not use hormonal application, no SBA labeling was observed between 1 and 3 days of culture (Fig. 5A–C); however, beginning on days 5 (Fig. 5D) and 8 (Fig. 5E) and up to day 13 (Fig. 5F), a faint label was detected. SBA label was detected as faint spots within single cells beginning on day 5 (Fig. 5D) and in some cell islets from day 8 onward (Fig. 5E).

Conversely, the series with IMDM medium that contained aldosterone (Fig. 5G–L) showed a completely different result. Starting from day 1 (Fig. 5G) through day 13 (Fig. 5L), a continously increasing amount of SBA-label was detected. The development began on single cells with a puntate pattern at day 2 (Fig. 5H), increased predominantly at the luminal plasma membrane of cells that formed tubes during days 3 (Fig. 5I) and 5 (Fig. 5J), was finally found in the whole cytoplasm of developed tubular cells by day 8 (Fig. 5K), and remained visible up to day 13 (Fig. 5L).

### *Dose-dependent action of aldosterone*

A fourth set of experiments was performed to elucidate whether aldosterone acts on tubule formation in a dose-dependent fashion (Fig. 6).

Aldosterone was used in a concentration range from $1\times10^{-10}$ M to $1\times10^{-5}$ M (Fig. 6). The low dose of $1\times10^{-10}$ M (Fig. 6A) did not stimulate the development of tubules. In contrast, concentrations of $1\times10^{-9}$ M (Fig. 6B) and $1\times10^{-8}$ M (Fig. 6C) induced growth of SBA-labeled cells that formed long rows and even small clusters but no tubules. The best results were obtained with use of $1\times10^{-7}$ M (Fig. 6D) and $1\times10^{-6}$ M (Fig. 6E). Numerous intensively SBA-labeled tubules with a smooth surface were obtained. Surprisingly, the application of $1\times10^{-5}$ M (Fig. 6F) did not further stimulate the development of SBA-labeled tubules; rather, this dose reduced the number and intensity of these tubules.

### *Antagonists prevent development*

A further set of experiments was performed to interfere with the morphogenic action of aldosterone on the base of the mineralocorticoid receptor. The effect of aldosterone ($1\times10^{-7}$ M) could be completely blocked by the application of $1\times10^{-4}$ M spironolactone (Fig. 6G). No SBA-labeled tubules were observed after application of aldosterone ($1\times10^{-7}$ M) in combination with $1\times10^{-4}$ M canrenoate (Fig. 6H). Thus, both antagonists abolish the morphogenic action of aldosterone.

### *Criteria of tubule differentiation*

The last set of experiments was performed to determine the degree of cellular differentiation in generated tubules. SBA-labeling is a practical method for illuminating the overall distribution of developed tubules (Fig. 7A). Compared with immunologic markers, the use of SBA saves at least 2 h of incubation time. However, SBA labeling does not provide information about functional development. Thus, we used immunohistochemical markers to elucidate typical features of developed tubules cultured in the presence of $1\times10^{-7}$ M aldosterone for 13 days (Fig. 7B–F). Mab anti-cytokeratine 19 (Fig. 7B) and mab anti-TROMA-1 (Fig. 7C) demonstrated tubules with typical collecting duct features. The label with mab anti-sodium potassium adenosine triphosphatase showed the appearance of an important functional feature, as found within the adult collecting duct of the kidney (Fig. 7D). Primary appearance of functional polarization was detected with mab anti-occludin (Fig. 7E). Use of this antibody revealed the development of tight junctions and the possible sealing of the tubular epithelium. Labeling the cultures with mab anti-laminin $\gamma1$ indicated the development of a basal lamina (Fig. 7F). Thus, the application of aldosterone stimulates the renal stem cells to form numerous

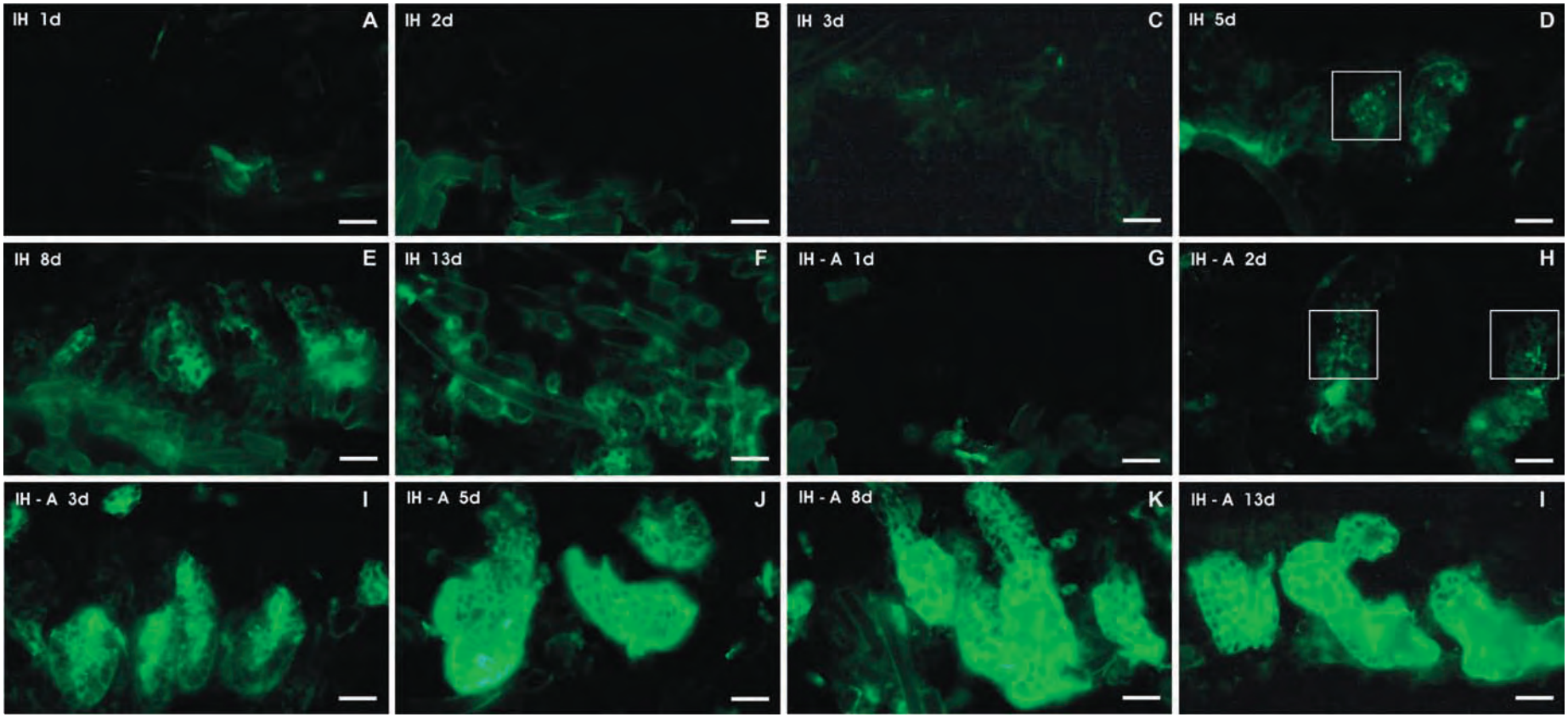


**FIG. 5.** Time-dependent development of soybean agglutinin (SBA)–labeled tubules cultured for 1, 2, 3, 5, 8, and 13 days at the interphase of an artificial interstitium without aldosterone (**A–F**) and after $1\times10^{-7}$ M aldosterone administration (**G–L**). Cultures without aldosterone application did not develop tubules. Administration of aldosterone shows no reaction after 1 day (**G**), while after 2 days a punctuated pattern within cell rows becomes visible (**H**). After 3 days, the primary cellular staining of developing tubules becomes visible (**I**). Increasing cytoplasmic SBA labeling of developing tubules is obvious after 5 days of culture (**J**). Polarized tubules become visible after 8 days of culture (**K**). Developed tubules have a smooth surface (after 13 days of culture) (**L**). Bar = 20 μm. Color images available online at www.liebertpub.com/ten

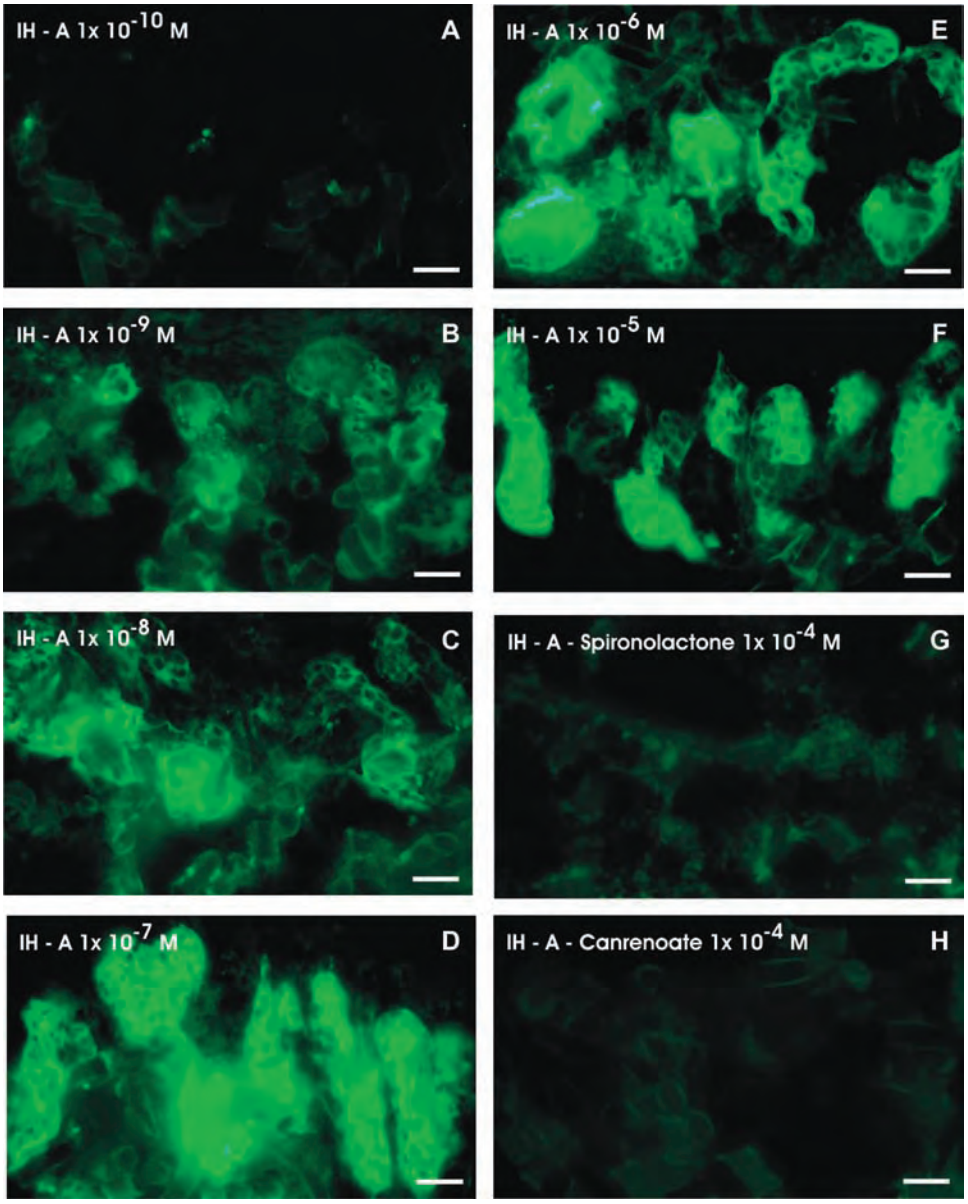


**FIG. 6.** Development of soybean agglutinin (SBA)–labeled tubules cultured for 13 days with different aldosterone concentrations. Application of $1\times10^{-10}$ M aldosterone does not show tubule development (**A**). Administration of $1\times10^{-9}$ M aldosterone leads to few labeled cell rows (**B**). Use of $1\times10^{-8}$ M aldosterone shows initial development of single tubules (**C**). $1\times10^{-7}$ M (**D**) and $1\times10^{-6}$ M (**E**) aldosterone results in the optimal development of numerous tubules. As compared with parts D and E, application of $1\times10^{-5}$ M aldosterone leads to a significant decrease in tubule development (**F**). In contrast, application of aldosterone ($1\times10^{-7}$ M) in combination with antagonists such as spironolactone ($1\times10^{-4}$ M) (**G**) or canrenoate ($1\times10^{-4}$ M) (**H**) completely abolishes the development of SBA-labeled tubules. Bar = 20 μm. Color images available online at www.liebertpub.com/ten

polarized collecting duct tubules, while omittance of the steroid hormone does not.

## DISCUSSION

While the cellular biological interactions during nephron induction have been intensively investigated, little information is available about basic mechanisms of the 3-dimensional development of tubules under *in vivo* and *in vitro* conditions.[15,16] For example, the morphogenetic factors that trigger the appearance of the renal tubular system, including the segmentation of the later proximal tubule, the loop of Henle, the distal tubule, the connecting tubule, and finally the heterogeneously composed collecting duct, are unknown. It is still unclear why parts of the proximal and distal

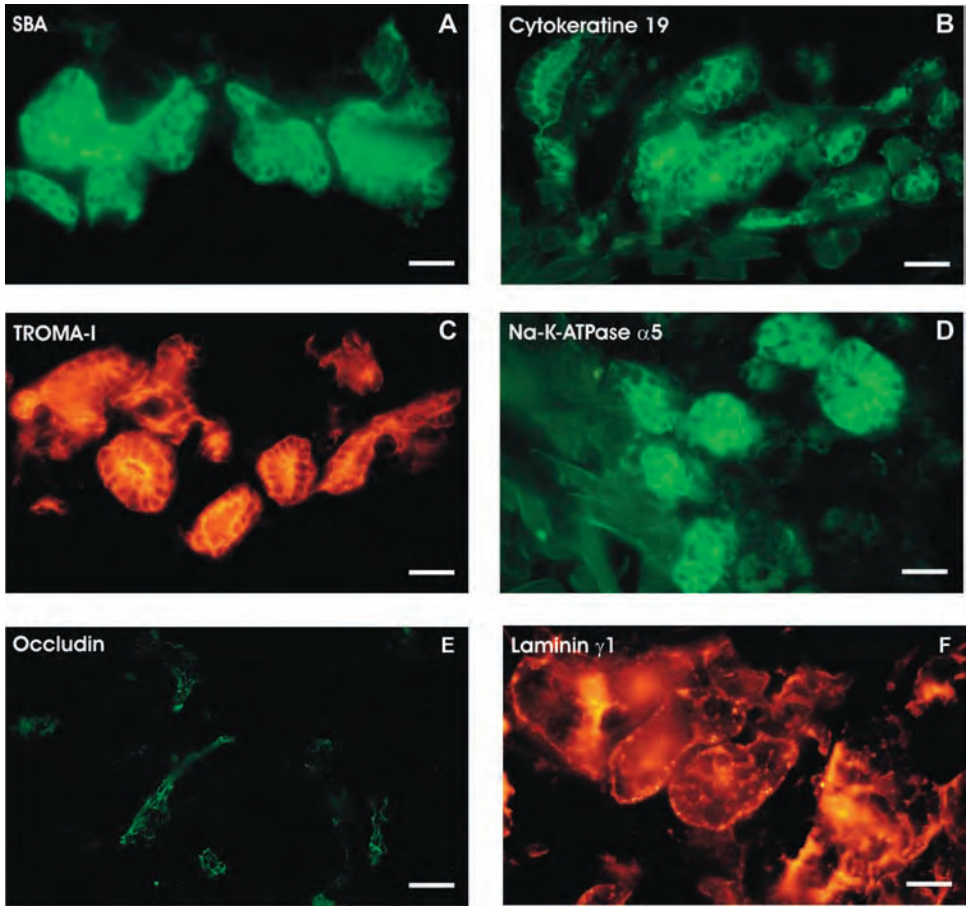


**FIG. 7.** Development of collecting duct–specific features in tubules cultured for 13 days in Iscove's modified Dulbecco's medium containing $1\times10^{-7}$ M aldosterone. Shown are soybean agglutinin–labeled tubules with a smooth surface (**A**) and immunofluorescence labeling with cytokeratine 19 (**B**), TROMA-1 (**C**), sodium potassium adenosine triphosphatase (**D**), occludin (**E**), and laminin $\gamma1$ (**F**). Bar = 20 μm. Color images available online at www.liebertpub.com/ten

tubule develop convolutes while all the other segments show a straightforward course.

### *Spreading of cells*

To investigate the development of a tubular segment under *in vitro* conditions, one can follow different culture strategies. In the past, tubules were isolated by microdissection or biochemical fractionation.[17–20] The specimens were then placed at the bottom of a culture dish and supplied by a medium containing, in most cases, serum. This classic type of culture promotes perfect cell monolayers but hampers the development of tubular structures.[21,22] When medium that contains serum or growth factors is applied the cells do not stay within the isolated tubule but emigrate and spread over its own basal lamina or on the bottom of the culture dish. Surprisingly, only few cells remain inside the isolated tubular segment. Thus, with this type of culture the isolated tubular segment does not remain in its original form and emigrated cells do not show all their original features since they dedifferentiate.[23,24] It is unknown whether the spreading cells stop cooperating with the basal lamina or whether the cells multiply too fast to synthesize enough extracellular matrix proteins for the construction of a new basal lamina during elongation of the tubular segment. Thus, we have found no reports describing cultures of isolated but intact tubules over prolonged periods.

### *Coating of cells*

An alternative strategy to micro-dissected tubules within a culture dish is the 3-dimensional culture of isolated renal cells,[25] cell lines,[26,27] or micropieces of embryonic tissue.[4,28,29] For this kind of culture, the cells in the isolated tissue are coated by a layer of extracellular matrix proteins, agar, or agarose, respectively. Embedded in collagen, Madin-Darby canine kidney (MDCK) cells, for example, start with migration, then form cell rows, and eventually build tubular structures.[30] However, the disadvantage of this technique is the need for coating with the not completely defined extracellular matrix compounds such as Matrigel (Becton Dickson, Franklin Lakes, NJ) and the application of serum-

containing media. Furthermore, the unstirred layers of medium in the stagnant environment within the culture dish or filter insert cause a deleterious accumulation of metabolites. Thus, the number of tubules available for cellular biological analysis is limited.

### *Perfusion culture in combination with an artificial interstitium*

To overcome these problems, improved culture conditions, such as a permanent perfusion of medium, have been applied.[31] To improve the culture conditions for generation of renal tubules, an advanced technique was developed.[13,32] With this process, embryonic renal tissue was microdissected without enzymatic disintegration and cultured between 2 layers of a polyester fleece within a perfusion culture container. Fresh culture medium without serum supplementation was continuosly pumped at a flow rate of 1 mL/h through the culture container. The tissue or polyester fleece was not coated with extracellular matrix proteins. Because of the limited size of embryonic mouse or rat specimens, we selected the neonatal rabbit as a cellular biological model, since even after birth the embryonic cortex contains numerous stem cell niches in their original extracellular environment.[7,33] The embryonic tissue layer is easily accessible for isolation and can be harvested in sufficient amounts for perfusion culture or further cellular biological analysis.

### *Morphogenic modulation with steroid hormones*

One would assume that growth factors such as fibroblast growth factor, transforming growth factor-$\alpha$, glial cell line–derived neurotrophic factor, hepatocyte growth factor, or vascular endothelial growth factor (VEGF) stimulate the development of tubules as described elsewhere.[34] However, recent experiments with endothelial growth factor did not confirm an improvement in tubule growth.[32] In contrast, by using the new culture technique, in the present study we demonstrate that application of aldosterone stimulates embryonic renal cells to form numerous polarized tubules derived from renal stem cells (Figs. 4G, 5L, 6D, 6E, 7A). This development could be inhibited by the application of antagonists such as spironolactone (Fig. 6G) or canrenoate (Fig. 6H). Immunohistochemistry further revealed that aldosterone promotes the generation of renal collecting duct–derived tubules as recognized by SBA (Fig. 7A), cytokeratine 19 (Fig. 7B), and TROMA-1 (Fig. 7C) labeling. Immunolabeling with mab anti-laminin $\gamma$1 (Fig. 7F) showed the development of a basal lamina, while mab anti-occludin (Fig. 7E) revealed the development of tight junctions. Mab anti-sodium potassium adenosine triphosphatase demonstrated the primary appearance of an important functional feature as found within the adult kidney (Fig. 7D). All these findings support the assumption that the tubules develop an apico-basal polarization.

The present experiments show that the application of a glucocorticoid such as dexamethasone instead of aldosterone (Fig. 4B) or in combination with aldosterone (Fig. 4H) does not further improve the development of tubules. In contrast, the application of dexamethasone stimulates numerous cells to spread over polyester fibers (Fig. 4B). In addition, under this hormonal treatment, tubules do not increase in number; rather, large cell clusters are formed between the fibers. Although the total mass of SBA-labeled cells increases, dexamethasone does not support the development of tubules. To our knowledge it is a new finding that the appearance of cell clusters (Fig. 4B, C, H,I) competes with the development of tubules (Figs. 4G, 5L, 6D, 6E, 7A). At present, we can only speculate about the reasons that aldosterone generates tubules while dexamethasone induces cell clusters.

Recent publications support the view that external as well as resident renal stem cells are involved in kidney regeneration after injury.[35,36] Newly developed tubules can arise from exogenous and endogenous cell populations in the case that these cells proliferate and differentiate. To realize this growth in a coordinated fashion, the action of signaling molecules is essential. Our experiments support the concept that aldosterone is able to direct such development. Whether this action of aldosterone is genomic or nongenomic remains to be investigated.[37,38]

### *Conclusions*

Our present experiments provide important new information on the development and long-term maintenance of renal tubules in long term perfusion culture. Aldosterone stimulates the development of tubules derived from renal stem cells in a concentration-dependent fashion. In contrast, dexamethasone leads to a morphogenic change by reducing development of tubules increasing the formation of cell clusters.

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