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Induced repatterning of type XVIII collagen expression in ureter bud from kidney to lung type: association with sonic hedgehog and ectopic surfactant protein C

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SUMMARY

Epithelial-mesenchymal tissue interactions regulate the formation of signaling centers that play a role in the coordination of organogenesis, but it is not clear how their activity leads to differences in organogenesis. We report that type XVIII collagen, which contains both a frizzled and an endostatin domain, is expressed throughout the respective epithelial bud at the initiation of lung and kidney organogenesis. It becomes localized to the epithelial tips in the lung during the early stages of epithelial branching, while its expression in the kidney is confined to the epithelial stalk region and is lost from the nearly formed ureter tips, thus displaying the reverse pattern to that in the lung. In recombinants, between ureter bud and lung mesenchyme, type XVIII collagen expression pattern in the ureter bud shifts from the kidney to the lung type, accompanied by a shift in sonic hedgehog expression in the epithelium. The lung mesenchyme is also sufficient to induce ectopic lung surfactant protein C expression in the ureter bud. Moreover, the shift in type XVIII collagen expression is associated with changes in ureter development, thus resembling aspects of early lung type epigenesis in the recombinants. Respecification of collagen is necessary for the repatterning process, as type XVIII collagen antibody blocking had no effect on ureter development in the intact kidney, whereas it reduced the number of epithelial tips in the lung and completely blocked ureter development with lung mesenchyme. Type XVIII collagen antibody blocking also led to a notable reduction in the expression of *Wnt2*, which is expressed in the lung mesenchyme but not in that of the kidney, suggesting a regulatory interaction between this collagen and Wnt2. Respecification also occurred in a chimeric organ containing the ureter bud and both kidney and lung mesenchymes, indicating that the epithelial tips can integrate the morphogenetic signals independently. A glial cell line-derived neurotrophic factor signal induces loss of type XVIII collagen from the ureter tips and renders the ureter bud competent for repatterning by lung mesenchyme-derived signals. Our data suggest that differential organ morphogenesis is regulated by an intra-organ patterning process that involves coordination between inductive signals and matrix molecules, such as type XVIII collagen.

Key words: Patterning, Organogenesis, Kidney development, Lung development, Type XVIII collagen, Endostatin, Frizzled, Sonic hedgehog, Mouse

INTRODUCTION

A typical feature of organogenesis is that the process depends on tissue interactions that in most cases occur between the epithelium and the mesenchyme (Saxén, 1987; Thesleff et al., 1995; Vainio and Müller, 1997). Classical grafting experiments have suggested that in many of the model systems the mesenchyme may direct epithelial morphogenesis, but more recent studies have also suggested an active role for the epithelium in the control of morphogenesis (Mina and Kollar, 1987; Sengel, 1990; Roberts et al., 1998).

The embryonic kidney and lung are useful models for studying the molecular mechanisms of morphogenetic tissue interactions. Both undergo a characteristic epithelial branching morphogenesis that starts with the formation of an epithelial bud that later contributes to differences in the form of these organs by means of differential branching (Saxén, 1987; Hogan et al., 1998; Metzger and Krasnow, 1999). The branching of the epithelial bud in the lung is stereotypic and asymmetric. The first three secondary buds in the right bronchus of the adult mouse develop to form the four lobes, whereas the left bronchus generates only one lobe. In the kidney, the ureter bud

invades the mesenchyme, after which it divides and follows the formation of the first few branches. This takes place in a characteristic dichotomous manner, which is different from that of the lung type (Saxén, 1987).

Analyses of the inducers involved in many systems, including the lung and kidney, have revealed that a fairly small number of signaling families may be implicated, such as epidermal growth factor (EGF), fibroblast growth factor (FGF), hedgehog, transforming growth factor beta (TGFβ) and Wnt (Hogan, 1999). It also seems to be typical for the signals to operate as a network and for their activities to be antagonized by binding factors that modulate diffusion or receptor activation, for instance (Perrimon and Duffy, 1998; Hogan, 1999; Perrimon and McMahon, 1999).

The extracellular matrix is also an important component in morphogenesis. It not only provides a physical substratum for the spatial organization of the cells in order to regulate morphogenetic features such as epithelial branching (Grobstein, 1967; Bernfield and Banerjee, 1982; Stopak and Harris, 1982), but it may also play a more active role in inductive tissue interactions by controlling the spread and activities of growth factors. Type XVIII collagen is a recently identified basement membrane zone component characterized by the occurrence of short and long N-terminal variants (Rehn et al., 1994; Saarela et al., 1998) transcribed from two widely separated promotors (Rehn et al., 1996). Type XVIII collagen may also play a role in morphogenesis, as its longest variant has a domain similar to the frizzled proteins implicated as putative receptors in Wnt signaling and as antagonists when secreted (Rehn and Pihlajaniemi, 1995; Rehn et al., 1998; Wodarz and Nusse, 1998). Although a proteolytic fragment of type XVIII collagen named endostatin is a potent anti-tumorigenic peptide (O'Reilly et al., 1997), the function of type XVIII collagen during embryogenesis is not known.

We report here on a patterning process that involves type XVIII collagen and correlates with a change in the epigenesis of the ureter bud from a development pattern of the kidney type to one resembling the early lung type, mediated by instructive induction from the lung mesenchyme. The data support a model in which differential organ morphogenesis is regulated by an intra-organ patterning process that involves both structural components such as type XVIII collagen and inductive signals that control their gene expression.

MATERIALS AND METHODS

Mouse strains, organ culture conditions and tissue recombinants

The organ cultures were performed with lung and metanephric kidney tissues isolated from embryonic day (E) 10.5 to E12.5 embryos of CD-1 and/or ROSA-26 mouse strains (Soriano, 1999, Jackson Laboratory, USA). The vaginal plug was used as a criterion for mating (0.5 days of gestation) in order to obtain timed embryos, but the number of tail somites (TS) and the morphology of the epithelial bud were also used as criteria for determining the developmental stage. The organ cultures were performed as previously described (Vainio et al., 1993; Thesleff and Sahlberg, 1999).

For all the tissue recombination experiments, the organ rudiments were incubated for 2 minutes in 3% pancreatin/trypsin (GibcoBRL, UK) in Tyrode´s solution. After mechanical separation of the epithelium and mesenchyme, the tissues were washed in 20% fetal bovine serum (FBS, GibcoBRL) in DMEM to remove residual enzyme activity and placed on ice until used. One lung mesenchyme and one ureter bud were used for each recombinant. The left and right lung mesenchymes and the mesenchyme around the tips and more cortical areas were found to induce branching in a similar manner in the recombinants (data not shown). Once this was known, mesenchyme was taken for the experiments at random. For the homotypic lung recombinants and heterotypic recombinants the tracheal mesenchyme was removed prior to recombination (Hogan, 1999; Shannon, 1994; Shannon et al., 1998). However, when the capability of the tip mesenchyme for inducing ectopic buds and collagen XVIII expression was being investigated, the distal tip lung mesenchyme was grafted onto an area from the tracheal mesenchyme that had been only partially removed to expose the tracheal epithelium. For the generation of chimeric organs, different proportions of the kidney mesenchyme were replaced with lung mesenchyme, recombined mechanically with a ureter bud that had branched once (T-shape in appearance, see Fig. 1F) and cultured as indicated under Results.

The ELQ and Q36.4 antibodies against type XVIII collagen have been described elsewhere (J. Saarela, M. Rehn, S. Vainio and T. Pihlajaniemi, unpublished). ELQ recognizes all three isoforms, whereas Q36.4 is directed against the two longest ones. Tests were performed to determine the active concentrations of the ELQ antibody required for the blocking experiments in organ culture (data not shown) and thereafter a concentration of 120 μg/ml was used. For the recombinants, lung mesenchymes were preincubated for 1 hour after separation in a medium including 120 μg/ml of ELQ, recombined and subcultured in the presence of the antibody. Antibody binding was visualized by adding peroxidase-coupled anti-rabbit IgG as a secondary antibody and stained.

The hanging drop assay has been described earlier (Vainio et al., 1992). The isolated ureter buds were washed before and after incubation in a hanging drop with 35 μl of medium containing 100 ng/ml of human recombinant glial cell line-derived neurotrophic factor (GDNF) (PeproTech, USA) or bovine serum albumin (BSA, Sigma, USA). Unless otherwise indicated, the culture time for the whole organs and recombinants was 3 or 5 days. A minimum of six recombinants were examined in each experiment. For better visualization of gene expression and branching profiles, E11.5 organs were cultured either 2-6 or 36 hours before staining as whole mounts. The affi-gel blue agarose beads (100-200 mesh, diameter 75-150 nm; BioRad, UK) used to generate GDNF (100 ng/μl), FGF2 (500 ng/μl), FGF7 (500 ng/μl), FGF10 (100 ng/μl) and BSA were prepared as described (Vainio et al., 1993; Sainio et al., 1997; Park et al., 1998).

Whole-mount and section immunostaining

Type XVIII collagen was detected using the ELQ and Q36.4 antibodies at 20 μg/ml. The Troma I antibody against cytokeratin Endo A was from the Developmental Studies Hybridoma Bank (USA) and the antibody against mouse pulmonary surfactant protein C (SP-C), m-20, from Santa Cruz Biotechnology, Inc. (USA). The secondary antibodies used were peroxidase-conjugated donkey anti-rabbit IgG and donkey anti-rat IgG, TRITC-conjugated donkey anti-rabbit IgG and Biotin SP-conjugated mouse anti-goat IgG (Jackson Immuno Research Laboratories, USA). Normal bovine serum was also from Jackson Immuno Research Laboratories and normal goat serum from DAKO (Denmark).

The peroxidase-conjugated whole-mount immunostaining was essentially based on the protocol previously described (Marti et al., 1995). Diaminobenzidine (DAB) (ZYMED, USA) was used as a substrate for the color reaction. For immunohistochemistry, samples were fixed with 4% paraformaldehyde (PFA) overnight and 8 μm paraffin-embedded serial sections were cut. The SP-C antigen was localized (Hsu et al., 1981) and the samples were photographed on Ektachrome Tungsten 64 Collagenor slide film (Kodak, USA) or with a digital camera (Leica, Germany) using an Olympus SZH10

Research Stereo Microscope. The composites were generated using the Adobe Photoshop v5.0 program. A Leitz confocal microscope (Leica, Germany) was used for monitoring fluorescence in the explants. The image analysis for monitoring the epithelial branching profiles of the organs and recombinants was performed with the Scion Image program, which was also used to generate the skeletons. The epithelial skeletons were superimposed using the macromedia Freehand 8 program.

Probes, whole mount *in situ* hybridization and staining of the explants

Probes specific for all mRNAs encoding the mouse gene for a1 (XVIII) collagen chain (Rehn and Pihlajaniemi, 1995) were used. The other probes were for sonic hedgehog (Shh) (Bellusci et al., 1997a), *Wnt11* (Kispert, et al., 1996), *Wnt2* (McMahon and McMahon, 1989), *Wnt7b* (Parr et al., 1993), *Sox9* (Wright et al., 1995), *Fgf10* (Bellucsi et al., 1997b), *Bmp4* (Bellusci et al., 1996), *Gli1*, *Gli2* and *Gli3* (Motoyama et al., 1998), and *CC10* (*Utg* – Mouse Genome Informatics; Whitsett, 1998). All of these were obtained as gifts. A probe for mouse *SP-C* (*Sftpc* – Mouse Genome Informatics) was obtained as an expressed sequence tag cDNA (Gbacc AA511605, Genome Systems, USA) and was confirmed by sequencing. The section and whole-mount in situ hybridization of cultured explants were performed largely as described previously (Parr et al., 1993; Wilkinson and Nieto, 1993; Kispert et al., 1998). The double staining of mRNA and protein was also based on the protocol described by Parr et al. (Parr et al., 1993), with some modifications. Cultured samples were processed as for whole-mount in situ hybridization but with the proteinase K treatment steps and postfixation step omitted. The samples were washed in PBS after the BM purple alkaline phosphatase substrate (Roche) for the color reaction, after which the whole-mount immunostaining protocol proceeded directly without the hydrogen peroxidase blocking step. The X-Gal staining of cultured whole-mount samples were performed according to Nonchev et al. (Nonchev et al., 1996) and those for section samples according to Bobola et al. (Bobola et al., 1995). The double staining with X-Gal and the antibodies were accomplished by first performing X-Gal staining and then the whole-mount immunostaining. For histological analysis, the whole-mount stained explants were embedded in glycolmethacrylate (JB-4; Polysciences, USA) and sectioned at 8-10 μm. Apoptosis was analyzed using an in situ cell detection kit (Boehringer Mannheim). BrdU labeling and detection was carried out using a cell proliferation kit (RPN 20, Amersham Life Science, UK) according to Sainio et al. (Sainio et al., 1997).

RESULTS

Type XVIII collagen expression is localized to different epithelial regions in the embryonic lung and kidney and correlates with their early epithelial morphogenesis

The function of type XVIII collagen is not known, but the presence of frizzled and endostatin domains in it suggests regulatory roles in addition to possible structural ones. Our expression studies indicated that at the initiation of lung and kidney organogenesis (E10.5), when the epithelial bud grows into the mesenchyme, type XVIII collagen mRNA is present throughout the epithelial bud from the tip to the stalk in both of these organs (Fig. 1A,B). Type XVIII collagen immunostaining of the section samples demonstrated that the protein is present in the basal side of the epithelium (Fig. 1C,D) and in some endothelial cells of the lung mesenchyme (Fig. 1C). During the formation of the first branches, type XVIII collagen expression in the lung became localized to the epithelial tips and was lost from the stalk area (Fig. 1E,G), while the opposite pattern was observed in the kidney (Fig. 1F,H). This differential expression pattern persisted during the later developmental stages (Fig. 1I,J). The differential expression patterns of type XVIII collagen, as summarized schematically in Fig. 1K, were further compared with those of type IV collagen, which showed no difference in epithelial expression patterns between the kidney and lung (data not shown).

In the cultured lung, several secondary lateral epithelial side buds grow out of the two primary branches, initially in an unbranched state (Fig. 1L,N,P,R,T). As at least three side branches usually grow, the branching will hereafter be called the 1-3 type, i.e. three side branches develop from one previous one. In the kidney, on the other hand, the secondary branching is mainly dichotomous after formation of the first primary branch and tends to follow the 1-2 type, in which each tip generates two new ones (Fig. 1 M,O,Q,S,U; Saxén, 1987). These two early epithelial morphogenetic types are summarized schematically in Fig. 1V. Altogether, the differences in type XVIII collagen expression pattern between the lung and kidney correlate with the type of early epithelial development.

At E11.5 and during 24 hours of subculture, the branches of the lung develop according to the 1-3 type and in an invariant manner in terms of position (Fig. 1L,N), whereas a few other types of branching also take place at the later stages (Fig. 1P). On the other hand, side branches appear in the kidney sample, too, after culturing for 48 hours (Fig. 1Q). We therefore performed a timecourse of lung and kidney types of branching. Altogether, 49 E11.5 lungs and 38 E.11.5 kidneys cultured for 3, 24, 48 and 72 hours were analyzed in terms of their type of epithelial branching. Statistical analyses of those cultured for 72 hours revealed that over 80% of the lung branches were of the 1-3 type, i.e. at least three lateral epithelial buds were growing out of a primary branch. This type of branching, in which the majority of branches are of the 1-3 type, is referred as early ‘lung-type’ branching. By contrast, an average of 88% of the ureter branches in the kidney were dichotomous, i.e. each epithelial tip had generated two new tips. This was defined as early ‘kidney-type’ epithelial development.

The lung mesenchyme is sufficient to completely respecify type XVIII collagen expression in the ureter bud from the kidney to the lung type

The differential expression of type XVIII collagen during epithelial morphogenesis suggests a role for epithelial-mesenchymal interactions in the process of type XVIII collagen patterning, and this was tested by the tissue recombination approach shown schematically in Fig. 2A-C. Control experiments showed that separation and recombination of the respective epithelial bud and mesenchyme restored the lung type (tip, Fig. 2D) or kidney type (stalk, Fig. 2E) of type XVIII collagen expression.

In heterotypic recombinants between the lung mesenchyme and the ureter bud, the kidney-type pattern of type XVIII collagen expression was observed for a lag period of 1-3 days (data not shown), after which expression disappeared from the stalk and was instead localized in the epithelial tip cells, constituting the early lung type. This was observed both with the antibody ELQ to all type XVIII collagen variants (Fig. 2F) and the anti-long Q36.4 antibody (Fig. 2G), which specifically recognizes only the two longest type XVIII collagen variants (J. Saarela, M. R., S. V.

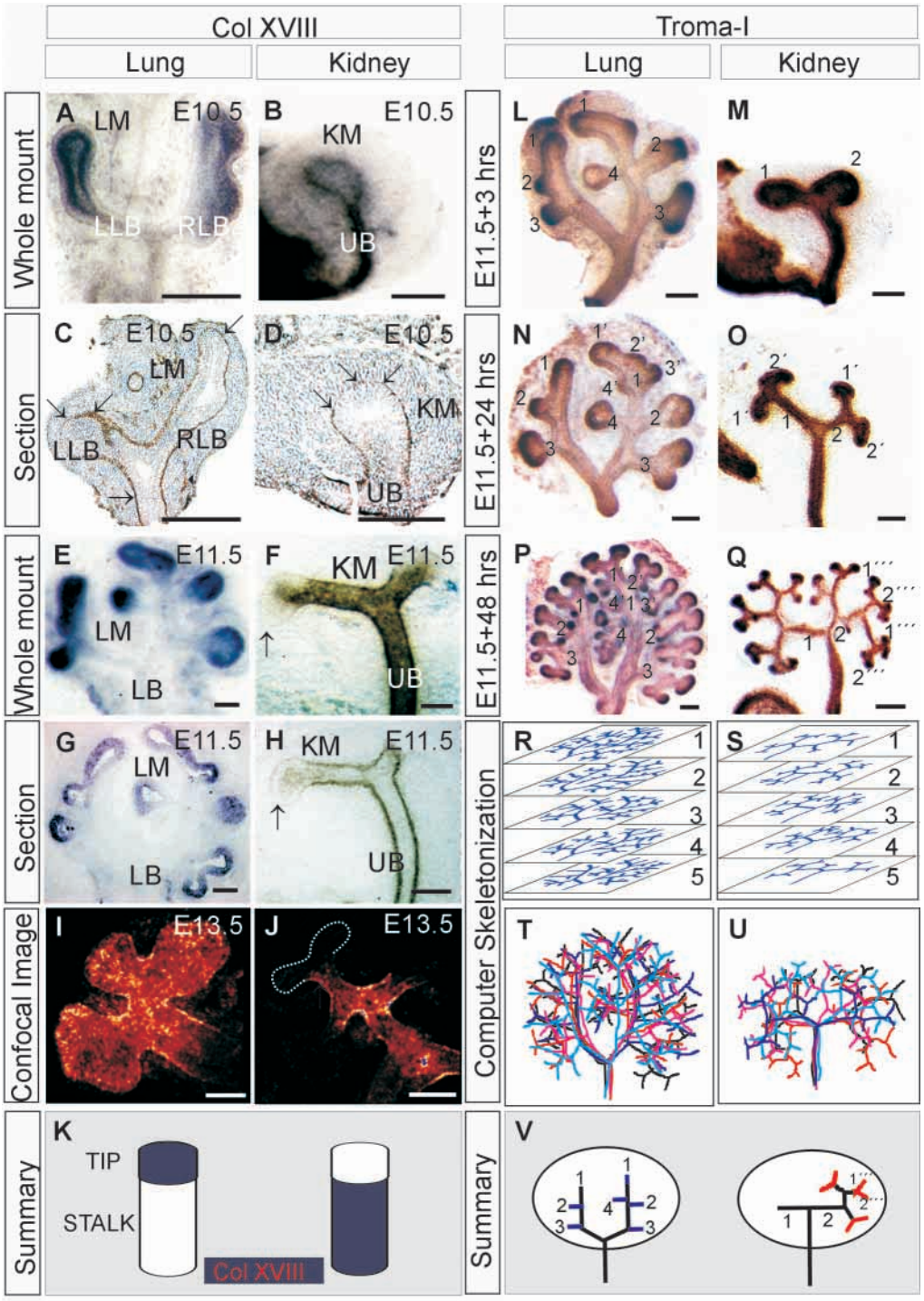

Fig. 1. Type XVIII collagen expression becomes localized to different epithelial regions in the kidney and lung and correlates with their epithelial morphogenesis. (A-J) Lung and kidney specimens at E10.5 (A-D), E11.5 (E-H) and E13.5 (I, J) are depicted, and the localization of type XVIII collagen mRNA (A,B,E,G) and protein (C,D,F,H,I,J) is shown on a whole-mount (A,B,E,F,I,J) or section basis (C,D,G,H). Type XVIII collagen is expressed throughout the epithelial bud of the lung (A,C, arrows) and kidney (B,D, arrows) at E10.5. At E11.5 expression is localized to the presumptive bronchial tips in the lung (E,G), while it is lost from the ureter tips in the kidney and is confined to the epithelial stalk region (F,H, arrows). At E13.5 it persists in the epithelial tip of the lung (I) and the epithelial stalk of the kidney (J). (K) A schematic summary of the complementary expression profiles of type XVIII collagen in the lung and kidney. (L-Q) Epithelial branching patterns in the lung (L,N,P; three left and four right-side branches are marked) and kidney (M,O,Q) after 3, 24 and 48 hours culturing were revealed using a Troma I antibody against cytokeratin. (R,S) Epithelial primary budding occurs remarkably invariantly in the early lung and follows 1-2 type branching in the early kidney, even under organ culture conditions, as demonstrated in a superimposed skeletons generated from epithelia by image analysis. (T-V) The skeletons are gathered from a number of samples, and the separate epithelial trees, depicted in different colors, are shown one upon another (T,U), and summarized in V (blue, 1-3 type branching, i.e. at least three branches; red, 1-2 type branching). 1‴ or 2‴ indicates that the tip has branched four times. LB, lung bud; LM, lung mesenchyme; KM, kidney mesenchyme; UB, ureter bud. Scale bars: 100 μm.

and T. P., unpublished). Interestingly, even the long variants were respecified to a lung-type location, in the epithelial tip cells (Fig. 2G), even though these two isoforms are not expressed in the normal early embryonic lung (data not shown). Hence, repatterning also occurred with the variants directed by separate promotors (Rehn et al., 1996), suggesting that the signals involved in the patterning process also integrate variant-specific mechanisms of type XVIII collagen gene regulation.

Reprogramming of early epithelial morphogenesis from the kidney type towards the lung type

To test whether the respecification of type XVIII collagen expression in the ureter bud was accompanied by other properties associated with the lung, we first monitored epithelial morphogenesis by image analysis. As expected, the early lung type of epithelial branching (mostly 1-3 type branching) and the kidney type (typically 1-2 type branching)

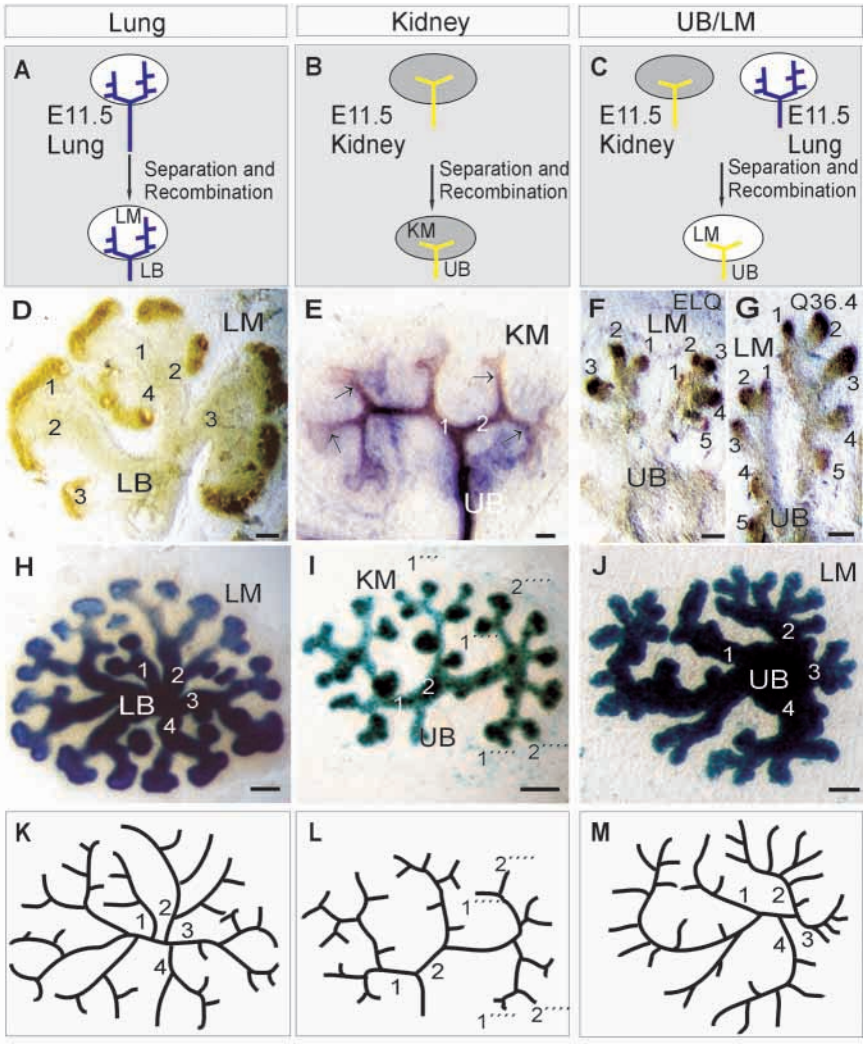

Fig. 2. Repatterning of type XVIII collagen expression in the ureter bud by lung mesenchyme is associated with an early morphogenetic shift from the kidney type to the early lung type. (A-C) Design of the experiments used to analyze the roles of epithelial-mesenchymal interactions in the control of type XVIII collagen expression and changes in early epigenesis (A,B) Homotypic or (C) heterotypic recombinants between E11.5 ureter buds and lung mesenchyme cultured for 3-5 days. The homotypic recombinants retain the lung type of collagen XVIII expression (D, tip) or the kidney type (E, arrows point to the region where Troma I staining appears, owing to the loss of type XVIII collagen expression from the tip) and the early epithelial branching pattern (H). In the heterotypic recombinants, type XVIII collagen expression is reversed completely from the kidney type to the lung type, and this is seen with both the ELQ (F) and the Q36.4 antibodies (G). The branching of the ureter bud is also changed from the kidney type towards the lung type (J,M). To examine the possibility of cell carryover, epithelial buds from ROSA-26 mice (n=32) were combined with wild-type mouse mesenchyme, but no cell contamination was observed adjacent to the epithelium (H-J). The computer-generated skeletons (K-M) obtained from image analysis of the explants in (H-J) also demonstrate the shift in the type of early epithelial branching morphogenesis to resemble early lung-type epigenesis. Culture times are 72 hours (D,E), 96 hours (H,I), 120 hours (F,G), and 144 hours (K). LB, lung bud; LM, lung mesenchyme; KM, kidney mesenchyme; UB, ureter bud. Scale bars: 100 μm.

were observed in the homotypic lung and kidney recombinants, respectively (Fig. 2D,E,H,I), while in all the recombinants between the lung mesenchyme and ureter bud in which morphogenesis had begun (n=38), we observed branching in the ureter epithelium that resembled that of the early invariant lung more than that of the kidney type (Fig. 2J). More specifically, 84% of the branches in the recombinant samples cultured for 120 hours (n=23, 235 branches calculated) showed 1-3 type branching and only 16% showed 1-2 type branching. This was also evident when the computer-generated skeletons of the epithelial branches were compared (Fig. 2K-M). To control for the possibility of cellular carryover, the same experiments were performed using wild-type lung ureter bud tissues from ROSA-26 mice, which constitutively express the *lacZ* gene (Soriano, 1999). No contaminating mesenchymal cells were observed adjacent to the epithelium (Fig. 2H-J).

Type XVIII collagen is necessary for ureter morphogenesis in heterotypic recombinants and type XVIII collagen antibody blocking leads to downregulation of *Wnt2*

The role of the type XVIII collagen repatterning process in reprogramming the ureter branching profile was tested using the anti-all type XVIII collagen (ELQ) antibody. Lung organogenesis was unchanged between experiments performed with (Fig. 3A,B) or without (not shown) 120 μg/ml of pre-immune IgG in the culture medium, while the same concentration of the anti-all ELQ antibody in the culture media reduced lung development (Fig. 3C) but had no effect on kidney development (data not shown). The number of lung epithelial tips increased in the pre-immune IgG-treated explants between 24, 48 and 72 hours of subculture, but the treatment with anti-all ELQ antibody resulted in an average of 34% fewer lung epithelial tips than in the control samples after 72 hours (Table 1). The secondary antibody did not recognize the IgG-treated controls (Fig. 3D), but typical binding to branching tips, demonstrating localization of type XVIII collagen, was seen in the ELQ-cultured lungs (Fig. 3E,F). Taken together, these observations suggest that the antibody was functional and could be used to assay the functions of type XVIII collagen in tissue recombinants as well. To monitor what signals could be involved in the functioning of type XVIII collagen we screened for the expression of three genes implicated in lung development, *Wnt2*, *Fgf10* and *Shh*, after type XVIII collagen antibody blocking. *Wnt2* expression was markedly reduced (Fig. 3G-J), whereas no such distinct decrease was detected in the expression of *Fgf10* (Fig. 3K,L) or *Shh* (Fig. 3M,N).

Table 1. Reduction in the number of lung epithelial tips in the presence of antibody against type XVIII collagen

	Control IgG		Type XVIII collagen antibody		
Incubation time (hours)	Number of cultures	Number of lung tips (average±s.d.)	Number of cultures	Number of lung tips (average±s.d.)	Reduction in number of tips relative to control (%)
24	8	9.0±0.76	8	8.4±1.5	6.9
48	8	18±3.8	8	15±2.1	17
72	8	43±5.9	8	29±3.3	34

Embryonic lungs were microdissected at E11.5 and placed into organ culture with or without normal IgG, which served as a control, normal media and ELQ, which recognized all the isoforms of type XVIII collagen containing the endostatin and frizzled domains (Rehn and Pihlajaniemi, 1995). The branching or endoderm-derived alveolar epithelium was monitored at three time points (24, 48 and 72 hours) by counting the number of epithelial tips in all of the explants. The reduction in the number of branches was calculated as a percentage of the number of branches observed in the controls at the same time points.

Furthermore, the smaller size of the lung cultured in the presence of anti-type XVIII collagen antibody suggested changes in cell proliferation and/or apoptosis. IgG-treated control lungs demonstrated apoptosis in the distal tip mesenchyme but not in the epithelium (Fig. 3O), whereas in the ELQ-treated lungs, the whole mesenchyme and epithelium were undergoing apoptosis (Fig. 3P). Consistent with the increase in apoptosis, the proliferation that was taking place in the epithelium and mesenchyme in the IgG-cultured lungs (Fig. 3Q) was reduced in the ELQ-treated explants (Fig. 4R). As demonstrated in Fig. 3C, less advanced branching was also seen in the section samples treated with type XVIII collagen antibody (Fig. 3E,F,I,J,L,N,P,R) than in those treated with IgG (Fig. 3D,G,H,K,M,O,Q).

Analysis of the recombinants indicated that branching of the ureter bud was unchanged within the lung mesenchyme in the presence of preimmune IgG (Fig. 3S,T) the 21 out of 36 explants (58%) that had initiated growth representing an average value for ureter growth without any IgG treatment (data not shown). Unexpectedly, the anti-all type XVIII collagen antibody almost completely blocked morphogenesis in the heterotypic tissue explants (Fig. 3U), in that only one out of the 37 explants (2.7%) had initiated development (Table 2). This suggests that type XVIII collagen itself is necessary in the ureter bud for the initiation of epithelial development in heterotypic recombinants.

Table 2. Type XVIII collagen is needed for ureter bud epigenesis, as judged by the blocking of development after treatment with the ELQ antibody against it

	Control IgG	Type XVIII collagen antibody
Branching cases/cases tested	21/36	1/37
Branching percentage	60%	2.7%

Lung mesenchyme and ureter bud were separated after pancreatin-trypsin treatment of the microdissected organs, as described in Materials and Methods. The lung mesenchymes were preincubated with 120 μg/ml of the ELQ antibody before recombination with the ureter bud for subculture. The same amount of preimmune IgG was used as a control. The proportion of explants that had initiated epithelial growth and branching was calculated.

Transient exposure to GDNF is sufficient to induce complete repatterning of type XVIII collagen expression in the ureter bud

We next set out to identify the signals involved in the control of type XVIII collagen repatterning in the explants. In the embryos at the 13 and 15 tail somite stages (approx. E10.5-E10.75), the ureter bud had formed from the Wolffian duct and was still expressing type XVIII collagen uniformly throughout the ureter tip, although the bud had not yet initiated its first branch (Fig. 1B,D, cf. 1F,H). Loss of type XVIII collagen from a tip may be necessary for the induction of branching and for the competence of the ureter bud for reprogramming by the lung mesenchyme. Consistent with this possibility, the ureters of the 13 and 15 tail somite embryos did not initiate branching with lung mesenchyme, although they did so in response to a kidney mesenchymal signal, GDNF (data not shown), in agreement with Sainio et al. (Sainio et al., 1997). As expected, type XVIII collagen expression persisted throughout the ureter bud with BSA-soaked beads at the 13 and 15 tail somite stages (Fig. 4A), but a GDNF-soaked bead caused a loss of type XVIII collagen expression from the ureter tips within 16 hours (Fig. 4B) and branching of the bud was initiated at 48 hours (Fig. 4C).

Having developed the assay system for monitoring the induction of ureter bud branching, we were able to define the minimum time required for GDNF-mediated induction in order to monitor the time that triggered only one branch but not yet any type XVIII collagen repatterning response. For these experiments, the ureter bud was separated from the kidney mesenchyme at the 13-15 tail somite stage and preincubated for 20 minutes to 6 hours in a hanging drop with GDNF. It was then washed to remove GDNF and recombined with lung mesenchyme, as described earlier. A 30-minute transient preincubation with GDNF prior to recombination induced a 100% branching response, whereas a 20-minute preincubation gave no response. However, a longer preincubation of the ureter bud with GDNF reduced the number of cases that had initiated epithelial branching in the recombinants (Fig. 4D). Interestingly, even the 30 minute pre-incubation with GDNF was sufficient to initiate loss of type XVIII collagen, followed by its lung-type repatterning with lung mesenchyme (Fig. 4E). Hence, we conclude that GDNF is an important signal for the epithelial competence of ureter bud to lead to respecification of type XVIII collagen with subsequent lung mesenchyme-derived signals.

Ectopic *sonic hedgehog* expression correlates with respecification of type XVIII collagen in the ureter bud

The repatterning of type XVIII collagen expression apparently involves factors that act in the ureter bud to mediate responses to mesenchymal repatterning signals. Screening was therefore initiated for the secreted factors implicated in lung and kidney

development. Of these factors, the expression of the morphogen *Shh* (Bellusci et al., 1997a) correlated with type XVIII collagen expression in the lung and kidney (Fig. 5A,B, cf. Fig. 1E,F). Ectopic type XVIII collagen expression in the tissue recombinants was also accompanied by a change in *Shh* expression in the distal tip cells (Fig. 5C), and thus *Shh* in the epithelial bud was repatterned completely by the lung mesenchyme in a similar manner to type XVIII collagen. Several other epithelial markers, including Wnt7b, HNF3β, Wnt11 and Sox9 (Fig. 5D-O), and also Gli1, Gli2 and Gli3, and Bmp4 (data not shown), failed to demonstrate such repatterning responses in the recombinants.

The distal tip mesenchyme induces ectopic branch formation and type XVIII collagen expression, but mesenchymal signals such as FGF10 and Wnt2b cannot do this

Our attempts to identify the mesenchymal signals involved in the repatterning of type XVIII collagen and *Shh* expression in the epithelium led to the conclusion that FGF10 is essential for lung organogenesis and is expressed specifically in mesenchymal cells (Bellusci et al., 1997b; Min et al., 1998; Park et al., 1998; Sekine et al., 1999) but does not appear to be lung specific. It was also expressed weakly in the kidney mesenchyme, and its expression persisted adjacent to the ureter bud in the recombinants (Fig. 5P-R). *Wnt2b* was also present in mesenchymal cells in both organs and remained

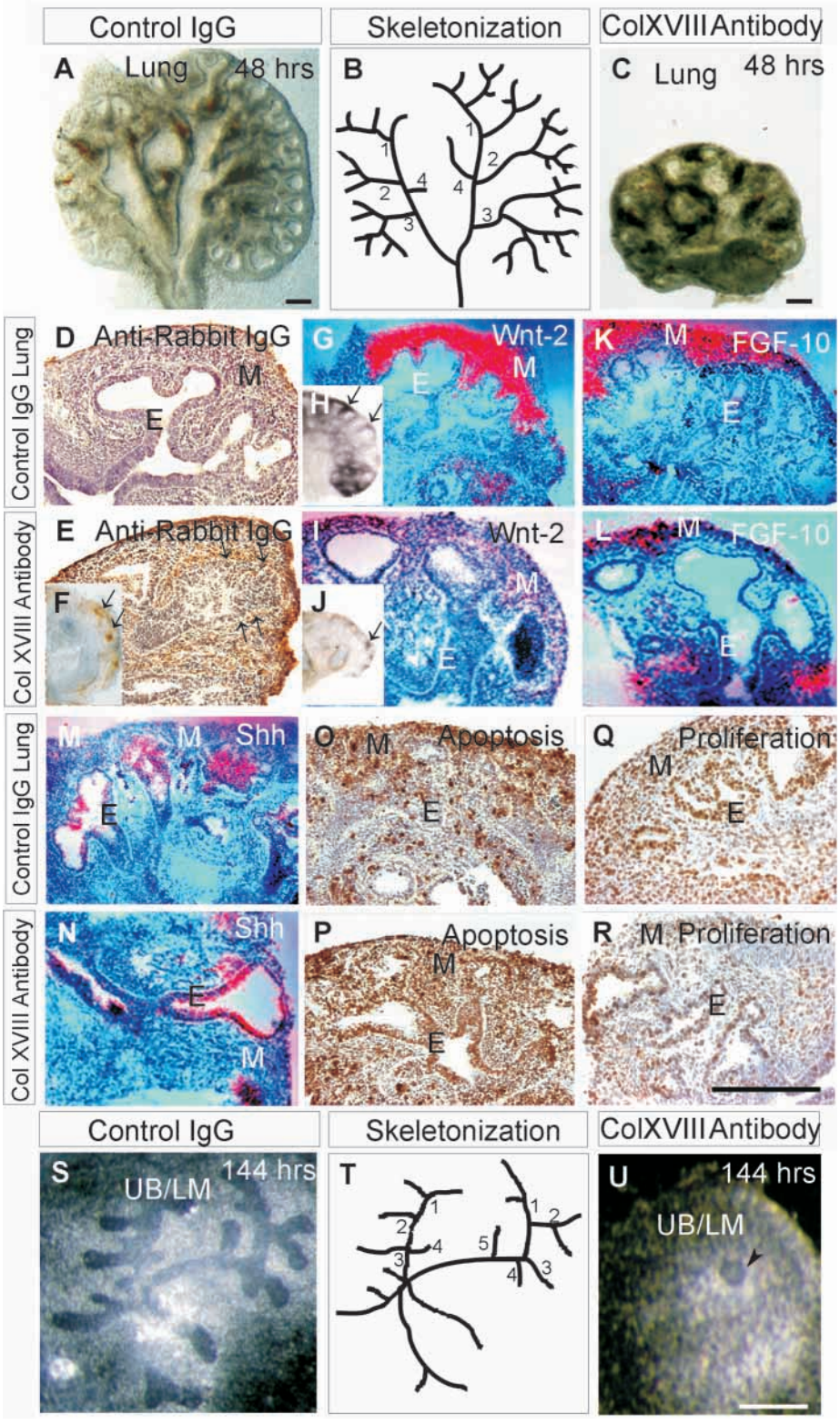

Fig. 3. Type XVIII collagen is needed for epithelial epigenesis. Control IgG (A,B,D,G,H,I,K,M,O,Q,S,T) or ELQ antibody against type XVIII collagen (C,E,F,I,J,L,N,P,R,U) was added to the culture medium at a concentration of 120 μg/ml and the samples were subcultured as whole organs for 48 hours (A-R) or as heterotypic tissue recombinants for 144 hours (S-U). Organogenesis of the lung (A,B) remains unchanged in the presence of the control IgG, whereas the ELQ antibody reduces the epithelial branches in the cultured lung bud (C). The antibody blocking (D-F) followed by binding of the secondary antibody indicate the presence of type XVIII collagen mainly in the epithelial tips (arrows in E,F). *Wnt2* expression is reduced in the ELQ-treated samples (G-J, arrows), whereas the expression of *Fgf10* (K,L) and *Shh* (M,N) remains mostly unchanged. (H,J) Whole mounts. Apoptosis mainly takes place in the mesenchyme in the IgG-treated samples (O), while both epithelial and mesenchymal cells undergo apoptosis in the ELQ-treated lung samples (P). Proliferating cells, i.e. cells that incorporate BrdU, are localized in the epithelium and mesenchyme of the IgG-treated lungs (Q), and the proliferation has decreased after ELQ-treatment (R). The ELQ antibody almost completely blocks growth of the ureter cultured together with lung mesenchyme (S-U). The arrowhead in U indicates the remnants of the ureter bud. An extreme example of reduced branching is shown (U). E, epithelium; LM, lung mesenchyme; M, mesenchyme; UB, ureter bud. Scale bars: 100 μm.

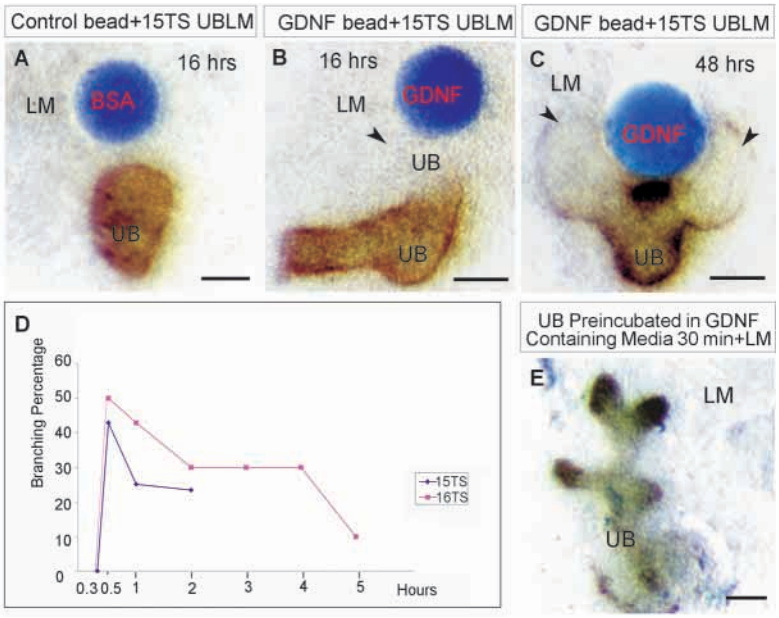

Fig. 4. GDNF signals make the ureter bud competent to respond to signals from the lung mesenchyme. To assay the competence of the ureter bud to respond to the lung mesenchyme, the buds were separated from kidneys of 15 tail somite embryos and combined with lung mesenchyme. (A-C) A BSA-soaked bead close to the ureter bud does not have any effect on type XVIII collagen expression at 16 hours, and the ureter bud degenerates (A), but a GDNF-soaked bead first causes a reduction in type XVIII collagen expression in the region of the ureter tip (B, arrowhead), and then induces ureter branching within 48 hours (C). (D) The ureter bud was separated from embryos with 15 (blue line) or 16 (red line) tail somites (around E10.5-11.0), exposed to GDNF in a hanging drop culture for the times indicated, washed and recombined for subculture with the lung mesenchyme from E11.5 embryos. Given up to 20 minutes of preincubation with 100 ng/ml GDNF, the ureters of the 15 (blue line) and 16 (red line) tail somite embryos did not initiate branching with the lung mesenchyme, but surprisingly, the ureter bud achieved its maximal response after 30 minutes, at which point all 15 explants analysed had initiated branching. Longer incubations with GDNF (30 minutes-6 hours) gradually reduced the proportion of cases that had initiated branching. (E) A 30 minute transient exposure of the ureter bud to GDNF in a hanging drop before recombination with the lung mesenchyme is sufficient to cause complete repatterning of type XVIII collagen expression from the kidney type to the lung type in response to the lung mesenchyme. LM, lung mesenchyme; TS, tail somites; UB, ureter bud.

expressed in the recombinants (data not shown), whereas *Wnt2* (McMahon and McMahon, 1989) was expressed only in the lung mesenchyme in both homotypic and heterotypic recombinants (Fig. 5S-U).

The role of the distal tip mesenchyme and specific growth factors in bud formation and in promoting type XVIII collagen expression in the lung epithelium was further tested using the tracheal tube as a model system (Shannon, 1994). The distal tip mesenchyme induced ectopic tracheal epithelial budding and type XVIII collagen expression, suggesting a role for type XVIII collagen in epithelial budding (Fig. 5V-X), whereas neither FGF2 (Fig. 5Y), FGF7 (Fig. 5Z), FGF10 (Fig. 5Z′) beads nor Wnt2b cells (data not shown) alone could replace the distal tip mesenchyme in these functions. These beads were also unable to reprogram the expression of type XVIII collagen in the ureter bud of recombinants when implanted in the lung mesenchyme (data not shown).

The lung mesechyme induces ectopic *SP-C* gene expression in the ureter bud

The distal lung bud epithelium expresses SP-C, a marker of type II pneumocytes (Meneghetti et al., 1996), which may be regulated by the transcription factors HNF3β and Nkx2.1 (Ikeda et al., 1995; Whitsett, 1998). The SP-C mRNA (Fig. 6A) and protein (Fig. 6D) were expressed in the embryonic lung but not in the kidney (Fig. 6B,E), whereas other differentiation markers, Nkx2.1 and CC10 (Whitsett, 1998), were present in both of these organs (data not shown) and are not organ-specific in this context. *SP-C* gene expression was also induced in the ureter bud when it was grown together with the lung mesenchyme (Fig. 6C), whereas no significant changes were recorded in the expression of the genes for HNF3β (Fig. 5G-I), Nkx2.1 or CC10 (data not shown). Adjacent serial sections of ureter buds prepared from ROSA-26 transgenic mice that express *lacZ* constitutively (Fig. 6F) were also stained with SP-C antibodies (Fig. 6G) to exclude epithelial carryover from the lung. Thus, the lung mesenchyme may function as an instructive inducer tissue and change the status of cell differentiation in the ureter bud.

The ureter bud is competent to integrate different early morphogenetic instructions simultaneously from mesenchymal cells and develop into a chimeric organ

At E11.5 the ureter bud of the kidney has branched once and is T-shaped (see Fig. 1F). We therefore assayed whether this bud could integrate two morphogenetic instructions simultaneously, by replacing various proportions of the kidney mesenchyme with lung mesenchyme (Fig. 6H) and using type XVIII collagen as a marker of differential morphogenesis. In such explants, developing as chimeric organs, one branch retained the kidney type of morphogenesis, whereas the other side branched in a manner resembling that of the early lung (at least three side branches; Fig. 6I), in the same manner as the ureter bud/lung mesenchyme recombinants. Thus the ureter bud is capable of integrating different early morphogenetic instructions from mesenchymal cells, a finding that is supported by data indicating that type XVIII collagen expression was localized in the epithelial tips on the side that had interacted with the lung mesenchyme (Fig. 6J). Tissues of ROSA-26 mice were again used to control for the possibility of cell carryover, but no signs of contamination were detected (Fig. 6I).

DISCUSSION

Epithelial ureter bud reprogramming by the lung mesenchyme

The mechanisms by which the rather limited number of embryonic signaling gene families form networks and regulate

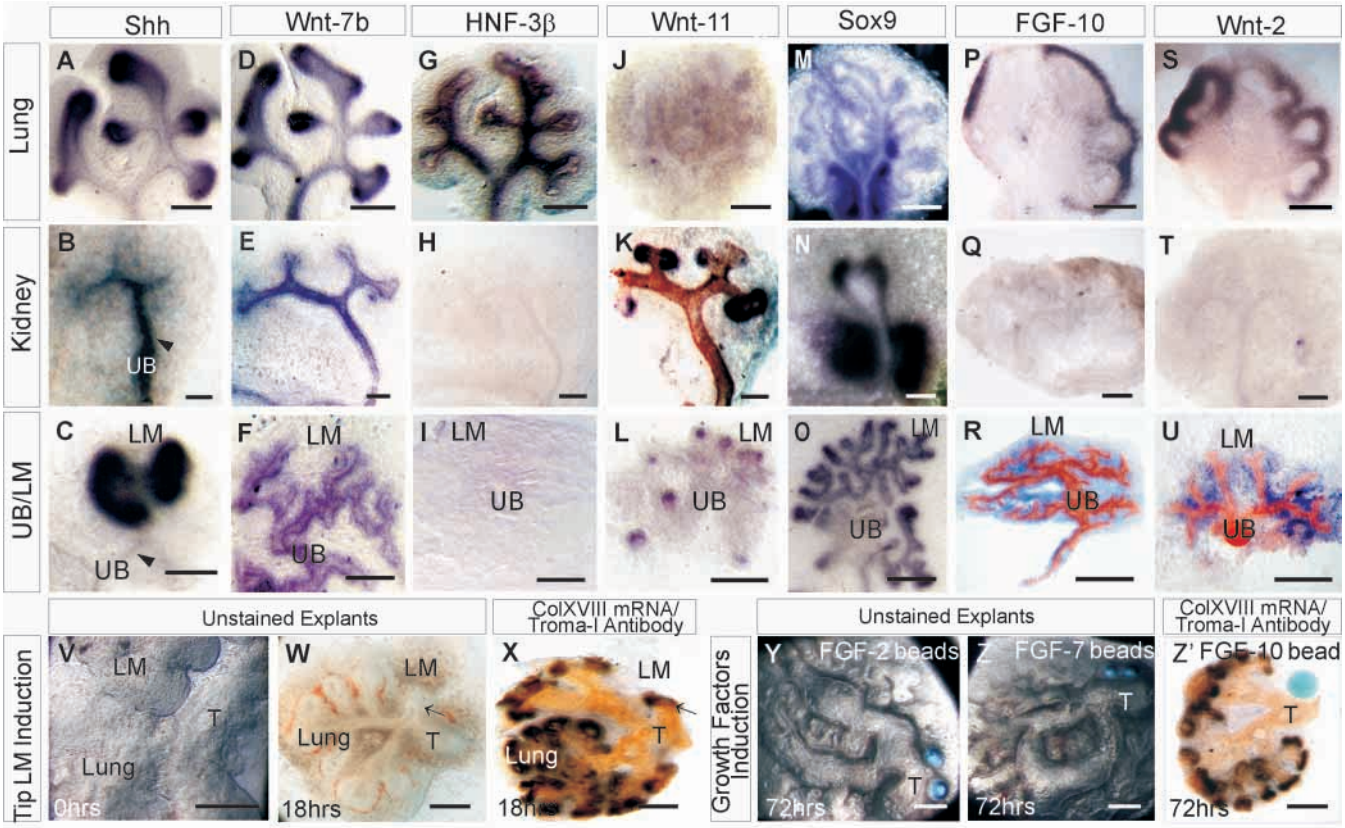

Fig. 5. Only the expression of *Shh* is repatterned in the same way as type XVIII collagen expression in heterotypic recombinants. The distal tip mesenchyme induces ectopic tracheal epithelial budding and type XVIII collagen expression. Organs (E11.5) cultured for 3-12 hours and ureter bud/lung mesenchyme recombinants cultured for 96 or 120 hours were obtained as depicted in Fig. 2 and the expression of certain genes involved in kidney or lung development was analyzed in whole mounts. (A,B) *Shh* was expressed in the bronchial epithelial tips (A) and the ureter stalk region (arrowhead in B). It was then repatterned to the ureter tip region and disappeared from the stalk area in the recombinants (arrowhead in C). (D-F) A bronchial tip marker, *Wnt7b* (D), was also expressed in the stalk area of the kidney (E) and mainly remained in the stalk in the recombinants (F). The gene for HNF3β was expressed throughout the lung epithelium (G), but not in the kidney (H) or in the recombinants (I). *Wnt11*, which was not expressed in the lung (J), is a marker of the ureter tip, where type XVIII collagen expression is lost, as revealed by a double localization of type XVIII collagen protein (brown) and *Wnt11* mRNA (dark purple). (K) *Wnt11* remained weakly expressed in the tips of the ureter buds in the tissue recombinants (L), and *Sox9* was not present in the bronchial tips (M) but was expressed in the ureter tips (N) and was maintained in these in the tissue recombinants (O). *Fgf10*, which was expressed in the lung mesenchyme adjacent to the distal epithelial tip region (P), was also weakly expressed in the mesenchymal cells of the embryonic kidney (Q) and was detected in the mesenchyme of the recombinants (R blue; Troma I staining to identify the ureter branches, orange). *Wnt2* was specifically expressed in the lung mesenchyme (S), and was not detected in the kidney (T). In the mesenchyme of the recombinants *Wnt2* remained expressed (U, blue; Troma I staining, orange). The distal tip lung mesenchyme recombined with the tracheal epithelium induces ectopic epithelial budding (V,W, arrow) and type XVIII collagen staining (X). FGF2 (Y), FGF7 (Z) or FGF10 (Z') agarose beads adjacent to the tracheal epithelium are not sufficient induce similar budding or type XVIII collagen expression. LM, lung mesenchyme; T, trachea; UB, ureter bud. Scale bar 100 μm.

the spatial organization of cells to create different forms via morphogenesis remain poorly understood, even though a number of factors have been identified and shown to be involved in various developmental model systems (Hogan, 1999). To address these questions, we used two embryonic model systems, the lung and the kidney, organs that undergo characteristic forms of epithelial branching morphogenesis. The results show that the expression of type XVIII collagen correlates with the type of epithelial development. It is occurring throughout the epithelial bud at the initiation of kidney and lung organogenesis but subsequently being restricted to the epithelial tips in the lung, whereas it is localized in the epithelial stalk region in the kidney and is lost from the epithelial tip area, which branches dichotomously. Hence, type XVIII collagen is distributed in the opposite epithelial regions after the initiative stages of organogenesis and its localization correlates with differences in epithelial development, suggesting specific roles in this development.

The characteristic modes of expression of type XVIII collagen in the lung and kidney epithelium provided an opportunity to study morphogenetic interactions in tissue recombinants between the lung mesenchyme and the ureter bud. Based on these experimental studies, we propose that the lung mesenchyme is able to reprogram the development of ureter bud epigenesis towards the early lung type, on the following grounds: (1) the embryonic lung mesenchyme completely respecified type XVIII collagen expression in the ureter bud from the epithelial stalk to the distal tip, i.e. from the kidney type to the lung type; (2) the shift in type XVIII collagen expression correlated with shifts in the expression of

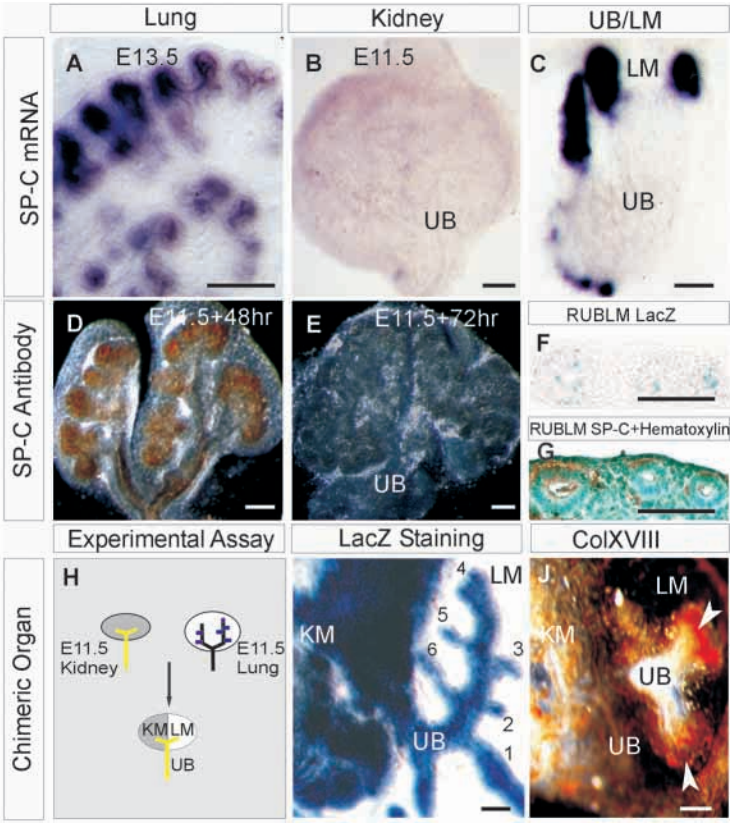

Fig. 6. Induction of *SP-C* in ureter epithelial cells of heterotypic recombinants and formation of a chimeric organ. Samples for organ cultures (A,B,D,E) and tissue recombinants (C,F,G) were obtained as shown in Fig. 2. SP-C mRNA (A) and protein (D) were expressed in the embryonic lung epithelial tips but not in the kidney (B,E). In the recombinants between the ureter bud and the lung mesenchyme, the expression of *SP-C* is again induced in the ureter bud (C). Adjacent serial sections of ROSA-26 transgenic mice stained for β-galactosidase (F) and with SP-C antibodies (G) served to exclude epithelial carryover from the lung. The SP-C antibody staining (brown) is seen in the epithelial cells. In order to determine whether the ureter bud can integrate two morphogenetic instructions simultaneously, portions of kidney mesenchyme were replaced with lung mesenchyme and combined with a T-shaped ureter bud. (H). The half of the bud surrounded by lung mesenchyme branched in a manner resembling the early lung-type pattern (I, six lateral branches are marked), while the other half developed with the kidney. Samples of the kidney mesenchyme and ureter bud of ROSA-26 mice were used in I to control for cellular carryover or cell migration from the kidney mesenchyme. (J) Type XVIII collagen is expressed in the ureter bud tips in the same manner as in the lung epithelium when surrounded by lung mesenchyme (arrowheads in J, compare with Fig. 1I). The samples presented in C,J had been cultured for 96 hours, and the one in I for 120 hours. LM, lung mesenchyme; RUBLM, ureter bud from a Rosa 26 mouse combined with lung mesenchyme from a wild-type mouse; UB, ureter bud. Scale bars 100 μm.

Shh and *SP-C*; and (3) epithelial morphogenesis changed from mostly dichotomous branching towards the lung type, in which several lateral side branches grow out of the primary epithelial buds. Thus, the lung mesenchyme seems to act as an instructive inducer of the ureter bud and is sufficient to respecify and reverse these marker gene patterns in recombinants. The data suggest involvement of an intra-organ patterning process induced by the lung mesenchyme.

GDNF is a necessary signal for competence of the ureter bud to respond to lung mesenchyme-derived signals

Tissues that are derived from a different axial level with different Hox codes (De Robertis et al., 1989) apparently share enough signals and transmission pathways for interactions, so that it is likely that the repatterning of type XVIII collagen and the early changes in epithelial epigenesis occur as a result of more than one signal. The repatterning occurred in the context of the whole epithelial bud, as was observed in a developing chimeric organ consisting of lung and kidney mesenchyme, suggesting roles for both local and long-range signals, and indicating that the epithelial branches can integrate these early morphogenetic signals independently.

The ureters of 13 and 15 tail somite embryos in the recombinants did not branch or repattern type XVIII collagen expression in the absence of factors initially of kidney mesenchyme origin. The interactions of an unbranched ureter bud with lung mesenchyme depend on preincubation with glial-derived neurotrophic factor (GDNF), and can hence be considered a competence factor for lung mesenchymal signaling. Interestingly, a GDNF-releasing bead caused repression of type XVIII collagen expression in the ureter tip cells, and this was followed by activated branching. Reduction in the amount of type XVIII collagen in the ureter tip may therefore be necessary for the initiation of dichotomous branching and may point to a permissive role for GDNF.

GDNF may render the ureter competent by inducing expression of an epithelial receptor for a mesenchymal signal common to both the lung and kidney. This may be followed by induced expression of another signal that interacts with the lung mesenchyme in a manner consistent with the sequential and reciprocal signaling mechanisms of epithelial-mesenchymal tissue interactions (Thesleff et al., 1995). GDNF induces renal expression of *Wnt11* and *Sox9*, both of which are present in the ureter tip (Pepicelli et al., 1997). Additional signals other than GDNF are apparently involved in regulating these genes, however, as our data show that their expression was still maintained by lung mesenchyme that was devoid of GDNF. We conclude that GDNF fulfills the criteria for an initial mesenchymal-derived signal and that it is sufficient to alter the epithelial competence with respect to other subsequent signals that regulate the expression of type XVIII collagen.

Wnt2 is a candidate for the lung-specific mesenchymal signal needed for epithelial development

When GDNF-induced competence has been achieved in the ureter bud, the initial signals that modify the status of the bud epithelium are apparently derived from the lung mesenchyme. The distal tip mesenchyme was found to promote ectopic tracheal epithelial budding, and type XVIII collagen expression was induced in the ectopic buds, indicating that the

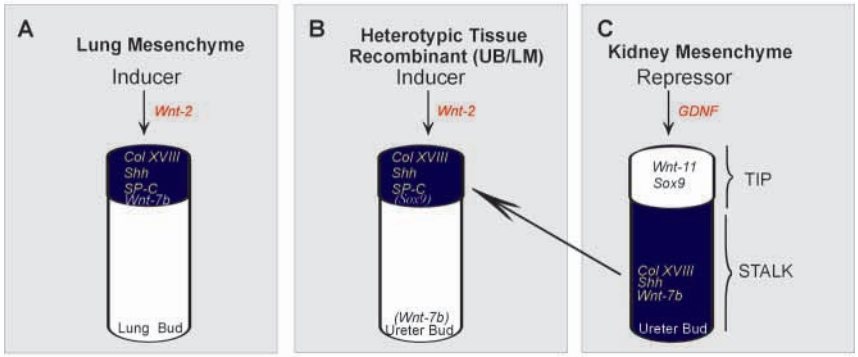

Fig. 7. Schematic summary and model of some molecular mediators in the experimental repatterning process. Lung and kidney organogenesis is regulated by sequential and reciprocal morphogenetic inductive tissue interactions (A,C). Lung mesenchyme was combined with ureter bud (B). Type XVIII collagen was initially present throughout the epithelial buds, but in the lung (A) its expression became localized to the epithelial tip region, while in the kidney (C) it was confined to the stalk of the ureter. In the recombinants, the type XVIII collagen and a morphogen, Shh, were repatterned from the stalk to the tip region (arrow from C to B) and correspondingly became expressed as in the lung bud. This process was accompanied by ectopic expression of *SP-C* (B), a marker of type II pneumocytes and early epigenesis resembling the lung type. GDNF is an essential signal for the competence of the ureter bud for responding to lung mesenchyme signaling, whereas *Fgf10* and *Wnt2b* are present in both of the mesenchymes. Since expression of the latter two persists in the recombinants, they may be common epithelial regulators. On the other hand, Wnt2 may promote lung-specific signaling in the mesenchyme and is lost from lungs incubated with the type XVIII collagen antibody ELQ.

distal tip mesenchyme contains factors sufficient to promote these phenomena. However, factors such as Wnt2b, FGF2, FGF7 and FGF10 did not induce budding or type XVIII collagen expression, which suggests a role for other factors or need for a combination of factors in the process.

Culture of the lungs in the presence of a type XVIII collagen antibody leads to reduced epithelial branching, which is accompanied by increased apoptosis and decreased proliferation. Expression of *Wnt2*, which appears to be lung-specific and remains expressed in the heterotypic recombinants, is also dramatically reduced in lungs treated with the antibody, while expression of *Shh* and *Fgf10* is not. Hence Wnt2 may function as a lung-specific signal and there may be a regulatory interaction between Wnt2 and type XVIII collagen. Wnt2 may also act as a growth factor inducing proliferation, because a decrease in its expression is correlated with reduced proliferation. The mechanisms and significance of the regulatory interaction between Wnt2 and type XVIII collagen remain to be demonstrated, as no lung phenotype has been detected in *Wnt2* knockout mice (Monkley et al., 1996).

Type XVIII collagen in the epithelium may contribute to regulation of differential epithelial morphogenesis

Type XVIII collagen was localized in the epithelial tip region of the early lung, whereas it was lost in these areas of the kidney. As an indication of the role of this collagen type in epithelial development in the lung, we observed an average of 34% lower numbers of epithelial tips when the explants were cultured in the presence of antibodies against it. As the repatterning process led to a similar lung-type expression of type XVIII collagen in the recombinants, we expected the response to the antibody to be similar to that seen in the lung. The effect of type XVIII collagen antibodies on the development of the recombinant epithelium was even more striking, however, as they almost completely blocked epithelial morphogenesis in the recombinants but not in the kidney. The data suggest an important role for type XVIII collagen in the chain of molecular events associated with the reprogramming of ureter bud development. Additional support for a role for type XVIII collagen in epithelial development was gained from the fact that its expression in the tracheal epithelium was induced by the distal lung mesenchyme and that blocking of its function led to a marked decrease in *Wnt2* expression.

What could be the role of type XVIII collagen? The longest variant of this collagen monitored here contains a frizzled motif that is homologous to the frizzled proteins implicated in Wnt signaling (Rehn et al., 1998; Wodarz and Nusse, 1998). We found that *Wnt11* and the gene for type XVIII collagen are mutually exclusive in their expression patterns in the kidney, the latter being lost from the newly formed tips in which the former occurred. Hence downregulation of type XVIII collagen could lead to the diffusion of some Wnts to the adjacent mesenchyme and consequently induce tubules in a Wnt-dependent manner (Stark et al., 1994; Kispert et al., 1998; Vainio et al., 1999). It is not known how the loss of type XVIII collagen from the ureter tips is regulated, but certain metalloproteases such as MMP9 are also expressed in the kidney and may be involved in the degradation process (Tanney et al., 1998).

The expression of type XVIII collagen at the epithelial tip in the lung could be involved in binding Wnts to the bronchial epithelial tips and concentrating them there in order to promote their typical morphogenesis at early developmental stages. Recent studies have suggested that type XVIII collagen is also a proteoglycan (Halfter et al., 1998). As proteoglycans are necessary for Wnt signaling (Reichsman et al., 1996; Häcker et al., 1997; Lin and Perrimon, 1999), type XVIII collagen may have a dual function in such signaling, that of antagonizing Wnts with its frizzled domain and that of activating Wnt signaling, e.g. in the form of Wnt2, with its glycosaminoglycan domains. Taken together, the localization of XVIII collagen and the regulation of its processing may have morphogenetic consequences, but this aspect requires further investigation.

We have been able to demonstrate an intra-organ patterning process that involves type XVIII collagen, an extracellular matrix protein that contains endostatin and frizzled domains. We also showed that repatterning of type XVIII collagen expression was accompanied by ectopic expression of lung *SP-C* and the morphogen *Shh*, and that this was associated with changes in the early epigenesis of the ureter bud towards the early lung type (. 7). Our data support a model according to which differential form may be regulated by patterning cues exchanged during

morphogenetic inductive tissue interactions, and that these localized signaling activities of growth factors involve extracellular matrix molecules such as type XVIII collagen.

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