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SBA-Positive Fibers between the CD Ampulla, Mesenchyme, and Renal Capsule

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Abstract. During kidney development, the CD shows two peculiarities. First, the tip of the CD ampulla is always found at a specific distance from the organ capsule. Second, the CD growth occurs as a perfectly straight elongation. It is unknown whether the CD-specific growth is dependent on hormonal action or on structural elements. Histochemical experiments on neonatal rabbit kidney yielded new insight into the interface of the CD ampulla and the surrounding nephrogenic mesenchyme. Incubation of tissue sections with soybean lectin (SBA) showed the existence of fibers extending in a radial course from the ampullar tip through the mesenchyme toward the organ capsule. SBA labeling did not colocalize with collagen type I, III, IV, V, and VI, laminin, fibronectin, and tenascin. It is assumed that while the kidney increases in volume the structural fixation of the ampullar tip by the SBA-positive fibers causes CD ampullae to maintain a constant distance from the organ capsule. The connection would explain the linear extension of the CD in relation to the organ capsule. In addition, the presented data suggest that the SBA-positive fibers between ampullar tip and organ capsule create a structural microcompartmentation of the nephrogenic zone.

Ingrowth of the ureteric bud into surrounding nephrogenic mesenchyme marks the onset of metanephros development (1). The first dichotomous branching determines the kidney poles. Subsequent branchings form the three-dimensional structure of the later renal pelvis. In contrast to these early structural branchings, later branchings of the uretric bud give rise to nephrons. The initial CDs with their blindly ending ampullae develop from the uretric bud, and the process of nephrogenesis sets in. The CD ampullae induce competent cells within the surrounding mesenchyme, which condensate to form comma-shaped and later S-shaped bodies (1,2). By further dichotomous branching and successive elongation of the CD, the ampullae are pushed further into the uninduced mesenchyme toward the capsule, where the next generation of nephrons is generated. Cellular interactions during CD arborization (branching morphogenesis) and nephrogenesis have been studied extensively by morphologic (3–6), cell biologic (7–11), and molecular biologic (12–13) methods.

Throughout metanephros development the elongation and branching of the CD determines the layout for the three-dimensional histoarchitecture of the kidney. In a corticomedullary section of the neonatal rabbit kidney, the directional growth of the CD straight toward the capsule contrasts with the surrounding highly convoluted tubules (Figure 1a). Impaired CD development leads to a reduced number of CD ampullae (14), which in turn leads to a decrease in nephron formation (8), segmental atrophy (Ask-Upmark-syndrome) (15), and eventually to the formation of cysts (16). It could be demonstrated in renal tissue cultures that transforming growth factor–β (TGF-β) leads to an increased branching rate of the CD (17), whereas TGF-α leads to a decrease (18). HGF represents another inhibiting signal via its receptor cMET. These signals are transduced by protein kinase C, a cAMP-dependent protein kinase and phosphoinositol-3-kinase (19,20).

There must be a specific reason why the CD exhibit directional growth from the medulla out to the cortex corticis while neighboring nephron segments, such as proximal and distal tubule, form convolutes. Directional growth of the CD cannot be explained by action of morphogens, growth factors, or extracellular matrix alone, but there must be a master-system that determines the histoarchitectural layout in form of a superordinated morphogenic field (21,22). Directional growth as observed in the CD is unique for the kidney and can be found neither in excretory ducts of salivary glands nor in bile duct, bronchial branching, or duct system of the exocrine pancreas. All these organs develop by branching morphogenesis alone, whereas the kidney is formed by a combination of branching morphogenesis and nephrogenesis.

In recent years knowledge of molecular interactions between nephron inducer and the surrounding nephrogenic mesenchyme has increased (Table 1). However, there is little data on morphologic features of the interface between these tissues (3,5,6). It has been assumed in the past that nephrogenic mesenchyme is randomly arranged around the tip of the CD. It

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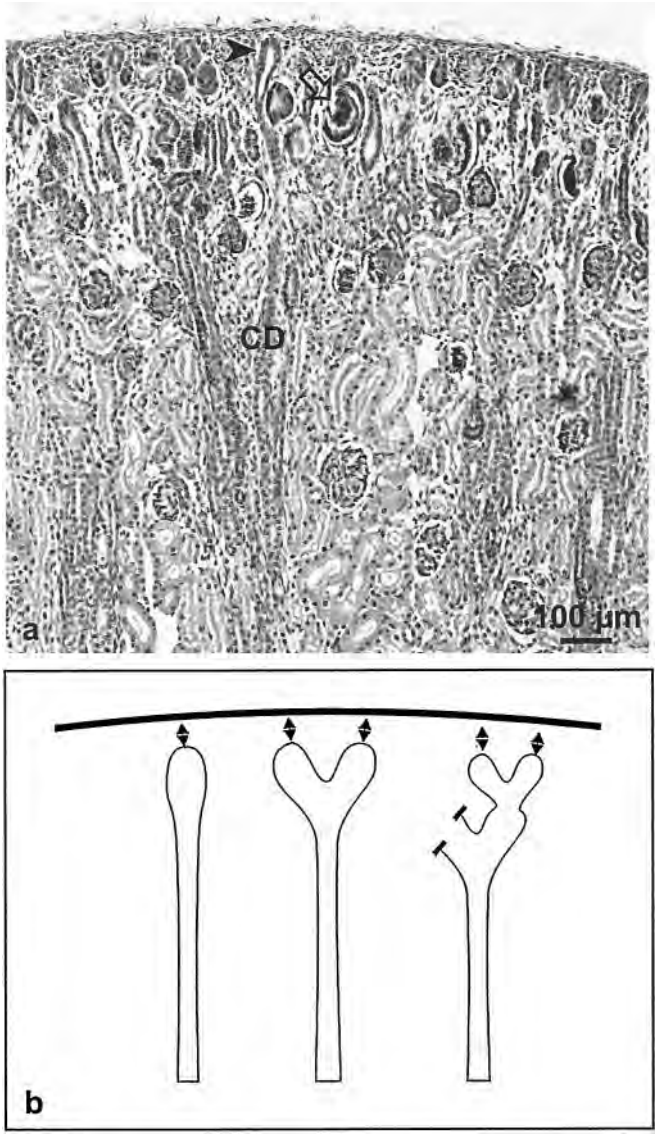

Figure 1. Hematoxylin-eosin–stained section of the cortex of neonatal rabbit kidney. (a) The maturing collecting duct (CD) ends in an ampulla (black arrowhead) underneath the organ capsule. Laterally a developing nephron (arrow) can be observed. (b) Schematic drawing illustrating how individual ampullae as well as dichotomous branchings maintain a constant distance from the organ capsule.

has also been assumed that the ampulla finds its way by itself. Using morphologic and immunohistochemical methods we could demonstrate for the first time that a structural connection exists between the ampullar tip and the renal capsule. As the kidney increases in size, we assume that this mechanism could help the ampullar tips maintain their orientation toward the capsule. This could explain the linearity of growth that is observed in CD.

Material and Methods

Tissue Preparation and Light Microscopy

One-day-old New Zealand rabbits were anesthetized with ether and killed by cervical dislocation. Both kidneys were removed immediately. The kidneys were then cut precisely along the corticomedullary axis. Human renal tissue was obtained from an anencephalic male fetus at week 14 of pregnancy and from a fetus at week 20 of pregnancy. Neonatal rabbit kidney and human fetal kidney (week 20 of pregnancy) were fixed in paraformaldehyde and embedded in paraffin. Sections were subsequently stained with hematoxylin-eosin.

Lectin Incubation

Corticomedullary oriented cryosectins (8 μm) of neonatal rabbit and human fetal kidney (week 14 of pregnancy) were prepared using a cryomicrotome (Microm, Heidelberg, Germany). Fixation in ice-cold ethanol was followed by washing steps in phosphate-buffered saline (PBS). The sections were then incubated in blocking solution (PBS + 1% bovine serum albumin [BSA] + 10% horse serum) for 30 min. The fluorescein-isothiocyanate (FITC)–conjugated lectin Soybean Agglutinin (SBA; Vector Laboratories, Burlingame, VT) was applied 45 min in blocking solution 1:4000. SBA preferentially binds to oligosaccharide structures with terminal α- or β-linked N-acetylgalactosamine and to a lesser extent galactose residues. After several washes in PBS, the specimens were embedded with Slow Fade Light Antifade Kit (Molecular Probes, Eugene, OR) and analyzed using an Axiovert 35 microscope (Zeiss, Oberkochen, Germany).

Co-Incubation Experiments

Co-incubation experiments were performed with SBA and mouse monoclonal antibodies, which recognize specifically rabbit type I and III collagen (Medicorp, Montreal, Canada). To reveal the basal aspect of the CD ampulla, mab anti-P_{CD}Amp 1 (6) was applied together with FITC-conjugated soybean agglutinin. Corticomedullary oriented cryosectins (8 μm) of neonatal rabbit kidneys were fixed in ice-cold ethanol and rinsed three times with PBS. Sections were blocked with 10% horse serum and 1% BSA in PBS. Type I collagen antibody diluted 1:800 or type III collagen antibody diluted 1:4000 were applied for 1 h. After rinsing three times with PBS containing 1% BSA, the sections were incubated with Texas Red conjugated donkey/anti mouse IgG (diluted 1:200; Jackson Immunoresearch, West Grove, PA) as secondary antibody together with FITC-conjugated SBA (diluted 1:4000) for 45 min. After washing in PBS, the sections were embedded with a Slow Fade Light Antifade Kit (Molecular Probes). The incubated sections were examined with an Axiovert 35 microscope (Zeiss, Oberkochen, Germany).

Culture Experiments

Cortical explants from kidneys of newborn New Zealand rabbits (up to 1-d-old) were isolated according to methods described earlier (25). The explants consisted of a piece of capsule fibrosa with adherent CD ampullae, S-shaped bodies, and nephrogenic blastema, which were mounted in tissue carriers. The tissue was placed in a 24-well plate containing Iscove's modified Dulbecco's medium (IMDM; Life Technologies-BRL Life Technologies, Eggenstein, Germany) including 10% FBS (Boehringer, Mannheim, Germany) for 24 h in an incubator (5% CO_2/95% air). The tissue carriers were subsequently transferred into a perfusion culture container (Minucells and Minutissue, Bad Abbach, Germany). IMDM (order #21980-032; Life Technologies BRL-Life Technologies) containing aldosterone (1×10^{-7} M; Sigma-Aldrich-Chemie, Deisenhofen, Germany) and 1% antibiotic-antimycotic solution (Life Technologies BRL-Life Technologies) was continuously perfused for 14 d at a rate of 1 ml/h with an IPC N8 peristaltic pump (Ismatec, Wertheim, Germany). The waste medium was collected separately in bottles.

Table 1. Cellular and extracellular molecules occurring at the interface between mesenchyme and ampulla tip in the developing kidney[a]

Mesenchyme		Ampulla Tip	
Cellular	Extracellular	Cellular	Extracellular
Glypican-3 (24)	Fibronectin (5)	Integrin α3a (14)	Osteopontin (45)
Angiopoietin-1 (25)	Tenascin (37)	Wnt-11 (7)	P_{CD}Amp1 (6)
Wnt-4 (7)		Calbindin-D_{28K} (38)	Laminin α1 (3)
Hoxa11 (26)		BMP-7 (9)	Galectin-3 (46)
Hoxd 11 (26)		Pax-2 (39)	Hyaluronan (47)
Glypican-5 (27)		LIF (10)	Nephronectin (29)
BMP-7,-2 (9)		Integrin β1 (40)	Laminin α5 (23)
NCAM (28)		Integrin α6 (4)	collagen type IV (5)
Integrin α8 (29)		c-met (15)	
Pax-2 (30)		c-ret (41)	
Syndecan-1 (31)		FGF-2 (42)	
Syndecan-2 (32)		Ret (12)	
sfrp-1,-2 (33)		c-ros (43)	
WT1 (16,34)		E-cadherin (12)	
BCL2 (16,34)		TROMA-I (44)	
LIFR (10)		Sox9 (44)	
GNDF (35)			
HGF (11)			
Polycystin (36)			

[a] Only publications showing clear illustrations are cited.

Microdissection Procedure

CD ampullae with S-shaped bodies from cortical explants of newborn New Zealand rabbit kidneys were microdissected under optical control of a KL 1500 stereomicroscope (Leica, Solms, Germany). The microdissected material was transferred to a microscope slide and fixed in ice-cold ethanol. After incubation with FITC-conjugated SBA (diluted 1:4000 in blocking solution), the specimens were covered by a cover glass and examined with an Axiovert 35 microscope (Zeiss, Oberkochen, Germany).

Results

Light microscopical analysis of the embryonic cortex of neonatal rabbit kidney shows that CD exhibit linear growth from the medulla to the subcapsular region (Figure 1a). CD run evenly spaced and in parallel. The ampullar tips are found at an average distance of 20 μm beneath the capsule (Figure 1b). This distance is maintained whether ampullae are in the process of branching or not.

Light microscopical analysis shows a wide cleft around the CD ampullae spatially separating the nephron inducer and the competent mesenchyme (Figure 2, a and d). This cleft is not species-specific but can be observed in neonatal rabbit kidney (Figure 2a) and in embryonic human kidney, week 20 of pregnancy (Figure 2d), mouse and rat as well (1,10,16). There is no close contact between the two tissues. It is obvious that contacts between mesenchymal cell processes and epithelial cells are not frequent. Earlier investigations by Lehtonen *et al.* (48) and by our group (6) have demonstrated the existence of a characteristic extracellular matrix at this site. The basal aspect of the CD ampulla differs from the matrix surrounding other tubules. Measurements yielded that the matrix layer around the ampullar tip averages 1.6 μm (0.2 to 3.1 μm) in thickness.

The CD ampulla plays an important role during the histoarchitectural layout of the organ during kidney development. As the kidney increases in size, the necessary elongation of the CD is driven by cell divisions in the ampullar neck zone. The CD ampulla maintains a constant distance to the renal capsule and does not deviate from linear elongation (Figure 1). Obviously, this is possibly due to a structural connection between the CD ampulla and the renal capsule. Present immunohistochemical incubations with soybean agglutinin (SBA) show the existence of fibers running from the ampullar tip to the capsule. These fibers can be demonstrated in neonatal rabbit (Figure 2, b and c) as well as in human embryonic kidney, week 14 of pregnancy (Figure 2, e and f).

Analyzing the cortex of neonatal rabbit kidney after SBA incubation reveals that the fiber network is exclusively found between the CD ampulla, the surrounding mesenchyme, and the covering organ capsule (Figure 3a). Downward to the ampullar neck and shaft, no SBA-positive fibers are visible. Around the cortical (Figure 3a) and medullary CD (Figure 3b), no SBA-positive fibers can be detected. The interstitium of matured kidney is completely free of SBA-positive fiber material. Only in the renal pelvis, some SBA positive fibers can be seen beyond the epithelium (Figure 3c).

Earlier immunohistochemical assays showed that P_{CD}Amp1

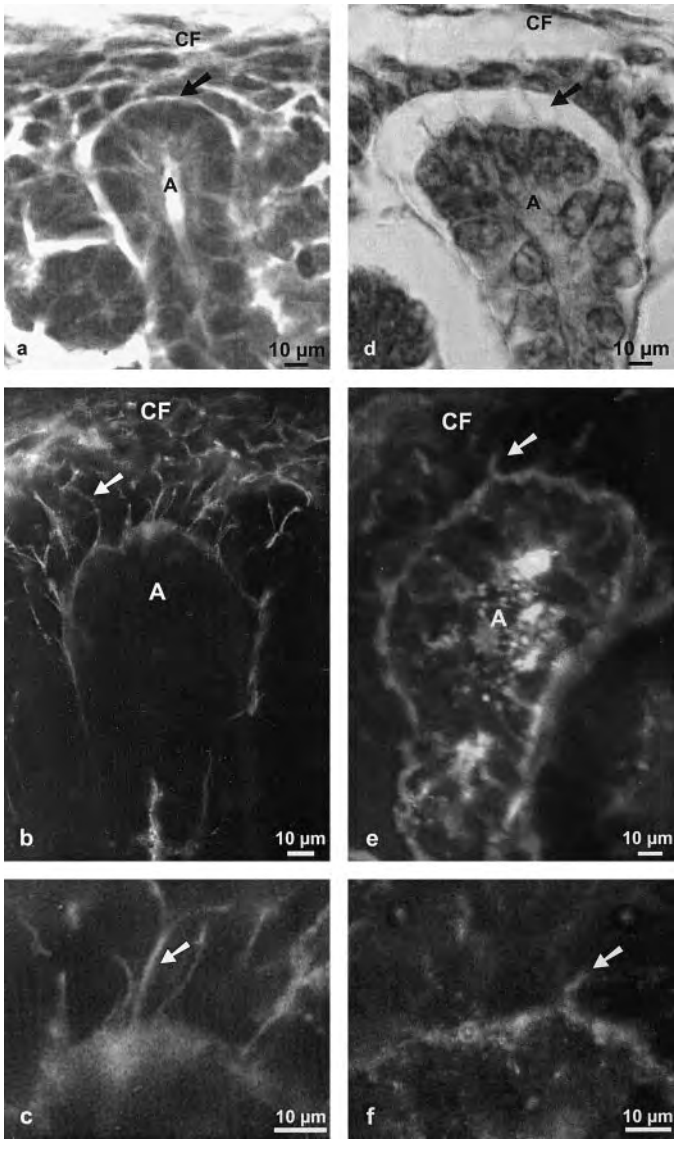

Figure 2. Microscopical micrographs of the epithelial-mesenchymal interface. A light-microscopically visible cleft (arrow) between CD ampulla and the surrounding mesenchyme exists both in the neonatal rabbit kidney (a) and in the embryonic human kidney (week 20 of pregnancy) (d). Histochemical incubation with soybean lectin (SBA) revealed that SBA-positive fibers (arrow) extend through this cleft toward the organ capsule in neonatal rabbit kidney (b) as well as in embryonic human kidney (e). Details illustrating SBA-positive fibers (arrow) at the epithelial-mesenchymal interface in neonatal rabbit kidney (c) and in human embryonic kidney (f). A, CD ampulla; CF, fibrous organ capsule.

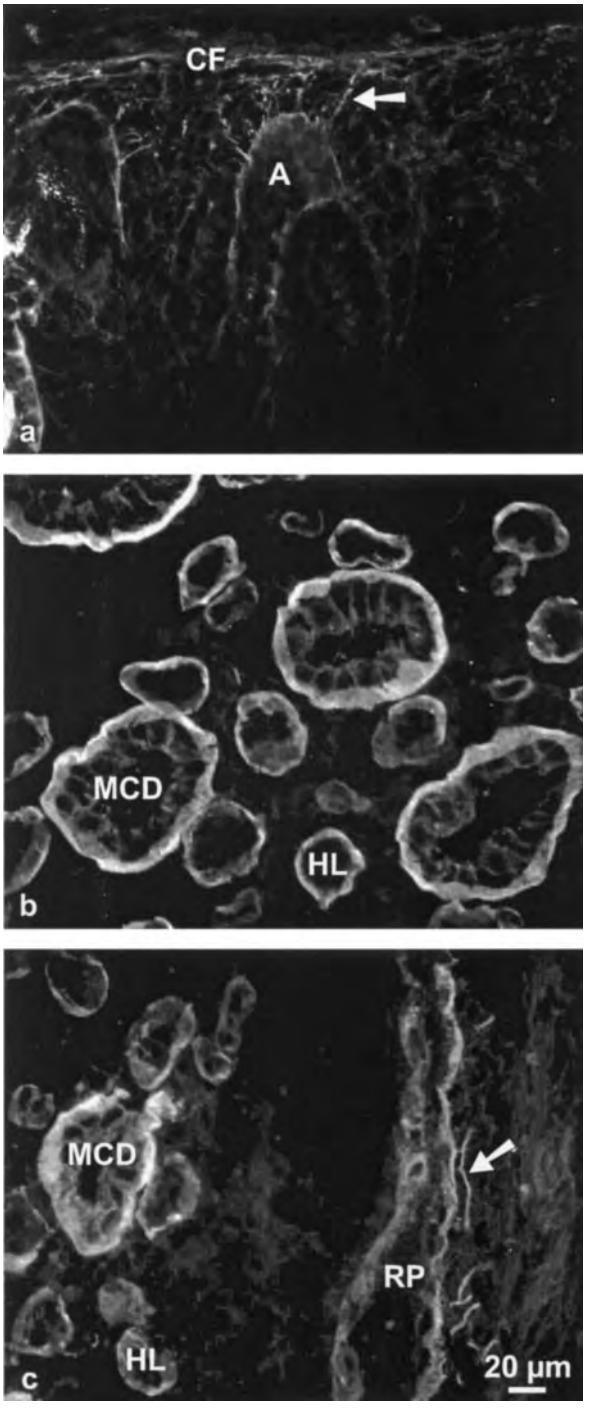

Figure 3. Distribution of SBA labeling in neonatal rabbit kidney. (a) Embryonic zone: the lectin particularly labels fibers (arrow) at the basal aspect of the CD ampulla (A) that head for the organ capsule (CF). SBA-positive fibers are absent in half-matured tissue. (b) Matured zone: the basal aspect of medullary CD (MCD) and thin limbs of loop of Henle (HL) reacts strongly with SBA. (c) Renal pelvis region: SBA binds to epithelial cells of the renal pelvis. Few fibers (arrow) are strongly positive for SBA in the tissue that surrounds the renal pelvis epithelium (RP).

(6) is colocalized with osteopontin (45) in the extracellular matrix around the ampulla. Both proteins are strongly expressed at the ampullar tip while their expression decreases along the maturing CD. Several other proteins and proteoglycans can be found at the basal aspect of the CD ampulla, whereas only tenascin and fibronectin are expressed in the surrounding mesenchyme (Table 1). We consequently investigated whether the SBA-positive fibers contain known proteins at this site. Double-labeling of the tissue shows that the extending fiber material is not positive for P_{CD}Amp1 (Figure 4a), collagen type I (Figure 4c), and collagen type III (Figure 4e),

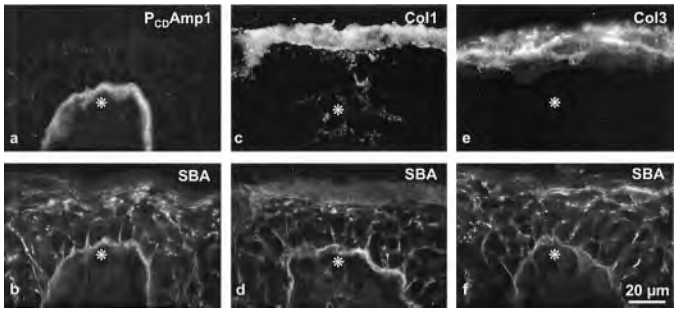

Figure 4. Colocalization experiments of SBA-positive fibers with P_{CD} Amp1, collagen type I and III. (a) P_{CD} Amp1 is expressed at the basal aspect of the CD ampulla (asterisks). (b) No colocalization could be observed with extending SBA-positive fibers. Collagen type I (c) and III (e) antibodies react strongly with fibers located in the region of organ capsule. However, a reaction with SBA-positive fibers present between CD ampulla and the organ capsule could not be observed (d and f).

but it is positive for SBA (Figure 4, b,d, and f). Further colocalization experiments revealed that SBA-positive fibers do not colocalize with collagen IV, V, and VI, laminin, fibronectin, and tenascin (no figure).

Microdissection experiments were performed to answer the question of whether SBA-positive fibers are visible when the ampullae are microsurgically isolated by forceps (Figure 5, a and b). Whole mount labeling of an isolated dichotomous branched CD ampulla shows numerous fibers at the outside of the ampullar tip. After preparation, the SBA-positive fibers remain attached to the surface and are not lost in the surrounding mesenchyme (Figure 5a). Higher magnification of the explants gave the impression that the fibers are not randomly distributed but focused to individual focal points in the basement membrane (Figure 5b). Culture experiments should further reveal how far SBA-positive fibers are maintained or lost during culture. The tissue explants demonstrated that the majority of cultured ampullae lost the SBA-positive fibers during a 14-d perfusion culture period (Figure 5c). However, only in a few ampullae could SBA-positive fibers be detected with reduced fluorescence signal compared with freshly isolated tissue.

Discussion

Induction of nephrons is triggered between the ampulla tip of the CD and the surrounding nephrogenic mesenchyme. Although the initial inductive signal is not yet discovered, subsequent cell biologic interactions between both tissues have been investigated in great detail (7,9,11,12,13,19,26,39). It is further unknown to date whether nephrogenesis is initiated solely by an exchange of soluble factors (10,33) or by direct cell-cell contacts, as demonstrated in transfilter experiments (49), or by a combination of both.

The present morphologic results of embryonic renal tissue show that mesenchymal cells are not randomly distributed around the ampulla tip (Figure 2) and that a distinct space is present at the interface of the CD ampulla and the surrounding mesenchyme (Figure 2,a and d). SBA-positive fibers are demonstrated to protrude from the basal aspect of the CD ampulla tip through the mesenchyme toward the capsula fibrosa (Figure 2, b, c, e, and f). The fibers originate from the ampulla tip, a region where WNT-11 (7), ret (12,41), osteopontin (45), and P_{CD}Amp 1 (6) are exclusively expressed (Figure 6a).

Experiments of Mounier *et al.* (5) in human fetal kidney revealed that type I and III collagens are absent in the mesenchyme surrounding the ampulla tip. These findings are confirmed by our results in neonatal rabbit kidney (Figure 4, c and e). In addition, type I and III collagens do not colocalize with SBA-positive fibers (Figure 4, d and f). Further colocalization experiments revealed that SBA-positive fibers do not colocalize with collagen IV, V, and VI, laminin, fibronectin, and tenascin (no figure). It is a new finding that fibers labeled by SBA originate radially from the ampullar tip, through the surrounding mesenchyme, and toward the capsula fibrosa (Figure 6a). In our opinion, these fibers lead to a structural microcompartmentation between the nephron inducer and the surrounding mesenchyme (Figure 2, b and e). In addition, the described fibers may help answer questions concerning cellular communication and spatial relations during nephrogenesis. For example, mechanical fixation of the ampullar tip at the capsule by the SBA-positive fibers could maintain a constant spatial relation between CD ampulla and mesenchyme throughout organ growth. It is known that longitudinal growth of tubules takes place along a basement membrane that displays embryonic characteristics and contains α1-laminin, α5-laminin (3,23), and fibronectin (5). However, it is unknown how the direction of tubular growth is determined. It can be assumed that the CD ampulla is attracted by secreted factors or hormonal action from the surrounding mesenchyme to grow toward the organ capsule. Factors like HGF, which promotes CD growth, could act in this fashion (11). In this case, the hormone-secreting cells have to remain at a constant distance to the ampulla. The maintenance of this position nearby the ampulla could be attributed to extracellular structures, which are revealed by the SBA labeling. Straight tubular elongation requires synchronized cell divisions to occur in opposing walls. If more divisions occur in one wall site, a change of growth direction would inevitably result. The growing duct would slant toward the side where fewer divisions take place. Therefore modulation of the frequency of cell division could very well be a mechanism to determine directional growth and convoluted growth. Regulation of cell division in combination with a structural connection in form of SBA-positive fibers would lead to the perfect linear growth.

Further, cellular processes such as filopodia and lamellopodia could use these extracellular fibers as guide structures to grow along (Figure 6b). Microscopic processes originating from ampullar epithelial cells could follow the three-dimensional matrix scaffold to reach mesenchymal cells and exchange morphogenic signals (15). Such cellular processes have not been discovered in the embryonic kidney so far. However, transfilter tissue culture experiments have demonstrated the formation of processes between spinal cord and nephrogenic mesenchyme during induction. If pore size is reduced so that

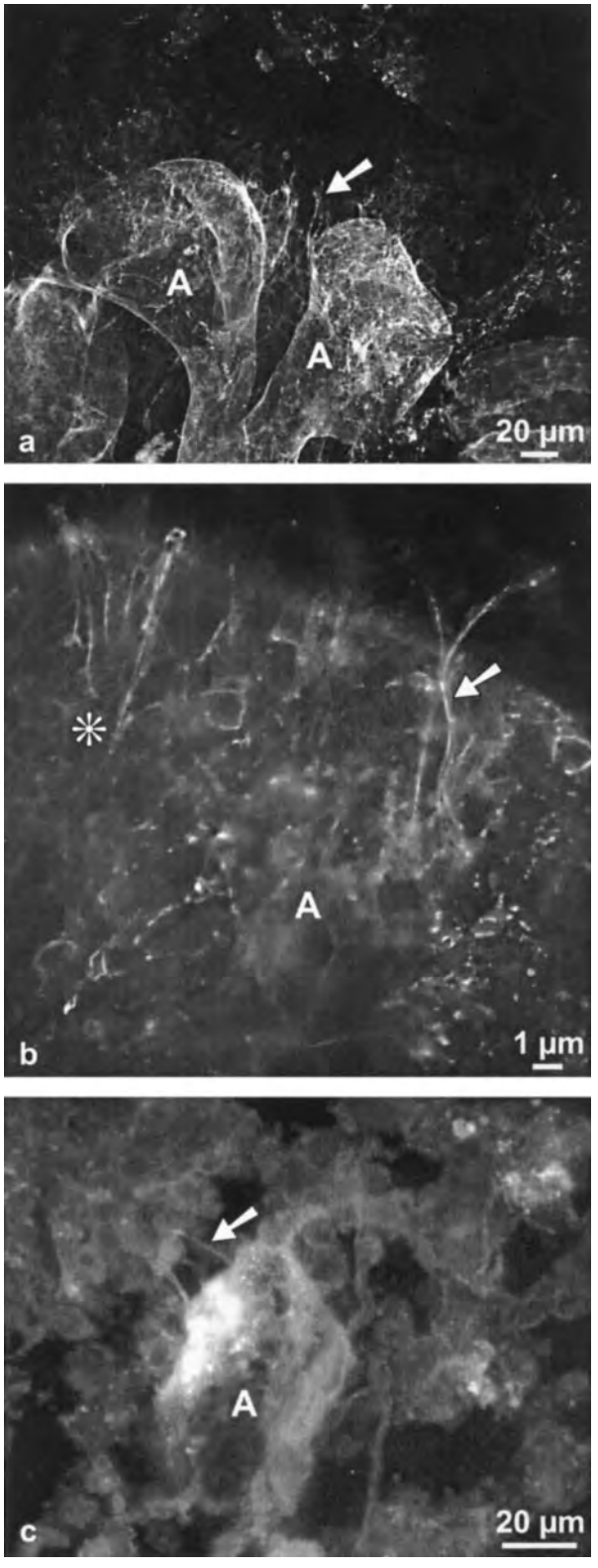

Figure 5. SBA incubation of microdissected CD ampulla (A). (a) Both ends of a branching CD are labeled with SBA. The SBA-positive fibers remain fixed at the basal aspect of the CD ampulla. (b) Higher magnifications show fibers that originate from the CD ampulla (asterisks). (c) Culture experiments: after 14 d of culture under serum-free conditions, the CD ampullae (A) and fibers (arrow) are still present in the renal cortical explants. However, the signal for SBA binding to the fibers appears reduced.

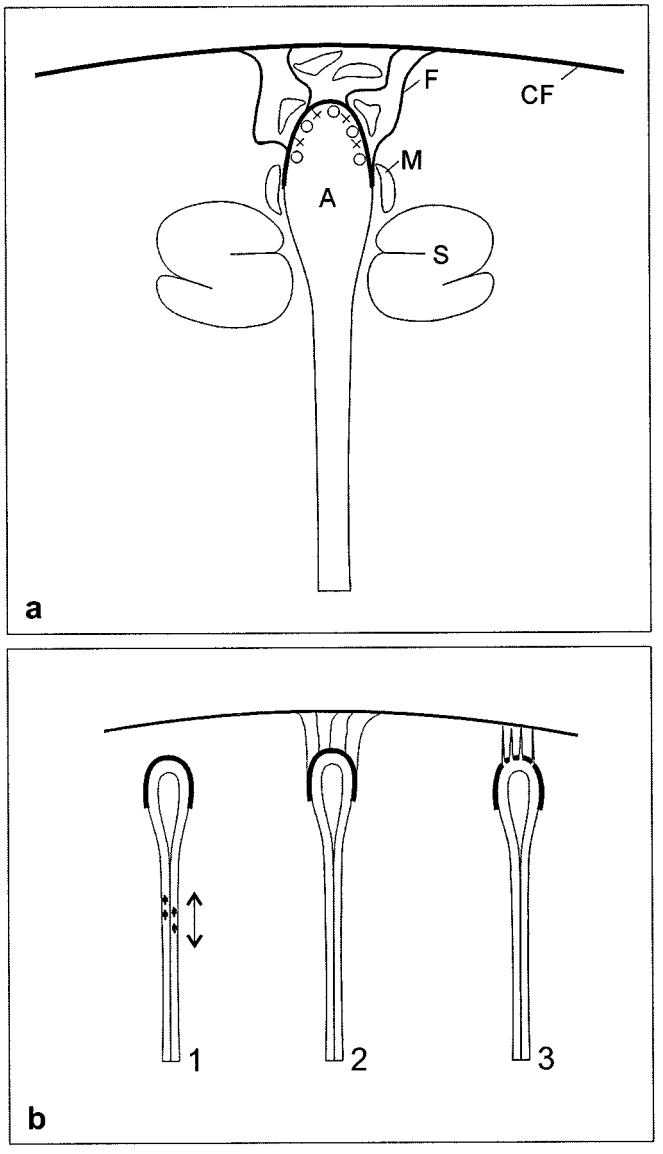

Figure 6. (a) Schematic representation of structural elements between the CD ampulla (A), the nephrogenic mesenchyme (M) and the organ capsule (CF). SBA-positive fibers (F) are present between the basal aspect of the ampulla and the capsule. Along their course, the fibers come in contact with mesenchymal cells. The ampullar tip expresses WNT-11 (o) and ret (x). S, S-shaped-body. (b) Hypothetical illustration of growth in renal CD. (1) The CD elongates by cell divisions in the ampullar neck region. (2) During kidney development, fibers could cause a connection between the ampullar tip and the renal capsule. (3) Cellular processes, such as filopodia and lamellopodia, could use these extracellular fibers as guide structures.

processes cannot establish cell-cell contacts, no induction of tubules takes place (49).

Our current data show for the first time structural elements between the CD ampulla, the nephrogenic mesenchyme, and the organ capsule. Ongoing experiments will show when these fibers appear during development, which kind of cell synthesizes them, what their molecular makeup is, and how the fibers are rearranged during dichotomous branching of the CD ampulla.

Acknowledgments

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