Bmp4 and Fgf10 play opposing roles during lung bud morphogenesis

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SUMMARY

Morphogenesis of the mouse lung involves reciprocal interactions between the epithelial endoderm and the surrounding mesenchyme, leading to an invariant early pattern of branching that forms the basis of the respiratory tree. There is evidence that Fibroblast growth factor 10 (Fgf10) and Bone Morphogenetic Protein 4 (Bmp4), expressed in the distal mesenchyme and endoderm, respectively, play important roles in branching morphogenesis. To examine these roles in more detail, we have exploited an in vitro culture system in which isolated endoderm is incubated in Matrigel™ substratum with Fgf-loaded beads. In addition, we have used a Bmp4lacZ line of mice in which lacZ faithfully reports Bmp4 expression. Analysis of lung endoderm in vivo shows a dynamic pattern of Bmp4lacZ expression during bud outgrowth, extension and branching. In vitro, Fgf10 induces both proliferation and chemotaxis of isolated endoderm, whether it is derived from the distal or proximal lung. Moreover, after 48 hours, Bmp4lacZ expression is upregulated in the endoderm closest to the bead. Addition of 30-50 ng/ml of exogenous purified Bmp4 to the culture medium inhibits Fgf-induced budding or chemotaxis, and inhibits overall proliferation. By contrast, the Bmp-binding protein Noggin enhances Fgf-induced morphogenesis. Based on these and other results, we propose a model for the combinatorial roles of Fgf10 and Bmp4 in branching morphogenesis of the lung.

Key words: Bmp4; Fgf10, Lung, Respiratory epithelium, In vitro culture, Endoderm, Branching morphogenesis, Tissue culture

INTRODUCTION

The embryonic mouse lung provides a model system for exploring the molecular and cellular principles underlying branching morphogenesis, a process common to the formation of a number of organs (Gumbiner, 1992; Hogan, 1999; Metzger and Krasiw, 1999). The early (pseudoglandular) lung is composed of three cell layers: an inner endodermal epithelium, a splanchnic mesodermal mesenchyme and an outer mesothelium. Starting at E10.5, reciprocal interactions between the epithelial and mesodermal layers lead to branching morphogenesis, a reiterative process in which a network of airway tubes is generated by the successive and invariant branching of the epithelium.

Grafting experiments have shown that secreted signals from the mesenchyme control branching morphogenesis (Alescio and Cassini, 1962; Taderera, 1967; Wessels, 1970; Shannon, 1994; Shannon et al., 1998). For example, when grafted adjacent to E11.5 tracheal endoderm denuded of mesoderm, distal mesenchyme induces ectopic endodermal budding. The endoderm in these ectopic buds adopts both the morphology and gene transcription patterns characteristic of distal cells, suggesting that mesenchymal signals stimulate changes in cell shape, movement and differentiation of the underlying epithelium (Shannon, 1994). Prime candidates for mediating these epithelial-mesenchymal interactions are members of two highly conserved families, the Fibroblast growth factors (Fgfs) and the Bone morphogenetic proteins (Bmps). In numerous other systems, including the limb and feather bud, Fgf and Bmp signals act together in regulatory networks during epithelial-mesenchymal interactions (Thelesleff et al., 1995; Martin, 1998; Normaly and Morgan, 1998; Tickle, 1999). Moreover, two members of these families, Fgf10 and Bmp4, are expressed in dynamic and complementary patterns in the lung. While Fgf10 is expressed in discrete domains in the early distal mesenchyme, in a pattern anticipating the sites of bud formation, Bmp4 expression is localized to the tips of endodermal buds (Bitgood and McMahon, 1995; Belluscet al., 1996, 1997b; Park et al., 1998).

Several lines of evidence implicate the Fgf family of ligands and receptors in lung development. In vitro, purified Fgf10 promotes the growth and branching of distal lung endoderm (Belluscet al., 1997b; Park et al., 1998). Fgf10 is required for lung development in vivo, since mouse mutants homozygous for a null allele do not generate primary lung buds from the ventral foregut (Min et al., 1998; Sekine et al., 1999). While the role of Fgf7 is less clear, this family member is expressed at low levels in the early lung, and can also support growth of lung endoderm in vitro (Cardoso et al., 1997; Belluscet al., 1997b; Park et al., 1998). Of the two Fgf receptors expressed in the endoderm during branching morphogenesis, Fgfr2 is ubiquitously detected, while Fgfr4 expression is confined to the distal tips of lung buds (Cardoso et al., 1997). To date, no evidence for Fgf10 signaling through Fgfr4 has been found.
However, both Fgf10 and Fgf7 signaling is mediated by Fgfr2 (Beer et al., 1997; Xu et al., 1998), and a requirement for Fgfr2(IIIb) in lung development has been demonstrated using both knockout and dominant negative approaches (Peters et al., 1994; Celli et al., 1998; De Moerlooze et al., 2000). Chimeras generated by aggregating Fgfr2 null ES cells with wild-type tetraploid embryos also phenocopy Fgf10 homozygous null embryos (Arman et al., 1999).

Despite the identification of Fgfs as positive mediators of distal mesenchyme signaling, a number of questions remain. First, since Fgf10 homozygous null mice completely lack primary lung buds, the precise role of the protein during branching morphogenesis is unclear. Several hypotheses are possible: in the simplest case, Fgf10 may be required as a permissive mitogenic factor for lung endoderm. Alternatively, or in addition, Fgf10 may promote directional growth or migration of individual buds. The Drosophila tracheal system provides precedence for Fgf signaling in the complex processes of cell migration and chemotaxis, and work in the mouse supports a chemotactic role for Fgf10 in the lung (Klambt et al., 1992; Sutherland et al., 1996; Park et al., 1998; for review, see Metzger and Krasnow, 1999). Finally, in combination with Bmp4, Fgf10 may also regulate endoderm differentiation, particularly by maintaining or promoting a distal cell fate (Weaver et al., 1999). Previous transgenic work using the lung-specific Surfactant protein-C (Sp-C) promoter has revealed roles for Bmp4 in proximodistal differentiation of lung endoderm, and in branching morphogenesis at late stages of development (Bellusci et al., 1996; Weaver et al., 1999). However, because high levels of expression from the Sp-C promoter are achieved only during midgestation, analysis of the role of Bmp4 in the earliest events of lung branching has not been possible. This difficulty is compounded by the early lethality of the Bmp4 null homozygotes, which die before the onset of lung development (Winnier et al., 1995; Lawson et al., 1999). Nevertheless, based on the complementary expression patterns of Fgf10 and Bmp4, we and others have hypothesized that Fgf10 protein in the mesenchyme locally induces Bmp4 transcription in the underlying endoderm, and that the resulting combination of Fgf10 and Bmp4 signaling in the distal endoderm contributes to the process of branching morphogenesis (Hogan et al., 1997; Lebeche et al., 1999). In order to dissect further the roles of these proteins, we have exploited a combination of two experimental tools: a tissue culture system for studying lung endoderm proliferation and morphogenesis in vitro (Nogawa and Ito, 1995), and a Bmp4lacZ ‘knock-in’ line of mice in which lacZ functions as a reporter for Bmp4 expression (Lawson et al., 1999). Based on the results obtained with this approach, we propose a model for the combinatorial roles of Fgf10 and Bmp4 in the early steps of branching morphogenesis.

MATERIALS AND METHODS

Lung explant cultures

Embryos from ICR mice (Harlan-Sprague-Dawley, Indianapolis) were dissected at E11.5. Noon on the day of the vaginal plug is E0.5. Distal lung tips (mesenchyme and endoderm), were isolated using tungsten needles (Bellusci et al., 1997b). To isolate endodermal buds, lungs were treated with pancreatin/trypsin solution (Hogan et al., 1994) for 5 minutes on ice and mesenchyme removed using tungsten needles.

Growth Factor Reduced Matrigel™ ( Collaborative Biomedical Products, Bedford, MA) was diluted 1:1 in culture medium (50% DMEM:50% Ham’s F12, 0.1% BSA, 0.05 U/ml penicillin, 0.05 µg/ml streptomycin). Isolated buds were embedded in MatrigelTM mix one bead diameter (125-175 µm) from an Fgf-loaded bead. After polymerization of the MatrigelTM at 37°C, explants were covered with culture medium and cultured at 37°C at 5% CO2 for up to 4 days.

Recombinant human BMP4 ( batch 4739-79/3178-163) and recombinant human NOGGIN (lot 5239-148; Genetics Institute, Cambridge, MA) were added to the culture medium in some experiments. Due to a limited source of NOGGIN, experiments using this protein were repeated only twice.

Bmp4lacZ expression in vivo and in explant cultures

Lungs from mice heterozygous for a Bmp4lacZ reporter allele (Lawson et al., 1999) were isolated at stages between E10.5 and E12. Isolated endoderm was embedded in MatrigelTM, fixed in 4% paraformaldehyde and reacted with X-gal as described (Lawson et al., 1999).

For explant studies, embryos heterozygous for the Bmp4lacZ allele were genotyped by β-galactosidase staining of the hindlimbs. Isolated tissue was embedded in MatrigelTM one bead diameter from an Fgf10-loaded bead and cultured as described above. Cultures were harvested at 0, 24, 48, 72 and 96 hours, fixed in 4% paraformaldehyde for 15 minutes and reacted with X-gal.

Preparation of protein carrying beads

Acrylic beads with immobilized heparin (Sigma) were rinsed with PBS three times, and beads approximately 125-175 µm in diameter were manually collected under the dissection microscope. 50 beads were soaked in 0.25 µ purified recombinant Fgf protein: either purified recombinant human Fgf10 (a generous gift of Dr Nobuyuki Itoh) or Fgf7 (Sigma). Beads were rotated at room temperature for 1 hour in a siliconized microfuge tube.

Cell proliferation analysis

Isolated E11.5 endoderm buds were placed on micropore filters (Costar, #110414) floating on undiluted DMEM and covered with Matrigel™, as described by Nogawa et al. (1998). The labeling reagent, 5-bromo-2′-deoxyuridine (BrdU) and 5-fluoro-2′-deoxyuridine (Amersham, Buckinghamshire, England), was diluted 1:1000 in DMEM. Buds were labeled for 1 hour at 37°C, washed with phosphate-buffered saline with 0.1% Triton X-100 and fixed in 10% formalin for 1 hour. Proliferating cells were detected using the Cell Proliferation Kit (Amersham), with modifications as described (Nogawa et al., 1998).

RESULTS

Fgf10 promotes lung endoderm proliferation and migration in vitro

In whole-lung cultures, Fgf10 directs the outgrowth of distal epithelial buds (Park et al., 1998). To examine the response of isolated lung endoderm to a distant source of Fgf10, we designed an in vitro migration assay. Mesenchyme-free distal buds were placed approximately 150 µm from a heparin bead loaded with purified, recombinant Fgf10 protein and cultured in Matrigel™ for up to 72 hours in serum-free medium (Fig. 1).

During the first 24 hours, the cut edge of the bud seals and the endoderm extends toward the source of Fgf10 (Fig. 1B). This migration continues over the next 48 hours (Fig. 1C,D),
with the endoderm eventually partially engulfing the bead (Fig. 1D). While the bud proliferates over the course of 72 hours, net movement is also observed so that the rear end of the bud is translocated from its initial starting position (asterisks, Fig. 1). While only 45% (n = 51/111) of buds reached the bead within 24 hours, 74% (n = 75/101) touched it by 48 hours and 93% (n = 44/47) by 72 hours in culture. This assay suggests that Fgf10 acts as both a mitogen and a chemoattractant for distal lung endoderm.

In control assays, buds cultured with a PBS-soaked control bead, or in 10% serum-containing medium without Fgf, do not proliferate or migrate toward the bead, and die within 24 hours (data not shown). Beads soaked with Fibroblast Growth Factor 7 (Fgf7), a protein closely related to Fgf10, promote proliferation of isolated endoderm, but do not exert a chemoattractive effect, results consistent with previous findings (Park et al., 1998).

In vivo, branching of the lung endoderm must be coordinated with outgrowth and proliferation of the surrounding mesenchyme. When isolated distal mesenchyme was exposed to Fgf10 beads in Matrigel(TM) matrix, the cells died during the 72 hour culture period (n = 58; data not shown). Beads soaked with Fibroblast Growth Factor 7 (Fgf7), a protein closely related to Fgf10, promote proliferation of isolated endoderm, but do not exert a chemoattractive effect, results consistent with previous findings (Park et al., 1998).

Dynamic expression of Bmp4lacZ in emerging lung buds in vivo

To follow Bmp4 expression during branching of the lung epithelium, we used a Bmp4-lacZ reporter line in which the pattern of X-gal-positive cells reproduces previously published in situ expression patterns of Bmp4 (Lawson et al., 1999; N. Ray Dunn and B. L. M. H., unpublished observations). We have previously reported expression of Bmp4lacZ in both lung endoderm and mesenchyme (Weaver et al., 1999). Removal of the mesenchyme from an E11.5 lung reveals that, in the endoderm, X-gal-positive cells are restricted to distal tips, emerging lateral buds and dichotomously dividing buds (Fig. 2A). Because branching morphogenesis in the early lung is stereotyped, dynamic changes in individual buds can be followed by observing the lungs of a series of embryos at different stages. Fig. 2B-K illustrates the expression of Bmp4lacZ in isolated endoderm from representative buds at progressive stages of outgrowth from E10.5 to E11.5. In the developing right lobe (Fig. 2B-F), the first (cranial) bud emerges dorsolaterally from the primary bud, in a region where Bmp4lacZ expression is low (Fig. 2B, arrow). Once the bud is established, X-gal-positive cells are observed throughout the extending bud (Fig. 2C). By E11.5, discrete proximal (stalk) and distal (tip) regions of the epithelium are established, with Bmp4lacZ transcription confined specifically to the distal region. As this bud dichotomously branches (Fig. 2D-F), Bmp4lacZ expression is downregulated in the central region (which will become the junction between the two future stalks), but is maintained in the two new extending distal buds.

A similar dynamic expression pattern is seen in the three lateral buds generated sequentially in the developing left lobe (Fig. 2G-K). X-gal-positive cells are initially observed in the distal epithelium as the primary bud extends (Fig. 2G) and strong expression of Bmp4lacZ is maintained in the distal tip (asterisks in Fig. 2G-K). The initiation of the first lateral bud (future bud no. 1, arrow in Fig. 2H), is associated with low levels of X-gal staining in the budding region. Expression is also low in the early second bud (no. 2; Fig. 2I), but Bmp4lacZ transcription is upregulated throughout the bud as it extends (Fig. 2J). This broad expression of Bmp4lacZ is gradually refined to the distal region of the two buds and excluded from the more proximal tissue (stalk; Fig. 2I-K). This dynamic cycle of Bmp4lacZ transcription is repeated in the third bud (no. 3; Fig. 2I-K).

Localized Bmp4lacZ expression is maintained in vitro

We then examined expression of Bmp4lacZ during the in vitro migration assay. Distal endodermal buds from Bmp4lacZ heterozygous mice were exposed to Fgf10-soaked beads, then fixed and stained for X-gal activity at 24 hour intervals. Representative results, shown in Fig. 3, demonstrate that Bmp4lacZ transcription is maintained over 3 days with Fgf10 beads. At the time of isolation, cells positive for X-gal are localized to the distal tip of the bud and are absent in more proximal stalk (n = 21/21; Fig. 3A). As the lung bud grows out, Bmp4lacZ is maintained in the most distal tissue when assayed...
at 24 hours (n=48/51), 48 hours (n=35/43) and 72 hours (n=25/26; Fig. 3B-D). Throughout the culture period, however, the stalk tissue does not express \textit{Bmp4 lacZ}.

**Fgf10 induces an ectopic domain of Bmp4 in reversed buds**

The patterns of Fgf10 and Bmp4 expression seen in vivo suggest that Fgf10 in the mesenchyme locally induces \textit{Bmp4 lacZ} transcription in the underlying endoderm. In whole lung cultures, \textit{Bmp4} is upregulated near a distally implanted Fgf10 bead (Lebeche et al., 1999). To test whether Fgf10 can act directly on the endoderm to regulate \textit{Bmp4}, we asked whether Fgf10 can induce \textit{Bmp4 lacZ} expression in two cell populations that normally are devoid of X-gal-positive cells: stalk endoderm and bronchial endoderm (Fig. 2A). To test stalk endoderm, which lies between the distal tip and the primary bronchial endoderm, heterozygous \textit{Bmp4lacZ} endodermal buds were cultured with Fgf10 beads as in Fig. 3. However, the orientation of each bud was reversed, such that the bud stalk, which lacks X-gal-positive cells, was placed facing the bead (Fig. 4A). After 24 hours, the stalk tissue extended toward the bead, and an ectopic domain of X-gal-positive cells was observed in the extending stalk (n=17/24; Fig. 4B). During the next 2 days, the bud continued to grow toward the bead and to partially engulf it. During this process, \textit{Bmp4 lacZ} upregulation was observed close to the bead at both 48 (n=21/24) and 72 hours (n=18/19; Fig. 4C,D). In contrast, \textit{Bmp4 lacZ} was downregulated in the tail end of the extending bud, previously the site of high endogenous expression (Fig. 4C, arrow in D).

**Fgf10 induces ectopic Bmp4 in isolated bronchial endoderm**

The E11.5 bronchial endoderm normally never buds in vivo, and does not express \textit{Bmp4 lacZ} in vivo or in vitro (Figs 2A, 4E). To ask whether ectopic Fgf10 can induce budding and \textit{Bmp4lacZ} transcripts in this proximal tissue, we tested the response of bronchial endoderm to Fgf10 beads. Isolated bronchial endoderm did not express \textit{Bmp4lacZ} at the start of culture (n=0/15 X-gal positive; Fig. 4E). Within 24 hours,
endoderm began to migrate towards the bead, but X-gal-positive cells were rarely observed \((n=3/20; \text{Fig. 4F})\). However, X-gal-positive cells were detected after 48 hours \((n=16/20; \text{Fig. 4G})\), and \textit{Bmp4\textsuperscript{lacZ}} transcripts were maintained in tissue close to the bead at 72 hours \((n=17/19; \text{Fig. 4H})\).

\textbf{Bmp4 inhibits outgrowth of isolated lung endoderm}

To examine the combinatorial effect of Bmp4 and Fgf10 on isolated endoderm, we tested the response of lung buds to Fgf10-loaded beads in the presence of exogenous Bmp4. The addition of 30 ng/ml or 50 ng/ml recombinant human Bmp4 to the culture medium at the time that the cultures are set up alters the response of buds to Fgf10 beads (compare Fig. 5A-C to D-I). While these buds do not appear consistently different from controls during the first 24 hours (Fig. 5A,D,G), by 48 hours, the epithelium develops a more dense appearance and a compressed morphology, in contrast to the translucent quality and expanded lumina of control buds (Fig. 5B,C,H,I). By 72 hours, this morphology is consistently observed at 30 ng/ml \((n=16)\) and 50 ng/ml \((n=17)\) Bmp4. Due to slight variations in experimental conditions, variable morphology of control cultures is observed, particularly after 4 days of culture (compare Figs 1D, 3D and 5C). Doses of 5 ng/ml \((n=24)\) and 0.5 ng/ml \((n=9)\) Bmp4 have no appreciable effect (data not shown). In control experiments, the addition of 30-100 ng/ml BSA has no significant effect on growth or chemotaxis of lung endoderm (data not shown).

While Fgf10 is both a mitogen and a chemoattractant in vitro, Fgf7 promotes proliferation without promoting significant migration of lung endoderm (Park et al., 1998; \textit{Fig. 6}). To test whether Bmp4 treatment specifically inhibits chemotaxis, and not other responses to Fgf signaling, lung endoderm was exposed to beads loaded with Fgf7, rather than Fgf10. As before, no significant difference in bud growth was observed after 24 hours (Fig. 6A,D,G). By 48 hours, however, Bmp4-treated buds adopt a dense appearance and...
compressed morphology, similar to that observed after Bmp4 treatment of buds exposed to Fgf10 beads (Fig. 6B,D,H). This morphology was consistently observed by 72 hours at concentrations of 30 ng/ml or 50 ng/ml Bmp4 (n=23) or 50 ng/ml Bmp4 (n=9), while doses of 5 ng/ml (n=12) and 0.5 ng/ml (n=8) had no effect.

We then asked what would happen if endogenous Bmp4 levels were decreased. To achieve this goal, the Bmp antagonist Noggin (Zimmerman et al., 1996) was added to the medium of buds cultured with Fgf10 or Fgf7 beads. In these experiments, Noggin (100 ng/ml or greater) promotes the outgrowth of buds exposed to 30 ng/ml Bmp4 and an Fgf10 bead (G-I), compared to Fgf10-treated cultures alone (D-F). In controls, high levels of BrdU incorporation are observed nearest the Fgf10 bead (brackets), while low levels of staining are seen throughout the bud (D-F). In buds treated with 30 ng/ml Bmp4, proliferation is reduced (G-I). Buds were cultured for 47 hours adjacent to Fgf10-soaked beads on Matrigel™-coated filters, and then exposed to BrdU for 1 hour. Incorporation of BrdU was visualized by whole-mount immunostaining. Scale bar, 150 µm.

Fig. 7. Effect of exogenous Bmp4 on Bmp4lacZ expression and proliferation in Fgf10-treated buds. In buds treated with 30 ng/ml Bmp4 and an Fgf10 bead, distal localization of Bmp4lacZ expression is maintained at 24 (A), 48 (B) and 72 (C) hours. Proliferation of distal bud endoderm is reduced in cultures treated with 30 ng/ml Bmp4 and an Fgf10 bead (G-I), compared to Fgf10-treated cultures alone (D-F). In controls, high levels of BrdU incorporation are observed nearest the Fgf10 bead (brackets), while low levels of staining are seen throughout the bud (D-F). Bmp4 inhibits proliferation but does not alter Bmp4lacZ expression domain

Bmp4lacZ expression serves as a marker of distal endoderm, in vivo and in vitro (Weaver et al., 1999; Fig. 2). We asked whether exogenous Bmp4 treatment altered the cell fate of lung endoderm by examining Bmp4lacZ expression in treated buds. After exposure to 30 ng/ml Bmp4 and Fgf10 beads, no change in expression is observed. Despite severe morphological changes in bud architecture, X-gal-positive cells are observed in the distal tip, but not in more proximal stalk tissue, at 24 (n=19/20), 48 (n=17/20) and 72 (n=12/17) hours (Fig. 7A-C). These results suggest that Bmp4 treatment does not alter the proximodistal character of lung endoderm during culture. Furthermore, these data illustrate that Bmp4 transcription is not autoregulated in the lung, since treatment with Bmp4 protein does not affect Bmp4lacZ transcription over a 72 hour period.

To assess the proliferation of Bmp4-treated buds, a whole-
In this study, we show that a single isolated lung bud, when vitro Fgf10 promotes outgrowth of lung epithelium in precisely temporally and spatially restricted in vivo. antagonist, observe that Fgf10 treatment induces the expression of its own Bmp4 treatment. By contrast, depletion of endogenous Bmp4 a positive mediator of bud extension, causing net movement of epithelium by promoting both proliferation and chemotaxis. The mitogenic effects of Fgf10 are antagonized in vitro by antagonist Noggin potentiates budding. We also observe that Fgf10 treatment induces the expression of its own antagonist, Bmp4, in isolated endoderm. Taken together, these results suggest a model for how budding from an epithelial tube is precisely temporally and spatially restricted in vivo.

**Fgf10 promotes outgrowth of lung epithelium in vitro**

In this study, we show that a single isolated lung bud, when exposed to a gradient of Fgf10, can migrate over 150 μm toward the Fgf10 source (Fig. 1). This finding confirms the conclusions of Park and colleagues (1998) that, in addition to acting as a mitogen in the early lung, Fgf10 has a role in guiding the extension of bud epithelium. In their experiments with intact lung cultures, Park et al. (1998) observed that Fgf10 promotes extension of distal epithelium, while proximal endoderm does not respond. In contrast to their results, we find that early lung epithelium from both proximal and distal regions is competent to respond to an Fgf10 signal, when isolated from the mesenchyme (Fig. 4). Our results suggest that Fgf10 is a potential promoter of budding through the lung; however, in vivo, the proximal mesenchyme restricts budding of proximal epithelium (Park et al., 1998).

Fgf signaling has previously been implicated in cell migration in other model systems (for review, see Montell, 1999). In *C. elegans*, mutations in an Fgf ligand and receptor prevent the migration of sex myoblasts to the future gonad (Burdine et al., 1998) and the *Drosophila* tracheal system, which has respiratory functions similar to the lung, requires Fgf signaling to guide the directional migration of tracheal cells (Metzger and Krason, 1999). Recent research in the chick limb illustrates that Fgf4, which is secreted from the apical ectodermal ridge, stimulates the migration of underlying mesenchymal cells (Li and Muneoka, 1999). By contrast, the response of lung epithelium to Fgf10 involves the coordinated movement of an entire epithelial sheet, containing hundreds of cells, towards an Fgf10 source. How this sheet of cells monitors the Fgf gradient over a distance of 150 μm remains unknown. It is tempting to speculate that lung buds, like the mouse limb bud and *Drosophila* imaginal disc cells, project cytonemes, an actin-based type of cellular projection previously shown to respond to Fgf (Ramirez-Weber and Kornberg, 1999).

While lung epithelium is capable of extending in response to Fgf10, lung mesenchymal cells are refractive to an Fgf10 source. At this stage, both FgfR1 (Peters et al., 1992) and FgfR2-IIIc (Arman et al., 1999) are expressed in lung mesenchyme. However, in vitro, when distal buds (mesenchyme and endoderm together) are cultured in an Fgf10 gradient, the extending epithelium leaves the surrounding mesenchyme behind during its journey toward the bead (Fig. 1). Since Fgf10 does not promote chemotaxis or growth of the mesenchyme, regulatory networks between the epithelium and the mesenchyme must coordinate the growth of these two cell layers in vivo (see Fig. 8).
**Bmp4lacZ expression during branching morphogenesis**

To correlate events of branching morphogenesis with the expression of Bmp4lacZ, we first followed the temporal and spatial localization of X-gal-positive cells in developing endoderm in vivo using a Bmp4lacZ reporter line (Lawson et al., 1999). At the region of lateral bud initiation, levels of Bmp4lacZ transcripts are low. As the bud extends, levels gradually increase and are highest at the tip, eventually demarcating the distal region from the non-expressing or low-expressing proximal stalk region. Thus, high expression of Bmp4lacZ is always associated with periods of bud extension, but not with lateral bud initiation. This suggests that Bmp4 is not required for bud outgrowth, but may have other roles in branching morphogenesis.

During early events of branching morphogenesis, we observe two styles of branching: lateral branching and dichotomous branching. Despite the morphological differences between lateral and dichotomous branches, cyclical and dynamic expression of Bmp4lacZ transcription is observed in buds of both types (Fig. 2). Therefore, the action of Bmp4 does not appear to be responsible for distinguishing between these types of branching pattern, but rather is common to all branching events.

In addition to endodermal expression of during branching morphogenesis, Bmp4lacZ is also detected transiently in the lung mesenchyme prior to budding of the endodermal primordia and ventrally during the early stages of lung development (E10.5-E13.5; Weaver et al., 1999). Since this expression pattern does not coincide temporally or spatially with lateral and dichotomous branching, there is no evidence for a role for mesenchymal Bmp4 expression in these processes. Rather, mesenchymal Bmp4 may be involved in setting up the initial lung bud field and/or dorsoventral patterning of the lung at early stages.

**Regulation of Bmp4lacZ by Fgf10 signaling**

Fgf10 is expressed dynamically in the lung mesenchyme (Bellusci et al., 1997b) in a domain complementary to that of Bmp4lacZ, making it a key candidate for transcriptional regulation of Bmp4lacZ. Here we demonstrate that Fgf10 can induce Bmp4lacZ expression in lung endoderm in the absence of mesenchyme. In response to Fgf10, Bmp4lacZ expression is observed in two endodermal tissues that normally do not express Bmp4lacZ at detectable levels: stalk endoderm and proximal bronchial endoderm (Fig. 4). Induction of Bmp4lacZ occurs more rapidly in stalk endoderm, which lies at an intermediate level on the proximodistal axis of the lung, than in the proximal bronchial endoderm. However, within 48 hours of exposure to purified Fgf10, X-gal-positive cells are evident in both tissues (Fig. 4).

Once Bmp4lacZ has been induced, it is unclear whether Fgf10 is further required to maintain expression. One observation, however, may shed light on this issue. When exposed to an Fgf10 bead, stalk endoderm extends and an ectopic domain of Bmp4lacZ is induced near the bead. During this same culture period, the endogenous domain of Bmp4lacZ, which lies furthest from the bead, is lost (Fig. 4). This suggests that endoderm receiving little or no Fgf10 signal does not transcribe Bmp4lacZ.

While migration appears to be a direct outcome of Fgf10 signaling, induction of Bmp4lacZ transcription probably lies considerably downstream of these initial events. In bronchial endoderm cultures, extension is seen within 24 hours, while X-gal-positive cells are observed only after an additional day of Fgf10 exposure (Fig. 4). Even when bronchial endoderm is placed directly next to an Fgf10 bead, X-gal-positive cells are not detected within the first 24 hours (M. W. and B. L. M. H., unpublished results). One interpretation of these findings is that Bmp4 expression is not a direct response to Fgf signaling. Rather, it is secondary to some change in cell behavior, such as an alteration in cell shape, or a change in cell fate from proximal to distal. Furthermore, these results demonstrate that Bmp4 is not required for outgrowth of lung endoderm in vitro, since tissue completely lacking expression of Bmp4lacZ can migrate toward an Fgf10 source (Fig. 4).

**Bmp4 antagonizes Fgf mediated outgrowth of lung endoderm**

After about 24 hours, exogenous Bmp4 inhibits the outgrowth and alters the morphology of lung endoderm in vitro. By contrast, reducing levels of endogenous Bmp4 with the Bmp antagonist Noggin promotes budding and outgrowth (Figs 5, 6). These results, coupled with the localized expression pattern of Bmp4 in the early lung (Fig. 2), suggest that one function of Bmp4 in vivo is to locally inhibit lung budding.

Extension of lung endoderm occurs by both proliferation and chemotaxis (Figs 1, 7), so Bmp4 treatment may affect either, or both, of these processes. Our BrdU incorporation data show that Bmp4 treatment decreases the overall proliferation of lung endoderm (Fig. 7). Importantly, however, the effect appears to be specific, reducing the appearance of a distal region of high proliferative activity, while incorporation of BrdU continues within the main body of the bud. Moreover, Bmp4-treated buds continue to express Bmp4lacZ, suggesting that the cultured tissue remains healthy and that the effects that we observe are not a result of Bmp4 toxicity (Fig. 7). In contrast to the tooth (Vainio et al., 1993), Bmp4 does not appear to be autoregulated in lung endoderm, since Bmp4 treatment does not alter the domain of Bmp4lacZ expression in these buds.

Although the molecular mechanism of Bmp4 action remains unclear, it is interesting to note that the protein has the same effect on lung endoderm treated with either Fgf10 or Fgf7. Though these two growth factors cause different responses in lung endoderm, they are thought to signal through the same receptor isoform, FgfrIIIB (Bellusci et al., 1997b; Park et al., 1998). In the feather bud, exogenous Bmp signaling has been shown to inhibit expression of Cek-1, an Fgf receptor expressed in budding regions, and to promote the expression of Cek-3, which is expressed in interbud regions (Normaly and Morgan, 1998). Notably, Fgfr2 is expressed ubiquitously in the lung endoderm, while Fgfr4 is specifically localized to the distal endodermal tips (Cardoso et al., 1997). It is conceivable that Bmp4 modulates the expression or activity of these two receptors, thus independently affecting both proliferation and chemotaxis.

The effect of exogenously added Bmp4 on cell proliferation in vitro does raise a paradox. In vivo (Fig. 2A), and in endodermal buds exposed to Fgf10 alone (Figs 3, 4), the level of endogenous Bmp4lacZ expression is highest in the distal cells, where cell proliferation is also highest (Fig. 7). One possibility is that in vivo an inhibitor of Bmp4 activity is produced at the distal tip, modulating very precisely the
availability of active Bmp. As a precedent for this idea, the
transcripts for the Bmp-binding protein Follistatin are found in
the distal tips of extending feather buds, and application of
exogenous Follistatin promotes ectopic feather buds (Patel et
al., 1999). While we have shown expression of Noggin in lung
mesenchyme (Weaver et al., 1999), no expression of the Bmp
antagonists Cerberus, DAN or Follistatin has been detected in
the lung endoderm (M. W. and B. L. M. H., unpublished results).
Alternatively, our results may reflect the response of a bud when it is exposed to a uniform high level of exogenous
Bmp4, as opposed to a gradient. In vivo, distal cells are most
likely exposed to a gradual increase in local Bmp4 levels, in
combination with a variable level of Fgf signaling. Distal cells
may therefore continually monitor local changes in the balance
between Bmp4 and Fgf10 signaling, which are not adequately
reflected in our in vitro culture system.

Fgf10 and Bmp4 in branching morphogenesis

A requirement for Fgf10 in lung bud initiation has already been
demonstrated (Min et al., 1998; Sekine et al., 1999). Based on
the results presented here, as well as recent findings of others
(Park et al., 1998; Lebeche et al., 1999), we propose a model for
the roles of Fgf10 and Bmp4 in lung branching
morphogenesis. Once a bud has been initiated, a local domain
of Fgf10 in the mesenchyme directly promotes both the
proliferation and chemotaxis of the underlying epithelium and
the bud extends (Fig. 8A). As extension occurs, distal
derm cells detect high levels of Fgf10, which indirectly
induce Bmp4 expression (Fig. 8B). The Bmp4 protein
produced in the tip acts as a lateral inhibitor of budding, thus
ensuring a single extending bud, rather than a cluster of buds,
in response to mesenchymal Fgf10. Only at discrete distances
from the tip, where Bmp4 levels are low but Fgf10 expression
is maintained, can a new bud form (Fig. 8C). In this way, a
combination of Bmp4 and Fgf10 signaling contributes to the
patterning of the early lung.

According to this model, the generation of lateral or
dichotomous branches is controlled by the local pattern of
Fgf10 in the mesenchyme. In both types of branch, budding
occurs in regions where Bmp4 levels are low but Fgf10 levels
are maintained. In a lateral branch, Fgf10 is asymmetrical
expressed as the bud extends, leading to an asymmetrical bud
(Fig. 8C). Symmetrical expression of Fgf10 in distinct domains
on either side of a bud, however, leads to paired buds and a
dichotomous branch (Fig. 8D, brackets). How the complex
expression domain of Fgf10 is controlled is not known. Hox
genes are expressed in overlapping domains in the lung
(Cardoso, 1995), and may form a grid controlling Fgf
expression (Hogan, 1999; Metzger and Krashov, 1999).
Alternatively, or in addition, Fgf10 levels may be modulated
by Shh expression in the lung endoderm. Both in vivo and in
vitro evidence suggests that Shh downregulates Fgf10
expression in lung mesenchyme (Belluscio et al., 1997a;
Lebeche et al., 1999).

In the chick feather bud and the chick limb, Bmps have been
shown to modulate outgrowth promoted by a stimulatory Fgf
signal (Niswander and Martin, 1993; Jung et al., 1998;
Normaly and Morgan, 1998; Pizette and Niswander, 1999).
Our work suggests that Bmp4 and Fgf10 form a conserved
regulatory network that patterns the branching mouse lung. In
vitro culture of the lung holds great promise for further
investigation of the molecular and cellular mechanisms
underlying branching morphogenesis.

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