Endothelial Cell–Specific Deletion of Transcription Factor FoxM1 Increases Urethane-Induced Lung Carcinogenesis

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Abstract

Vascular endothelial cells provide essential support to the tumor microenvironment, but little is known about the transcriptional control of endothelial functions during tumorigenesis. Here we define a critical role for the Forkhead transcription factor FoxM1 in modulating the development of tumor-associated endothelial cells. Pulmonary tumorigenesis induced by urethane administration was compared in mice genetically deleted for FoxM1 in endothelial cells (enFoxm1−/− mice). Notably, lung tumor number and size were increased in enFoxm1−/− mice. Increased tumorigenesis was associated with increased proliferation of tumor cells and increased expression of c-Myc and cyclin D1. Furthermore, perivascular infiltration by inflammatory cells was elevated and inflammatory cells in BAL fluid were increased. Expression of Flk-1 (vascular endothelial growth factor receptor 2) and FoxF1, known regulators of pulmonary inflammation, was decreased in enFoxm1−/− mice. siRNA-mediated knockdown of FoxM1 in endothelial cells reduced endothelial-specific expression of Flk-1 and FoxF1 expression, which was driven by direct transcriptional induction by FoxM1 as target genes. Endothelial specific deletion of FoxM1 in vivo or in vitro also decreased expression of Sfrp1 (secreted frizzled-related protein 1), a known inhibitor of canonical Wnt signaling, in a manner that was associated with increased Wnt signaling. Taken together, our results suggest that endothelial-specific expression of FoxM1 limits lung inflammation and canonical Wnt signaling in lung epithelial cells, thereby restricting lung tumorigenesis. Cancer Res 71(1): 40–50. ©2011 AACR.

Introduction

Foxm1 (previously known as HFH-11B, Trident, Win, or MPP2) is a member of the Forkhead family of transcription factors that share homology in the winged-helix/Forkhead DNA-binding domain (1, 2). Foxm1 expression is induced during cellular proliferation in a variety of cell types, including epithelial and endothelial cells (3, 4). Foxm1 promotes G1/S and G2/M phases of cell cycle by directly activating transcription of regulatory genes such as Cdc25B, cyclin B1, Aurora B kinase, and Polo-like kinase 1 (5, 6). Foxm1 decreases nuclear levels of the CDK inhibitor proteins p21CIP1 and p27KIP1 by regulating their degradation through the ubiquitin ligase complex (7). Foxm1 is overexpressed in a variety of highly proliferative human cancers, including lung adenocarcinomas and squamous cell carcinomas, as well as many other solid tumors (reviewed in refs. 7, 8 and 9). Overexpression of Foxm1 significantly increased the number and size of tumors induced by the 3-methylcholanthrene (MCA)/butylated hydroxytoluene (BHT) lung tumor induction/promotion protocol (10). Deletion of Foxm1 from type II lung epithelial cells using a Cre (Cre recombinase)/LoxP system significantly decreased the number and size of lung adenosas induced by either MCA/BHT or urethane (11). These data indicate that Foxm1 expression in tumor cells is essential for progression of chemically induced lung cancer in vivo. However, the role of Foxm1 in tumor-associated endothelial cells remains unknown.

There is increased evidence that multiple cell types and processes contribute to the microenvironment associated with carcinogenesis. Supporting cells in the tumor microenvironment include inflammatory cells, endothelial cells, and stromal fibroblasts. Nontumor cells contribute both positive and negative growth signals to the tumor. These cells produce a variety of growth factors, chemokines, and matrix-degrading enzymes that enhance the proliferation and invasion of the tumor. Little is known about the molecular mechanisms controlling the cross-talk between tumor cells and other cells of tumor microenvironment.

Inflammation and angiogenesis are 2 major processes in tumor microenvironment that drives tumorigenesis (12). Activated inflammatory cells stimulate growth and progression of tumor cells. Inflammation, recurrent cell injury, and associated compensatory cell proliferation promote the growth of malignant cells (13). Inflammatory cells also induce angiogenesis and extracellular matrix remodeling, further destabilizing epithelial cells during malignant transformation. Tumor-associated angiogenesis is regulated by a number of signaling...
increased peripheral pulmonary capillaries (17). Foxm1 deletion from endothelial cells delayed lung repair by lipopolysaccharide (LPS; ref. 18). Although Foxm1 increases the expression of VEGF-A in glioma cells (16), indicating the important role of Foxm1 in VEGF signaling and angiogenesis. Foxm1 is necessary for the formation of the pulmonary vasculature during embryogenesis, and Foxm1−/− mice exhibited severe defects in the formation of peripheral pulmonary capillaries (17). Foxm1 deletion from endothelial cells delayed lung repair after injury by lipopolysaccharide (LPS; ref. 18). Although these studies indicate that Foxm1 transcription factor is critical for the development and repair of lung vasculature, the role of Foxm1 in tumor-associated endothelial cells remains unknown.

In the present study, we utilized a genetic mouse model in which the Foxm1 gene was deleted from endothelial cells (Tie2-Cre/Foxm1fl/fl or enFoxm1−/− mice). Surprisingly, numbers and sizes of lung tumors were increased in enFoxm1−/− mice following urethane induction. Increased tumorigenesis in enFoxm1−/− lungs was associated with increased lung inflammation and increased canonical Wnt signaling.

Materials and Methods

Transgenic mice and lung tumorigenesis

The Foxm1fl/fl C57BL/6 mice (19) were bred with Tie2-Cre C57BL/6 mice to generate Tie2-Cre/fl−/Foxm1fl/fl (enFoxm1−/−) double transgenic mice. Foxm1fl/fl and Tie2-Cre/fl− single transgenic mice were used as controls. Tumorigenesis was induced using either urethane protocol or MCA/BHT protocol as previously described (11). For urethane tumorigenesis, induced using either urethane protocol or MCA/BHT protocol transgenic mice were used as controls. Tumorigenesis was determined by immunostaining and qRT-PCR within the last 6 months. Human endothelial cell line HMVEC-L was bought from American Type Culture Collection 1 month before the experiments. MFLM-91U or HMVEC-L cells were transfected with 100 nmol/L of either Foxm1-specific siRNA (siFoxm1) or mutant control siFoxm1 (6), using Lipofectamine 2000 reagent (Invitrogen) in serum-free media for 72 hours as described (6).

Luciferase (LUC) reporter driven by a −1.5-kb Flk-1 promoter (Flk-1–LUC) was generated as described in Supplementary Materials and Methods. A −2.7-kb Foxf1-LUC vector was described previously (24). MFLM-91U cells were transfected with cytomegalovirus (CMV)-Foxm1b or CMV-empty plasmids, as well as with LUC reporters driven by either Flk-1 or Foxf1 promoters. CMV-Renilla was used as an internal control to normalize transfection efficiency. Dual luciferase assay (Promega) was performed 24 hours after transfection as described previously (25). Chromatin immunoprecipitation (ChIP) assay was conducted as described in Supplementary Materials and Methods and by Wang and colleagues (6).

Statistical analysis

We used Microsoft Excel Program to calculate standard deviation and statistically significant differences between samples using the Student’s t Test. P < 0.05 values were considered statistically significant.

Results

Urethane induced tumor formation after deletion of Foxm1 from endothelial cells

To determine the role of Foxm1 in endothelial cells during lung tumorigenesis, we utilized a double transgenic mouse model in which the Foxm1 gene is selectively deleted from endothelial cells (Tie2-Cre/fl−/Foxm1fl/fl or enFoxm1−/− mice).

Immunohistochemistry and immunofluorescence

Paraffin (5 μm) sections were stained with hematoxylin and eosin (H&E) for morphologic examination or immunostained with antibodies (listed in Supplementary Materials and Methods) as described previously (10). Sections from β-gal–stained lungs were immunostained with either Ki-67 or prosurfactant-associated protein C (pro-SPC) and counterstained with nuclear fast red (Vector Labs). Immunofluorescent staining was performed using antibodies listed in Supplementary Materials and Methods.

Quantitative real-time RT-PCR

Total lung RNA was analyzed by quantitative real-time RT-PCR (qRT-PCR) using StepOnePlus Real-Time PCR system (Applied Biosystems). Samples were amplified with TaqMan Gene Expression Master Mix (Applied Biosystems) combined with inventoried TaqMan gene expression assays (Supplementary Table S1).

siRNA transfection, luciferase assay, and chromatin immunoprecipitation assay

Mouse endothelial cell line MFLM-91U was provided by Akeson and colleagues (23). MFLM-91U cells express endothelial-specific VEGF-R1 and VEGF-R2, Tie-1, and Tie-2 as demonstrated by immunostaining and qRT-PCR within the last 6 months. Human endothelial cell line HMVEC-L was bought from American Type Culture Collection 1 month before the experiments. MFLM-91U or HMVEC-L cells were transfected with 100 nmol/L of either Foxm1-specific siRNA (siFoxm1) or mutant control siFoxm1 (6), using Lipofectamine 2000 reagent (Invitrogen) in serum-free media for 72 hours as described (6).
Previous studies demonstrated that in this mouse line, Foxm1 was efficiently deleted in endothelial cells without affecting epithelial and inflammatory cells of the lung (18). Tie2 promoter expressed Cre that excised exons 4 to 7, encoding the DNA-binding domain and transcriptional activation domain of the Foxm1 protein. Consistent with published studies (18), Foxm1 mRNA was significantly decreased in total lung RNA from enFoxm1mice (Fig. 1A). Foxm1 mRNA levels in control Foxm1fl/fl and Tie2-Cre+ lungs were similar (data not shown). Lung tumorigenesis was induced using urethane injections. Thirty weeks after initial urethane injection, increased numbers of lung tumors were seen in enFoxm1 mice (Fig. 1B). Tumor diameters in enFoxm1 mice were significantly increased (Fig. 1C). The tumors in enFoxm1 mice were lung adenomas (Fig. 1D). To identify cell origin of the lung tumor cells, immunohistochemical staining was conducted using antibodies against either pro-SPC, a type II alveolar epithelial marker, or Clara cell-specific protein (CCSP). All tumors in control and enFoxm1 mice were positive for thyroid transcription factor 1 (TTF1) and SPC and negative for CCSP. Both

Figure 1. Deletion of Foxm1 from lung endothelial cells increases tumor formation. A, qRT-PCR analysis of Foxm1 mRNA expression in control Foxm1fl/fl and enFoxm1 mice. RNA was isolated from the total lung. β-Actin mRNA was used for normalization. B, increase in the total number of urethane-induced tumors in enFoxm1 mice. Foxm1fl/fl and control Foxm1fl/fl mice were administered 6 weekly urethane injections and lungs were harvested 30 weeks after initial urethane injection. C, increased diameter of tumors in enFoxm1 mice. Mean number of tumors (±SE) per lung and mean tumor diameter (±SE) were calculated from n = 12 mouse lungs per group. D, H&E staining shows an increase in the size of lung tumors (Tu) in enFoxm1 mice. Tumors in both enFoxm1 and control mice are positive for thyroid transcription factor 1 (TTF1) and SPC and negative for CCSP. Magnifications: D, 100×; insets, 400×. **, P < 0.01.
enFoxm1−/− and control tumors expressed TTF1 (Fig. 1D), a lung epithelial specific transcription factor that is widely expressed in non–small cell lung cancers (NSCLC) in mice and humans (26, 27). Furthermore, increased lung tumorigenesis was observed in enFoxm1−/− mice after MCA/BHT treatment (Supplementary Fig. S1), a well-known protocol for lung tumor initiation/promotion (28), indicating that the effect of Foxm1 deletion in enFoxm1−/− mice is not limited to urethane tumorigenesis. These data showed that deletion of Foxm1 from endothelial cells increased lung tumor burden after tumor induction.

**Proliferation of tumor cells in enFoxm1−/− lungs is increased**

Immunohistochemical staining with proliferation-specific Ki-67 antibodies showed no significant differences in the cellular proliferation between untreated control and enFoxm1−/− lungs (Fig. 2A and B). However, 30 weeks after urethane treatment, numbers of Ki-67–positive cells in enFoxm1−/− tumors were significantly increased (Fig. 2B), which is consistent with increased tumor sizes in these mice (Fig. 1C). Moreover, qRT-PCR analysis revealed that the expression of c-Myc and cyclin D1, known markers of proliferating cells, was significantly increased in lungs from enFoxm1−/− mice compared with control mice (Fig. 2C and D). Thus, increased tumor formation in enFoxm1−/− lungs resulted from increased proliferation.

**Urethane induces chronic lung inflammation in enFoxm1−/− lungs**

Conditional deletion of Foxm1 from endothelial cells did not influence the lung morphology and lung inflammation was not detected in untreated enFoxm1−/− mice (Fig. 3A), consistent with previously published studies (18). However, 30 weeks after urethane treatment, severe perivascular inflammation was observed in enFoxm1−/− mouse lungs but not in urethane treatment, numbers of Ki-67–positive cells in enFoxm1−/− tumors were significantly increased (Fig. 2B), which is consistent with increased tumor sizes in these mice (Fig. 1C). Moreover, qRT-PCR analysis revealed that the expression of c-Myc and cyclin D1, known markers of proliferating cells, was significantly increased in lungs from enFoxm1−/− mice compared with control mice (Fig. 2C and D). Thus, increased tumor formation in enFoxm1−/− lungs resulted from increased proliferation.

![Image](https://example.com/image.png)

**Figure 2.** Increased proliferation of tumor cells in enFoxm1−/− lungs. enFoxm1−/− and control Foxm1fl/fl lungs were harvested 30 weeks after initial urethane injection and used for immunohistochemistry with Ki-67 antibodies or to isolate total lung mRNA for qRT-PCR. A, increased proliferation of tumor (Tu) cells in enFoxm1−/− lungs. B, enFoxm1−/− lungs have increased number of Ki-67–positive tumor cells compared with control mice. Ki-67–positive cells were counted in 10 random microscope fields of control or enFoxm1−/− tumors. Increased mRNA levels of c-Myc (C) and cyclin D1 (D) in enFoxm1−/− lungs after urethane treatment were shown by qRT-PCR. β-Actin mRNA was used for normalization. Magnifications: A, 100×. *, P < 0.05; **, P < 0.01.

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Increased perivascular leukocyte infiltration is associated with increased tumor sizes in enFoxm1−/− mice. A, H&E staining of lungs from enFoxm1−/− and control Foxm1fl/fl mice 30 weeks after urethane treatment. Lung inflammation was not observed in untreated enFoxm1−/− or control mice. B and C, increased infiltration of inflammatory cells around bigger tumors (Tu) and vessels in enFoxm1−/− lungs 30 weeks after urethane treatment. Perivascular infiltration of inflammatory cells is shown with arrows. Higher magnification of the representative vessels from control and enFoxm1−/− lungs (bottom). V, blood vessel. Magnifications: A–C, 100×; C (bottom), 1,000×. D, percentage of vessels exhibiting leukocyte infiltration was determined in 10 random microscope fields and presented as mean ± SD.
the control lungs (Fig. 3B–D). Large tumors found in enFoxm1−/− lungs were generally located near the blood vessels at sites of leukocyte infiltration (Fig. 3B). These results are consistent with previously published studies showing that chronic inflammation promotes tumor growth (29).

Chronic lung inflammation in human patients is associated with the increased risk of developing lung cancer (30, 31). Thus, we assessed the presence of inflammatory cells at the early stage of tumor induction with urethane. Mice were treated with 3 weekly injections of urethane and lungs were harvested 3 days after the last urethane injection. Increased focal inflammation was seen in the perivascular regions in enFoxm1−/− lungs compared with control lungs (Supplementary Fig. S2), suggesting the increased inflammatory response to urethane treatment. These results were further supported by an increase in total number of inflammatory cells in BAL fluid from urethane-treated enFoxm1−/− mice compared with control mice (Supplementary Fig. S2). No differences in the total number of BAL cells were observed in enFoxm1−/− and control mice without urethane treatment (Supplementary Fig. S2). Taken together, these data showed that urethane increased pulmonary inflammation in enFoxm1−/− mice, which was not resolved even by 30 weeks after urethane treatment.

**Decreased expression of Flk-1 and Foxf1 in enFoxm1−/− lungs**

Because Foxm1 induces proliferation of endothelial cell during embryonic development and LPS lung injury (17, 18), we examined whether endothelial specific deletion of Foxm1 influences tumor-mediated angiogenesis. PECAM-1 staining was not different in enFoxm1−/− and control tumors (Supplementary Fig. S3A). Likewise, qRT-PCR of the total lung RNA showed no differences in PECAM-1 mRNA in enFoxm1−/− and control lungs (Supplementary Fig. S3B).

To identify potential Foxm1 targets, expression of several genes critical for lung inflammation and tumor formation was examined. While mRNA levels of VEGF-A, IL-6, tumor necrosis factor α, lymphoid enhancer factor, and β-catenin were not influenced by Foxm1 deletion (Supplementary Fig. S3D), those of Flk-1 (VEGF-R2) mRNA and protein were decreased in enFoxm1−/− tumors (Fig. 4A and B, top). The specific decrease of Flk-1 protein in Foxm1-deficient endothelial cells was shown by colocalization experiments using antibodies against endothelial-specific protein vWF or Sox-17 (Fig. 4A, bottom), the latter of which is a transcription factor exclusively expressed in endothelial cells of adult lung (32). Interestingly, Foxm1 protein was not detected in endothelial cells of enFoxm1−/− tumors (Supplementary Fig. S4A and B), confirming an efficient deletion of Foxm1 from endothelium. Furthermore, Foxf1 mRNA, encoding a transcription factor selectively expressed in endothelial cells (33, 34), was significantly decreased in enFoxm1−/− lungs (Fig. 4B). Foxf1 protein was not detected in endothelial cells of enFoxm1−/− tumors as shown by colocalization with CD34 or SOX17 antibodies (Fig. 4A, bottom). Because deficiency of either Flk-1 or Foxf1 in mice was associated with severe lung injury and inflammation (35–38), the reduced expression of these genes may contribute to the increased inflammation seen in enFoxm1−/− lungs following urethane treatment. Interestingly, the decreased Flk-1 and Foxf1 mRNAs were also observed in enFoxm1−/− lungs prior to urethane treatment (Fig. 4B), suggesting that enFoxm1−/− mice are predisposed to pulmonary inflammation induced by urethane.

**Foxm1 directly induces transcriptional activity of Flk-1 and Foxf1 promoters**

Decreased expression of Flk-1 and Foxf1 in enFoxm1−/− lungs suggests that these genes are targets of Foxm1. To determine whether Foxm1 regulates expression of these genes *in vitro*, HMVEC-L cells were transfected with short interfering RNA (siRNA) specific to the human Foxm1 mRNA (siFoxm1) or with mutant siFoxm1, containing 5 mutations in recognition sequence (9). Seventy-two hours after siRNA transfection, total RNA was prepared from the HMVEC-L cells and analyzed for Foxm1 expression by qRT-PCR. siFoxm1 transfection efficiently reduced Foxm1 mRNA (Fig. 4C). Consistent with our *in vivo* studies (Fig. 4A), Foxm1 depletion in cultured endothelial cells significantly decreased Flk-1 and Foxf1 mRNAs (Fig. 4C).

Because Foxm1 deficiency was associated with decreased Flk-1 and Foxf1 expression in *in vivo* and *in vitro* (Fig. 4A–C), we investigated whether Foxm1 transcriptionally activated promoter regions of these genes. The potential Foxm1 DNA-binding sites were identified in the −1.5-kb promoter region of the mouse Flk-1 gene and −2.7-kb promoter region of the mouse Foxf1 gene (Fig. 4D). ChIP assays were used to determine whether Foxm1 protein directly binds to the promoter regions of these 2 genes in mouse MFLM-91U endothelial cells. Foxm1 protein specifically bound to both the Flk-1 and Foxf1 promoter regions as demonstrated by the ability of siFoxm1 to reduce binding of Foxm1 protein to the promoter DNA (Fig. 4D, left). To determine whether the Foxm1-binding sites were transcriptionally active, cotransfection experiments were conducted using CMV-Foxm1b expression vector (17) and LUC reporter constructs driven by either Flk-1 or Foxf1 promoter regions. Cotransfection of the CMV-Foxm1b expression vector significantly increased activity of both reporters when compared with CMV-empty vector (Fig. 4D). These results show that Foxm1 directly binds to and transcriptionally activates the mouse Flk-1 and Foxf1 promoter regions, indicating that these endothelial genes are direct Foxm1 targets.

**Canonical Wnt signaling is increased in enFoxm1−/− lungs after urethane treatment**

Because deletion of Foxm1 from endothelial cells caused increased proliferation of tumor cells following urethane injury (Fig. 2B), we focused on molecular mechanisms involved in the cross-talk between epithelial and endothelial cells in enFoxm1−/− lungs. *Secreted frizzled-related protein 1* (*Sfrp1*) mRNA, a Wnt signaling inhibitor, was significantly decreased in enFoxm1−/− lungs at 3 and 30 weeks after urethane treatment (Fig. 5A). Diminished expression of *Sfrp1* was also found in lungs of untreated enFoxm1−/− mice (Fig. 5A), coinciding with decreased Foxm1 mRNA levels.
Figure 4. Decreased expression of Flk-1 and Foxf1 in enFoxm1−/− lungs and cultured endothelial cells. A, enFoxm1−/− mice had decreased Flk-1 protein levels in tumors (Tu) compared with control Foxm1fl/fl tumors (top). The decrease in Flk-1 and Foxf1 protein levels in enFoxm1−/− tumors was specific to endothelial cells (bottom). Immunostaining was conducted using antibodies against Flk-1 (red) and either endothelial specific vWF or SOX17 (green). Endothelial specific CD34 or SOX17 (green) antibodies were used to colocalize with Foxf1 (red). The nuclei were counterstained with DAPI (blue). Arrowheads indicate endothelial cells. B, enFoxm1−/− mice showed decreased Flk-1 and Foxf1 mRNAs either prior to or after urethane treatment. qRT-PCR was done using total lung RNA from either untreated mice or mice harvested 30 weeks after urethane treatment. Mouse β-actin mRNA was used for normalization. C, Foxm1 depletion in HMVEC-L cells reduced Flk-1 and Foxf1 mRNA expression. HMVEC-L human endothelial cells were mock transfected (MOCK) or transfected with siRNA duplex specific for Foxm1 mRNA (siFoxm1). Human β-actin mRNA was used for normalization. D, Flk-1 and Foxf1 are direct transcriptional targets of Foxm1. A schematic drawing of promoter regions of the mouse Flk-1 and Foxf1 genes. Locations of potential Foxm1 DNA-binding sites are indicated (gray boxes). ChIP assay showed that Foxm1 protein binds to promoter regions of Flk-1 and Foxf1 genes. Foxm1 binding to genomic DNA was normalized to IgG control antibodies. Diminished binding of Foxm1 to the endogenous mouse promoter regions of the Flk-1 and Foxf1 genes was observed after siFoxm1 transfection in MFLM-91U endothelial cells. Luciferase assay showed that Foxm1 induced the transcriptional activity of Flk-1 and Foxf1 promoters. MFLM-91U cells were transfected with CMV-Foxm1b expression vector and LUC reporter driven by either −1.5-kb mouse Flk-1 or −2.7-kb mouse Foxf1 promoter regions. CMV-empty plasmid was used as a negative control. Cells were harvested at 24 hours after transfection and processed for dual luciferase assays to determine LUC activity. Transcriptional activity of the mouse Flk-1 and Foxf1 promoters was increased by CMV-Foxm1b transfection. Magnifications: A: top, 100×; middle, 400×; bottom, 1,000×. *, P < 0.05.
Because 2 potential Foxm1 DNA-binding sites were identified in the −1.0-kb promoter region of the mouse Sfrp1 gene (Fig. 5B), we used ChIP assay to determine whether Foxm1 protein directly binds to the mouse Sfrp1 promoter. Foxm1 specifically bound to the mouse Sfrp1 promoter region as demonstrated by the ability of siFoxm1 to reduce binding of Foxm1 protein to the Sfrp1 promoter DNA (Fig. 5B).

Decreased activity of Sfrp1 was associated with the activation of canonical Wnt signaling in the lung (39). To assess canonical Wnt signaling, enFoxm1/C0/C0 mice were bred with...
TOPGAL transgenic mice that are frequently used as in vivo reporter for canonical Wnt activity (22), enFoxm1<sup>-/-</sup>/TOPGAL mice and control Foxm1<sup>fl/fl</sup>/TOPGAL or enFoxm1<sup>-/-</sup>/TOPGAL mice were treated with 6 weekly injections of urethane. Increased TOPGAL activity was observed in hyperplastic epithelial regions of enFoxm1<sup>-/-</sup> mice during initial stages of lung tumorigenesis (Fig. 5C, top). Type II lung epithelial cells expressing pro-SPC were frequently found in regions with increased TOPGAL activity (Fig. 5C, bottom). Likewise, Ki-67 was frequently colocalized with β-gal in enFoxm1<sup>-/-</sup>/TOPGAL lungs (Fig. 5C, middle), indicating increased proliferation rates in enFoxm1<sup>-/-</sup> epithelium. Finally, consistent with increased canonical Wnt signaling, the increase in nuclear localization of β-catenin was detected in enFoxm1<sup>-/-</sup> tumors (Fig. 5D). Thus, Foxm1 deletion from endothelial cells led to the decreased expression of the Wnt inhibitor Sfrp1, causing activation of canonical Wnt signaling and increased proliferation of epithelial cells.

**Discussion**

Although Foxm1 is known as a proliferation-specific transcription factor, recent studies suggest the importance of Foxm1 in other cellular functions, including cell migration, invasiveness, vascular permeability, angiogenesis, surfactant homeostasis, oxidative stress, and inflammation (16–18, 25, 40–43). The significance of the work presented here is the finding that Foxm1 is involved in the cross-talk between endothelial cells and other respiratory cell types during formation of lung cancer (Fig. 6). Foxm1 expression in endothelial cells is critical for proliferation of lung tumor cells via regulation of canonical Wnt signaling (through Sfrp1 and Foxf1) and pulmonary inflammation (through Flik1 and Foxf1).

Foxm1 is ubiquitously expressed in proliferating cells of different origin and is known to play a critical role in cell-cycle progression by directly activating transcription of cell-cycle regulatory genes such as Cdc25, cyclin B1, Aurora B kinase, and Polo-like kinase 1 (5, 6). Previous studies showed that Foxm1 deficiency caused decreased proliferation of endothelial cells during lung development (17) and during acute lung injury induced by inflammatory mediator, LPS (18). Thus, Foxm1 is critical for proliferation of endothelial cells and may contribute to vessel formation in cancer lesions. Surprisingly, despite Foxm1 deficiency in endothelial cells, enFoxm1<sup>-/-</sup> mice developed increased numbers of lung tumors after urethane exposure. Although lungs of untreated enFoxm1<sup>-/-</sup> mice had normal morphology, urethane treatment caused severe pulmonary inflammation, which was characterized by perivascular infiltration of inflammatory cells and increased numbers of inflammatory cells in BAL fluid. The direct relationship between inflammation and cancer is widely accepted: inflammation promotes tumor growth (29). Therefore, increased numbers of lung tumors in enFoxm1<sup>-/-</sup> mice may be a consequence of persistent pulmonary inflammation after urethane exposure. Interestingly, enFoxm1<sup>-/-</sup> mice exhibited impaired endothelial cell repair and increased vascular permeability following acute lung injury with LPS (18). Decreased vascular repair after urethane treatment may contribute to augmented lung inflammation and increased tumorigenesis in enFoxm1<sup>-/-</sup> mice.

We found that Flik1 protein and mRNA in the enFoxm1<sup>-/-</sup> lungs were decreased. The decline in pulmonary expression of Flik1 in aged humans and animals was associated with more severe lung injury, increased inflammation, and higher mortality (38). Decreased Flik1 expression was found in patients with bronchopulmonary dysplasia, a chronic lung injury that is associated with persistent pulmonary inflammation (44). Consistent with these studies, reduced Flik1 expression in urethane-treated enFoxm1<sup>-/-</sup> lungs may promote pulmonary inflammation. We also found that expression of Forkhead...
protein, Foxf1, was decreased in enFoxm1−/− endothelial cells. Haploinsufficiency of Foxf1 gene in mice caused abnormal lung capillary development and pulmonary edema (36, 37). Inactivating mutations or deletions in FOXF1 gene locus were found in human patients with alveolar capillary dysplasia with misalignment of pulmonary veins, a devastating developmental disorder with a mortality rate of 100% in the first months of life (45). Foxf1 deficiency caused increased pulmonary inflammation after chemically induced or allergen-mediated lung injury, indicating an important role of Foxf1 in the pathogenesis of pulmonary inflammation (20, 36). Thus, the increased lung inflammation in urethane-treated enFoxm1−/− lungs could be a consequence of decreased Foxf1 expression. An important contribution of the present study is demonstration that both Flk-1 and Foxf1 genes are direct targets of Foxm1. Thus, Foxm1 is likely to regulate lung inflammation by inducing expression of Flk-1 and Foxf1 in endothelial cells.

Because endothelial specific disruption of Foxm1 increased proliferation of epithelial derived tumor cells, we focused on molecular pathways involved in the cross-talk between endothelial and epithelial cells during tumor formation. Sfrp1, a known inhibitor of the canonical Wnt signaling, was decreased in enFoxm1−/− lungs. Originally identified as a developmentally active pathway, the canonical Wnt pathway has recently been linked to the pathogenesis of lung cancer, NSCLC in particular (46). The Wnt inhibitors, Sfrp1, WIF, and DKK3, were shown to be decreased in NSCLC (47, 48). In the present study, we demonstrated that deletion of Foxm1 in endothelial cells in vivo and in vitro reduced Sfrp1 mRNA. Foxm1 specifically binds to the Sfrp1 promoter region, indicating that the Sfrp1 gene is a direct target of Foxm1. Moreover, we provided evidence that Wnt signaling is activated in enFoxm1−/− respiratory epithelial cells, which could be a direct consequence of decreased inhibition by Sfrp1. We also found an increase in epithelial proliferation in enFoxm1−/− lungs, a finding consistent with previous studies showing that canonical Wnt signaling induces cellular proliferation and expression of genes critical for the cell-cycle progression (49). Because the pivotal role of Wnt activation in cancer has been already established (46), increased Wnt signaling in enFoxm1−/− lungs may contribute to aberrant proliferation of lung epithelial cells and increased tumor formation. Interestingly, Foxf1 transcription factor was downregulated in enFoxm1−/− lungs and in Foxm1-depleted cultured endothelial cells. Recent studies demonstrated that Foxf1 controls proliferation of epithelial cells by limiting mesenchymal to epithelial signaling during gut development (50). The loss of Foxf1 from intestinal mesenchymal cells increased canonical Wnt signaling and led to hyperproliferation of intestinal epithelium (50). Therefore, the decreased expression of Foxf1 in enFoxm1−/− lungs could contribute to the activation of Wnt signaling and induction of epithelial proliferation.

Taken together, our data suggest that increased tumorigenesis in enFoxm1−/− mice is the result of increased lung inflammation and activation of canonical Wnt signaling, both of which are known to promote tumorigenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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