The Forkhead Box M1 Transcription Factor Is Essential for Embryonic Development of Pulmonary Vasculature*

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Transgenic and gene knock-out studies demonstrated that the mouse Forkhead Box m1 (Foxm1 or Foxm1b) transcription factor (previously called HFH-11B, Trident, Win, or MPP2) is essential for hepatocyte entry into mitosis during liver development, regeneration, and liver cancer. Targeted deletion of Foxm1 gene in mice produces an embryonic lethal phenotype due to severe abnormalities in the development of liver and heart. In this study, we show for the first time that Foxm1−/− lungs exhibit severe hyper trophy of arteriolar smooth muscle cells and defects in the formation of peripheral pulmonary capillaries as evidenced by significant reduction in platelet endothelial cell adhesion molecule 1 staining of the distal lung. Consistent with these findings, significant reduction in proliferation of the embryonic Foxm1−/− lung mesenchyme was found, yet proliferation levels were normal in the Foxm1-deficient epithelial cells. Severe abnormalities of the lung vasculature in Foxm1−/− embryos were associated with diminished expression of the transforming growth factor β receptor II, a disintegrin and metalloprotease domain 17 (ADAM-17), vascular endothelial growth factor receptors, Polo-like kinase 1, Aurora B kinase, laminin α4 (Lama4), and the Forkhead Box fl transcription factor. Cotransfection studies demonstrated that Foxm1 stimulates transcription of the Lama4 promoter, and this stimulation requires the Foxm1 binding sites located between −1174 and −1145 bp of the mouse Lama4 promoter. In summary, development of mouse lungs depends on the Foxm1 transcription factor, which regulates expression of genes essential for mesenchyme proliferation, extracellular matrix remodeling, and vasculogenesis.

Lung development in mouse embryos begins at 9.5 days post coitum (dpc)1 when the foregut endoderm invades the splanchnic mesenchyme and undergoes dichotomous branching (1). After the conducting airways are formed, lung growth continues by septation of peripheral sacculles to form terminal alveoli. The alveoli are lined by Type I and Type II epithelial cells and are vascularized by mesenchyme-derived endothelial cells, creating an extensive capillary bed to facilitate efficient gas exchange. Lung development depends on mesenchymal-epithelial cell signaling mediated by Sonic hedgehog (Shh) (2), transforming growth factor β (TGF-β) (1), bone morphogenetic protein-4 (3, 4), hepatocyte growth factor (5), fibroblast growth factor 10 (6, 7), and fibroblast growth factor 7 (8–10). These signaling proteins regulate branching morphogenesis and vasculogenesis of the lung by inducing expression of cell type-specific transcription factors (1, 11–15).

The lung mesenchyme undergoes vasculogenesis (formation of blood vessels de novo) and angiogenesis (branching of preexisting blood vessels) in a process requiring appropriate levels of vascular endothelial growth factor (VEGF), which stimulates mesenchyme proliferation and differentiation toward endothelial cell lineage (16, 17). Targeted disruption of the VEGF or VEGF receptor type I (Flt1) or type II (Flk1) causes an embryonic lethal phenotype displaying impaired blood-island formation and delayed endothelial cell differentiation, leading to abnormal blood vessel development (18–21). Overexpression of VEGF in the respiratory epithelium stimulates vasculogenesis in transgenic mouse lungs but results in aberrant vessel formation and increased expression of Flk1 (22). Differentiation of pulmonary mesenchyme also depends on proper expression of extracellular matrix proteins, including laminins, collagens, and integrins (1). Mice deficient in the laminin α4 (Lama4) gene exhibit impaired microvessel maturation and hemorrhage (23). Likewise, the disruption of Lama2 protein function causes defects in cell adhesion of a subpopulation of embryonic mesenchymal cells bearing a myofibroblast phenotype (24).

The Forkhead Box (Fox) proteins are an extensive family of transcription factors that share homology in the Winged Helix/Forkhead DNA binding domain (25–27). Fox proteins Foa2, Fox1, Fox1, and Foxp play important roles in regulating transcription of genes involved in branching lung morphogenesis and vasculogenesis during lung development (11, 12, 28–33). Expression of the Foxm1 transcription factor (previously known as HFH-11B, Trident, Win, or MPP2) is induced during

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‡ The abbreviations used are: dpc, days post coitum; Foxm1, Forkhead Box m1; LUC, luciferase; Foxm1−/−, Foxm1 null allele; WT, wild type; EMSA, electrophoretic mobility shift assay; Foxf1, Forkhead Box fl; H&E, hematoxylin and eosin staining; VEGF, vascular endothelial growth factor; Pecam-1, platelet endothelial cell adhesion molecule 1; TGF-β, transforming growth factor β; αSM, α-smooth muscle actin; Lama2, laminin α2; Lama4, laminin α4; Col12a1, procollagen type XII α1; CMV, cytomegalovirus; RT, reverse transcriptase; BrdUrd, 5-bromo-2′-deoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; TUNEL, terminal deoxynucleotidyltransferase UTP nick end labeling; BCP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, nitro blue tetrazolium.

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cellular proliferation and extinguished in terminally differentiated cells (34–37). Partial hepatectomy experiments demonstrated that mice with postnatal hepatic-specific deletion of the Foxm1 fl/fl (LoxP-targeted) allele exhibited a significant reduction in hepatocyte DNA replication and mitosis, which was associated with altered expression of proteins that limit Cdk1 and Cdk2 activity required for normal cell cycle progression (38). We recently demonstrated that Alb-Cre Foxm1 fl/fl hepatocytes are highly resistant to developing hepatocellular carcinoma following diethylnitrosamine/phenobarbital liver tumor induction (39). The mechanism of resistance to hepatocellular carcinoma development is associated with defects in cellular proliferation due to an aberrant increase in hepatocyte nuclear levels of Cdk inhibitor p27Kip1 protein and diminished expression of the M-phase-promoting Cdc25B phosphatase (39). Interestingly, Foxm1−/− embryos die in utero between 13.5 and 18.5 dpc due to severe defects in development of the embryonic liver and heart (40, 41). Foxm1−/− livers displayed abnormal accumulation of polyplloid hepatoblasts resulting from diminished DNA replication and a failure to enter mitosis. This was associated with diminished protein levels of the Polycomb kinase 1 (Plk-1) and Aurora B kinase (40), both of which phosphorylate regulatory proteins essential for orchestrating mitosis and cytokinesis (42, 43). Foxm1 is required for differentiation of hepatoblast precursor cells toward biliary epithelial cell lineage, because Foxm1−/− livers fail to develop intrahepatic bile ducts (40). Although it is well established that the Foxm1 protein is essential for hepatocyte proliferation and differentiation, the role of Foxm1 during embryonic lung development remains uncharacterized.

We previously reported on generating transgenic mice in which the Rosa26 promoter drives Foxm1 expression in all cell types and demonstrated that the Foxm1 is essential for cell proliferation required to repair lung injury in response to butylated hydroxytoluene treatment (44). Premature Foxm1 expression was associated with increased proliferation levels of pulmonary endothelial, epithelial, and smooth muscle cells as well as an earlier expression of the cell cycle promoting cyclin A2 and cyclin B1 (44). In this paper, we demonstrated that Foxm1−/− lungs displayed severe abnormalities in the development of pulmonary microvasculature that were associated with diminished pulmonary platelet endothelial cell adhesion molecule 1 (Pecam-1), TGF-β receptor type II, a disintegrin and metalloprotease domain 17 (ADAM-17) protein, VEGF receptors, Plk-1, Aurora B kinase, Lama4, and the Foxf1 transcription factor. Foxm1 is essential for proliferation of lung mesenchyme and vascular smooth muscle cells during embryonic lung development. Cotransfection experiments demonstrated that the Foxm1 gene is a direct transcriptional target for Foxm1 transcription factor. These results suggest that Foxm1 regulates pulmonary genes essential for mesenchyme proliferation, extracellular matrix remodeling, and vasculogenesis during lung development.

MATERIALS AND METHODS

Foxm1−/− Mice—We previously described the generation of the Foxm1−/− mice in which the targeted allele lacked the DNA binding and transcriptional activation domains (40). These Foxm1−/− mice were bred for four generations into the C57BL/6 mouse genetic background. Foxm1−/− mice were mated in the evening, and vaginal plugs were checked in the morning. The noon of day of appearance of a vaginal plug was designated as 0.5 dpc. Dams were killed by carbon dioxide asphyxiation. Quantification of expression levels was determined with Tiff files of ethidium bromide-stained gels by using the BioMax 1D program (Eastman Kodak Co.) as described previously (47).

Rnase protection assay was performed with [32P]UTP-labeled antisense RNA synthesized from Flk1 or cyclin T1 plasmid template with T7 RNA polymerase as previously described (48). RNA probe hybridization was performed with BCIP/NBT substrate as described previously (31). We counted the number of large pulmonary vessels (arteries and veins with diameter ≥25 μm) in >400 microscope field using H&E and Pecam-1-stained lung sections from 15.5- and 17.5-dpc Foxm1−/− and WT embryos, respectively, as described previously (40). Five random lung sections were counted in four different mouse lungs to calculate the mean number of large vessels ± S.D.

Foxa2 staining was performed using mouse monoclonal antibodies against Foxa2 (1:5, clone 4C7, University of Iowa Developmental Studies Hybridoma Bank, Iowa City, IA) followed by anti-mouse antibody conjugated with biotin, avidin-horseradish peroxidase complex, and diaminobenzidine substrate (all from Vector Laboratories). For immunofluorescent detection of asM, antibody-antigen complexes were detected using fluorescein isothiocyanate anti-goat secondary antibodies and then counterstained with DAPI as described previously (39). To measure apoptosis in WT and Foxm1−/− embryonic lungs, a TUNEL assay was performed using ApoTag Red in situ apoptosis detection kit from Intergen (Purchase, NY) according to the manufacturer’s recommendations (39).

Affymetrix cDNA Array Analysis, Reverse Transcriptase (RT)-PCR, and Northern Blot Protection Assay—Total mouse lung RNA was prepared from 14.5-dpc lungs of WT or Foxm1−/− embryos using RNA-STAT-60 (Tel-Test “B” Inc., Friendswood, TX). To avoid individual variations, we combined 10 μg of RNA from three distinct embryonic lungs as described previously (46). Synthetic embryonic mouse lung cDNAs with CyDye nucleotides (Cy3 or Cy5), hybridization of Affymetrix GeneChip® Mouse Genome 430A array, scanning, and analysis of cDNA microarrays were performed by the Functional Genomics Facility at the University of Chicago (Chicago, IL) as described previously (46). In Table I, we summarized our focus on characterization of 23 genes whose expression levels were altered >2.5-fold in Foxm1−/− lungs compared with WT lungs. To verify expression levels of these genes, we used total lung RNA isolated from two distinct 14.5-dpc embryos to perform RT-PCR analysis. A set of primers described previously (44) and sense and antisense primers were used for amplification: ADAM-17, 5′-TGATCTTGTGTCACAGAC-3′ and 5′-GTAATTTGTAGTGTCCT-C-3′; Lama2, 5′-TGTCGTTGGGATCTTGATGC-3′ and 5′-CAAGAGTGTCAACTCACTT-3′; polyclonal type XI α1 (Col12a1), 5′-GACTTCCCTACACTGATAACAGCC-3′ and 5′-GTCGACAGGAGAGGAGGATGAAG-3′; integrin β1, 5′-ATTGGGTCTGATGATGTCG-3′ and 5′-CCAGACGTCGGTGCTGAACTG-3′; midkine, 5′-GTCAGCAGACCAAGATCAAGACAG-3′ and 5′-GTCGACAGGAGAGGAGGATGAAG-3′; smooth muscle calponin (1:50, clone C-20, Santa Cruz Biotechnology); Plk-1 kinase (1:100, F-8, Santa Cruz Biotechnology); Aurora B kinase (1:100, clone 6, BD Transduction Laboratories); Pecam-1 (1:500, clone MEC 13.3, Pharmingen) and goat polyclonal antibodies against laminin α4 (1:100, V-20, Santa Cruz Biotechnology). Antibody-antigen complexes were detected using biotinylated secondary antibody and horseradish peroxidase-conjugated streptavidin and then counterstained with DAPI as described previously (39).
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RESULTS

Foxm1−/− Embryonic Lungs Displayed Hypertrophy of Pulmonary Arteries—Foxm1−/− mice were bred to generate Foxm1−/− embrionys, which were used to examine whether Foxm1 deficiency causes gross morphological defects in lung development. Embryonic 18.5-dpc Foxm1−/− and WT lungs were fixed, paraffin-embedded, sectioned, and stained with H&E. Although Foxm1−/− 18.5-dpc lungs exhibited normal size, lobular architecture, and sacculation compared with WT or Foxm1+/− littermates (Fig. 1, A–B, and data not shown), Foxm1−/− lungs exhibited hypertrophy of vascular smooth muscle cells of the pulmonary arteries (Fig. 1, C–D), as evidenced by the immunohistochemical staining with one of the following antibodies specific for smooth muscle cells: SM (Fig. 2, A–B), smooth muscle calponin (Fig. 2, C–D), and smooth muscle myosin heavy chain (Fig. 2, E–F). We also compared the size of arterial muscle layer in WT and Foxm1−/− 18.5-dpc lungs to demonstrate that Foxm1 deficiency is associated with a 5-fold increase in arterial muscularity (Fig. 1). Interestingly, many extrapulmonary arteries including the carotid artery and aorta in Foxm1−/− embionys exhibited severe hypertrophy of smooth muscle cells (Fig. 2, G–H, and data not shown).

These Foxm1−/− vascular smooth muscle cells displayed abnormally large DAPI-stained nuclei (Fig. 2, B and H) and diminished pulmonary expression of Plk-1 and Aurora B kinase (Fig. 1, E–H and J), a finding consistent with a polyploid genotype resulting from inhibition of mitosis with premature initiation of DNA replication (38, 40). Interestingly, smooth muscle cells of the pulmonary bronchi in Foxm1−/− embionys displayed normal morphology and SM staining (Fig. 2, I–J, and data not shown), suggesting that there is a compensation for Foxm1 function in airway smooth muscle cells.
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Foxm1−/− Lungs Exhibit Defects in Development of Pulmonary Microvasculature—Mouse 17.5-dpc lung mesenchyme undergoing extensive migration and differentiation to form peripheral pulmonary capillaries (1, 15). Therefore, we used lung sections from 17.5-dpc Foxm1−/− or WT mouse embryos to perform immunostaining with endothelial-specific Pecam-1 antibody to determine the role of Foxm1 in lung capillary development. Although a similar Pecam-1 staining was observed in pulmonary arteries of WT and Foxm1−/− embryos, we observed a significant reduction in the number of large pulmonary vessels compared with aged-matched WT embryonic lungs (Fig. 3, A–B and E–F). In contrast, significant decreases in Pecam-1 staining in the peripheral capillaries of Foxm1−/− lungs suggested that their lung microvasculature was disrupted (Fig. 3, A–B). Consistent with these results, pulmonary levels of the endothelial-specific VEGF receptor Flk1 mRNA were significantly reduced in Foxm1−/− lungs as determined by the RNase protection assay (Fig. 3G). Interestingly, cell death did not contribute to decreased vasculogenesis in Foxm1−/− lungs because they do not display elevated levels of apoptosis compared with WT littermates as determined by the TUNEL assay (Fig. 3, C–D). These results suggest that Foxm1 is essential for proper development of pulmonary vasculature from lung mesenchyme.

Foxm1−/− Lungs Exhibited Defects in Mesenchyme Proliferation—Foxm1−/− 15.5-dpc lungs exhibited normal tubular structure and the expression levels of epithelial-specific Foxa2 protein compared with WT lungs (Fig. 4, G–H). Although the pulmonary mesenchyme in Foxm1−/− embryos displayed normal staining for the mesenchyme-specific protein vimentin (Fig. 4, I–J), diminished numbers of mesenchymal cells were observed in distal regions of the Foxm1−/− lungs (Figs. 4, A–D, and 5A) without significant change in the number of epithelial cells (Fig. 5A). To determine the role of Foxm1 in mesenchyme proliferation, we measured DNA replication rates in 15.5-dpc Foxm1−/− and WT lungs by injecting BrdUrd into pregnant female mice 2 h prior to harvesting the embryos. Immunohistochemistry with BrdUrd antibody demonstrated that Foxm1−/− lungs exhibited diminished Flk1 expression. Total lung RNA was prepared from WT and Foxm1−/− 17.5-dpc mouse embryos and used to analyze for Flk1 and cyclophilin mRNA levels by RNase protection assay. Each individual sample was normalized to its corresponding cyclophilin level as described under “Materials and Methods.” Numbers represent averages of Flk1 mRNA levels with respect to WT embryonic lungs. Abbreviations: Br, bronchi; Ar, artery. Magnification: ×200 (A–D).

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Fig. 2. Smooth muscle specific staining in WT or Foxm1−/− arteries. A–B, embryonic lungs from WT and Foxm1−/− 18.5-dpc embryos were fixed, paraffin-embedded, sectioned, and then immunohistochemically stained with antibody specific for αSM followed by fluorescein isothiocyanate-conjugated secondary antibody and DAPI counterstaining (A–B and G–H). C–J, lung sections were also stained with monoclonal antibodies specific for smooth muscle calponin (C and D), smooth muscle myosin heavy chain (E and F) or αSM (U and J). Staining was visualized by secondary antibody conjugated with alkaline phosphatase and BCIP/NBT substrate followed by counterstaining with nuclear fast red. Pulmonary (B, D, and F) and carotid (H) arteries (Ar) of the Foxm1−/− embryos display severe hypertrophy of vascular smooth muscle cells. Arrowheads show the size of arterial muscle layers. The airway smooth muscle cells of pulmonary bronchi (Br) exhibited normal αSM staining (I–J). Magnifications: ×400 (A–H) and ×200 (I and J).

Fig. 3. Foxm1−/− lungs exhibit defects in development of pulmonary vasculature. A—B, Foxm1−/− embryos display reduced Pecam-1 staining. Embryonic lungs from WT (A) and Foxm1−/− (B) 17.5-dpc embryos were fixed, paraffin-embedded, sectioned, and then immunohistochemically stained with Pecam-1 antibody followed by biotinylated secondary antibody, avidin-alkaline phosphatase complex, and diaminobenzydine. Magnifications: ×200 (A–B). C–D, Foxm1−/− lungs display reduced Flk1 mRNA levels with respect to WT embryonic lungs. Numbers represent the mean plus or minus the standard deviation. A p value of 0.05 is shown with an asterisk. E–F, Foxm1−/− lungs displayed a reduced number of pulmonary blood vessels. H&E- and Pecam-1-stained lung sections from 15.5- and 17.5-dpc Foxm1−/− and WT embryos were used to count the number of pulmonary vessels (arteries and veins with diameter >25 μm) in ×400 microscope field. Five random lung sections were counted in four different mouse lungs to calculate the mean number of large vessels ± S.D. A p value of 0.05 is shown with an asterisk. G, Foxm1−/− lungs exhibit diminished Flk1 expression. Total lung RNA was prepared from WT and Foxm1−/− 17.5-dpc mouse embryos and used to analyze for Flk1 and cyclophilin mRNA levels by RNase protection assay. Each individual sample was normalized to its corresponding cyclophilin level as described under “Materials and Methods.” Numbers represent averages of Flk1 mRNA levels with respect to WT embryonic lungs. Abbreviations: Br, bronchi; Ar, artery. Magnification: ×200 (A–D).
these genes in Foxm1−/− embryonic lungs (Fig. 5C). These results demonstrate that Foxm1 deficiency reduces proliferation of mesenchymal cells in the developing lung.

Affymetrix Microarray Identified Diminished Expression of Genes Essential for DNA Replication and Mitosis in Foxm1−/− Lungs—To determine additional target genes regulated by Foxm1 in embryonic lungs, we performed Affymetrix gene array analysis. Affymetrix cDNA probes were synthesized from total RNA prepared from WT or Foxm1−/− 14.5-dpc lungs, which represent a developmental stage preceding the major vascular defects in Foxm1−/− lungs. This analysis allowed the identification of 23 genes in which expression levels were altered ≥2.5-fold in Foxm1−/− lungs compared with WT lungs (Table I). Altered Foxm1−/− expression levels of nine genes were verified by RT-PCR analysis. Affymetrix and RT-PCR analysis confirmed undetectable levels of Foxm1 mRNA in Foxm1−/− lungs (Table I and Fig. 5D). Consistent with decreased cellular proliferation in Foxm1−/− lungs (Fig. 4, E–F), we observed a reduced expression of S-phase promoting cyclin...

**FIG. 5.** Foxm1−/− lungs displayed altered gene expression during embryonic development. A, diminished number of mesenchymal cells in Foxm1−/− lungs. H&E-stained sections from WT and Foxm1−/− 15.5-dpc lungs were used to count the number of mesenchymal and epithelial cells in 10 random ×400 microscopy fields. Mean ± S.D. was calculated using three different WT or Foxm1−/− embryos. B, reduced DNA replication in Foxm1−/− lung mesenchyme. BrdUrd-stained WT and Foxm1−/− 15.5-dpc lungs were used to count the number of positive cells/1000 mesenchymal or epithelial cells. Mean ± S.D. was determined using ten ×400 microscopy fields in each of three WT or Foxm1−/− embryos. A p value ≤ 0.05 is shown with an asterisk. C–D, altered gene expression in Foxm1−/− lungs. 14.5-dpc WT and Foxm1−/− embryonic lungs were dissected and used for preparation of total RNA. RT-PCR was performed with primers specific to Plk-1, Aurora B, Foxm1, Pecam-1, Foxf1, ADAM-17, integrin β1, midkine, Col12a1, Lama2 and Lama4, and cyclophilin. Each individual sample was normalized to its corresponding cyclophilin level. Values are means ± S.D.
sistent with diminished levels of the TGF-β receptor type II in Foxm1−/− lungs (Table I), we observed a 60–80% reduction in pulmonary levels of procollagens type V α2 and type XII α1 and cadherin 11 genes (Table I and Fig. 5D), all of which are known downstream targets for TGF-β signaling pathway (66–68). Interestingly, we also observed a 60% reduction in Foxm1−/− expression levels of Smad3 (Table I), an intracellular mediator of the TGF-β signaling pathway (69). These results suggest that Foxm1−/− lung phenotype is associated with diminished TGF-β signaling and reduced expression of extracellular matrix proteins that are regulated by the TGF-β signaling pathway. Finally, increased expression of several genes was observed in Foxm1−/− lungs including the DNA repair enzyme AP endonuclease 2, intracellular signaling proteins adenylyl cyclase 3, and phospholipase Cβ, and the homeobox transcription factors HoxC9 and paired mesoderm homeobox 2b (Pmx2b) (Table I).

### Table I

| Gene name                          | GenBankTM number | Foxm1−/−/WT lung RNA |
|------------------------------------|------------------|----------------------|
| **Transcription factors and cell cycle regulators** |                  |                      |
| Forkhead Box m1 (Foxm1, HFH-11B, Trident) | NM_008021        | Not detected         |
| Cyclin B1                          | NM_007629        | 0.27                 |
| Cyclin A2                          | NM_009828        | 0.40                 |
| Cdc25c phosphatase                 | NM_009860        | 0.43                 |
| Forkhead Box f1 (Foxf1; HFH-8; Frec-1) | NM_010426        | 0.18                 |
| Paired mesoderm homeobox 2b (Pmx2b) | NM_008888        | 3.3                  |
| Homeobox C9 (HoxC9)                | NM_008272        | 3.0                  |
| **Receptors and intracellular signaling proteins** |                  |                      |
| TGF-β receptor type 2 (Tgfr2)      | S69114           | 0.06                 |
| Frizzled-6 (Fzd6; negative regulator of Wnt pathway) | BC026150 | 0.15                 |
| Pecam-1                            | NM_008816        | 0.3                  |
| VEGF receptor 1 (Flt1)             | NM_010228        | 0.38                 |
| Smad3                              | AR008192         | 0.43                 |
| AP endonuclease 2 (Ape2)           | AB072498         | 13.0                 |
| Adenyl cyclase 3 (Adcy3)           | AF458089         | 5.7                  |
| Phospholipase Cβ (Plcd)            | NM_019676        | 3.5                  |
| **Extracellular cell signaling and secreted proteins** |                  |                      |
| A disintegrin and metalloprotease domain 17 (Adam-17; TACE) | BE890579 | 0.09                 |
| Laminin α4 (Lama4)                 | U69176           | 0.15                 |
| Laminin α2 (Lama2)                 | U12147           | 0.29                 |
| Procollagen type XII α1 (Col12a1)  | AW412729         | 0.19                 |
| Procollagen type V α2 (Col5a2)     | AV293424         | 0.38                 |
| Bone morphogenetic protein 5 (Bmp5) | NM_007555        | 0.31                 |
| Integrin β1 (fibronectin receptor β) | BM120341       | 0.33                 |
| Cadherin 11                         | NM_009866        | 0.41                 |

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Embryonic lung expression profile of Foxm1−/− versus WT mouse embryos at 14.5 dpc eDNA probes were synthesized from 10 µg of RNA from either wild-type (WT) or Foxm1−/− 14.5-dpc lungs and then hybridized to Affymetrix mouse Gene Chip. Values represent a fold change in RNA expression of Foxm1−/− lungs compared with WT lungs. Genes in normal type exhibited diminished expression in Foxm1−/− lungs, and genes in italic typeface displayed increased expression in Foxm1−/− lungs.

**Foxm1 Directly Regulates Mouse Lama4 Promoter—Because Foxm1−/− lungs exhibited significantly reduced Lama4 mRNA and protein levels (Table I and Figs. 5D and 6), we investigated whether Foxm1 transcriptionally regulates the Lama4 promoter region. Two potential Foxm1 DNA binding sites were identified in −1.2-kb promoter region of the mouse Lama4 gene (−897/−911 and −1174/−1145 bp) (Fig. 7A), the latter of which contains two overlapping Foxm1 binding motifs (Fig. 7C). Cotransfection experiments were performed in mesenchymal U2OS cells using CMV-Foxm1 expression vector and LUC reporter driven by the −1200-bp Lama4 promoter region. Cotransfection of the Foxm1 expression vector increased the expression of the −1200-bp Lama4-LUC reporter plasmid (Fig. 7B), suggesting that the Foxm1 protein is a transcriptional activator of Lama4 gene. Furthermore, deletions of −1140/−1200, −920/−1200, or −880/−1200-bp Lama4 regions were equally sufficient to reduce the ability of Foxm1 to activate transcription of the Lama4 promoter in cotransfection experiments (Fig. 7B), indicating that the −1140/−1200-bp Lama4 region contains a functional Foxm1 binding site. To determine whether Foxm1 protein directly binds to DNA in the Lama4 promoter region, we performed EMSA with nuclear protein extract from Foxm1-expressing U2OS cells and two distinct oligonucleotides (−1180/−1139 and −891/−917 bp) that contain potential Foxm1 binding sites in the promoter region of the mouse Lama4 gene. Although the −891/−917-bp oligonucleotide did not bind to endogenous Foxm1 protein (data not shown), two overlapping Foxm1 binding sites in the −1180/−1139 bp region formed specific Foxm1 protein-DNA complexes (Fig. 7C), as demonstrated by the disruption of the Foxm1 protein-DNA complex with either Foxm1 antibody or cold competitor oligonucleotide (Fig. 7C). The formation of Foxm1 protein-DNA complexes was not inhibited by the addition of either antibody to the platelet-derived growth factor receptor α chain or nonspecific oligonucleotide, which contains a binding site for SP1 transcription factor (Fig. 7C). These results indicate that the endogenous Foxm1 protein specifically binds to DNA in −1180/−1139-bp Lama4 promoter region, which is required for transcriptional induction of the Lama4 promoter by Foxm1.
**FIG. 6. Foxm1 deficiency is associated with reduced expression of Lama4 in developing lungs.** Embryonic lungs were dissected from WT (A, C, and E) and Foxm1−/− (B, D, and F) mouse embryos at 15.5 (A–D) or 18.5 dpc (E and F). Lung paraffin sections were immunohistochemically stained with Lama4 antibodies and counterstained with nuclear fast red. Lama4 protein is expressed in subsets of both mesenchymal and epithelial cells of the distal lung regions of WT 15.5-dpc embryos (A and C) as well as in endothelial cells of blood vessels in WT 18.5-dpc lungs (E, shown with arrowheads). Lama4 expression is decreased in mesenchyme of Foxm1−/− 15.5-dpc lungs (D) and endothelial cells of pulmonary arteries in Foxm1−/− 18.5-dpc lungs (F). The percent of Lama4-positive mesenchymal cells in 15.5-dpc lungs was determined using five random fields of view in each of the three WT or Foxm1−/− embryos (G). Values are means ± S.D. A p value ≤ 0.05 is shown with an asterisk. Abbreviations: Br, bronchi; Ar, artery. Magnifications: ×200 (A and B); ×630 (C and D); and ×400 (E and F).

**DISCUSSION**

Foxm1−/− embryos die in utero between 13.5 and 18.5 dpc because of severe defects in liver morphogenesis due to diminished hepatoblast DNA replication and a failure to enter mitosis causing polyploid phenotype (40). In this study, we demonstrate that embryonic Foxm1−/− lungs exhibit a severe hypertrophy of arteriolar smooth muscle cells and a paucity of distal mesenchymal cells resulting from diminished proliferation of lung mesenchyme. The hypertrophy of vascular smooth muscle cells is consistent with decreased expression of the Polo-like kinase 1 and Aurora B kinase (Ref. 40 and this report), which contribute to a failure to complete mitosis and the reinitiation of the S-phase, resulting in a polyploid phenotype (42, 43). An alternative explanation for muscle hypertrophy may result from severe heart defects in Foxm1−/− embryos, which cause a diminished circulating output and altered signaling during development of blood vessels. The finding that many extrapulmonary arteries in Foxm1−/− embryos exhibited the hypertrophy of smooth muscle cells provides further support for this concept.

Foxm1−/− lungs displayed reduced levels of Cdc25C phosphatase and cyclin B1, the latter of which is a direct transcriptional target for Foxm1 (57). Progression into mitosis requires the activation of Cdk1 through assembly with cyclin B regulatory subunit and the removal of Cdk1 inhibitory phosphates at Thr-14 and Tyr-15 by the Cdc25B and Cdc25C phosphatases (70–72). Therefore, diminished pulmonary levels of cyclin B1 and Cdc25C can decrease Cdk1 activation and M-phase progression, causing the hypertrophy of vascular smooth muscle cells in Foxm1−/− lungs.

Proliferation defects in Foxm1−/− mesenchyme were also associated with reduced expression of S-phase promoting cyclin A2, which activates Cdk2 protein required for DNA replication (73, 74). Cdk2 complexes with either cyclin E or cyclin A cooperate with cyclin D-Cdk4/6 to phosphorylate the retinoblastoma protein, which releases the bound E2F transcription factor and allows it to stimulate expression of genes required for DNA replication (75, 76). Therefore, defects in DNA replication in Foxm1−/− lungs may be a direct consequence of the reduced pulmonary levels of cyclin A2. This result is consistent with previously published data (44) demonstrating that transgenic overexpression of Foxm1 in Rosa26-Foxm1 transgenic mice is associated with increased cyclin A2 levels and premature proliferation of different lung cell types following butylated hydroxytoluene lung injury. In the lung mesenchyme of Foxm1−/− embryos, we also found significant reduction in the expression of the mitotic regulators Aurora B kinase and Polo-like kinase 1, a finding consistent with diminished progression into mitosis as reported in our previous developmental studies of embryonic Foxm1−/− livers (40). Our results suggest that Foxm1 controls the expression of pulmonary cell cycle regulatory pathways required for proliferation of pulmonary mesenchymal cells and vascular smooth muscle cells during lung morphogenesis (Fig. 8).

Foxm1−/− lungs displayed normal branching and acculation, and the pulmonary levels of epithelial-specific Foxa2 protein were normal in Foxm1−/− embryos. Furthermore, detectable BrdUrd levels were observed in Foxm1−/− pulmonary epithelium, suggesting that the function of Foxm1 protein in proliferation may be compensated, thus allowing normal epithelial proliferation during branching lung morphogenesis. Interestingly, Foxm1−/− lungs displayed reduced expression of the TGF-β receptor type II and Smad3 genes, suggesting that Foxm1−/− lung phenotype is associated with reduced TGF-β signaling. Consistent with this hypothesis, we observed diminished pulmonary levels of procollagens type V α2 and type XII α1 and cadherin 11 genes, all of which are known downstream targets for TGF-β signaling pathway (66–68). Published studies demonstrated that the TGF-β inhibits pulmonary branching morphogenesis in culture (1), and abrogation of the TGF-β receptor II signaling, either with antisense oligonucleotides or with blocking antibodies, stimulates pulmonary epithelial proliferation and lung morphogenesis (77, 78). Furthermore, abrogation of Smad2 and Smad3 expression results in a strong gain of function phenotype for epithelial proliferation and lung branching in embryonic lung culture experiments (79). Because lung branching morphogenesis was not affected in Foxm1−/− embryos, diminished TGF-β signaling may provide a compensatory mechanism to stimulate epithelial proliferation in Foxm1−/− lungs. Whether Foxm1 directly or indirectly regulates the expression of the TGF-β receptor II and Smad3 in pulmonary epithelial cells remains to be determined.

In the canalicular stage (16.5–17.5 dpc) of lung morphogenesis, the lung mesenchyme undergoes extensive differentiation toward endothelial cell lineage. In addition to proliferation defects in 17.5-dpc Foxm1−/− lung mesenchyme, we found severe defects in the formation of pulmonary microvasculature. Foxm1−/− lungs exhibited reduced Pecam-1 staining, diminished number of pulmonary blood vessels, and decreased mRNA levels of VEGF receptor type II (Flk1), which is essen-
For antibody reactions, we preincubated nuclear extracts with 1 C, 500-fold molar excess) but not nonspecific Sp1 oligonucleotide to interfere with the formation of Foxm1 protein-DNA complexes (shown with arrows). Two overlapping Foxm1 binding sites. Specificity of the Foxm1 protein-DNA complexes was demonstrated by the ability of the cold competitor DNA to displace the radiolabeled probe. U2OS cells, which express endogenous Foxm1 protein. EMSA was performed with the endogenous Foxm1 protein binds to its potential binding sites in lungs. Cells were harvested at 36 h after transfection and processed for dual luciferase assays to determine luciferase activity. Transcriptional induction is expressed as a fold increase relative to CMV-empty vector (S.D.). A

![Diagram of Foxm1 regulation](http://www.jbc.org/)

**Fig. 8.** Schematic diagram showing the regulation of lung mesenchyme development by Foxm1. Immature lung mesenchyme undergoes proliferation and differentiation toward endothelial, vascular smooth muscle, and airway smooth muscle cell lineages. Foxm1 is essential for proliferation of endothelial and vascular smooth muscle cells but not for epithelial or airway smooth muscle cells. Foxm1 positively regulates differentiation of pulmonary mesenchyme during the formation of lung microvasculature.

capillary development, pulmonary edema, and perinatal lethality were found in the ~55% newborn Foxf1−/− mice, which exhibited an 80% reduction in wild-type pulmonary Foxf1 levels. These defects in newborn Foxf1−/− lungs were associated with diminished expression of Flk1, Pecam-1, and other genes required for development of pulmonary vasculature (31), which is partially similar to gene expression defects in Foxm1−/− lungs (Table I and Figs. 3 and 5D). Interestingly, the development of alveolar capillaries and expression of these lung genes was unchanged in 40% newborn Foxf1−/− mice that had near WT pulmonary levels of Foxf1 mRNA, indicating that the WT Foxf1 levels are critical for lung vasculogenesis (31). These studies suggest that reduction of Foxf1 levels in newborn Foxf1−/− embryos may contribute to vascular defects during lung morphogenesis.

**Foxm1−/−** lungs displayed severe reduction in pulmonary levels of Lama2 and Lama4 genes, both of which are essential for mesenchyme differentiation during lung development (23, 24). Lama4 levels were severely reduced in both lung mesenchyme and mesenchyme-derived endothelial cells of Foxm1−/− lungs, and this was associated with severe vascular defects. Because Lama4−/− mice displayed impaired microvessel maturation and vascular leakage (23), diminished Lama4 expression may contribute to vascular abnormalities and edema seen in Foxm1−/− embryos (Ref. 40 and this report). We also demonstrated that Foxm1 protein induces transcriptional activity of the mouse Lama4 promoter in cotransfection experiments, suggesting that the Lama4 gene is a direct transcriptional
target of Foxm1 during lung development. Further support for this concept provides the fact that endogenous Foxm1 protein specifically binds to the −1180/~1139-bp region of the mouse Lama4 promoter and that retention of these sequences are essential for transcriptional activation by Foxm1. This is the first evidence that proliferation-specific Foxm1 transcription factor may directly regulate the expression of extracellular matrix proteins.

In summary, Foxm1−/− embryonic lungs exhibited diminished mesenchyme proliferation, hypertrophy of smooth muscle cells of pulmonary arteries, and severe abnormalities in development of pulmonary microvasculature. The Foxm1−/− lung phenotype was associated with diminished pulmonary levels of Pecam-1, ADAM-17, Foxf1, Plk-1, Aurora B, and Lama4 proteins, and reduced TGF-β signaling. Cotransfection experiments demonstrated that Lama4 gene is a direct transcriptional target for Foxm1 protein. These results suggest that Foxm1 is essential for lung morphogenesis by regulating the expression of genes required for extracellular matrix remodeling, mesenchyme proliferation, and vasculogenesis.

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