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Original Article

Patterning parameters associated with the branching of the ureteric bud regulated by epithelial-mesenchymal interactions

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ABSTRACT The mechanisms by which the branching of epithelial tissue occurs and is regulated to generate different organ structures are not well understood. In this work, image analyses of the organ rudiments demonstrate specific epithelial branching patterns for the early lung and kidney; the lung type typically generating several side branches, whereas kidney branching was mainly dichotomous. Parameters such as the number of epithelial tips, the angle of the first branch, the position index of the first branch (PIFB) in a module, and the percentage of epithelial module type (PMT) were analysed. The branching patterns in the cultured lung and kidney, and in homotypic tissue recombinants recapitulated their early *in vivo* branching patterns. The parameters were applied to heterotypic tissue recombinants between lung mesenchyme and ureteric bud, and tip number, PIFB and PMT values qualified the change in ureter morphogenesis and the reprogramming of the ureteric bud with lung mesenchyme. All the values for the heterotypic recombinant between ureteric bud and lung mesenchyme were significantly different from those for kidney samples but similar to those of the lung samples. Hence, lung mesenchyme can instruct the ureteric bud to undergo aspects of early lung-type epithelial morphogenesis. Different areas of the lung mesenchyme, except the tracheal region, were sufficient to promote ureteric bud growth and branching. In conclusion, our findings provide morphogenetic parameters for monitoring epithelial development in early embryonic lung and kidney and demonstrate the use of heterotypic tissue recombinants as a model for studying tissue-specific epithelial branching during organogenesis.

KEY WORDS: *Patterning, lung development, kidney development, branching morphogenesis, image analysis*

Introduction

The early stages in the morphogenesis of several organs such as the kidney, lung and salivary gland share common features, including condensation of the mesenchymal cells and thickening, folding or branching of the epithelial sheets (Thesleff *et al.*, 1995; Pohl *et al.*, 2000, Vainio and Lin, 2002). The lung primordium, for example, which appears in mouse embryos at E9.5, originates as an epithelial bud on the endoderm of the primitive foregut. Shortly after its appearance, it develops into the prospective trachea and two lung buds. In the kidney, the collecting duct system develops from the ureteric bud, which forms as an outgrowth from the caudal end of the Wolffian duct at E10.5. The ureteric bud immediately invades the metanephric mesenchyme, signals emanating from which induce further formation of the ureteric bud (Saxén, 1987).

Classic tissue recombination studies have demonstrated that the mesenchymal cells typically direct epithelial morphogenesis and determine the form of developing organs (Davies and Bard, 1996; Gilbert, 2000). The specificity of this mesenchymal influence on epithelial morphogenesis nevertheless varies between the primordia of different organs and is stage-dependent. The metanephric mesenchyme is quite unique in its capacity to support the

Abbreviations used in this paper: CV, coefficient of variation; DAB, Diaminobenzidine; GDNF, glial cell line-derived neurotrophic factor; GFP, green fluorescent protein; LB/LM, lung bud and lung mesenchyme; PIFB, position index of the first branch; PMT, percentage of the module type; TAS, tail somites; UB/KM, ureteric bud and kidney mesenchyme; UB/LM, ureteric bud and lung mesenchyme.

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growth and branching of the ureteric bud, and no other epithelial tissue branches with the kidney mesenchyme (Grobstein, 1955; Saxén, 1987). The lung mesenchyme, on the other hand, is an example of a permissive tissue, and can induce the growth of several epithelia, such as the lung and ureteric bud (Shannon, 1994; Sainio *et al.*, 1997).

Epithelial cells are also restricted in their differentiation potential depending on their developmental stage and origin in the embryo. The early lung epithelium can be induced to grow and differentiate to distinct fates by several heterologous embryonic mesenchymal tissues (Shannon *et al.*, 1998). At the later stage of development, however, the interactions between the epithelial and mesenchymal tissue become more specific, and only the pulmonary mesenchyme can support subsequent branching and morphogenesis of the bronchi. Early growth and branching of the ureteric bud, on the other hand, can be supported by the lung mesenchyme after the formation of the first epithelial branch (Kispert *et al.*, 1996; Sainio *et al.*, 1997). The ureteric bud may also be sufficiently plastic to be induced to other cell fates, since the lung mesenchyme is able to induce ectopic expression of lung surfactant C (Lin *et al.*, 2001a), a marker of type II pneumocytes (Meneghetti *et al.*, 1996), in the ureteric bud. The lung mesenchyme is also able to induce a shift in the expression of type XVIII collagen and sonic hedgehog in the ureteric bud from the kidney to the lung type, i.e. from the stalk region to the tips of heterotypic recombinants. This is accompanied by obvious morphological changes in ureter development (Lin *et al.*, 2001a).

Mesenchymal cells within a certain tissue are also specific in their mode of supporting epithelial branching, the mesenchymal cells adjacent to the growing epithelial tips in the lung being able to support branching, whereas the tracheal mesenchyme inhibits it (Wessells, 1970; Cardoso, 2000). The results of tissue separation and recombination experiments have also suggested that the programme for epithelial branching may, at least partially, be in the epithelium itself. In support of this, branching of an isolated renal epithelium has been achieved in culture (Perantoni *et al.*, 1991; Qiao *et al.*, 1999).

To explain the capacity of mesenchymal cells to support epithelial development in various tissue recombinants, mesenchyme-common and mesenchyme-specific factors have been postulated to be involved (Grobstein, 1967; Saxén, 1987). Hence, the interactants may express general factors that are being expressed by several developing tissues and promote overall growth, or else they may only be expressed in a given organ and contribute to a tissue-specific mode of epithelial branching. Fibroblast growth factor 10 (FGF-10), for example, which promotes epithelial growth in the lung (Min *et al.*, 1998; Weaver *et al.,* 2000), is also expressed in the kidney. *Wnt-2*, on the other hand, is expressed in the lung mesenchyme but not in the kidney and could carry lung-specific characters of the epithelium (Lin *et al.*, 2001a). It is likely that other factors are also involved, however, as no lung-type phenotype has been reported in *Wnt-2* knock-out (Monkley *et al.*, 1996).

The shapes of different parenchymal organs undergoing epithelial branching may be explained by two fundamental characteristics of the epithelial rudiments, their branching pattern and the rate of proliferation of the epithelial cells (Sainio *et al.*, 1997). However, even though a number of factors that are expressed in organs undergoing epithelial branching, such as the kidney and lung, have been identified (for databases, see http://www.ana.ed.ac.uk/anatomy/database/lungbase/ lunghome.html), the mechanisms that lead to formation of an organ-specific pattern of epithelial branches is still poorly understood (see also Hacohen *et al.*, 1998). In order to monitor differential epithelial branching, we followed in this work the sequential appearance of the branching profiles of ureter and lung epithelial buds during early stages of organogenesis and developed novel parameters to characterize epithelial morphogenesis for molecular analysis. Parameters such as the number of tips, the percentage of the module type (PMT) and the position index of the first branch (PIFB) demonstrate features of epithelial branching and point out the differences in branching between the kidney and lung epithelium. These parameters were also used to follow the capacity of the lung mesenchyme to induce the morphogenesis of the ureteric bud in experimental tissue recombinants. These studies demonstrate what kind of morphological changes are taking place due to epithelial-mesenchymal interactions in ureteric bud when the bud is recombined with lung mesenchyme. The recombination system and the parameters obtained can be used to study the mechanisms that control organ-specific epithelial branching morphogenesis in more detail.

TABLE 1

	Lung				Kidney			
	Left	Right	Right/Left	P value	Posterior	Anterior	Anterior/Posterior	P value
E10.75	468±56 (n=12)	537±51	1.15	P<0.01	66±12 (n=6)	133±53	2.0	P<0.05
E11.5	623±48 (n=5)	754±71	1.21	P<0.01	148±36 (n=6)	264±50	1.78	P<0.001

The first branch is asymmetric in the lung and kidney. The average lengths (±SD) of the first left and right epithelial branches (in μm) at two stages of development of the embryonic lung and kidney are shown. The P value describes the significance of the difference in length between the right and left or anterior and posterior branches.

Results

Identification of the Characteristic Epithelial Branching Modules in the Early Developing Lung and Kidney

To obtain reference points for the experimental studies on epithelial branching morphogenesis, we first monitored the dynamics of the generation of epithelial buds in the mouse embryonic lung and kidney from the initiation of organogenesis, stage 1-2 tail somites (TAS), onwards. The embryonic lung initiates its bud formation earlier than the kidney. At 1-2 TAS (E10) the lung epithelium had formed two primary buds (Fig. 1A). The sign of the initiation of lateral branching in the right lung bud was seen at 4-5 TAS (Fig. 1B), and these lateral branches sprouted almost simultaneously at 4-9 TAS (Fig.1 B,C). A fourth bud appeared interiorly and emerged from the right main bud at 11-12 TAS (Fig. 1D). The left lung bud remained without forming any clear lateral side buds until E11 (18-20 TAS), when two lateral buds began to emerge from it (Fig. 1E). A third bud appeared at E11.5 (21-24 TAS) (Fig. 1F). The skeletonised images of different stages of the early lungs

TABLE 2

	E10.75	E11.5	E11.5+24 h
Lung	2.29±0.58 (n=24)	2.83±0.70 (n=10)	3.00±0.84 (n=20)
Kidney	1.47±0.16 (n=9)	1.71±0.25 (n=7)	1.69±0.24 (n=14)
P value	<0.001	<0.001	<0.001

The position index of the first branch (PIFB) is higher in the early embryonic lung than in the kidney.

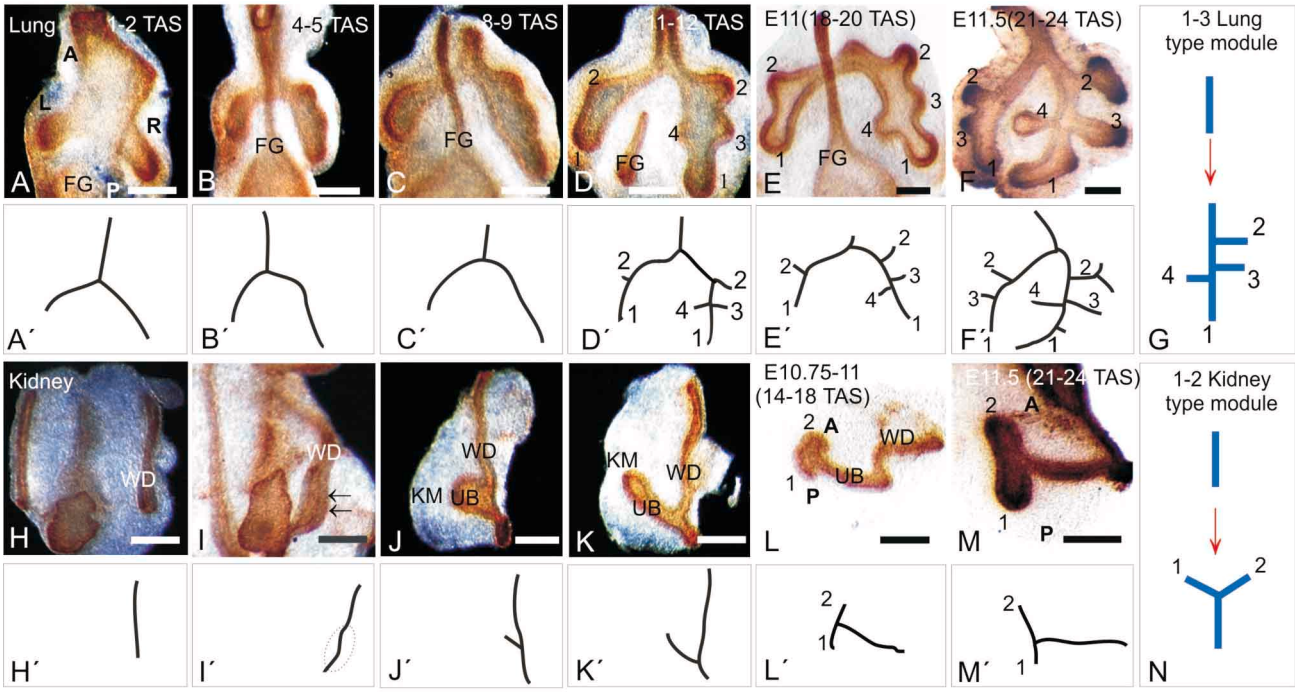

Fig. 1. The pattern of epithelial branching in the embryonic lung and kidney is already different at the early stages of organogenesis. *The Troma-I antibody was used to visualize the epithelial bud in the lung (A-F) and kidney (H-M)* in vivo*, and the stained epithelium was skeletonised with a Scion image program. The 1-12 tail somites (TAS) correspond to E10-E10.75.* **(A)** *In the lung, organogenesis is initiated by formation of two endoderm-derived epithelial buds (1-2 TAS). The right one will generate the first four lateral epithelial side-buds during 4-12 TAS* **(B-D)***, which form more or less simultaneously. The numbers indicate the order in which the tips appeared. Note that the left side of the lung is delayed in its development relative to the right side. The epithelial buds generated at 11-12 TAS grow unbranched at 18-20TAS* **(E)***. The formation of several lateral epithelial branches is apparent in both lobes at 21-24 TAS* **(F)** *and constitutes a lung type of branching module, which is summarized as a 1-3 type in* **(G)***. Branching is counted as taking place according to the 1-3 type module when at least three buds are generated from the main stem. Initiation of kidney organogenesis is delayed relative to the lung. At 1-2 TAS the Wolffian duct still grows caudally* **(H)** *and at 4-5 TAS* **(I)** *it has started to grow dorsally (arrows) into the adjacent metanephrinc mesenchyme. At 8-12 TAS the ureteric bud (UB) is clearly distinguishable but has not yet initiated branching* **(J,K)***. At 14-18 TAS (E10.75-E11), the UB of the kidney has initiated its first dichotomous branch* **(L)***, to generate a typical T-shaped bud at 21-24 TAS (E11.5)* **(M)***. The branching pattern typical of the kidney is summarized as a 1-2 type in* **(N)***, indicating that each epithelial tip in the kidney branches dichotomously to generate two subsequent epithelial tips. (A´-F´, H´-M´) are epithelial skeletons produced by image analysis and correspond to the epithelial branching patterns shown in (A-F, H-M). Abbreviations: FG, Foregut; WD, Wolffian Duct; TAS, Tail Somite; UB, Ureter Bud; KM, Kidney Mesenchyme; L, left; R, right; A, anterior; P, posterior; HG, Hindgut. Scale bar, 200 μm.*

(Fig. 1, A'-F') demonstrate that a lung epithelial bud typically generates several lateral side branches, with at least three of them from the main primary epithelial bud. Such epithelial branching is thought to represent 1-3 type branching, and a stem with lateral sub-branches will be referred to below as the lung-type branching module (Fig. 1G).

No signs of ureteric bud formation from the Wolffian duct were yet to be seen at the stages when the first two lung buds had already been generated (Fig. 1H). At the 4-5 TAS stage the posterior end of the Wolffian duct enlarged from its dorsal part (Fig. 1I, arrows), and at 8-12 TAS a ureteric bud became clearly distinguishable and it appeared in an unbranched form in the surrounding kidney mesenchyme (Fig. 1 J,K). At 14-16 TAS the tip of the ureteric bud enlarged (Fig. 1L) and at 18-24 TAS (E11-11.5) it proceeded to generate its first T-shaped branch (Fig. 1M). The mode of branching of the epithelial bud in the kidney was clearly different from that of the lung, even at these early developmental stages. The first epithelial bud in the kidney branched dichotomously, the tip thus generating two new epithelial tips. Based on the skeletonised micrographs of the processed images from the kidneys at the early stages (Fig 1, H´-M´), we propose that the kidney-type epithelial branching represents a 1-2 type branching, so that dichotomously branched units can be referred to as representing the kidney-type branching module (Fig. 1N).

Sequential Generation of the Lung-Type Branching Module In Vitro *and* In Vivo *during Early Lung Development*

To investigate whether the 1-3 type branching module observed in the early lung also held good during later developmental stages, we investigated the epithelial branching patterns of both cultured and *in vivo* samples. Organs isolated at E11.5 were subcultured for 3, 24, 48 and 72 h and their epithelial structures were stained and their morphology analyzed. In most cases the subsequent epithelial buds continued to generate at least 3 lateral side branches, similar to the early stages (Fig. 2 A-C). During progressive formation of the epithelial branches, however, a few newly formed branches appeared in the 1-2 type mode, but these typically generated more side branches later, and the module was then judged to represent the 1-3 branching type (Fig. 2 A-C). The lungs cultured for various lengths of time were thus composed to major extent of lung-type modules.

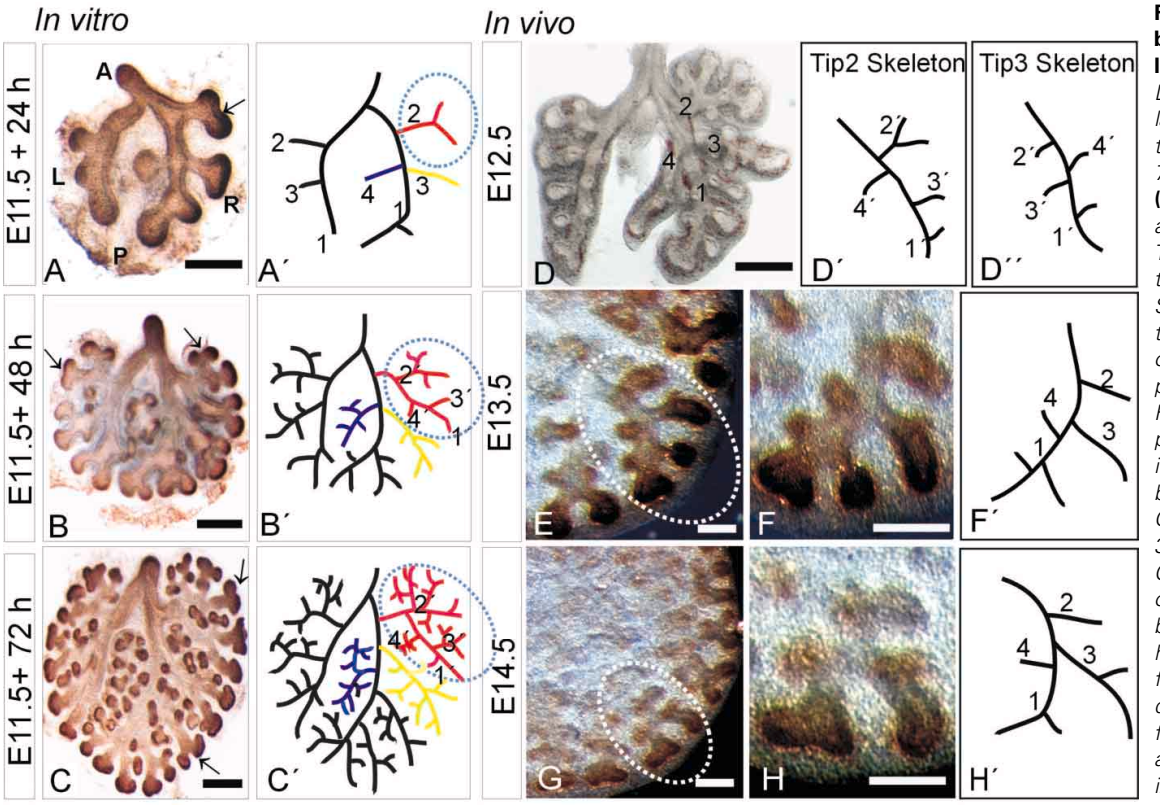

Fig. 2. The *in vitro* mode of branching pattern in the lung mimics that *in vivo*. *Lung buds were either isolated at E11.5 and subcultured for 24 h* **(A)**, *48 h* **(B)** *or 72 h* **(C)** *or isolated at E12.5* **(D)**, *E13.5* **(E)** *and E14.5* **(G)** *and stained directly with Troma-I antibody (A-C, E-H) to visualize the epithelial tree. Skeletonization of the epithelial branches was carried out with the Scion image program (A´-C´, D´, D´´, F´, H´). In the cultured lungs, the primary lateral branches seen in (A) undergo secondary branching during culture (B, C), mainly according to the 1-3 pattern. The arrows in (A-C) indicate a few examples of transient 1-2 type branches.* **(A´-C´)** *Branches have been marked with different colours to indicate the developmental history of the founder branches seen in A´ and the 1-3 lung-type branching module. The pattern of generation of epithelial side-branches in culture is similar to that* in vivo *(D-H), where the 1-3 lung-type module is detectable at the periphery of the growing lung at E12.5* **(D´,D´´)**, *E13.5* **(F´)** *and E14.5* **(H´)**. *F and H are the enlarged structures indicated by the circles in E and G, respectively. L, left; R, right; A, anterior; P, posterior. Scale bar, 200 μm.*

To study the branching patterns more carefully, the complete epithelial tissue was skeletonized with the image analysis program and the developmental history of each module was followed (Fig. 2 A´-C´). Hence the branches generated during subsequent developmental stages could be traced back to the early bud from which they had appeared. The data indicated that the 2nd and 3rd epithelial branches in the lung mainly repeated the 1-3 lung type of branching, but at later stages, after culturing for 72 h, the structure of the lung became too complex to distinguish the branching patterns accurately and the culture conditions apparently started to limit the overall formation of the organ. In order to avoid this problem and to study the later stages of branching in more detail as well, we dissected four main branches of the right lung individually from an E12.5 embryo (Fig. 2D). These branches were cultured for three days and the sub-branches generated were monitored. The branching pattern of the second and third branches to form in the cultures continued to follow the 1-3 type of development (data not shown), suggesting that the branching development typical of the early lung bud persisted into the later stages of organogenesis.

To analyze whether the branching pattern observed in the organ culture system was similar to that occurring in an *in vivo* situation, lungs from E12.5, E13.5 and E14.5 embryos were separated out, fixed and processed for whole-mount immuno-staining to reveal the epithelial branches (Fig. 2 D-H). At these stages the epithelial branches on the surface of the lung were still easily distinguishable. As during the earlier developmental stages, the 1-3 type of branching module was distinguished (Fig. 2 D-D´´, F-F´, H –H´). Thus the lung type of epithelial branching pattern generated *in vitro* is similar to that formed *in vivo*.

The Ureteric Bud branches in Organ Culture in a manner that is typical of the Kidney and clearly distinct from the Embryonic Lung

After the formation of the first epithelial branch in the kidney, the mode of generation of the subsequent epithelial branches was followed further in organ culture. Epithelial branching in kidneys at E11.5 cultured for 24-72 hours showed to major extent the 1 - 2 type branching pattern, in which each epithelial tip generated two new epithelial tips (Fig. 3 A-C, A'-C') and correlated rather well to the *in vivo* situation (D-H'). In 72 h cultures, when the epithelium had branched for 5-6 generations, the average number of branched ureteric tips was 41 (N=5, N is the number of cases studied). This value was in agreement with the formula $2^{n,}$ in which n is the generation of branching, applied to the iterative bifid branching pattern by Al-Awqati and Goldberg (1998). However, as the branches are generated in non linear manner (see eg. Fig. 3C) this formula may not be fully applied to model early branching morphogenesis. A few lateral side branches also appeared from different grades of main stalk in the kidney, but they represented no more than a minority of the branches.

Specific Parameters for Lung-Type and Kidney-Type Epithelial Morphogenesis

To search for specific parameters for monitoring the characteristics of early kidney or lung-type epithelial branching, we measured

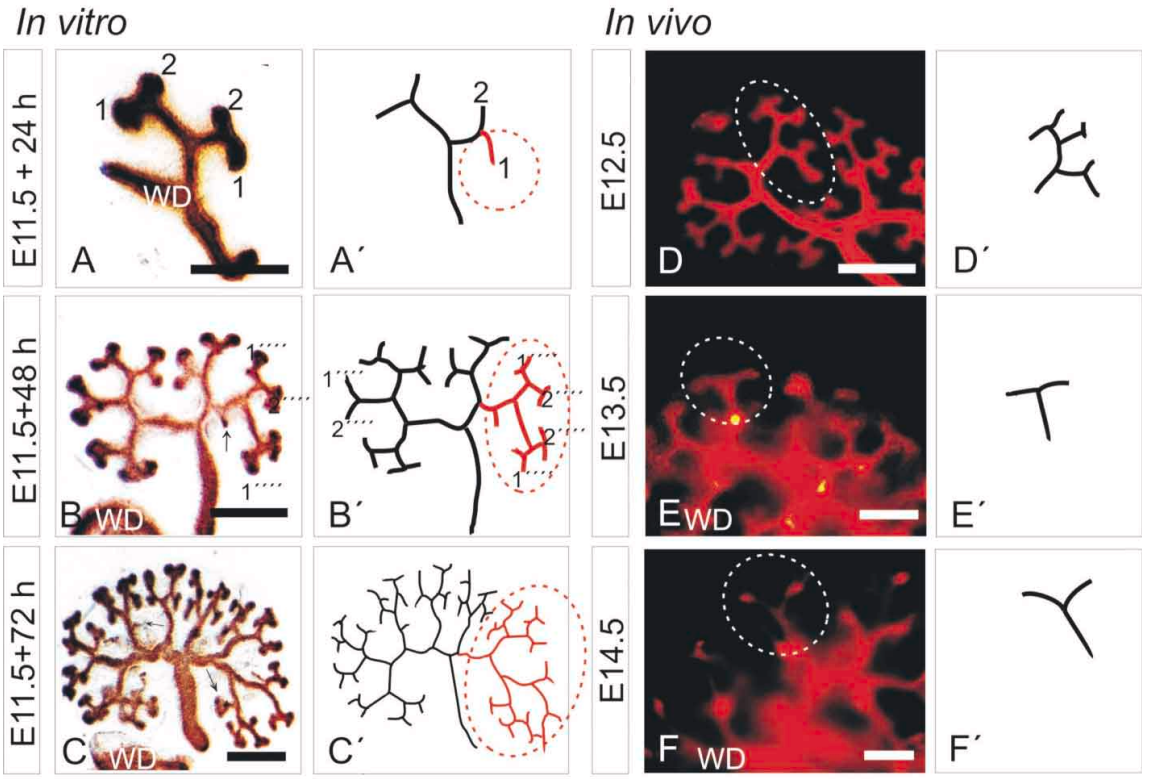

Fig. 3. Kidney development involves dichotomous epithelial branching. *Kidney rudiments were separated out at E11.5 and cultured for 24 h* **(A)**, *48 h* **(B)** *or 72 h* **(C)** *or isolated at E12.5* **(D)**, *E13.5* **(E)** *and E14.5* **(F)** *and stained with Troma-I antibody to visualize the epithelial branches. After the initiation of ureter branching, the subsequent branches are also generated dichotomously, with each tip dividing into two and thus generating two new tips, representing the 1-2 kidney type of branching module. This mode is typical at E11.5 + 24 h (A) and during subculture (B, C). A few lateral branches are marked with arrows (B, C). The developmental history of the branches generated can be followed in organ culture, and one branch is indicated in red in the skeletons generated by the Scion image program* **(A´-C´)**. *The skeletons in (A´-C´) correspond to the epithelial branching profiles of (A-C). The pattern of generation of epithelial branches in culture is similar to that* in vivo *(D-F), where the 1-2 kidney-type module is observed at the periphery of the growing kidney at E12.5* **(D´)**, *E13.5* **(E´)** *and E14.5* **(F´)**. *1´´´´ and 2´´´´ indicate that the bud has branched four times. A-C is the HRP-conjugated whole mount immunostaining and D-F is TRITC-conjugated immunofluorescent staining. WD, Wolffian Duct. Scale bar, 200 μm.*

the following values, 1) the length of the left and right, or anterior and posterior, epithelial branches, 2) the angle of the first branch (α or β), 3) the number of epithelial tips, 4) the position index of the first branch in one specific module (PIFB), i.e. the ratio between the total length of the branch and the length from the stalk to the position of the first branch, 5) the percentage of the given module type (PMT) (see Fig. 4 for descriptions of the parameters).

The lengths of the longest left and right epithelial branches in the developing lung and the lengths of the anterior and posterior branches in the kidney were measured at E10.5 and E11.5. Before the initiation of the second branch in lung, the right lung bud was significantly longer than the left (Table 1), and the anterior branch of the kidney was also longer than the posterior one at both time points. Thus, the first branch of the ureteric bud in the kidney was dichotomous but asymmetric. In addition, this value showed that the asymmetry was more evident in the developing kidney than in the lung.

The skeletonised images of the early lung and kidney rudiments were used to measure the angle of the first epithelial branch in the cultured explants. These studies revealed that the angle between the first left and right epithelial branches of the lung

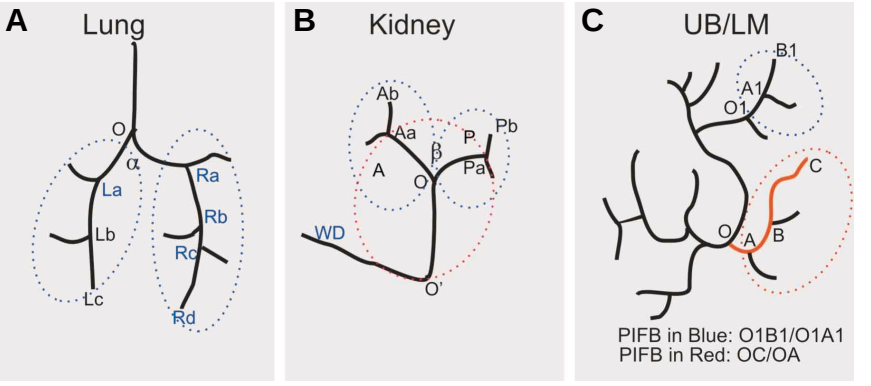

Fig. 4. Measurement of certain parameters in the lung and kidney to reveal differences in epithelial branching. *The epithelial branches encircled constitute the lung (1-3) or kidney type (1-2) of early branching module in* **(A)** *and* **(B)**. *α and β in (A) and (B) indicate the angles between the left and right side of the first epithelial branch in the lung and kidney. The length of the epithelium was measured from the stalk of the epithelium O to the points Ra, Rb, La, Lb and Lc for the lung (A) and Aa, Ab, Pa, Pb for the kidney (B). The values obtained from OLc/OLa, ORd/ORa, O´Aa, O´Pa, OAb/OAa and OPb/OPa represent the position index of the first branch (PIFB) in the lung and kidney, respectively.* **(C)** *Skeleton of one representative sample of UB/LM. In order to obtain PIFB values for the tissue recombinants, the lengths of branches were measured (Examples are shown in red and blue represent lung and kidney type modules) to obtain PIFB. As an example in red circle the PIFB is counted by OC/OA and in blue circle the PIFB is O1B/O1A.*

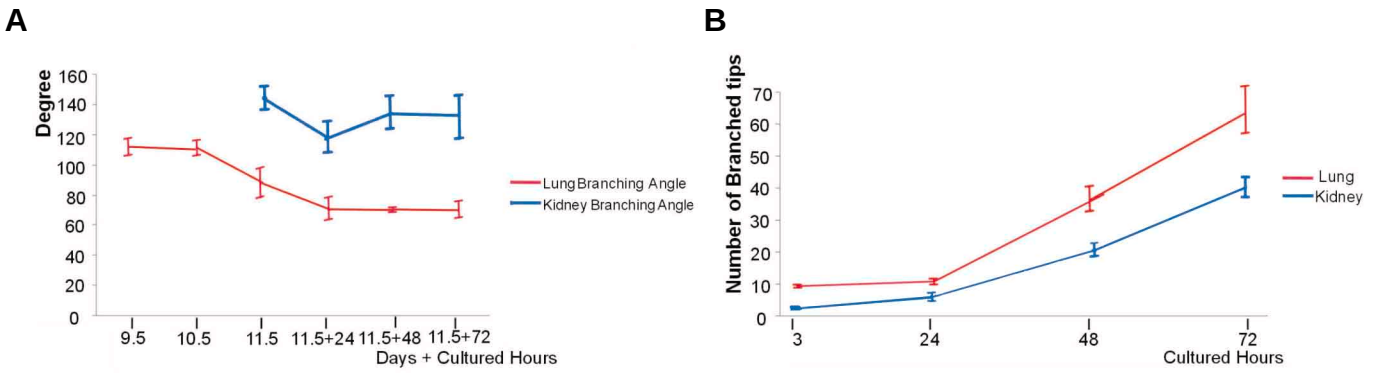

Fig. 5. Angle of the first branch and number of epithelial tips in the lung and kidney. (A) *The angle of the first branch is greater in the kidney than in the lung.* **(B)** *The developing lung bud has generated more epithelial branching tips (red line) than the kidney (blue line) at the time points indicated in culture.*

measured at different time points was constantly smaller than the corresponding angle in the kidney, being less then 120°, as opposed to between 130-160° in most kidney cases (Fig. 5A). The number of tips was also higher in the lung samples than in the kidney samples and their amount increased faster in the former (Fig. 5B).

We next measured the PIFB values in specific models in E10.5, E11.5 and E11.5 lung and kidney samples cultured for 24 h (Table 2). These were significantly higher for the lung than for the kidney at these early developmental time points ($P<0.001$), suggesting that the PIFB value can be used to identify the lung and kidney types of epithelial branching development. The PIFB value was also unchanged when the E11.5 kidney was cultured for 24 h suggesting that the proportions of the branches are maintained during early developmental stages.

Finally the percentages of the lung and kidney module types (PMTs) in the explants were analyzed from their skeletonized images, using the module types characterized earlier (Fig. 1) and this was based on visual inpesctions of generation of the modules during culture. Briefly, the branching module was considered to represent a kidney type if a dichotomous 1-2 type of epithelial branching pattern was seen. The lung type, a 1-3 type of branching pattern, was judged to be in question if three or more early or more advanced lateral buds were seen in the samples. Both kidney-type and lung-type modules were identified in each explant, and the kidney and lung-type modes were counted as percentages of the total number of modules. The criteria meant that the lung-type module was not exclusive, but was clearly dominant in the developing lung, and similarly the kidney-type module was dominant in the kidney (Table 3A).

Lung Mesenchyme is Sufficient to Induce Early Lung-Type Epithelial Development in a Ureteric Bud, as judged by Various Morphological Criteria

Recent data have shown that the ureteric bud is competent to develop with mesenchyme of non-kidney origin, such as lung mesenchyme (Kispert *et al.*, 1996; Sainio *et al.*, 1997; Lin *et al.*, 2001a), what type of changes were induced in ureter morphogenesis has not been well characterized. Recombination of a ureteric bud with a lung mesenchyme leads to morphological changes in the former (Fig. 6 A-C´). The novel parameters described above were used to qualify the changes in epithelial morphogenesis in the heterotypic tissue recombinants as well.

The epithelial morphogenesis of the heterotypic recombinants was compared with that of the homotypic recombinants between lung epithelium and mesenchyme and kidney epithelium and mesenchyme. Advantage was taken of the transgenic mice expressing green fluorescent protein (GFP) for monitoring epithelial branching during culture. As it was a case of comparing intact lung and kidney samples, the average number of epithelial tips in the homotypic recombinants between lung epithelium and lung mesenchyme (LB/LM) was significantly higher than that in the homotypic recombinants of ureteric bud and kidney mesenchyme (UB/KM) (Fig. 6D). The average number of epithelial tips in the heterotypic tissue recombinants of ureteric bud and lung mesenchyme (UB/LM), on the other hand, was significantly higher than that in the homotypic kidney recombinants, and close to that of the lung homotypic tissue recombinants (Fig. 6D).

TABLE 3

A

	1-3 type branching (%) (Early Lung Type)	1-2 type branching (%) (Early Kidney Type)
Kidney (n=15; 314 modules)	12	88
Lung (n=20; 244 modules)	81	19

B

	1-3 type branching (%) (Early Lung Type)	1-2 type branching (%) (Early Kidney Type)
UB/KM (n=10; 86 modules)	16	84
LB/LM (n=14; 94 modules)	66	34
UB/LM (n=15; 103 modules)	70***	30

The percentage of the module type (PMT) in intact cultured tissues (A) and in homotypic and heterotypic recombinants (B). In A, samples from E11.5 embryos cultured for 24, 48 and 72 h were used. PMT was based on visual inspection and was determined by considering the module to represent a kidney-type module when branching was dichotomous (1-2 type) and one tip generated two new tips, and a lung-type module (1-3 type) when at least 3 side branches were apparent. The appearance of modules was monitored in culture. The difference in PMT values between UB/LM and LB/LM was not significant ($P=0.19$), whereas the lung-type PMT was significantly higher in UB/LM than in UB/KM ($P<0.001$, indicated with ** * in the table). N means the number of samples studied.

PIFB values also indicated epithelial changes in the ureteric bud in the heterotypic recombinants. The PIFB value was 3.10±1.22 (n=96, where n is the number of modules analyzed) for the LB/LM and 2.03±0.67 (n=86) for the UB/KM, showing a similar difference as between the intact tissues (Fig. 6E). The PIFB of the UB/LM heterotypic recombinants was 3.22±1.81 (n=75), and hence was close to that of the LB/LM recombinants and significantly different from that of the UB/KM recombinants (Fig. 6E).

Analysis of the recombinants after culturing for 5 days revealed 66% lung-type modules in the homotypic lung recombinants and 84% kidney-type modules in the kidney homotypic recombinants (Table 3B). The homotypic lung and kidney recombinants maintain their lung and kidney-type branching modes to a major extent. In the heterotypic recombinants, 70% of the modules represented the lung type and 30% the kidney type. The lung type branching module was thus dominant. The PMT values for the heterotypic recombinant were significantly different from those for the homotypic kidney recombinants but similar to those for the homotypic lung recombinants (Table 3B), indicating reprogrammed epithelial development.

Altogether, we conclude that the lung-type branching was predominant in the heterotypic UB/LM tissue recombinants, supporting the conclusion that the lung mesenchyme is sufficient to reprogramme morphogenesis of the ureteric bud towards the early lung type.

Left and Right Lung Mesenchyme and Mesenchyme from the Epithelial Tip Region promote Ureter Growth and Branching in a Similar Manner

The branching of the epithelial bud in the developing lung is typically asymmetric (see Fig. 2D) and there are more epithelial tips in the right lobe of the lung at E12.5 when compared to the left lobe (Fig. 7D). If the corresponding mesenchyme and epithelium is separated at E11.5 and then recombined and subcultured the right mesenchyme, however, looses it potential to promote more epithelial tips when compared to the left lung tissue (Fig. 7D). To test further whether the mesenchyme from left and right parts of the lung could promote ureter branching differently, mesenchyme separated from the right or left side of the lung bud (E11.5) was combined with ureteric bud of embryos of the same age. If the mesenchyme of the lung was responsible for the control of asymmetric branching in the embryonic lung, the same asymmetry might be detected in the ureter branching of the recombinants. However, the results showed that as is the case in the lung mesenchyme from the right or left side promoted branching of ureteric bud (Fig. 7, A-C), but there was no statistically significant difference in the number of epithelial tips between these recombinants (Fig. 7D).

For the recombination experiments mesenchyme from the whole E11.5 lung expect the tracheal region is used. To test if the

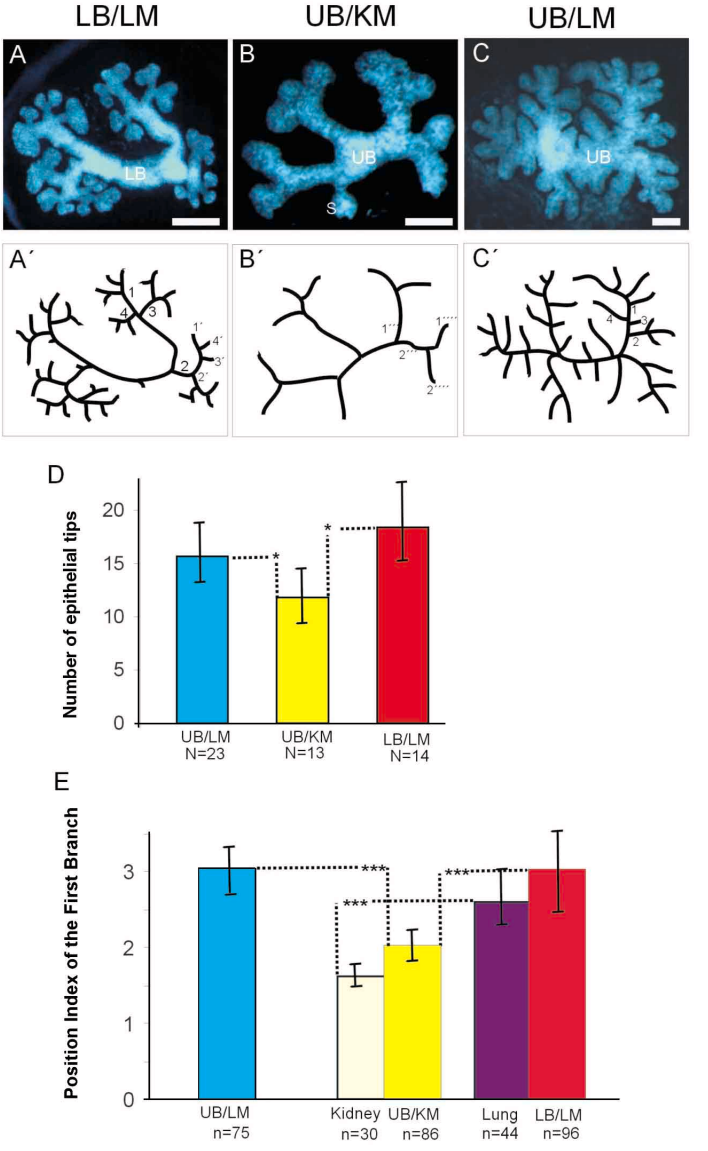

Fig. 6. The average number of epithelial tips and the position index of the first epithelial branch (PIFB) suggest that the lung mesenchyme respecifies ureter branching towards the lung type in cultures. *Lung epithelium and UB were separated from their respective mesenchymes from E11.5 embryos of transgenic mice expressing GFP under the CMVβ-actin promotor and were recombined with the corresponding mesenchyme separated from a wild type embryo at the same stage, and the recombinants were placed in culture for the times indicated. Homotypic recombinants between lung epithelial bud and lung mesenchyme* **(A)**, *cultured for 96 h, and ureter bud and kidney mesenchyme* **(B)**, *cultured for 96 h, are sufficient to reconstitute the lung 1-3 type* **(A´)** *and kidney type* **(B´)** *epithelial branching patterns. "S" signify for 'Stalk of ureteric bud' in B. Ureter tips marked with 1´´´, 2´´´ have branched three times and those marked 1´´´´, 2´´´´ four times. The ureter bud is also sufficient to branch with lung mesenchyme* **(C,C´)** *cultured for 120 h, doing so in the manner of the lung epithelium.* **(D)** *Average numbers of branching tips in UB/LM, UB/KM and LB/LM tissue recombinants cultured for 5 days. The average number of epithelial tips in the homotypic LB/LM recombinant is significantly higher than that in UB/KM (P<0.05), while that in the UB/LM heterotypic recombinant is close to the value for LB/LM and significantly higher than that for UB/KM (P<0.05). N: represents the number of case studied.* **(E)** *Measurement of the PIFB in the intact lung and kidney and in the various types of tissue recombinants indicates that it is very significantly higher in the lung and in the homotypic lung recombinants than in the kidney or the homotypic kidney recombinants (P<0.001). The PIFB value of the heterotypic tissue recombinants between the ureter bud and lung mesenchyme (UB/LM) is significantly higher than that of the kidney and UB/KM (P<0.001) and similar to that obtained for the lung and the LB/LM recombinants. N represents the number of modules studied. Scale bar, 200 μm.*

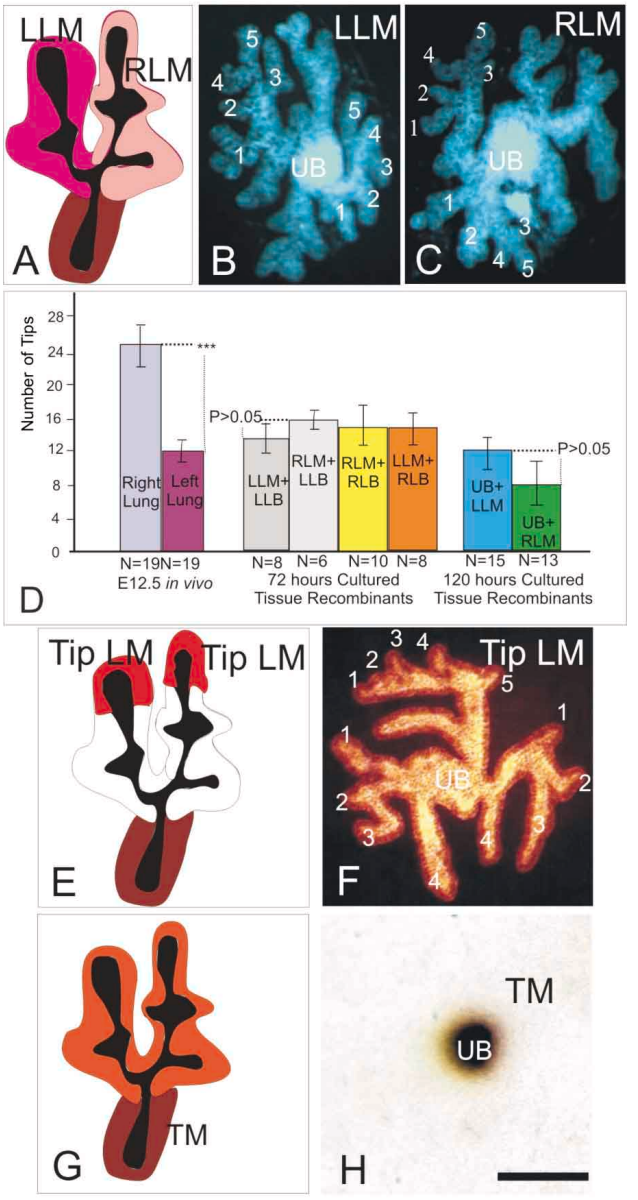

Fig. 7. The left and right lung mesenchyme are equal in their ability to promote branching of the ureter bud. *The ureter bud was separated out from E11.5 embryos of a transgenic mouse expressing GFP under the CMV β-actin promotor and recombined either with wild-type lung mesenchyme from the left or right side of an embryo at the same stage* **(A)**. *Both the left lung mesenchyme (LLM)* **(B)** *and the right lung mesenchyme (RLM)* **(C)** *have the potential to promote growth and branching of the ureter bud.* **(D)** *Counting of the average number of epithelial tips in the intact lobes of the right and left lung at E12.5 indicated a difference but when epithelium and mesenchyme are separated and recombined back together or to epithelial bud of the other lobe no significant difference in the induced tip numbers is seen (LLM+LLB, RLM+LLB, RLM+RLB, LLM+RLB). Left and right lung mesenchyme (LLM or RLB) support branching of ureteric bud (UB) with no significant difference in induced tip number. N represents the number of cases studied.* **(E)** *Lung mesenchymes adjacent to the secondary tips (red area) are sufficient to promote ureter growth and branching in the heterotypic tissue recombinants* **(F)**. **(G)** *Mesenchyme separated from the tracheal region (brown area) does not have the potential to promote ureter branching when these pieces of tissue are replaced in heterotypic tissue recombination* **(H)**. *The samples in (B), (C), (F) and (H) were cultured for 120 h. Scale bar, 200 μm.*

mesenchyme around the epithelial tip or closer to the stalk would have differences in their capacity to promote branching we specifically tested the potential of mesenchyme adjacent to the epithelial tip to promote branching of the ureteric bud (Fig. 7 D,E). As expected, the mesenchyme at the tip region was also sufficient to promote epithelial branching (Fig. 7F). Finally, the tracheal mesenchyme (Fig. 7G) had been shown to cease branching when placed adjacent to the epithelial tip region in the lung (Shannon, 1998), and as expected, the tracheal mesenchyme did not induce branching of the ureteric bud in the tissue recombinants (n=7) (Fig. 7H).

Discussion

Several factors have been proposed as being critical for establishing the pattern of branching during organogenesis, including the stereotype of the branching pattern, the number of generations, branch points and stems, the branching angles, the pace of development and fasciculation (for a review, see Al-Awqati and Goldberg 1998). Three successive patterns of branching have been found to take place during kidney organogenesis (Al-Awqati and Goldberg 1998 and references therein). First, the tip of each bifid branch can divide dichotomously into another bifid branch. In the human embryonic kidney, however, the branching occurs according to the terminal bifid model, which consists of a lateral branch dividing further into two terminal branches. During later divisions in human kidneys, several nephrons are induced simultaneously and their connecting tubules fuse and elongate to form arcades of several kinds. In the present study on mouse kidney development we show that the branching pattern of the kidney epithelium follows mainly the dichotomous 1-2 type during early organogenesis. This is supported by the average number of branching tips, the number obtained being fairly close to the expected value represented by the formula $2^{n,}$ which models the iterative bifid branching or dichotomous branching pattern (Al-Awqati and Goldberg 1998). On the other hand, the value does not follow the formula $2(2)^{n} - 2$, which models terminal bifid branching. However, the interpretation is complicated by the fact that branching does not proceed identically in different parts of the organ. In the early lung developing epithelium generates several lateral side branches according to the 1-3 type pattern, and does not appear to follow either iterative or terminal bifid branching.

To study further the difference in epithelial branching between the kidney and lung, we compared the numbers of epithelial tips, the left and right, or anterior and posterior lengths of the first branch, and the angle of the first epithelial branch. In addition, we developed values referred to as PIFB and PMT to represent characteristics of the branches. The applicable values were also used to monitor how the lung mesenchyme modifies the early growth and branching of the ureteric bud. From the early stages, E11.5, onwards, the lung generates relatively more epithelial tips than the kidney, and the difference becomes more pronounced after 48 h of subculture. Homotypic recombinant samples act similarly to intact tissues, the tissue recombinants between lung bud and lung mesenchyme containing significantly more branching tips than those between ureteric buds and kidney mesenchyme. The kidney and lung epithelia also differ in the symmetry of their branching and in the angle of the first branching, the ratio between anterior and posterior branches in the kidney being around two and the ratio between the right and left branches in the

lung being close to one. The angle of the first branch is less in lung samples cultured to two-dimensional form than in the corresponding kidney samples. The two latter parameters are not applicable to recombination samples, however, since the reference point stalk is lost upon operation.

The PIFB values for kidney and lung epithelia are informative, being significantly higher for the lung epithelium than for the ureteric bud throughout the period from E10.5 to E11.5 when cultured for 24 h *in vitro*. The same difference is also observed between the homotypic kidney and lung recombinants, indicating further that the recombination technique can be used to investigate epithelium-mesenchyme interactions. PIFB is defined by measuring the length of the longest epithelial branch, which is divided by the distance from the stem to the first branch in a branching module. Such an approach thus also takes into account the proportional growth of the epithelium.

Since the kidney and lung epithelia do not branch exclusively dichotomously and laterally, respectively (for a review, see also Davies and Davey, 1999), kidney and lung-type epithelial modules have also been defined and PMT values calculated. A new module was considered to be formed when the new tips had generated more than three tips. Analysis of PMT in kidney material placed in culture at E11.5 gave 12% 1-3 type branches, whereas the majority (88%) were dichotomous, i.e. of the 1-2 type. Our observations are consistent with time lapse photographs of cultured embryonic kidneys, where the epithelium is seen to express GFP under the Hox B7 promotor and mimic those seen *in vivo* (Srinivas *et al.*, 1999, see also: http://cpmcnet.columbia.edu/dept/genetics/kidney). In the lung, on the other hand, the 1-3 type of branching was exemplified in 81% of the modules. The PMT values of homotypic kidney and lung recombinants were again similar to those of the corresponding intact or cultured tissues. It is worth pointing out that PMT values can be monitored only at the early stages of morphogenesis, since later the organ structure becomes too complex for the interpretation of modules, neither can two-dimensional culture conditions fully represent the three-dimensionality of the generation of epithelial side branches. Despite these limitations, however, the branching profiles of *in vitro* samples correspond fairly well to those of *in vivo* samples, and modules were still detectable in freshly separated lung from E14.5 embryo. Thus several measurable parameters are available to investigate whether a particular instance of epithelial development represents the lung or kidney type.

Ureteric bud tissue separated from kidney mesenchyme at the stage of formation of the first branch survives and initiates epithelial growth when combined with lung mesenchyme (Kispert *et al.*, 1996; Sainio *et al.*, 1997; Lin *et al.*, 2001a), while lung mesenchyme is capable of reprogramming the expression of some genes of a ureteric bud toward that of the lung epithelium, when judged by the patterns of expression of a basement membrane component, type XVIII collagen, and the *Sonic hedgehog* gene (Lin *et al.*, 2001a). Lung mesenchyme is also sufficient to induce ectopic lung surfactant protein C expression in the ureteric bud. Both the number of epithelial tips and the PIFB values for recombinants between the lung mesenchyme and the ureteric bud demonstrate that lung mesenchyme also re-directs the morphogenesis of the kidney epithelium as was suggested by our earlier work (Lin *et al.,* 2001a). All three values are similar to those obtained for homotypic lung recombinants, and significantly different from the values for homotypic kidney recombinants. The additional results now in hand indicate that the development of a ureteric bud can indeed shift from the dichotomous kidney type of branching pattern towards one that resembles aspects of early lung-type epithelial morphogenesis.

In the heterotypic recombinants the ureteric bud expresses genes such as *c-ret* (Kispert *et al.*, 1996), *Wnt-11* and *Sox 9* (Lin *et al.*, 2001a), which are not expressed by lung epithelium, suggesting that the epithelial bud still retains some of its ureteric bud characteristics with lung mesenchyme. This may contribute to the restriction in the potential of the epithelium to interact with the lung mesenchyme during subculture. The ureteric bud may lack certain receptors for the signals that are expressed by the lung mesenchyme.

We have reported here on several morphological measurements carried out to characterize epithelial branching properties during early lung and kidney development. Several parameters were found to be distinct for the kidney and lung types of epithelial branching, and since the two-dimensional cultured samples mimic the *in vivo* system fairly well, the measurements were applied to homotypic and heterotypic tissue recombination model systems. The average number of branching tips, the PIFB and PMT values, and the pattern of conformity to either the kidney or lung type showed now in this paper that epithelial morphogenesis shifts towards the lung type in heterotypic tissue recombinants between the ureteric bud and lung mesenchyme. In addition, the tissue recombination technique was used to demonstrate that mesenchymal tissue separated either from the left or the right side of the early lung bud possesses a similar capacity to support early lung-type growth in the ureteric bud. The heterotypic tissue recombinants thus should serve as a useful model to study early steps in tissue morphogenesis experimentally.

Materials and Methods

Metanephric Kidney, Lung Organ Culture and Tissue Recombination Experiments

The CD-1 mouse line was from the Jackson Laboratory and the transgenic mouse expressing GFP mouse line was from Dr. A. Nagy (Samuel Lunenfeld Research Institute, Toronto). The organ culture and tissue recombination experiments were carried out as previously reported (Lin *et al.*, 2001a). The lungs were isolated from E9.5 to E14.5 embryos, and the urogenital block or kidney was isolated from E10.5 to E11.5 embryos. For better visualization of the branching profiles, the organ rudiments were cultured for 3 hours to flatten the samples. Kidney and lung rudiments were prepared from E11.5 embryos, cultured for 24, 48, and 72 h, and processed.The organ rudiments for all the tissue recombination experiments were incubated for two minutes in 3% pancreatin/trypsin (GibcoBRL) in Tyrode´s solution. During mechanical separation of the lung mesenchyme we routinely discarded the mesenchyme that surrounded the presumptive trachea. Each UB/LM recombinant involved one lung mesenchyme and one ureteric bud, but for the recombination between the ureteric bud and the left lung mesenchyme or right lung mesenchyme, two mesenchymes were used for one ureteric bud. For unknown reason the isolated ureteric bud does not branch well with kidney mesenchyme when recombined (Lin *et al.,* 2001b) and glial cell line-derived neurotrophic factor (GDNF) has been used as a factor to promote ureteric bud branching with kidney mesenchyme and to study functions of other growth factor on ureteric bud branching morphogenesis (Qiao *et al.,* 1999). Therefore, in UB/KM homotypic tissue recombinant

GDNF (100 μg/ml, PeproTech, USA) was used. Two kidney mesenchymes were used for one ureteric bud for UB/KM. For the recombination between the ureteric bud and tracheal mesenchyme, three tracheal mesenchymes were used for one ureteric bud. The bare area of the ureteric bud was scraped gently with the tip of a needle (0.7 X 40 mm, TERUMO). Inspection of the wall of such a ureteric bud showed a clear absence of residual metanephric cells. A minimum of six recombinations in each experiment was carried out, and the subculture time was five to six days.

Whole-Mount Immunostaining and Time Lapse Studies of GFP UB/KM and GFP UB/LM

HRP conjugated and fluorescence whole-mount immuno-staining was carried out according to Marti *et al.,* (1995) and Lin *et al.* (2001a). 3,3'-diaminobenzidine (DAB) (ZYMED, USA) being used as a substrate for the colour reaction. For the easier comparison of the samples after different hours of culture and also for the easier manipulation for immunostaining the E11.5 explants were cultured for 3 h. The Troma-I antibody against cytokeratin (Developmental Studies Hybridoma Bank) was used, and peroxidase-conjugated and TRITC-conjugated donkey anti-rat IgG (Jackson Immuno Research laboratories, Inc) as secondary antibodies. The samples were viewed under a Leitz CLSM confocal microscope (Leica, Germany) when ready, and photographed with a Leica digital camera (Leica DC 100S). The ureteric buds or lung buds for the time lapse studies were from a transgenic mouse expressing GFP in response to the CMV β-actin promotor generated by germline transmission of green fluorescent ES cells (Hadjantonakis *et al.*, 1998). GFP- UB/KM and GFP - UB/LM were taken out of the incubator every 24 hours and photographed with a Leica digital camera.

Image Analysis of Epithelial Branches during Morphogenesis

Epithelial branching morphogenesis was determined by counting the numbers of epithelial tips in the cultured explants at different points in time and by analyzing the epithelial structures on the whole mount explants using transillumination to visualize them. Digital photomicrography was used to acquire the images, which were then analysed using the Scion image analysis software (version beta 3b, Scion Corporation, NIH, USA). The main steps of the image analysis were the following: (1) segmentation of the epithelial branches and calculation of the skeleton, mainly after Gilbert *et al.*, (1996), and (2) measurement of the length and angle after spatial calibration of the pixels. The lengths were defined with the freehand line selection tool. The angle between two axes of first branching in different lung and kidney explant stages was measured using the angle measurement tool in the software.

The precision of the skeletonizing and of the measurements resulting from this was ensured as follows: a typical sample was repeatedly digitized 10 times after repositioning, refocusing and adjustment of the illumination, and the length and angle measurements were performed on each image after segmentation and skeletonization. The precision or coefficient of variation (CV) was calculated from the formula CV=SD/mean, where SD is the standard deviation of the 10 measurements. The precision was 0.017 for angle measurements and 0.025 for length measurements.

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