Haploinsufficiency of the forkhead gene Foxf1, a target for sonic hedgehog signaling, causes lung and foregut malformations

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SUMMARY

The murine Foxf1 gene, encoding a forkhead – or winged helix – transcription factor, is expressed in splanchnic mesenchyme during organogenesis. The concentration of expression to subepithelial mesenchyme suggested that Foxf1 is activated by paracrine signals from endodermal epithelia. Homozygous Foxf1-null mice die before embryonic day 10, owing to defects in extra-embryonic mesoderm, and do not provide any information about the role of Foxf1 in morphogenesis of endodermally derived organs. We show that, on CD1 genetic background, Foxf1 heterozygote perinatal mortality is around 90%. The haploinsufficiency causes a variable phenotype that includes lung immaturity and hypoplasia, fusion of right lung lobes, narrowing of esophagus and trachea, esophageal atresia and tracheo-esophageal fistula. Similar malformations are observed in mutants that are defective in the sonic hedgehog (Shh) signaling pathway, and we show that exogenous Shh activates transcription of Foxf1 in developing lung. Foxf1 mRNA is absent in the lungs, foregut and sclerotomes of Shh-/- embryos, but persists in tissues where indian hedgehog (Ihh) is expressed. In lung organ cultures, activation of Foxf1 by Shh is counteracted by bone morphogenetic protein 4 (BMP4). Fibroblast growth factor (FGF) 10 and FGF7 both decrease Foxf1 expression and we speculate that this is mediated by transcriptional activation of epithelial Bmp4 (in the case of FGF10) and by inhibition of Shh expression for FGF7.

Key words: Lung, Mouse, Foregut, Foxf1, forkhead

INTRODUCTION

Morphogenesis of the mammalian lungs and foregut is controlled by interactions between the endodermal epithelium and the surrounding mesenchyme. Grafting experiments have demonstrated the distinct inductive capacities of proximal and distal mesenchyme, derived from the trachea and the tips of the growing lungs, respectively (Wessells, 1970). In the last decade, several key components in the paracrine crosstalk between mesenchyme and epithelium have been identified.

Hedgehog proteins are secreted signaling molecules that are involved in many morphogenetic processes. In Drosophila, Hedgehog signaling is received by the cell surface receptor Patched and transduced by Smoothened, which modulates the transcription factor Cubitus interruptus (Ci) through control of its proteolytic processing (reviewed by Aza-Blanc and Kornberg, 1999). The essentials of this pathway appear to be shown if the effect is direct or mediated by other secreted proteins. Hedgehog proteins can either be activated, such as Fgf10, or repressed, like Shh, Wnt2 or Gli3. Other target genes encode paracrine factors that are known to take part in morphogenesis and can either be activated, such as Wnt2, or repressed, like Fgf10, by Shh (Belluscì et al., 1996; Lebeche et al., 1998; Pepicelli et al., 1998). Inactivation of Shh or components in its signal transduction pathway, such as Gli2 and Gli3, gives rise to various degrees of lung and foregut malformations, with fusion of lung lobes, hypoplasia and esophageal atresia or stenosis, but the mechanism behind these morphogenetic defects is unknown (Litingtung et al., 1998; Motoyama et al., 1998; Park et al., 2000; Pepicelli et al., 1998). Mesenchymal genes, whose expression depends on hedgehog signaling have been identified. Several of these encode proteins that themselves are part of the hedgehog signaling pathway (such as Ptc1, Gli1 and Gli3). Other target genes encode paracrine factors that are known to participate in morphogenesis and can either be activated, such as Ihh, or repressed, like Fgf10, by Shh (Belluscì et al., 1997a; Litingtung et al., 1998; Pepicelli et al., 1998). However, in many cases it remains to be shown if the effect is direct or mediated by other secreted proteins.

The forkhead genes Foxf1 (also known as FREAC1 or HFH8) and Foxf2 (also known as FREAC2 or LUN) encode
two transcription factors that are closely related with regard to primary structure, DNA binding specificity and expression pattern, but with distinct activating properties (Clevidence et al., 1994; Hellqvist et al., 1998; Hellqvist et al., 1996; Mahlapuu et al., 1998; Pierrou et al., 1994). Both genes are expressed during organogenesis in mesenchymal tissue derived from splanchic mesoderm and sclerotomes (Aitola et al., 2000; Mahlapuu et al., 2001; Mahlapuu et al., 1998; Peterson et al., 1997). In developing lung and in several other endodermally derived organs, Foxf1 and Foxf2 mRNA is concentrated in subepithelial mesenchyme, which has prompted the speculation that their expression is induced by epithelial paracrine signaling (Aitola et al., 2000; Mahlapuu et al., 1998). We recently reported inactivation of Foxf1 by gene targeting (Mahlapuu et al., 2001). However, homozygous Foxf1-null mice degenerate at the early somite stage and die before organogenesis begins; hence, they do not provide any information on the role of Foxf1 in foregut or lung development. We show that Foxf1 is activated by Shh signaling and that, on a specific genetic background, Foxf1 haploinsufficiency gives rise to lung and foregut malformations similar to those observed in Shh and Gli mutants.

MATERIALS AND METHODS

Mouse mutants
A targeting construct containing a total of 12 kb of the Foxf1 locus was made from 129/Sv genomic λ clones, in which the forkhead box of Foxf1 (from Sx1 to NoI) was replaced by a PGK-Neo cassette (Mahlapuu et al., 2001). The Foxf1 locus was targeted in two ES cell lines: RW4, derived from 129/SvJ, and E14, derived from 129/Ola. Germline transmission was obtained with clones derived from both cell lines and the targeted allele was maintained on mixed 129/Ola-C57Bl/6, 129/Ola-CD1, 129/Sv-CD1 and 129/Sv-C57Bl/6 backgrounds. Shh<+/− mice (Chiang et al., 1996) were obtained from The Jackson Laboratory, Maine (Stock Shh<tm1>, #JR3318).

In situ hybridization, immunohistochemistry, histology and skeletal preparations
Pregnant CD1 mice were sacrificed at different stages of gestation. The embryonic lungs were dissected in phosphate-buffered saline containing 0.1% Tween-20. The unfixed tissue was photographed immediately. For in situ hybridization and immunohistochemistry embryonic lungs were fixed in 4% paraformaldehyde at 4°C. In situ hybridization of whole-mount embryos and cryosections (8 μm) or paraffin sections (3 μm) was performed as previously described (Blixt et al., 2000) using the following probes. The Foxf1 probe consists of a 400 bp NoI/KspI cDNA fragment located immediately 3’ of the forkhead box. Plasmids used to generate probes for uteroglobin (Utg; also known as Cc10 or Ccsp) and surfactant proteins A, B and C (Sftpa, Sftpb, Sftpc) were kindly provided by Dr B. Stripf; for patched (Pch) by Dr M. P. Scott; for Foxa2 (HNF3β) and Bmp4 by Dr B. L. M. Hogan; and for sonic hedgehog (Shh) by Dr C. Betsholtz. The probe for vascular smooth muscle α-actin (Acta; now known as Acta2 – Mouse Genome Informatics) was generated from IMAGE cDNA clone No. 1224519. Immunostaining was performed with antibodies to platelet/endothelial cell adhesion molecule (Pecam; Pharmingen, clone MEC 13.3). Antibody binding was detected with biotinylated secondary antibodies and HRP-streptavidine amplified by TSA TM Biotin System (NEN Life Science Products). Histological sections (3 μm, paraffin) were stained with Eosin and Hematoxylin.

Bone and cartilage was visualized by staining with Alizarin Red and Alcian Blue (Hogan et al., 1994).

Whole lung organ cultures
Lung buds were dissected from E11.5 embryos and explants were placed on Millipore MF filters (Millipore), on top of stainless steel grids, in BGM medium (Life Technologies) supplemented with 0.2 mg/ml ascorbic acid, 50 U/ml penicillin/streptomycin and 0.1% BSA. About 3 ml of medium was added to each 30 mm dish to establish an air-fluid interface at the level of the explants. The cultures were maintained in 5% CO2, 100% humidity.

Localized effects of fibroblast growth factors (FGFs) in lung explant cultures were tested by implanting heparin beads (Sigma) impregnated with human recombinant FGF10 (100 μg/ml, R & D) or FGF7 (100 μg/ml, R & D) into the mesenchyme of E11.5 lungs, as described (Lebeche et al., 1999). The effect of human recombinant transforming growth factor β1 (TGFβ1; 7.5 μg/ml, R & D), TGFβ2 (50 μg/ml, R & D), bone morphogenetic protein 4 (BMP4; 100 μg/ml, R & D) and epidermal growth factor (EGF; 100 μg/ml, Sigma) in lung cultures was tested by grafting Affi-gel blue beads (BioRad) soaked in recombinant protein. Explants were cultured for 24 hours and fixed for whole-mount in situ hybridization.

Preparation of Shh-expressing cells
COS-7 cells were transfected with either sense or antisense (control) Shh expression plasmids (kindly provided by Dr J. Ericson) using LipofectAMINE (Life Technologies). After 24 hours, transfected cells were trypsinized and plated in bacteriological grade Petri dishes to generate aggregates of cells. Cell aggregates were implanted into E11.5 lung explants in whole lung organ cultures. Explants were incubated for 24 hours and fixed for whole-mount in situ hybridization.

RESULTS

Survival of Foxf1 heterozygotes depends on genetic background
A Foxf1-null mutant was created by homologous recombination in embryonic stem (ES) cells (Mahlapuu et al., 2001). Chimeric mice that passed the targeted allele through germline were generated with cell clones obtained from two different ES cell lines – RW4 and E14 – derived from different inbred strains of mice, 129/SvJ and 129/Ola. Offspring from matings between chimeric males and C57Bl/6 females had the expected allele frequencies, consistent with Mendelian inheritance of the Foxf1-null allele (48% heterozygotes, n=67; see also Table 1). This frequency remained stable (50%, n=117) when first generation heterozygous males were crossed with C57Bl/6 females. In contrast, when chimeras were mated with CD1 females the frequency of heterozygous offspring was only 14.6% (n=137), indicating that approximately 83% of heterozygotes died before or soon after birth. Crosses between

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<td>C57Bl/6</td>
<td>1 Born</td>
<td>35 (52%)</td>
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<td>2 Born</td>
<td>59 (50%)</td>
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<tr>
<td>CD1</td>
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<td>E18.5</td>
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first generation survivor heterozygous males and CD1 females resulted in an even higher heterozygote mortality, estimated to be 93% (6.7% heterozygous pups, \( n=105 \)). When fetuses were genotyped the day before birth, at E18.5, heterozygote frequency was normal (44%, \( n=110 \)), which, together with the heterozygous genotype of dead newborn pups, showed that the low heterozygote frequency is due to perinatal mortality and not early resorption. Knockout lines established from the RW4 and E14 cell lines gave similar heterozygote frequencies and passing the targeted allele several generations through C57Bl/6 before crossing with CD1 did not alter this. The elevated perinatal mortality of Foxf1 heterozygotes thus appears to reflect a sensitivity to Foxf1 gene dose in the CD1 strain.

**Foxf1 heterozygotes suffer from respiratory failure and foregut malformations**

Anatomical and histological analysis of E18.5 Foxf1 heterozygous fetuses from CD1 mothers revealed multiple malformations in the foregut and respiratory tract. The lungs are small and pale with hemorrhagic lesions along the edges (Fig. 1A). Instead of the four separate lobes of a normal right lung (Fig. 1B), various degrees of fusion between lobes are seen and one of the lobes – the accessory lobe – is consistently missing (Fig. 1A). Lung differentiation appears retarded; in some fetuses, formation of alveoli has not begun at E18.5, the lung mesenchyme is compact and looks undifferentiated (Fig. 1C,E). In others the histology looks normal, despite the smaller size and lobation defects of the lungs. In situ hybridization of E18.5 lung sections with probes for genes expressed in proximal (Utg) and distal (Sftpa, Sftpb, Sftpc) lung epithelium gave similar results in wild-type and Foxf1 heterozygotes, which indicates that proximo-distal epithelial differentiation proceeds normally in the mutant (Fig. 1G-J and data not shown). No major alterations in lung vascularization could be detected, as judged by the expression pattern of markers for endothelial cells (platelet endothelial cell adhesion molecule type 1; Pecam1; Fig. 1K,M) and vascular smooth muscle cells (Actvs; Fig. 1L,N). However, a lower level of Actvs expression suggests that vascular smooth muscle differentiation may be hampered; something that could explain the hemorrhagic lesions in mutant lungs. Based on these results, and the fact that a fraction of Foxf1 heterozygotes survive birth and reach maturity, we conclude that the mutants suffer from lung hypoplasia combined with a general and variable delay in lung maturation. The abnormal lobation indicates defects in lung branching morphogenesis.

In normal mouse development, the primordial lung buds and tracheal groove form around E9.5 as an epithelial outpouching from the ventral foregut, surrounded by splanchnic mesenchyme. The tracheo-esophageal septum divides the tracheal lumen and the foregut at E10.5, and by E11.5 trachea and esophagus are completely separated. The esophagus of E18.5 Foxf1+/− mutants has a narrow lumen (Fig. 2A), frequently merges with the trachea and sometimes ends before...
reaching the pharynx (esophageal atresia; Fig. 2C). The tracheal cartilage of Foxf1 heterozygotes is hypoplastic (Fig. 2A,C,D) and does not form the ventral rings typical of a normal trachea (Fig. 2B,E). The gastrointestinal tract posterior of the esophagus did not exhibit any anatomical or histological abnormalities.

Examination of the skeleton revealed that ribs are asymmetrically attached to the sternum in Foxf1 mutant embryos (Fig. 2F). As a consequence, the bilateral ossification centra in the sternebrae are misaligned at the midline (F).

Based on the observed defects, we conclude that respiratory failure caused by lung immaturity and inability to feed, as a result of constriction or atresia of the esophagus, contributes to elevated perinatal mortality of Foxf1 heterozygotes.

To investigate at what stage lung hypoplasia and lobation defects are first evident in Foxf1 heterozygotes, we examined bud branching at the early stages of lung development. Lung epithelium was visualized by whole-mount in situ hybridization with a Foxa2 probe. At E11.5, the left and right lungs are separated by the primitive bronchi, and primary epithelial buds have developed that will give rise to the four lobes of the right lung (Fig. 3B). At E12.5, the respiratory tree has branched extensively in the wild type and the four lobes of the right lung are clearly distinguishable (Fig. 3D). Branching in the Foxf1 mutant is considerably less advanced and separation of right lung lobes is incomplete (Fig. 3C). The mesenchyme surrounding the accessory lobe bud remains fused with that of the caudal lobe, which explains why a separate accessory lobe does not form (arrowhead in Fig. 3C).

**Foxf1 is a target for Shh signaling**

Foxf1 is expressed in the lung mesenchyme with the highest mRNA levels found adjacent to the epithelium (Mahlapuu et al., 1998). The expression pattern and the fact that Foxf1 heterozygosity affects airway morphogenesis indicate that Foxf1 may take part in epithelio-mesenchymal crosstalk. Several observations suggest a functional connection between Foxf1 and Shh. Shh is expressed throughout the epithelium (Bitgood and McMahon, 1995), but high level expression is restricted to the distal epithelium in the growing buds (Fig. 4A; Bellusci et al., 1996; Urase et al., 1996). Target genes for Shh signaling, such as Ptch, show a complementary expression pattern, which is most prominent in the mesenchyme that surrounds the distal epithelium (Fig. 4B; Bellusci et al., 1997a). To investigate if Foxf1 expression matches the sites of Shh secretion, we localized Foxf1 mRNA in E11.5 and E12.5 lungs with whole-mount in situ hybridization. The results show that...
change is induced by control cells (E,G). This pattern is similar to that of confined to mesenchyme surrounding the distal epithelium of mesenchyme throughout the airways, the highest level is extracellular Shh.

4E,G). This result shows that transcriptional activation of secretion cells, whereas no change in expression of either gene (Fig. 4D) and Foxf1 (F,G), whereas no change is induced by control cells (E,G).

although expression of Foxf1 is found in subepithelial mesenchyme throughout the airways, the highest level is confined to mesenchyme surrounding the distal epithelium of the buds (Fig. 4C). This pattern is similar to that of Ptc (Fig. 4B), which further supports the notion of a link between Shh signaling and Foxf1 expression.

To determine if ectopic Shh secretion can activate Foxf1 in lung mesenchyme, we used in vitro cultures of wild-type lung explants. COS-7 cells, transfected to secrete Shh, were implanted into the mesenchyme and the effects on expression of Foxf1 was monitored by in situ hybridization. The expression of Ptc (B) and the splanchnic mesenchyme surrounding the foregut of embryos showed that in E10.5 and E12.5 Shh−/− embryos, Foxf1 mRNA is absent from several of its normal sites of expression, including sclerotome and mesenchyme of trachea, esophagus, oral cavity and lungs (Fig. 5). The midgut and hindgut, however, retain Foxf1 expression pattern in Shh mutants (Fig. 5F,G). The lack of Foxf1 expression in the lung mesenchyme and the splanchnic mesenchyme surrounding the foregut of Shh−/− embryos, confirms that Shh signaling is required for activation of Foxf1 in these tissues.

**Foxf1 expression is downregulated by BMP4**

A number of paracrine factors have been described to participate in the epithelio-mesenchymal crosstalk that controls morphogenesis and gene expression during early lung development (see Hogan, 1999; Hogan and Yingling, 1998). In order to identify other potential regulators of Foxf1, we investigated the effect of candidate growth factors on the Foxf1 expression pattern in in vitro cultures of lung explants.

BMP4 is secreted by the distal epithelium of lung buds and inhibits epithelial branching and proliferation (Bellusci et al., 1996; Weaver et al., 1999). The distribution of Foxf1 mRNA, which is lower adjacent to the Bmp4-expressing epithelium at the distal face of the bud than in a zone just behind the tip (compare Fig. 6B,D,H with Fig. 6F; see also Fig. 7B), suggests BMP4 as a potential negative regulator of Foxf1. To investigate the effect of BMP4 on Foxf1 expression, we implanted BMP4-saturated beads in the mesenchyme of lung explants. As shown in Fig. 6A, exogenous BMP4 from a bead decreases Foxf1 expression in the surrounding mesenchyme. Epithelial BMP4 is thus likely to act as a negative regulator of mesenchymal Foxf1 expression. No change in Foxf1 mRNA level or distribution was induced by TGFβ1 or TGFβ2, two additional members of the TGFβ superfamily that have been implicated in lung morphogenesis (Lebeche et al., 1999; Zhao et al., 1996), or by EGF, which is also essential for normal lung development (Miettinen et al., 1997; data not shown).

FGF10 and FGF7 are secreted by mesenchymal cells and signal through the FGFR2-IIib receptor in the epithelium (Arman et al., 1999; De Moerloose et al., 2000; Igarashi et al., 1998). As mesenchymal factors, FGFs are less likely to regulate Foxf1 directly, but may do so indirectly through effects on epithelial gene expression. When added exogenously, in implanted beads, both FGF10 and FGF7 stimulate epithelial

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**Fig. 4.** Foxf1 is a target for Sonic hedgehog signaling in lung.

(A-C) Gene expression in E11.5 mouse lung examined by whole-mount in situ hybridization. Shh (A) is expressed throughout the lung epithelium, but the highest expression level is restricted to distal epithelium in the bulbous ends of the buds. Ptc (B), encoding the Shh receptor, and Foxf1 (C) are both expressed in mesenchyme adjacent to lung epithelium. The expression patterns mirror the distribution of Shh, with low levels along the proximal tubular epithelium and higher levels distally. (D-G) Exogenous Shh activates Foxf1. COS-7 cells transfected to secrete Shh (D,F) or control COS-7 cells (E,G) were grafted into the mesenchyme (location of grafts indicated by white stars) of wild-type lung explants, which were then cultivated for 24 hours. The expression of Ptc (D,E) and Foxf1 (F,G) were examined by whole-mount in situ hybridization. The mesenchyme immediately adjacent to the Shh-producing cells express elevated levels of both Ptc (D) and Foxf1 (F), whereas no change is induced by control cells (E,G).
proliferation and bud dilation, whereas the expression of Foxf1 in subepithelial mesenchyme next to the bead decreases (Fig. 6C,G). FGF7 downregulates Foxf1 in a wide area around the bead, adjacent to both proximal and distal epithelium (Fig. 6G). FGF7 also reduces expression of Shh in the epithelium (Fig. 6K; Lebeche et al., 1999) and Ptch in the mesenchyme (Fig. 6I). In the area influenced by exogenous FGF10, Foxf1 is downregulated in the regions where it is normally expressed at high levels – next to distal epithelium – but the basal expression along the proximal, tubular epithelium is not affected (Fig. 6C). Distal epithelium also responds to exogenous FGF10 by activating Bmp4 (Fig. 6E; Lebeche et al., 1999; Weaver et al., 2000).

DISCUSSION

Foxf1<sup>−/−</sup> embryos are deformed already at E8.5, never turn and die around E9.5, before organogenesis begins in the foregut (Mahlapuu et al., 2001). They cannot therefore be used to investigate the role of Foxf1 in lung and gut morphogenesis. However, the heterozygous phenotype described here gives some insight into the normal function of Foxf1 in this process. The fact that two independent targeted lines of mice, derived from ES cells of different genetic origin, produced the same phenotype and that the heterozygote frequency did not normalize during breeding, show that the observed defects are caused by the knockout. Foxf1 haploinsufficiency depends on genetic background, which suggests that either the expression level or the threshold concentration of Foxf1 required for normal development varies between the tested strains. Although the nature of this variability is not known, the observed defects demonstrate the importance of Foxf1 for normal foregut and lung development and also suggest links to upstream regulators.

The malformations seen in Foxf1 heterozygotes bear a striking resemblance to those described for mutants in the Shh signaling pathway. Mice homozygous for null mutations in Shh have foregut defects with esophageal atresia or tracheo-esophageal fistula; lung growth is retarded and branching morphogenesis is impaired, but proximo-distal differentiation of the airway epithelium is unaffected (Litingtung et al., 1998; Pepicelli et al., 1998). Gli transcription factors are expressed in the lung mesenchyme and are nuclear targets for hedgehog signaling transduced from the patched/smoothed receptor (Dominguez et al., 1996; Goodrich et al., 1996; Tabin and McMahon, 1997). Mutations in genes for Gli proteins give rise to various lung and foregut defects. The Gli2 knockout mice have narrowing of esophagus and trachea, tracheal cartilage dysplasia, small lungs with right lung lobe fusion and lack of accessory lobe (Motoyama et al., 1998). Heterozygosity for Gli3 in a Gli2<sup>−/−</sup> background adds esophageal atresia with tracheo-esophageal fistula to the malformations and aggravates the lung hypoplasia (Motoyama et al., 1998). The similarities
Fig. 6. Foxf1 expression is downregulated by BMP4 and indirectly by FGF7 and FGF10. Beads soaked in BMP4 (A), FGF10 (C,E) or FGF7 (G,LK) were implanted in the mesenchyme of wild-type lung explants, which were then cultivated for 24 hours. BSA-soaked beads served as a controls (B,D,F,H,J,L). Expression of Foxf1 (A-D,G,H), Bmp4 (E,F), Ptc (I,J) and Shh (K,L) was analyzed by whole-mount in situ hybridization. BMP4 reduces Foxf1 expression in mesenchyme adjacent to the bead (A). FGF10 attracts distal epithelium and stimulates its proliferation, which is seen as dilation of the bulbous part of buds close to the bead (C). The patches of elevated Foxf1 expression next to distal epithelium (D) are lost in the area influenced by exogenous FGF10 (C), whereas no effect is seen on the low level expression in mesenchyme along the proximal, tubular epithelium (C). A likely explanation for this inhibition is that it is mediated by BMP4, as Bmp4 is activated by FGF10 in distal, but not in proximal epithelium (E). FGF7 induces an even more pronounced epithelial proliferation and bud expansion (G,LK). Foxf1 is generally downregulated in the area influenced by FGF7 (G), which may be mediated by a decreased Shh signaling, as epithelial Shh expression is reduced by FGF7 (K). This interpretation is supported by the similarity in the effect of FGF7 on Foxf1 and another Shh-responsive gene, Ptc (I).

Fig. 7. Summary of paracrine interactions shaping the Foxf1 expression pattern in developing lung. (A) Activation of Foxf1 by Shh is based on both ectopic expression and loss-of-function experiments presented in this work. The inclusion of Gli2/3 in this pathway is inferred from their role in transducing the hedgehog signal and the similarity between Gli2/3 knockout phenotypes and the Foxf1 heterozygote phenotype. Inhibition by BMP4 refers to the effect of BMP4 beads on Foxf1 expression presented here. The role of FGF10 and FGF7 in regulation of Bmp4 and Shh, respectively, has been published by others (Lebeche et al., 1999) and has also been confirmed by us; the net effect of FGFs on Foxf1 expression was analyzed in this work. (B) High magnification view of lung explant hybridized with a Foxf1 probe. Foxf1 expression in subepithelial mesenchyme peaks at the transition between the proximal, tubular epithelium of the distal, bulbous part of the bud. The circular object is a bead implanted in the mesenchyme as a negative control and has no relevance for the expression pattern discussed here. (C) Model of paracrine signaling in a lung bud that provides a possible explanation for the observed distribution of Foxf1 mRNA. Proximal tubular epithelium secretes a low level of Shh (pink), which gives rise to a low level of Foxf1 expression (light blue) in the subepithelial mesenchyme. Shh secretion is higher in the distal epithelium (red), which activates high level expression of Foxf1 (dark blue). Fg10 is expressed in mesenchyme at the distal tip of the bud (black). In response to FGF10 signaling, Bmp4 is activated in the most distal epithelium (green). Secretion of BMP4 inhibits Foxf1 expression, which therefore drops again towards the tip of the bud (light blue).
of the mutant phenotypes suggest that Shh, Gli and Foxf1 are parts of the same signaling pathway. The mesenchymal expression of Foxf1, its activation by ectopic Shh secretion and loss of expression in Shh−/− lung bud mesenchyme place Foxf1 downstream of Shh. Ci, the Drosophila homolog of mammalian Gli genes, encodes a protein whose activity as transcription factor is regulated directly by hedgehog signaling (Dominguez et al., 1996). It is therefore likely that Foxf1 is downstream of Gli in mammals and perhaps directly transcriptionally activated by Gli proteins (Fig. 7A).

Shh is expressed in axial structures, floorplate and notochord (Echelard et al., 1993), where it is involved in somite patterning and formation of sclerotomes from cells of the ventromedial part of the somites (Fan et al., 1995; Fan and Tessier-Lavigne, 1994; Johnson et al., 1994). In Shh−/− embryos that develop to term, most sclerotomal derivatives, including the entire vertebral column, are absent (Chiang et al., 1996). However, sclerotomal differentiation in Shh mutant embryos is initiated normally, as judged by expression of the sclerotomal marker Pax1 (Chiang et al., 1996). Based on this, Shh is postulated to be essential for the maintenance, but not the initiation, of sclerotome differentiation (Chiang et al., 1996; Marcell et al., 1999). Foxf1 is not expressed in somites, but is turned on in sclerotomal cells as these separate from the dermomyotome (Mahlapuu et al., 2001). As development of the axial skeleton proceeds, Foxf1 is turned off in the condensed, chondrogenic mesenchyme of the future vertebra and remains expressed in the gradually narrowing intervertebral discs (Fig. 5B,C; Aitola et al., 2000; Mahlapuu et al., 2001; Mahlapuu et al., 1998). In Shh−/− embryos, no sclerotomal expression of Foxf1 is observed, which is consistent with our conclusion that Foxf1 is a target for Shh signaling. This also shows that although Pax1 is induced normally in the Shh mutant and apparently can be activated by a Shh-independent pathway, other sclerotomal markers – such as Foxf1 – are not. Thus, Shh is necessary for specific aspects of sclerotome induction and not just for maintenance.

The absence of Foxf1 expression in sclerotomes and in mesenchyme surrounding the foregut epithelium and lung buds of Shh−/− embryos, indicates that transcriptional activation of Foxf1 is a response to Shh signaling in these tissues. In contrast, the midgut and hindgut expression of Foxf1 is normal in the Shh mutant. The expression range of Foxf1 in gut mesenchyme of Shh−/− embryos exactly matches the expression of another member of the hedgehog family, Ihh. Ihh signals via the same receptor as Shh and is expressed in gut epithelium from the hindstomach to the rectum (Björgood and McMahon, 1995). It is thus possible that activation by hedgehog signaling is a general requirement for Foxf1 expression. Indeed, throughout embryonic development, expression of Foxf1 correlates with sites of hedgehog secretion. In addition to the gut, lungs and sclerotomes, Foxf1 is also expressed in the subepithelial mesenchyme of the oral cavity, the tongue and dental follicles (Aitola et al., 2000; Mahlapuu et al., 2001; Mahlapuu et al., 1998), all of which are proximal to sites of epithelial Shh production (Björgood and McMahon, 1995). At the early somite stage, Foxf1 is expressed in extra-embryonic mesoderm, including the yolk sac blood islands (Mahlapuu et al., 2001; Peterson et al., 1997); an expression pattern that it shares with Ptc1 (Maye et al., 2000) and which matches the secretion of Ihh from the yolk sac endoderm (Farrington et al., 1997; Maye et al., 2000). In the patterning of primitive mesoderm, Foxf1 expression characterizes the lateral plate mesoderm, which is adjacent to regions of visceral endoderm that produce Shh and Ihh (Echelard et al., 1993).

Asymmetry of rib attachment and ossification of the sternum is a common malformation in Foxf1 heterozygotes. This represents an exaggerated version of the ‘crankshaft sternum’ (Theiler, 1989), which can also be caused by inactivation of engrailed 1 (Wurst et al., 1994) and by experimental amniotic sac puncture (Chang et al., 1996). The connection between Foxf1 gene dose and sternal morphogenesis is not clear, as Foxf1 is expressed neither in the sternum, nor the costal, primordia. As discussed above, Foxf1 is expressed in sclerotomal mesenchyme and is turned off during chondroblast differentiation (Aitola et al., 2000; Mahlapuu et al., 2001; Mahlapuu et al., 1998). It is possible that a reduced expression level of Foxf1 could induce a subtle vertebral asymmetry that causes the left and right costal processes to grow at slightly different angles. Another possibility is that the composition of the amniotic fluid is altered in response to a lowered Foxf1 expression. Foxf1 is expressed in amnion (Peterson et al., 1997) and is essential for its development and function; in homozygous Foxf1 knockout embryos the mesodermal component of the amnion has severe defects in differentiation and cell adhesion (Mahlapuu et al., 2001). Finally, the skeletal defects may be secondary to the altered chest morphology that is a consequence of a considerable reduction in lung volume.

Bmp4 is expressed in the distal face of the epithelial buds of the developing lung and is induced by FGF10 signaling from the adjacent mesenchyme (Lebecche et al., 1999; Weaver et al., 2000). The stimulation of epithelial cell proliferation, caused by FGF10, is counteracted by BMP4 (Weaver et al., 2000). Locally applied BMP4 has an inhibitory effect on mesenchymal Foxf1 expression in lung explants. Lowering the Foxf1 expression by reducing its gene dose, as in Foxf1 heterozygotes, may therefore have similar effects as an increase in Bmp4 expression. In fact, epithelial overexpression of Bmp4 in transgenic mice results in lung hypoplasia, inhibition of branching, and pulmonary immaturity at birth (Belluscı et al., 1996).

FGF10 is produced by groups of mesenchymal cells located distally of the epithelial buds (Belluscı et al., 1997b) and activates Bmp4 expression in the adjacent epithelium. It functions as a mitogen and a chemotactant for epithelial cells and induce bud dilation when provided exogenously (Belluscı et al., 1997b; Park et al., 1998). Fgf10 is essential for lung bud formation and branching; Fgf10−/− embryos develop trachea, but no lungs (Min et al., 1998; Sekine et al., 1999). In response to implantation of a bead containing FGF10, mesenchymal cells adjacent to bud epithelium express less Foxf1. A likely mechanism behind the decrease in Foxf1 expression in response to FGF10 is enhanced inhibition by BMP4, as Bmp4 expression increases in bud epithelium next to the bead (Fig. 6A). This interpretation is supported by the observation that Foxf1 expression is only downregulated in the sites of high expression, next to the bud epithelium. Along the proximal, tubular epithelium of the future bronchi, Foxf1 expression is normal. This is exactly the pattern that would be expected if the effect was mediated by BMP4, as, unless the mesenchyme is removed experimentally, FGF10 activates
Bmp4 only in bud epithelium, not in proximal tubular epithelium (Lebeche et al., 1999; Weaver et al., 2000).

A second FGFR family member, FGFR7, is also produced by the lung mesenchyme. The effect on epithelium induced by FGFR7 is distinct from that of FGFR10, although both factors signal through the same receptor, FGFR2-IIIb (Arman et al., 1999; De Moerlooze et al., 2000; Igarashi et al., 1998). Shh expression is inhibited by FGFR7, but not by FGFR10 (Lebeche et al., 1999). Locally applied exogenous FGFR7 decreases Foxf1 expression. As Shh activates Foxf1, decreased activation by Shh signaling is a plausible mechanism behind the reduced expression of Foxf1 seen in response to exogenous FGFR7. A reduction in Ptc expression similar to that observed for Foxf1 in the area influenced by FGFR7 is consistent with this interpretation (Fig. 6f; Lebeche et al., 1999). The paracrine interactions thought to influence Foxf1 expression in the lung are summarized in Fig. 7A.

A low level of Foxf1 mRNA is found in a zone of mesenchyme that lines the proximal, tubular epithelium of the developing lung (Fig. 7B); an expression pattern that matches the basal level of Shh expression in this part of the epithelial tree. In the buds, the expression level of Shh is significantly higher, which will upregulate Foxf1 expression in the surrounding mesenchyme. At the same time, BMP4 secreted from the distal epithelium of the bud will inhibit Foxf1 expression. However, whereas Bmp4 expression is confined to the most distal part of the bud, Shh expression is elevated in the entire bulbous part and thus extends further proximally. These interactions may explain the observed distribution of Foxf1 mRNA, which peaks at the transition between the distal, bulbous and the proximal, tubular epithelium and then falls back to a lower level along the outer, distal face of the bud (Fig. 7B,C).

An important question that remains to be answered is what target genes are regulated by Foxf1 and the role that it plays in formation of the lung. The expression pattern suggests that Foxf1 could be involved in reshaping the growing bud into a narrower, tubular shape (Fig. 7B). Heterozygous lungs exhibit a general growth retardation, which suggests that Foxf1 promotes proliferation. Shh is a mesenchymal mitogen (Bellusci et al., 1997a) and this function may be mediated by Foxf1. The notion that Foxf1 can be involved in growth control is supported by the observation that proliferation of primitive streak mesoderm is reduced in Foxf1−/− embryos (Mahlapuu et al., 2001).

Their phenotypic similarity with Foxf1 heterozygotes suggests that Shh, Gli2 and Gli3 mutants owe many of their malformations to a reduction in Foxf1 expression, thus implicating Foxf1 as a key target of hedgehog signaling in lung and foregut morphogenesis. Although the phenotype depends on genetic background, it shows that Foxf1 gene dose can be crucial for developmental processes. In humans, foregut malformations including esophageal atresia, tracheo-esophageal fistula and lung abnormalities occur in 1 in 2000-5000 live births (Skandalakis and Gray, 1994). The human FOXF1 gene, located at 16q24 (Larsson et al., 1995), should be considered a candidate locus in familial cases of these malformations.

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