Fusion of lung lobes and vessels in mouse embryos heterozygous for the forkhead box f1 targeted allele

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Formation of the mouse lung begins at 9.5 days postcoitum (pc) as a ventral outpouching of the foregut endoderm that invades the adjacent mesenchyme. Reciprocal interactions between the splanchnic mesenchyme and the respiratory epithelium support dichotomous branching that forms the left and right primary bronchi (10, 27). By 11 days pc, four right and one left primary lung bronchi are formed that define the lung lobes. Subsequently the conducting airways are formed by the process of branching morphogenesis (10, 27).

The mammalian Fox genes are an extensive family of transcription factors (13) that share homology in the (HFH)-1 domain (also known as FoxJ1) have been implicated in lung-branching morphogenesis, cell differentiation, and lung-selective gene expression (6, 23).

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embryonic development, cellular proliferation, and metabolism (Reviewed in Refs. 6, 7, 12, 16, 23, 30). One of these family members, Foxf1 [also known as Hfh8 or forkhead-related-activator (Frec]-1], is first expressed during gastrulation in a subset of mesodermal cells arising from the primitive streak region that contributes to the extraembryonic and lateral mesoderm (24). Consistent with this early expression pattern, Foxf1(-/-) embryos die in utero from defects in extraembryonic and lateral mesoderm differentiation (19). During organogenesis, high levels of FoxF1 expression persist in the mesenchyme of the respiratory and gastrointestinal tracts. FoxF1 RNA is expressed at mesenchymal-epithelial interfaces involved in lung and gut morphogenesis (20, 24). In the adult mouse, FoxF1 RNA is detected in smooth muscle layers of pulmonary bronchioles, lamina propria of the stomach and intestine, and alveolar endothelial cells (15, 24).

Targeted ablation of the Foxf1 gene caused early embryonic lethality (19), whereas severe pulmonary abnormalities were observed in heterozygous Foxf1 mutant mice (14, 18). The winged-helix DNA binding domain of Foxf1 was replaced by an in-frame insertion of a nuclear localizing β-galactosidase (β-gal) gene, which disrupted the function of the mouse gene in vivo (14). Approximately 55% of newborn Foxf1(+/−) heterozygous mice died of severe lung hemorrhage. The severity of pulmonary abnormalities in the Foxf1(+/−) mice correlated with diminished levels of Foxf1 mRNA [designated as low-Foxf1(+/−) mice]. Defects in lung alveolarization and development of the alveolar capillaries were observed in the low-Foxf1(+/−) newborn mice. Lung hemorrhage in severely affected Foxf1(+/−) mice was coincident with disruption of the mesenchymal-epithelial cell interfaces in the alveolar and bronchial regions of the newborn lung and was associated with increased apoptosis and reduced surfactant protein B (SP-B) expression. Moreover, the lung defect associated with the Foxf1(+/+) mutation was accompanied by reduced expression of vascular endothelial growth factor (VEGF) receptor-2 [fetal liver kinase-1 (Flk-1)], BMP-4, as well as the lung Kruppel-like factor and T-box (Tbx2-Tbx5) transcription factors.

The mechanisms by which FoxF1 mediates changes in lung morphogenesis remain unclear; however, recent studies demonstrated that Foxf1(+/−) lungs exhibit lobe fusions and that pulmonary expression of Foxf1 was undetectable in Shh(-/-) mouse embryos, which suggests that Shh signaling is essential for Foxf1 expression (18). However, analysis of pulmonary genes with expression that was altered and associated with the lobular defect in Foxf1(+/−) embryonic lungs remains uncharacterized. The present study was conducted to determine the early developmental defects leading to lung fusions in the Foxf1(+/−) embryos and to identify potential genetic pathways influenced by diminished pulmonary levels of Foxf1. In situ hybridization of mesenchymal and epithelial marker genes [e.g., Foxf1, Patched (Ptc), Shh] revealed that as early as 11 days pc, defects were observed in Foxf1(+/−) embryonic lungs that were associated with reduced expression of FGF-10, BMP-4, and the Gli3 transcription factor.

MATERIALS AND METHODS

Heterozygous Foxf1 mice. The generation of Foxf1(+/−) mice was described previously (14). Because the nuclear-localizing β-gal gene is expressed at the targeted locus under control of FoxF1 regulatory sequences, β-gal enzyme staining was used for analysis of FoxF1 expression. Heterozygous male mice were originally kept in a 129/Black Swiss mouse background. For generation of the heterozygous embryos for morphological and RNA analysis, male breeder mice were mated with wild-type (wt) CD-1 female mice in the evening, and vaginal plugs were checked in the morning. Noon of the day of appearance of a vaginal plug was designated as 0.5 days pc. Dams were killed by carbon dioxide asphyxiation at various days of gestation, and the embryos were removed by cesarean section. Tail samples were used for genotyping by PCR analysis as described previously (14).

β-Gal staining. Staining for β-gal was performed according to Cleveline and colleagues (5) with a few modifications. Briefly, embryos and dissected lungs were fixed for either 30 min (11–14 days pc) or 1 h (older than 14 days pc) in 2% formaldehyde-0.2% glutaraldehyde [with 0.02% Nonidet P-40 (NP-40) and 0.01% sodium deoxycholate] in PBS at pH 7.8. Color development was done at 30°C in the presence of 5 mM potassium ferrocyanide, 5 mM ferricyanide, 2 mM magnesium chloride, 0.02% NP-40, 0.01% sodium deoxycholate, and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) as described previously (5). After staining was completed, samples were washed with PBS and postfixed in 4% paraformaldehyde (PFA) overnight at 4°C, paraffin embedded, and sectioned as described previously (14). For analysis of embryonic lung morphology, dissected lungs were fixed overnight in 4% PFA at 4°C and subsequently washed three times in PBS. Lungs were photographed using a Nikon dissecting microscope and the MDS-120 digital microscopy system (Kodak).

In situ hybridization with digoxigenin-labeled probes. Whole-mount lung in situ hybridization was carried out according to published protocols (9). Except for the FoxF1 (HFH-8) cDNA (24), the DNA clones used for generation of digoxigenin-labeled RNA probes were generated by RT-PCR of total RNA isolated from 9.5-day pc embryos or from adult mouse lungs. RT-PCR was performed with a Superscript One-Step RT-PCR kit (GIBCO-BRL) according to the manufacturer’s instructions. The recovered DNA fragments were subcloned in pBluescript SK (Stratagene), and the sequence of the clones was confirmed by dideoxy sequencing. T3 or T7 RNA polymerase in the presence of digoxigenin-labeled UTP generated sense and antisense riboprobes from linearized plasmid DNA by restriction-enzyme digestion and in vitro transcription. The relevant information regarding the DNA clones generated is listed in the following order: name of the gene, GenBank accession number, length of fragment generated, and DNA sequence amplified: Ptc, NM008957, 419 bp fragment, 3,450–3,954; Fgf10, NM008002, 480 bp, 80–609; Vegfa, M95200, 481 bp, 497–977; Bmp4, D14814, 318 bp, 7,101–7,127 and 8,131–8,423; Shh, X76290, 311 bp, 516–826; Gli1, AB025992, 529 bp, 2,934–3,462; Gli2, X99104, 453 bp, 1,040–1,502; and Gli3, NM008130, 516 bp, 1,044–1,560.

RNA extraction and RNase protection assay. Mouse lungs were dissected from 14-day-pc wt and Foxf1(+/−) embryos, and total lung RNA was prepared by an acid guanidiothiocyanate-phenol-chloroform extraction method using RNA STAT-60 (Tel-Test B, Friendswood, TX). The RNase protec-
tion assay was performed with \(^{32}\text{P}\)UTP-labeled antisense RNA synthesized from plasmid templates with the appropriate RNA polymerase as previously described (25). RNA probe hybridization, RNase ONE (Promega, Madison, WI) digestion, electrophoresis of RNA-protected fragments, and autoradiography were performed as described previously (15, 25). Cyclophilin probes were added to the same hybridization reaction for normalization of RNA among lung RNA samples. Quantitation was performed on scanned X-ray films using BioMax 1D image-analysis software (Kodak). The mouse FoxF1 RNase protection probe (Foxf1 cDNA nucleotides 437–816) was generated from Foxf1 cDNA sequences located outside of the FoxF1β-gal gene-targeting vector (14, 15) and hybridizes selectively to mRNA transcribed from the wt Foxf1 locus. Foxf1(+/−) 14-day-pc embryos were divided on the basis of pulmonary levels of Foxf1 mRNA using RNA protection assays: either high-Foxf1 levels (~50% of Foxf1 levels in wt lungs) or low-Foxf1 levels (20% of the Foxf1 levels in wt lungs). The low-Foxf1(+/−) mouse lung was associated with lung hemorrhage and perinatal lethality (14). RNase protection probes for Flk-1 and BMP-4 were described previously. These studies indicate that Foxf1 is expressed in mesenchymal at sites of interaction with epithelium.

RESULTS

β-Gal staining reveals mesenchymal expression in developing Foxf1(+/−) lung. In 9.5-day-pc Foxf1(+/−) embryos, β-gal staining derived from the Foxf1 gene promoter was observed in the mesenchyme of the foregut region containing the respiratory diverticulum that gives rise to both the upper and lower respiratory regions (Fig. 1A). High levels of β-gal staining persisted in the mesenchyme of the developing lung (10.5 days pc), oral cavity, intestine, and allantois (Fig. 1, B and D). In 12-day-pc Foxf1(+/−) embryos, high levels of β-gal staining were observed in the mesenchymal cells of the oral cavity, lung, stomach, intestine, and intersomitic arteries and in mesenchymal cells throughout the conducting airways of the lung (Fig. 1, C and E). More intense β-gal staining was evident in the mesenchyme juxtaposed to the bronchiolar epithelial cells (12 days pc; Fig. 1E), the former giving rise to the bronchiolar smooth muscle layer. These expression studies reveal that Foxf1 is expressed in lung mesenchyme at sites of interaction with epithelium.

Defect in primary lung-bud formation in Foxf1(+/−) embryonic lungs. By 11 days pc, the lung consists of five distinct lung buds, which form the five lobes that are characteristic of the adult mouse lung (Fig. 2, A and D). The formation of the four right lung buds occurs in the following sequential order: dorsal cranial, lateral middle, caudal, and ventral accessory bud (reviewed in Ref. 10). Furthermore, the right and left lungs possess several lateral buds that undergo branching morphogenesis but fail to develop into discrete lobes. As early as 10.5–11 days pc, the Foxf1(+/−) lungs displayed altered lung-bud orientation and diminished branching morphogenesis (Fig. 2, B, C, E, and F). These Foxf1(+/−) lung defects commonly included a caudal and lateral shift in the orientation of the accessory lobe (Fig. 2, arrowhead) and a lateral shift in the middle (Fig. 2, B, C, E, and F) and cranial lobe positions (data not shown). These studies indicate that
Fig. 2. Defective lung-bud formation in forkhead box (Foxf1) embryonic lungs. Early stages of branching morphogenesis are depicted in 10.5-day-pc (A–C) and 11-day-pc (D–F) mouse embryos. Lungs in A and D were from wild-type [wt, (+/+) embryos, and lungs in B, C, E, and F were from heterozygous [ht, (+/–)] embryos. Poorly developed lung buds and a diminished number of secondary lateral buds were observed in the left lobe from ht embryos. Wt embryonic lungs in A and D showed the stereotypical pattern of lung-bud growth: left (ll) primary bud grows out of the primary left bronchus. Right lung revealed the cranial (cr), middle (mi), caudal (ca), and accessory (ac) buds that give rise to the four right lobes. Ht Foxf1 lungs (B, C, E, F) showed variations from the normal pattern of lung-bud formation. Positioning of the accessory buds was abnormal (arrowhead). Magnification: ×63.

Fig. 3. Fusion of right lobes in embryonic Foxf1 (+/–) lungs. Defective lung-bud formation in 12-day-pc (C–F) and 13-day-pc (G and H) embryonic Foxf1 (+/–) lungs. Ventral (v) views show the left lung lobe on the right side (A, C, E, G); dorsal (d) views show the left lung lobe on the left side (B, D, F, H). Wt 12-day-pc lungs (A, B). A lateral shift in the position of the accessory lobe results in its partial fusion with the caudal lobe (E, G). Partial fusion of the cranial and middle lobes was observed in ht lungs (D, F, H); larger arrows indicate fused ht lung lobes. Sections through 14.5-day-pc wt (I) and ht (J) embryonic lungs showed abnormal fusion of cranial, middle, and caudal lobes. Magnification: A–H, ×34; I and J, ×50.
FoxF1 is required for location site and extent of branching morphogenesis of the lung buds.

Right-lobe fusions of the embryonic Foxf1(+/−) lung.

As a consequence of the early defect in lung-bud formation, pairwise ventral and dorsal views of either 12-day-pc (Fig. 3, C–F) and 13-day-pc (Fig. 3, G and H) Foxf1(+/−) lungs or 12-day-pc wt lungs (Fig. 3, A and B) revealed a range of fusion defects in the right Foxf1(+/−) pulmonary lobes. Approximately 90% of Foxf1(+/−) lungs displayed a fusion of the accessory lobe with the caudal lobe (Fig. 3, E–H) that resulted from a range of altered positions of the accessory lobe. Although this lobe-fusion defect was absent in 10% of Foxf1(+/−) lungs, the orientation of the accessory lobe was shifted caudally (Fig. 3, C and D). Furthermore, embryonic Foxf1(+/−) lungs displayed fusion of the right cranial and middle lobes, which was likely caused by a lateral shift in the orientation during lung development (Fig. 3, arrows). In severe forms, the Foxf1(+/−) lung-fusion defect was associated with an angular shift of the cranial and middle lobes, which merges them in the same plane (Fig. 4D; 18-day-pc Foxf1(+/−) lung). Cross sections of 14.5-day-pc wt embryonic lung demonstrated distinct separation of the five lung lobes and bronchi (see Fig. 3J). In contrast, examination of a severely fused Foxf1(+/−) embryonic lung at the same thoracic level revealed fusion of the cranial, middle, and caudal lobes associated with aberrantly juxtaposed secondary bronchi (see Fig. 3J).

The lobar fusion defect in Foxf1(+/−) lungs became more evident at later stages of development (18 days pc). Ventral and dorsal views of wt lung revealed the normal left lung lobe and the four distinct lobes of the right lung (Fig. 4, A and B) with the accessory lobe spanning the cranial and left lobes (Fig. 4A). Most of the Foxf1(+/−) embryonic lungs exhibited fusion of the accessory and caudal lobes and associated abnormal orientation of the accessory lobe (Fig. 4C). The dorsal view of a severely fused Foxf1(+/−) embryonic lung (Fig. 4D) showed complete joining of the cranial and middle lobes. Approximately 13% of Foxf1(+/−) lungs exhibited more severe malformations in which the cranial, caudal, and middle lobes were fused (data not shown; see Fig. 3J).

A subset of the Foxf1(+/−) newborn mice died from severe lung hemorrhage owing to this phenotype being associated with pulmonary levels of FoxF1 RNA that were 20% of wt expression (14). To determine whether the lethality of Foxf1(+/−) newborn mice was associated with the severity of lobe-fusion defects, we examined lung morphology of the surviving adult Foxf1(+/−) mice. In a manner similar to that observed in the embryonic Foxf1(+/−) lungs, fusion between the accessory and caudal lobes was observed most in lungs from

![Image](http://ajplung.physiology.org/)

Fig. 4. Mild and severe fusions of right lobes in 18-day-pc Foxf1(+/−) lungs. Ventral and dorsal views of 18-day-pc wt embryonic mouse lung displays the normal left lung lobe (ventral view left lung lobe, right) and the four distinct lobes of the right lung including cranial (cr), middle (mi), caudal, and accessory (ac) lobes (A and B). Most of the Foxf1(+/−) embryonic lungs exhibited fusion of the accessory and caudal lobes and associated abnormal orientation of the accessory lobe (C). Dorsal view of a severely fused Foxf1(+/−) embryonic lung showing complete fusion of the cranial and middle lobes (D). Magnification: ×10.
the adult Foxf1(+/−) mice (Fig. 5, A, C−F). Most adult Foxf1(+/−) mice possessed distinct cranial, middle, and caudal lobes and lacked the severely fused lobes that are typical of Foxf1(+/−) embryos. This is supported by the fact that 35–50% of the Foxf1(+/−) embryonic and newborn lungs displayed severe lung fusions, and only one severely fused lung was found several days postnatally (Table 1). We also found that the adult Foxf1(+/−) lungs exhibited FoxF1 mRNA levels that were similar to wt lungs (data not shown), which suggests that severe lung fusions were associated with low pulmonary FoxF1 mRNA. Taken together, these results suggest that lethality of Foxf1(+/−) newborn mice correlated with severe fusion/H11002/H11001 together, these results suggest that lethality of Foxf1(+/−) newborn mice correlated with severe fusion.

Fewer mesenchymal-epithelial interfaces in Foxf1(+/−) lungs. To examine the expression pattern of signaling molecules in the Foxf1(+/−) lungs, in situ hybridization analysis was performed on 11-day-pc lungs. Whole-mount in situ hybridization for Foxf1 and the putative Shh receptor Ptc were compared with 11-day-pc wt and Foxf1(+/−) heterozygous type (ht) lungs. FoxP1 and Ptc mRNA expression overlapped in the distal mesenchymal cells of the developing wt lung buds (Fig. 6, A and B, arrowheads). The defective lung-bud formation in Foxf1(+/−) mice was associated with fewer mesenchymal regions that expressed the Foxf1 and Ptc genes (Fig. 6, A and B). Likewise, embryonic Foxf1(+/−) lungs developed fewer distal bronchial epithelial regions expressing Shh and VEGFA (1, 22) compared with wt lungs (Fig. 6, C and D).

Diminished distal mesenchymal expression of FGF-10 in 10− to 11-day-pc Foxf1(+ −) lungs. Whole-mount in situ hybridization studies revealed that Foxf1(+/−) lungs exhibited diminished FGF-10 hybridization signals in the ventral view, which allowed visualization of the right, middle, cranial, and accessory lung buds (Fig. 6E). In contrast, FGF-10 RNA was unaltered in the Foxf1(+/−) distal lung mesenchyme in a dorsal view, which visualizes the left and caudal lung buds (Fig. 6F). Likewise, at 10 days pc, Foxf1(+/−) lungs exhibited diminished FGF-10 RNA in the distal mesenchyme in the ventral view (Fig. 6G) but not in the dorsal view (Fig. 6H). In light of the critical role of FGF-10 in lung-bud formation and branching morphogenesis, it is likely that reduced mesenchymal expression of FGF-10 in the developing Foxf1(+/−) lungs contributes to the defective bud formation of the right ht lung.

Whole-mount in situ hybridization of 12-day-pc wt and Foxf1(+ −) lungs was performed with Foxf1, Ptc, FGF-10, VEGFA, and BMP-4 probes and demonstrated decreased numbers of mesenchymal-epithelial interfaces, which is consistent with the branching abnormality (Fig. 7, A–E). A lateral shift in orientation of the accessory lobe was observed for all of the markers in the 12-day-pc Foxf1(+/−) lung (Fig. 7, arrowheads). Furthermore, 12-day-pc Foxf1(+ −) lungs displayed increased expression of FGF-10 in the distal mesenchyme of the accessory lobe compared to wt lungs, which suggests that the onset of FGF-10 expression was delayed in the Foxf1(+ −) lungs (Fig. 7C, arrowheads). Decreased hybridization of the transcription factors Gli1, Gli2, and Gli3 further supports the concept that the 12-day-pc Foxf1(+/−) lungs have reduced mesenchyme compared with wt lungs (Fig. 7, F–H).

Total lung RNA from 14-day-pc wt and Foxf1(+ −) embryos was used in RNase protection assays to quan-

Table 1. Classification of lung-lobe defects exhibited by Foxf1 (+ −) mice

<table>
<thead>
<tr>
<th>Foxf1 (+−) Lungs</th>
<th>n</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
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<tr>
<td>14 days postcoitum</td>
<td>20</td>
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<td>19 days postcoitum</td>
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<td>Newborn</td>
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<td>3 days postnatal</td>
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n, No. of Mice. Mild, fusion of accessory lung lobe and lungs possess distinct lung lobes; moderate, partial fusion of two or more lung lobes; and severe, almost complete fusion of two or more lung lobes.
titate FoxF1, Flk-1, BMP-4, Gli2, and Gli3 mRNA (Fig. 8). Consistent with previous studies (14), RNase protection assays with embryonic lung RNA with the FoxF1 probe identified low- and high-expressing populations of Foxf1 expression in Foxf1(-/-) lungs. Consistent with our previous findings (14), diminished levels of BMP-4 and Flk-1 mRNA were found in only the low-Foxf1(-/-) embryonic lungs (Fig. 8). Likewise, only the low-Foxf1(-/-) lungs displayed a 70% reduction in Gli3 mRNA levels (Fig. 8), a finding that is consistent with defective formation of the right medial, caudal, and accessory lung lobes in the Gli3(-/-) mice (8). Foxf1(-/-) lungs exhibited no significant changes in Gli2 mRNA levels (Fig. 8). Taken together, these studies indicate that the defective lung formation was associated with reduced expression of FoxF1, FGF-10, BMP-4, Flk-1, and Gli3 mRNA.

Defective orientation of vessels in Foxf1(+/-) embryos. Fusion of the Foxf1(+/-) embryonic lung lobes was associated with fusion of correspondingly large pulmonary blood vessels. The invariant relationships
among the major veins (white) and arteries (black) and the bronchial airway (red) are schematically shown in Fig. 9D. Foxf1(+/−) embryonic lungs (19 days pc) with mild fusion of the accessory and caudal lobes (Fig. 9B) showed misplacement and fusion of vessels. For example, instead of the middle lobe vein entering the ventral face of the middle lobe (Fig. 9A), it entered the cranial side of the middle lobe in Foxf1(+/−) embryonic lungs (Fig. 9B). Furthermore, even Foxf1(+/−) lungs with mild phenotype displayed fusion of the caudal and accessory lobe veins within the abnormally fused lung lobes (Fig. 9E). Foxf1(+/−) embryos with severe fusions of the right lung lobes also possessed complete fusion of the accessory and caudal pulmonary veins, which bifurcated within the fused lobe (schematically shown in Fig. 9F). In addition, small abnormal veins were found that connected the cranial and middle lobe veins (schematically shown in Fig. 9F, shared veins). In more severely affected Foxf1(+/−) lungs, multiple vein fusions were observed between the cranial and middle lobe veins as well as between the caudal and
shown to express reduced pulmonary levels of FoxF1 (14). The most prevalent Foxf1(+/−) lung defect consisted of a mild fusion between the caudal and accessory right lobes resulting in a lateral shift in the position of the accessory lobe. This mild lung-fusion defect was found in surviving adult Foxf1(+/−) mice in which wt levels of Foxf1 mRNA were observed in the lungs. Taken together, our results suggest that severe fusion of the right lung lobes is associated with significant reductions in pulmonary FoxF1 levels and suggest that its expression is critical for proper lung morphogenesis.

Although a recent study reported that Foxf1(+/−) CD-1 mouse embryos exhibit esophageal atresia, tracheosophageal fistula, skeletal abnormalities, and lung-lobe fusions, the Foxf1(+/−) lungs were not used to identify pulmonary genes with altered expression that was associated with the lobular fusion (18). Our current study carried out a developmental expression analysis of Foxf1(+/−) mouse lungs to identify genes with altered expression due to Foxf1 haploinsufficiency and that may contribute to the lung-fusion phenotype. In situ hybridization studies with mesenchymal (Foxf1, Ptc) and epithelial (Shh, Vgfl) marker genes revealed that as early as 11 days pc, Foxf1(+/−) lungs exhibited fewer mesenchymal-epithelial interfaces. We also found that severe lung-lobe fusions in Foxf1(+/−) mice were associated with reduced pulmonary expression of BMP-4, a signaling molecule that is essential for proper lung morphogenesis (28) and may therefore contribute to defective lobe formation in Foxf1(+/−) lungs. Diminished pulmonary expression of Foxf1 reduced lung-bud formation and branching morphogenesis in association with decreased mesenchymal expression of FGF-10 in the periphery of right middle, cranial, and accessory lobes at 10–11 days pc in Foxf1(+/−) embryos. In 12-day-pc Foxf1(+/−) lungs, we detected increased mesenchymal expression of FGF-10 in the distal tip of the accessory lobe bud; FGF-10 expression is delayed in the Foxf1(+/−) lungs. The initial positioning of the lung buds was defective in the Foxf1(+/−) lungs. Genetic and in vitro studies have implicated a role for FGF-10 as a chemoattractant that influences the location of lung-bud formation (3, 10, 17, 26, 28). It is therefore likely that the altered temporal expression pattern of FGF-10 in early embryonic Foxf1(+/−) lungs contributes to observed defects in branching and lobulation. Because Foxf1 expression is found at the onset of splanchnic mesoderm formation (24), our studies are also consistent with the possibility that wt FoxF1 levels are necessary for normal temporal expression of FGF-10 in the splanchnic mesenchyme. Alternatively, FGF-10 and Foxf1 may function in the regulatory loop that maintains their expression.

The zinc-finger Gli transcription factors are homologs of the Drosophila segment polarity gene cubitus interruptus, and, in response to Shh signaling through its putative receptor Ptc, the Gli proteins are transcriptionally active because they are not proteolytically cleaved (11, 21). Defective lung-bud formation in Foxf1(+/−) embryos were associated with changes

Fig. 8. Diminished Flk-1, BMP-4, and Gli3 mRNA in 14-day-pc Foxf1(+/−) lungs. Total RNA was prepared from 14-day-pc embryonic mouse lungs and analyzed for Foxf1, Flk-1, BMP-4, Gli2, and Gli3 mRNA levels by RNase protection assay. Each probe was combined with cyclophilin probe in the hybridization reaction and signals were normalized to corresponding cyclophilin levels (in the same lane) as described in MATERIALS AND METHODS. Numbers below each panel represent averages of normalized mRNA levels in Foxf1(+/−) lungs with respect to wt lungs ± SD. Foxf1(+/−) embryonic lungs were subdivided into two classes: high-Foxf1 levels (~50% of Foxf1 levels in wt lungs) or low-Foxf1 levels (~20% of the Foxf1 levels in wt lungs). Low-Foxf1(+/−) mouse lung was associated with lung hemorrhage and perinatal lethality as described previously (14).

in the expression of the Gli3 transcription factor, which has been implicated in lung-lobe formation and morphogenesis (6, 29). RNase protection assays demonstrated that low-Foxf1(+/−) lungs (14 days pc) displayed a 70% reduction in Gli3 mRNA. Decreases in Gli3 mRNA seen in Foxf1(+/−) lungs may play a role in the aberrant defects in lung budding. Defects in the right medial, caudal, and accessory lobe were observed in Gli3(−/−) mice (8). Furthermore, Foxf1(+/−) lungs possess reduced Shh signaling, because fewer Ptc-expressing mesenchymal cells were present in Foxf1(+/−) lungs. Potentially, the diminished number of mesenchymal cells expressing Ptc in Foxf1(+/−) embryonic lungs may lead to reduced Shh signaling and cause proteolytic cleavage of Gli proteins that in general act as transcriptional repressors of Gli target genes (11, 21). Taken together, our results suggest that the combination of diminished Ptc-mediated Shh signaling and reduction in Gli3 transcription-factor expression may contribute to the lung-lobe fusion defects in Foxf1(+/−) embryos.

We also observed that the pulmonary veins were misplaced in the Foxf1(+/−) lungs, and this defect was associated with the severity of the lung fusions. Our previous data (14) demonstrated that the peripheral pulmonary vasculature of Foxf1(+/−) mice was disrupted as assessed by platelet endothelial cell adhesion molecule (PECAM)-1 staining; however, PECAM-1 staining was normal in the large vessels. The defect in peripheral vasculature was associated with reduced expression of VEGFA and Flk-1 in the developing Foxf1(+/−) lungs. Whether the observed defects in orientation and fusion of large pulmonary vessels in Foxf1(+/−) mice was a direct consequence of the decreased FoxF1 mRNA is not entirely clear. Because the formation of the pulmonary vasculature is closely re-
lated to the growth of the conducting airway bronchial- oles, we propose that the defects in large vessels in the Foxf1(+/−) lungs were secondary to defects in the initial bud-site specifications.

In summary, we have shown that Foxf1(+/−) embryos exhibited defects in lung-bud formation and branching, which caused fusion of lung lobes and pulmonary veins. Foxf1(+/−) newborn mice with severely fused lung lobes died perinatally, and the lung defects correlated with the diminished pulmonary levels of FoxF1 expression. Early embryonic Foxf1(+/−) lungs exhibited diminished numbers of mesenchymal-epithelial interfaces that may disrupt formation of lung branch points. Expression of FGF-10, BMP-4, and the Gli3 transcription factor was decreased in lungs of Foxf1(+/−) embryos and may play a role in the disruption of branching morphogenesis that was seen in the haploinsufficient FoxF1 mice.

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