

Foxm1 transcription factor is required for lung fibrosis and epithelial-to-mesenchymal transition

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Alveolar epithelial cells (AECs) participate in the pathogenesis of pulmonary fibrosis, producing pro-inflammatory mediators and undergoing epithelial-to-mesenchymal transition (EMT). Herein, we demonstrated the critical role of Forkhead Box M1 (Foxm1) transcription factor in radiation-induced pulmonary fibrosis. Foxm1 was induced in AECs following lung irradiation. Transgenic expression of an activated Foxm1 transcript in AECs enhanced radiation-induced pneumonitis and pulmonary fibrosis, and increased the expression of *IL-1 β* , *Ccl2*, *Cxcl5*, *Snail1*, *Zeb1*, *Zeb2* and *Foxf1*. Conditional deletion of Foxm1 from respiratory epithelial cells decreased radiation-induced pulmonary fibrosis and prevented the increase in EMT-associated gene expression. siRNA-mediated inhibition of Foxm1 prevented TGF- β -induced EMT *in vitro*. Foxm1 bound to and increased promoter activity of the *Snail1* gene, a critical transcriptional regulator of EMT. Expression of *Snail1* restored TGF- β -induced loss of E-cadherin in Foxm1-deficient cells *in vitro*. Lineage-tracing studies demonstrated that Foxm1 increased EMT during radiation-induced pulmonary fibrosis *in vivo*. Foxm1 is required for radiation-induced pulmonary fibrosis by enhancing the expression of genes critical for lung inflammation and EMT.

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Introduction

Pulmonary fibrosis includes a heterogeneous group of lung disorders characterized by progressive and irreversible

destruction of lung architecture, disruption of gas exchange and death from respiratory failure (Wynn, 2011). Pulmonary fibrosis results from dysregulated repair of damaged tissue following a variety of damaging stimuli, including ionizing radiation (Mehta, 2005). Radiotherapy is often utilized for treatment of solid tumours in the thorax and a variety of metastatic tumours. Approximately 60% of patients with non-small-cell lung cancer (NSCLC) received radiation therapy for their cancer treatment (Kong *et al*, 2005). Ionizing radiation injures pulmonary epithelial and endothelial cells, and causes the release of pro-inflammatory cytokines that recruit macrophages and lymphocytes to the sites of injury (Wynn, 2011). Myofibroblasts produce collagen and extracellular matrix (ECM) proteins during the repair of the basement membranes following radiation-induced injury. During the normal healing process, alveolar-capillary permeability is restored and inflammation resolves. All too frequently, radiation to the thorax causes pulmonary fibrosis as lung injury, inflammation and remodelling persist (Hardie *et al*, 2010; Wynn, 2011). While various signalling pathways have been implicated in the pathogenesis of radiation-induced pulmonary fibrosis, the transcriptional programmes that drive disease progression are poorly understood.

Activated myofibroblasts play a central role during the pathogenesis of pulmonary fibrosis by synthesizing and depositing ECM proteins. Myofibroblasts are likely derived from various cells, including: (1) resident stromal fibroblasts, (2) bone-marrow-derived 'fibrocytes' and (3) alveolar type II epithelial cells, a subset of which undergo epithelial-to-mesenchymal transition (EMT) (Andersson-Sjoland *et al*, 2011; Wynn, 2011). Recent studies support the role of EMT in pulmonary fibrosis (Willis *et al*, 2005; Kim *et al*, 2006; Willis *et al*, 2006; Kim *et al*, 2009). During EMT, epithelial cells lose apical-basal polarity, basement membrane attachment and cell-cell contact. Epithelial cells gain mesenchymal characteristics associated with increased migratory behaviour, cytoskeletal rearrangements and their migration into the lung interstitium where they produce excess ECM. EMT is controlled by a network of signalling and transcriptional events mediated in part by TGF- β signalling (Thiery and Sleeman, 2006; Chapman, 2011). The TGF- β /Smad signalling pathway is required for both EMT and fibrosis in a variety of organs (Xu *et al*, 2009). Snail and Twist family members of transcription factors are important regulators of EMT, repressing E-cadherin and activating the mesenchymal transcriptomes (Kalluri and Weinberg, 2009).

Forkhead Box M1 (Foxm1) is a member of the Forkhead family of transcription factors that shares homology in the Winged Helix/*Forkhead box* DNA-binding domain. In proliferating cells, Foxm1 regulates the G₂/M transition of the cell cycle through transcriptional activation of Cdc25B phosphatase, Plk1, AuroraB kinase and Cyclin B1 (Costa *et al*, 2005). Consistent with its role in cell cycle progression, increased expression of Foxm1 was found in a variety of human

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tumours (Kalin *et al*, 2006; Yoshida *et al*, 2007; Wang *et al*, 2009). FoxM1 plays important roles during embryonic development, monocyte/macrophage recruitment, DNA repair, surfactant production, angiogenesis and formation of tight junctions during LPS-induced acute lung injury (reviewed in (Kalin *et al*, 2011) and (Kalinichenko *et al*, 2001; Kalin *et al*, 2008b; Wang *et al*, 2008b; Zhang *et al*, 2008; Wang *et al*, 2009; Ren *et al*, 2010; Balli *et al*, 2011a, b)). In the present study, we found increased Foxm1 in biopsy samples from human patients with interstitial pulmonary fibrosis (IPF). Since the role of Foxm1 in fibrotic diseases is unknown, we used gain-of-function and loss-of-function mouse models to identify the role of Foxm1 in radiation-induced pulmonary fibrosis. Expression of constitutively active Foxm1-ΔN mutant within type II epithelial cells increased fibrosis, whereas cell-specific ablation of Foxm1 from type II cells protected mice from the radiation-induced pulmonary fibrosis. We identified the Foxm1 as a novel potent mediator of fibrotic events following radiation injury supporting the potential utility of targeting Foxm1 for treatment of pulmonary fibrosis.

Results

Foxm1 is increased during radiation-induced pulmonary fibrosis

Thoracic irradiation causes pulmonary fibrosis in mice (Chiang *et al*, 2005). Mice were exposed to ionizing radiation directed to the thoracic region. Foxm1 mRNA progressively increased in the lung tissue following irradiation (Figure 1A). Foxm1 staining was increased in type II epithelial cells of the irradiated lungs as demonstrated by colocalization with SP-C, a marker of alveolar type II cells (Figure 1B). Foxm1 staining was also increased in lung tissue from patients with idiopathic pulmonary fibrosis compared to control lung tissue from organ donors (Figure 2A). Increased numbers of FoxM1-positive cells were identified throughout fibrotic regions, in sharp contrast to the paucity of Foxm1-stained cells in normal lungs from organ donors. Foxm1 protein levels were upregulated in type II epithelial cells of

the IPF fibrotic lesions, but not in the control lungs (Figure 2B).

Increased expression of Foxm1 in respiratory epithelial cells increased radiation-induced pulmonary fibrosis and inflammation

Since not all the patients develop pulmonary fibrosis after the same dose of irradiation, we tested whether increased activity of Foxm1 in type II alveolar cells is sufficient to induce pulmonary fibrosis. Transgenic mice were used in which a constitutively active form of Foxm1 (Foxm1-ΔN) was expressed under control of SP-C promoter (*SP-C-rtTA^{tg}/tetO-Foxm1-ΔN^{tg}/-* or *epiFoxm1-ΔN* mice, (Wang *et al*, 2010)). Doxycycline (Dox) was used to activate *epiFoxm1-ΔN* transgene in adult lungs (Supplementary Figure 1A and B). To induce lung fibrosis, the thoracic regions of Dox-treated *epiFoxm1-ΔN* and Dox-treated control mice were exposed to a single dose of 12 Gy ionizing radiation. As early as 3 months post radiation, increased focal collagen deposition was observed in peribronchial regions of Foxm1-ΔN mice (Supplementary Figure 2A). Lung collagen 1α1 (*Col1α1*), collagen 3α1 (*Col3α1*) and α-smooth muscle actin (α-SMA) mRNAs were induced in *epiFoxm1-ΔN* mice, findings consistent with increased ECM production and myofibroblast activation (Supplementary Figure 2B). Six months after irradiation, pulmonary fibrosis in *epiFoxm1-ΔN* mice was extensive, while few focal fibrotic regions were found in control mice (Figure 3A). Without irradiation, *epiFoxm1-ΔN* mice did not develop pulmonary fibrosis and inflammation (Figure 3A–C, Supplementary Figure 3C).

Collagen deposition was increased in *epiFoxm1-ΔN* lungs (Figure 3A, right panels and 3B). α-SMA and *Col3α1* mRNAs were increased in the *epiFoxm1-ΔN* lungs at 6 months after irradiation (Figure 3C). Increased fibrosis in *epiFoxm1-ΔN* lungs was associated with sustained pulmonary inflammation as demonstrated by increased numbers of interstitial F4/80-positive macrophages, perivascular CD3-positive lymphocytes (Supplementary Figure 3A) and increased bronchoalveolar lavage (BAL) cell counts (Supplementary Figure 3B). mRNAs encoding pro-inflammatory cytokines *Ccl2*, *Cxcl5* and *IL-1β*, all known mediators of pulmonary

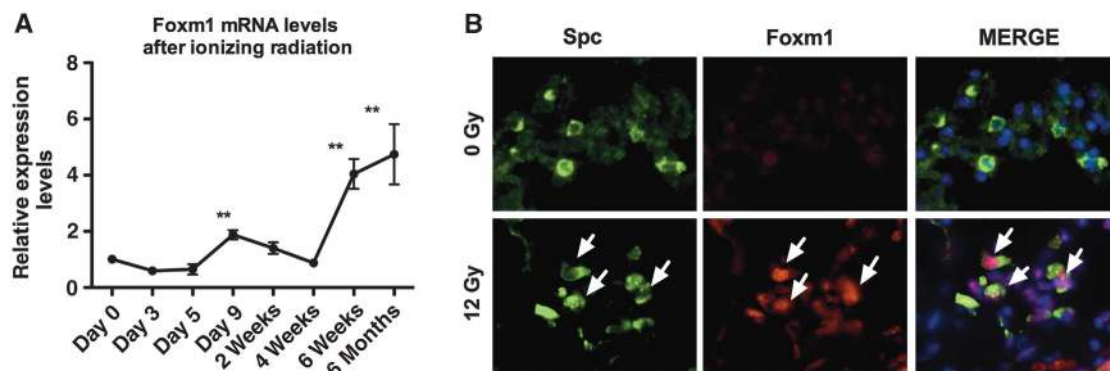


Figure 1 Thoracic irradiation increased expression of Foxm1 in the lung. The thoracic regions of C57BL/6 mice were exposed to 12 Gy ionizing radiation. (A) Foxm1 mRNA is increased after irradiation. At designated time points, whole-lung RNA was isolated and Foxm1 mRNA levels were evaluated by qRT-PCR. β-actin mRNA was used for normalization. Data represent means ± s.d. of three independent determinations using lung tissue from *n* = 3–5 mice/time point. (B) Foxm1 protein levels were upregulated in type II epithelial cells of the irradiated lungs. SPC-positive lung epithelial cells (green) co-expressed Foxm1 (red) 6 months after irradiation. Representative sections from five irradiated samples and three control samples are shown. A *P*-value <0.01 is shown with asterisks (**). Magnification is × 400.

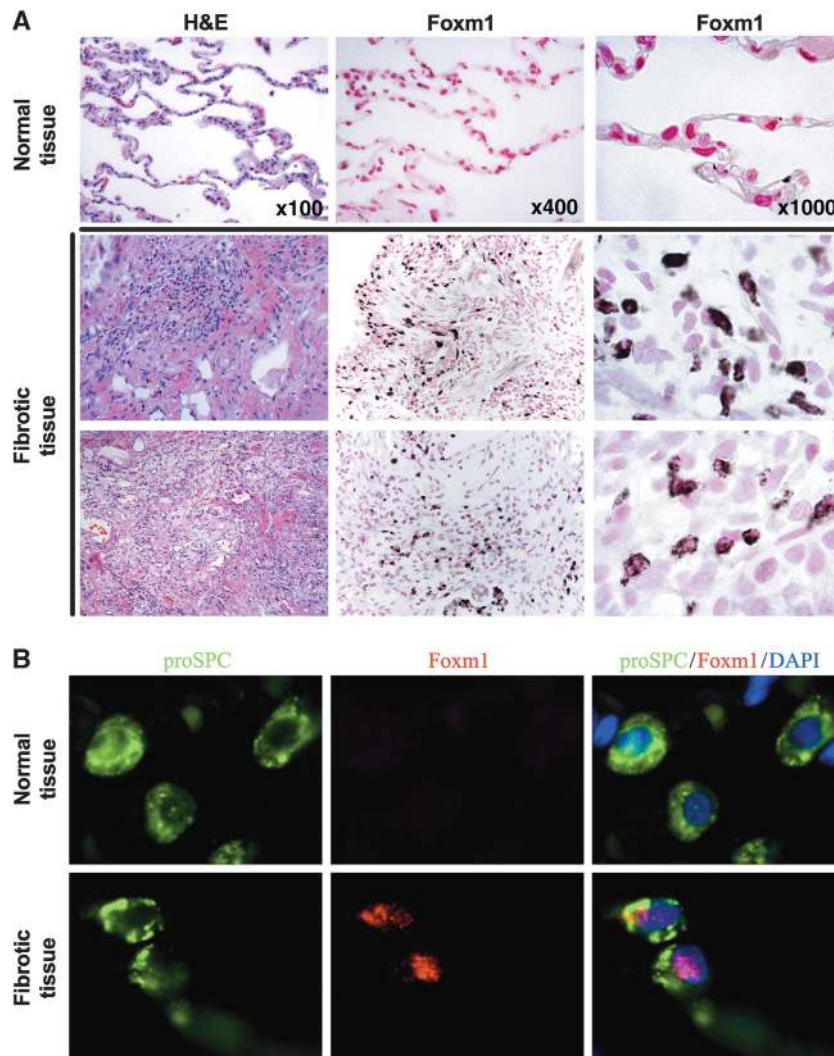


Figure 2 Foxm1 protein is highly expressed in fibrotic lesions of IPF patients. (A) Lung tissue sections from patients with IPF and control organ donors were stained with H&E or with an antibody specific for human Foxm1. Representative sections from 10 IPF and 6 normal control samples are shown. Magnification: left and middle panels, $\times 200$; right panels, $\times 1000$. (B) Foxm1 protein levels were upregulated in type II epithelial cells of the IPF fibrotic lesions, but not in the control lungs. Pro-SPC-positive lung epithelial cells (green) co-expressed Foxm1 (red) in IPF lungs. Representative sections from six IPF and three normal control samples are shown. Magnification is $\times 2000$.

inflammation and fibrosis (Rose *et al*, 2003; Wilson *et al*, 2010; Liu *et al*, 2011), were increased while *Ccl3* was not changed (Supplementary Figure 3C). Cotransfection of CMV-Foxm1b expression vector significantly increased transcriptional activity of the -1.3 Kb *Ccl2* and the -1.14 Kb *Cxcl5* promoter regions in a luciferase reporter assay *in vitro* (Supplementary Figure 3D).

Six months after irradiation, the number of Ki-67-positive cells was increased in the lungs of epiFoxm1- Δ N mice (Figure 3D). Since the Foxm1- Δ N was specifically overexpressed in type II cells and is known to regulate cellular proliferation, we performed colocalization experiments to identify proliferating cell types in the fibrotic lungs. The number of proliferating type II cells that were double-positive for both proSPC and Ki-67 was similar in epiFoxm1- Δ N and control mice (Supplementary Figure 4A and B). No changes in *Cyclin B1* and *Cyclin D1* mRNAs were observed in isolated type II cells (Supplementary Figure 4C), indicating

that overexpression of Foxm1 does not influence proliferation of type II cells in the fibrotic lung. In contrast, the number of proliferating myofibroblasts that were positive for Ki-67 and α -SMA was increased in epiFoxm1- Δ N mice (Supplementary Figure 4A and B). Increased proliferation of fibroblasts was likely a result of increased levels of profibrotic mediators IL-1 β , *Ccl2* and *Cxcl5* (Supplementary Figure 3C and D and (Moore *et al*, 2001; Quan *et al*, 2006; Ekert *et al*, 2011; Kawamura *et al*, 2012)), that contribute to the fibrotic phenotype in irradiated epiFoxm1- Δ N mice. Taken together, these data indicate that increased activity of Foxm1 in type II cells enhanced pulmonary inflammation and fibrosis after thoracic irradiation.

Foxm1 regulates genes associated with EMT

The role of EMT in the development of pulmonary fibrosis has been supported by a number of recent publications (Willis *et al*, 2005; Kim *et al*, 2006; Willis *et al*, 2006;

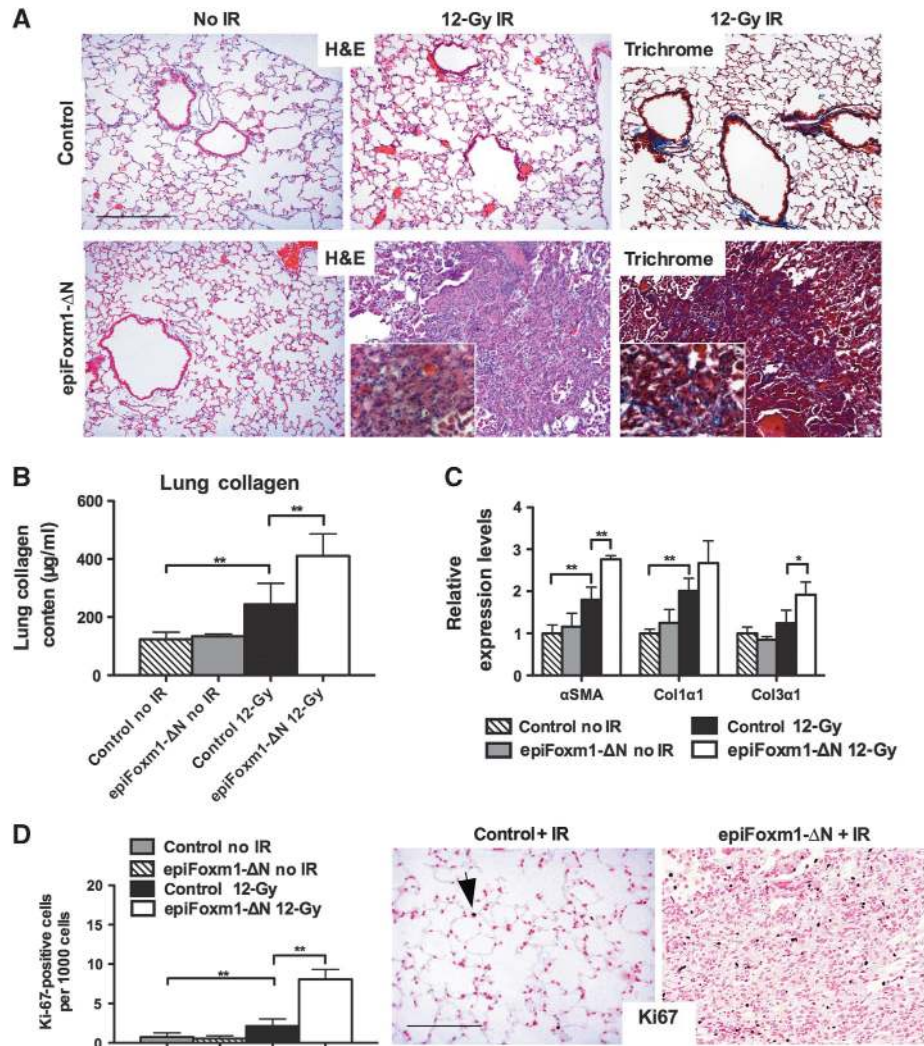


Figure 3 Aberrant expression of Foxm1 in AECs exacerbated pulmonary fibrosis following thoracic irradiation. The thoracic regions of transgenic mice expressing constitutively active Foxm1-ΔN mutant in AECs (*epiFoxm1-ΔN*) were irradiated with 12 Gy. (A). At 6 months post radiation, H&E staining (left panels) of lung tissue demonstrated minimal remodelling and fibrosis in control mice, but severe fibrotic lesions in *epiFoxm1-ΔN* mice (middle panels). Masson's trichrome staining (right panels) showed increased deposition of collagen in lungs of *epiFoxm1-ΔN* mice. Representative sections from at least seven mice per group are shown. (B) Increased collagen depositions in irradiated *epiFoxm1-ΔN* lungs were shown by quantitative Sircol collagen assay. (C). Increased mRNAs of α -SMA and *Col3α1* were found by qRT-PCR in *epiFoxm1-ΔN* mice at 6 months post radiation. β -actin mRNA was used for normalization. Data represent mean \pm s.d. of three independent determinations using lung tissue from $n = 5-7$ mice. (D) Cellular proliferation is elevated in irradiated *epiFoxm1-ΔN* lungs compared to control lungs. The number of Ki-67+ cells were counted and quantified per 1000 cells using 10 random microscope fields. A P -value < 0.01 is shown with (**) and P -value < 0.05 is shown with (*). Magnification: panels A, $\times 100$; inserts, $\times 400$; panels D, $\times 200$.

Demaio et al, 2011; Marmai et al, 2011). Since expression of Foxm1-ΔN in type II alveolar epithelial cells (AECs) exacerbated pulmonary fibrosis, we tested whether Foxm1 influences EMT. Three months after thoracic irradiation, *Snail1*, *Snail2*, *Zeb1*, *Zeb2*, *Twist2* and *Foxf1* mRNAs were increased in *epiFoxm1-ΔN* lungs (Figure 4A). The epithelial-specific marker *E-cadherin*, the loss of which is a key step during EMT (Thiery and Sleeman, 2006; Kalluri and Weinberg, 2009), was significantly decreased (Figure 4A). Six months post radiation, *Snail1*, *Zeb1*, *Zeb2*, *vimentin* and *fibronectin* mRNAs were increased and *E-cadherin* mRNA was decreased (Figure 4B). Colocalization experiments demonstrated the presence of Foxm1-ΔN transgenic protein in a subset of α -SMA-positive and vimentin-positive cells localized within fibrotic lesions (Figure 4C), suggesting that the

Foxm1-ΔN-expressing epithelial cells underwent EMT after irradiation.

Conditional deletion of Foxm1 from alveolar type II cells protects mice from radiation-induced pulmonary fibrosis

Foxm1 was conditionally deleted in the respiratory epithelium (*Spc-rtTA/tetO-cre/Foxm1^{fl/fl}*) termed *epiFoxm1 KO* mice (Kalin et al, 2008b)). *epiFoxm1 KO* and control mice were treated with Dox to induce Foxm1 deletion. Since we expected that Foxm1 deletion would decrease pulmonary fibrosis, the dose of thoracic irradiation was increased to 18 Gy. Six months after radiation, the collagen depositions in *epiFoxm1 KO* lungs were considerably smaller compared to controls (Figure 5A). α -SMA, *-Col1α1* and *-Col3α1* mRNAs

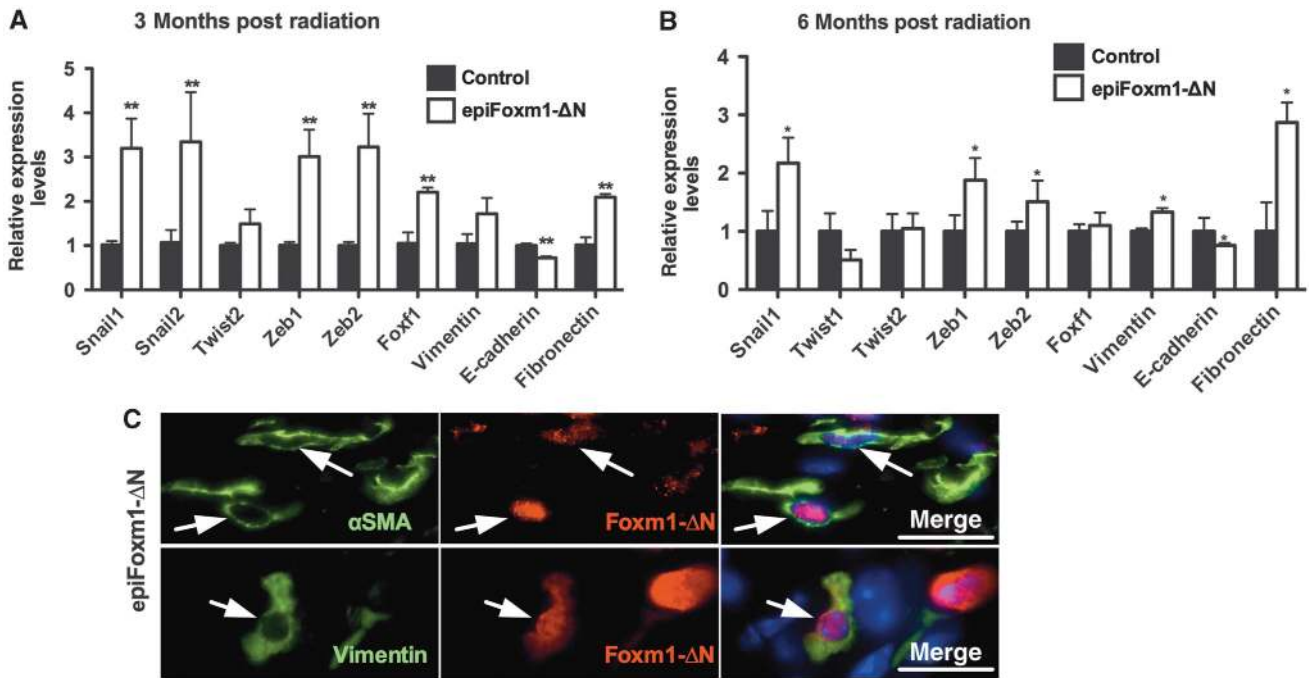


Figure 4 Foxm1 regulates epithelial-to-mesenchymal transition during radiation-induced pulmonary fibrosis. Aberrant overexpression of Foxm1 in AECs increased mRNA levels of EMT genes in epiFoxm1-ΔN lungs at 3 months (A) and at 6 months (B) following thoracic irradiation as demonstrated by qRT-PCR. β -actin mRNA was used for normalization. Data represent mean \pm s.d. of three independent determinations using lung tissue from $n = 5$ –13 mice per group. A P -value < 0.05 is shown with asterisk (*) and P -value < 0.01 is shown with (**). (C) Foxm1-positive epithelial cells (red) from irradiated epiFoxm1-ΔN lungs expressed the mesenchymal markers α -SMA and vimentin (green) at 6 months after radiation. Nuclei were stained with DAPI (blue). Magnification is $\times 1000$.

were decreased, consistent with decreased ECM production and myofibroblast activation (Figure 5B). Lung collagen content was decreased in irradiated epiFoxm1 KO mice (Figure 5C). Loss of Foxm1 was associated with decreased *Snail1*, *Twist1*, *Twist2*, *Foxf1*, *Zeb1* and *Zeb2* mRNAs (Figure 5D). Pro-inflammatory *Cxcl5*, *IL-1 β* and *TGF β* mRNAs were significantly reduced, whereas *Ccl2* and *Ccl3* mRNAs were not changed (Figure 5E). These data indicate that ablation of Foxm1 from type II cells diminished radiation-induced pulmonary fibrosis and decreased expression of genes associated with EMT and lung inflammation.

Expression of Foxm1 was inhibited by siRNA-mediated knockdown of Foxm1 in mouse lung epithelial MLE15 cells. A 60% reduction in *Foxm1* mRNA was associated with decreased expression of *Snail1*, *Zeb2*, *Twist2* and *Foxf1* mRNAs, whereas *Zeb1* mRNA was not changed (Figure 6A), implicating Foxm1 in the regulation of EMT *in vitro*.

Foxm1 is required for TGF β -induced EMT

To test whether Foxm1 is required for EMT, human epithelial A549 cells were treated with TGF- β 1 to induce EMT *in vitro* (Kasai *et al*, 2005). Consistent with the previous studies, TGF- β 1 increased mesenchymal markers fibronectin, α -SMA, SNAIL1, ZEB1, ZEB2 and decreased epithelial marker E-cadherin (Figure 6B). Knockdown of Foxm1 by siRNA prevented EMT as demonstrated by reduced protein levels of all mesenchymal markers, and increased level of epithelial E-cadherin in TGF- β 1-treated A549 cells (Figure 6B). Inhibition of Foxm1 prevented the induction of SNAIL1, ZEB1 and ZEB2 transcription factors critical for EMT

(Figure 6B). Foxm1 depletion did not alter SMAD2 or AKT phosphorylation (Figure 6C), suggesting that Foxm1 acts downstream of TGF- β 1/Smad and AKT signalling pathways. There were no differences in total levels of AKT and SMAD2 proteins in Foxm1-deficient cells (Figure 6C).

Since Foxm1 directly activated transcription of *Jnk1* (Wang *et al*, 2008a) and JNK1 has been shown to be required for TGF- β 1-induced EMT both *in vitro* and *in vivo* (Javelaud and Mauviel, 2005; Alcorn *et al*, 2009), JNK1 protein was examined by western blot. JNK1 was decreased in Foxm1-depleted cells, whereas JNK2 did not change (Figure 6C). Decreased phosphorylation of c-JUN (Figure 6C) was consistent with the loss of JNK1. To determine if Foxm1 influenced transcriptional activity of Smad during EMT, a CMV-Foxm1 expression vector was co-transfected with a Smad reporter (3TP-Luc) containing Smad-responsive elements (SRE) (Lange *et al*, 2009). Neither Foxm1 nor a Jnk1 inhibitor (SP10064) affected Smad transcriptional activity (Figure 6D) induced by TGF β 1. Thus, Foxm1 does not influence the transcriptional activity of Smad during EMT. Altogether, the loss of JNK1 and decreased expression of SNAIL1, ZEB1 and ZEB2 likely contributes to decreased EMT in Foxm1-deficient cells after TGF β treatment.

Foxm1 directly activates the Snail1 promoter

Since Foxm1 induced *Snail1* mRNA and protein *in vivo* and *in vitro* (Figures 4,5 and 6), we investigated whether *Snail1* is a direct transcriptional target of Foxm1. A potential Foxm1-binding site was identified within the -1.0 Kb promoter regions of mouse and human *Snail1* genes (Figure 7A). Chromatin immunoprecipitation (ChIP) assay was used to

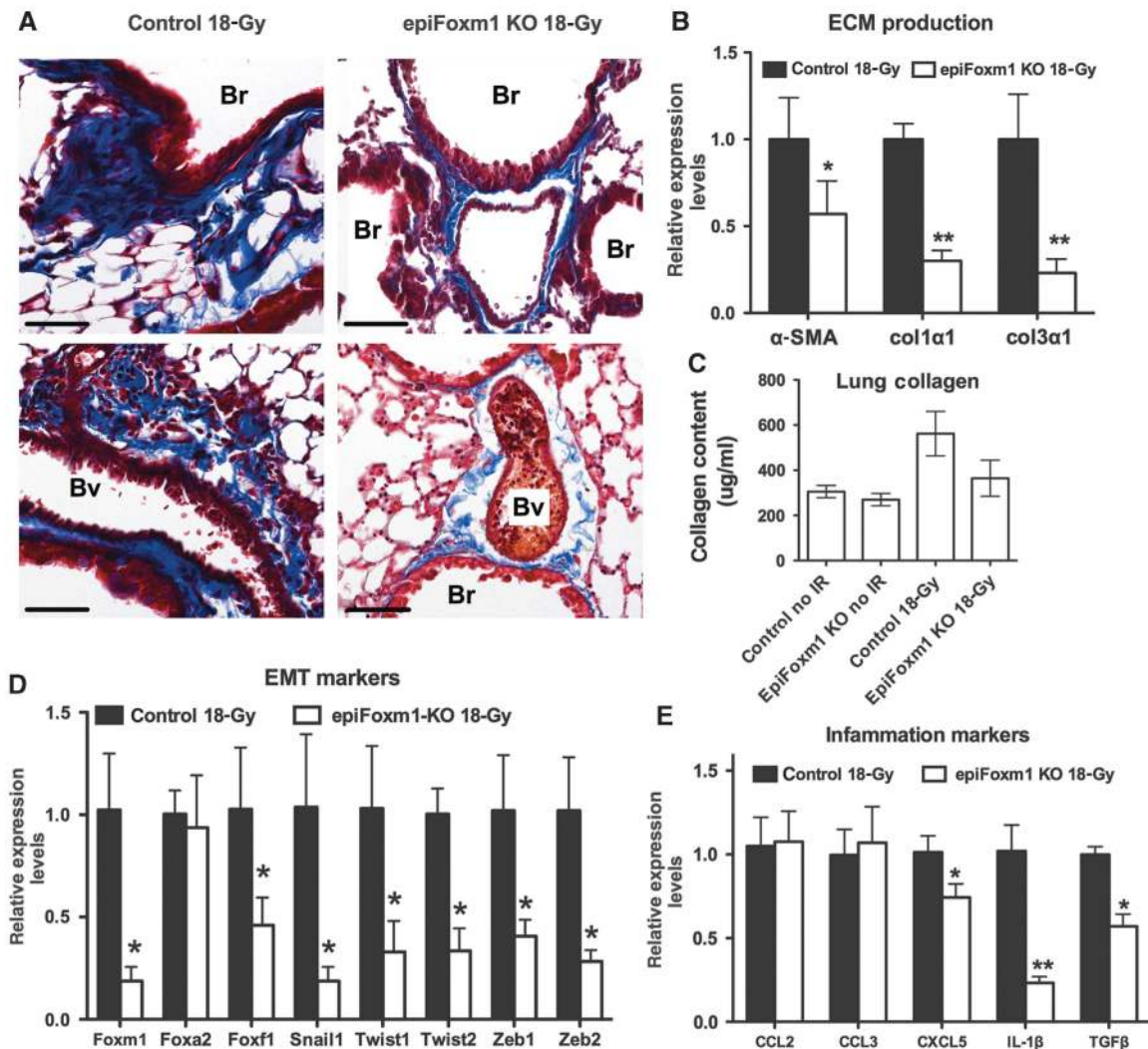


Figure 5 Ablation of Foxm1 from pulmonary epithelial cells prevented radiation-induced fibrosis. The thoracic regions of conditional knock-out mice (*epiFoxm1 KO*) and control mice were irradiated with 18 Gy. (A) At 6 month post radiation, Masson's trichrome staining of lung tissue was performed. Increased collagen depositions were found in the lungs of control mice ($n = 8$ mice), but not in *epiFoxm1 KO* mice ($n = 5$ mice). Magnification is $\times 400$. (B) Decreased mRNA levels of ECM genes in *epiFoxm1 KO* lungs at 6 months after thoracic irradiation were demonstrated by qRT-PCR. β -actin mRNA was used for normalization. (C) Decreased collagen depositions were found in irradiated *epiFoxm1 KO* lungs by quantitative Sircol collagen assay. mRNA levels of EMT genes (D) and pro-inflammatory genes (E) were decreased in irradiated *epiFoxm1-KO* mice as demonstrated by qRT-PCR. β -actin mRNA was used for normalization. Data represent mean \pm s.d. of three independent determinations using lung tissue from five to eight mice per group. A P -value < 0.05 is shown with (*) and P -value < 0.01 is shown with (**).

determine whether Foxm1 binds to the promoter region of *Snail1* gene in A549 cells. After TGF- β 1 treatment, the specific binding of Foxm1 protein to the *Snail1* promoter DNA was increased (Figure 7B). CMV-Foxm1b significantly increased the activity of a -720 bp *Snail1* promoter region in a luciferase reporter assay (Figure 7C). Site-directed mutagenesis of the Foxm1-binding site decreased the ability of Foxm1 to activate the -720 bp *Snail1* promoter in A549 cells (Figure 7C) and U2OS cells (Supplementary Figure 5), indicating that the $-483/-473$ *Snail1* promoter region contains a functional Foxm1-binding site. Thus, Foxm1 was capable of inducing transcriptional activity of the -720 bp *Snail1* promoter region in co-transfection experiments. Interestingly, TGF- β did not synergize with Foxm1 to enhance the -720 bp *Snail1* promoter activity (Figure 7C), because the *Snail1* promoter region lacks AP-1/4-binding sites that are required for activation of Snail1 by TGF β

(Peinado *et al*, 2003; Medici *et al*, 2006). Foxm1 did not activate the -1.2 kb *Zeb2* promoter (Supplementary Figure 5). Thus, Foxm1 directly bound to and induced the transcriptional activity of the *Snail1* gene, indicating that *Snail1* is the direct Foxm1 target. Finally, expression of Snail1 in Foxm1-deficient A549 cells restored TGF β -induced decrease in E-cadherin protein (Figure 7D and E), indicating that Foxm1 influenced EMT through Snail1.

Expression of activated Foxm1- Δ N mutant induced EMT during radiation-induced or bleomycin-induced pulmonary fibrosis

Since Foxm1 regulated the expression of EMT-associated genes *in vivo* (Figures 4 and 5) and *in vitro* (Figure 6), lineage-tracing experiments were utilized to determine whether epithelial cells expressing the activated Foxm1- Δ N mutant underwent EMT during pulmonary fibrosis.

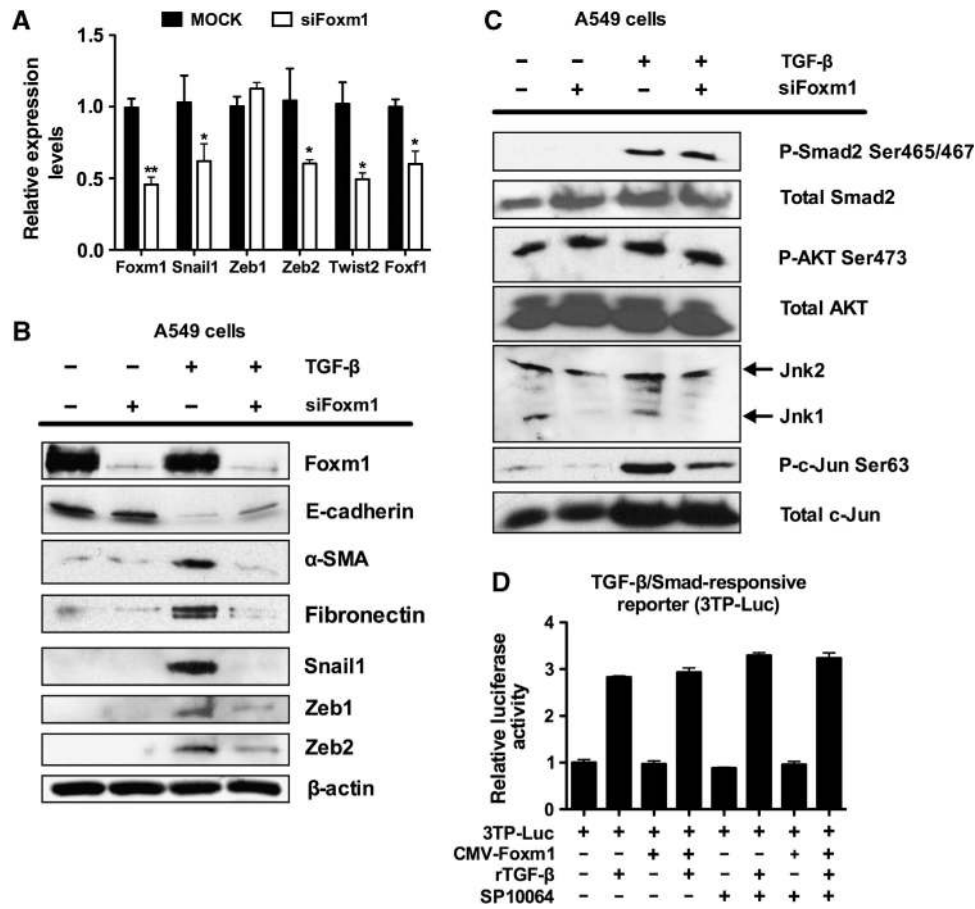


Figure 6 Foxm1 is required for EMT *in vitro*. (A) Depletion of Foxm1 from MLE15 cells was performed by siRNA transfection. At 48 h after transfection, RNA was isolated and examined by qRT-PCR. mRNAs of *Foxm1*, *Snail1*, *Zeb2*, *Twist2* and *Foxf1* were decreased compared to mock-transfected cells. Expression levels were normalized using β -actin mRNA. Data represent mean \pm s.d. of three independent determinations. (B, C) Foxm1 knockdown by siRNA prevented the TGF- β -induced EMT in A549 epithelial cells. siFoxm1 transfection decreased protein levels of Snail1, Zeb1, Zeb2, fibronectin and α -SMA, and increased E-cadherin, shown by western blot (B). Foxm1 depletion did not affect pSmad2, pAKT and Jnk2. Jnk1 and p-cJun were decreased in Foxm1-depleted cells, shown by western blot (C). Data represent one of three independent experiments. (D) Foxm1 does not show transcriptional synergy with TGF- β . U2OS cells were co-transfected with CMV-Foxm1 and 3TP-luc-containing SREs. Transfected cells were incubated with rTGF- β and with or without the Jnk1 inhibitor SP10064 for 24 h. Triplicate plates were used to calculate the mean \pm s.d. relative luciferase activity. Data represent one of two independent experiments. A *P*-value < 0.05 is shown with (*) and *P*-value < 0.01 is shown with (**). Source data for this figure is available on the online supplementary information page.

To permanently label epithelial cells expressing FoxM1- Δ N transgenic protein, SP-C-rtTA/TetO-GFP-FoxM1- Δ N double-transgenic mice (epiFoxm1- Δ N) were bred with TetO-Cre/Rosa26-loxP-STOP-loxP- β -galactosidase reporter mice (Rosa26R) to generate SP-C-rtTA/TetO-GFP-FoxM1- Δ N/TetO-Cre/Rosa26R (epiFoxm1- Δ N/Rosa26R) quadruple transgenic mice (Supplementary Figure 6A). In these mice, the Foxm1- Δ N-expressing epithelial cells were permanently labelled with β -galactosidase (β -gal) after Dox administration. Dox simultaneously induced expression of FoxM1- Δ N and Cre-mediated excision of the LoxP-STOP-LoxP cassette from the ROSA26 locus, resulting in β -gal and FoxM1- Δ N expression in the same cell. Mice without TetO-GFP-FoxM1- Δ N transgene (SP-C-rtTA/TetO-Cre/Rosa26R) were used as controls. β -gal activity was detected in a subset of airway Clara cells and type II AECs in both groups of mice (Supplementary Figure 6C), consistent with previous studies (Perl *et al*, 2002; Wang *et al*, 2010). Thoracic irradiation caused severe pulmonary fibrosis with extensive collagen deposition in *epiFoxm1- Δ N/Rosa26R* lungs (Supplementary Figure 6B). Epithelial cells undergoing EMT were identified by colocalization of β -gal with the

mesenchymal markers α -SMA (Figure 8A) or vimentin (Supplementary Figures 6D and 8). Consistent with the role of Foxm1 in EMT, β -gal was detected in 18.5% of α -SMA-positive and in 14% vimentin-positive fibroblasts of *epiFoxm1- Δ N/Rosa26R* lungs, compared to 4 or 3% of fibroblasts in control lungs (Figure 8B and Supplementary Figure 6E). To demonstrate that the role of Foxm1 in EMT is not limited to the radiation-induced fibrosis, pulmonary fibrosis was induced by bleomycin. Although at 21 days after injury, no differences were found in collagen content in bleomycin-treated *epiFoxm1- Δ N/Rosa26R* and control mice (Supplementary Figure 7A and B), the number of β -gal-positive fibroblasts was significantly increased in *epiFoxm1- Δ N/Rosa26R* lungs (Supplementary Figure 7C and D). Furthermore, quantitative flow cytometry analysis of bleomycin-treated *epiFoxm1- Δ N* lungs demonstrated significant increase in the number of cells expressing both proSPC and α -SMA proteins (Figure 8C and D). After isolation of type II cells followed by cell sorting for GFP, a purified population of GFP-positive (Foxm1-expressing) type II cells contained increased levels of *Vimentin* and *Snail1* mRNAs,

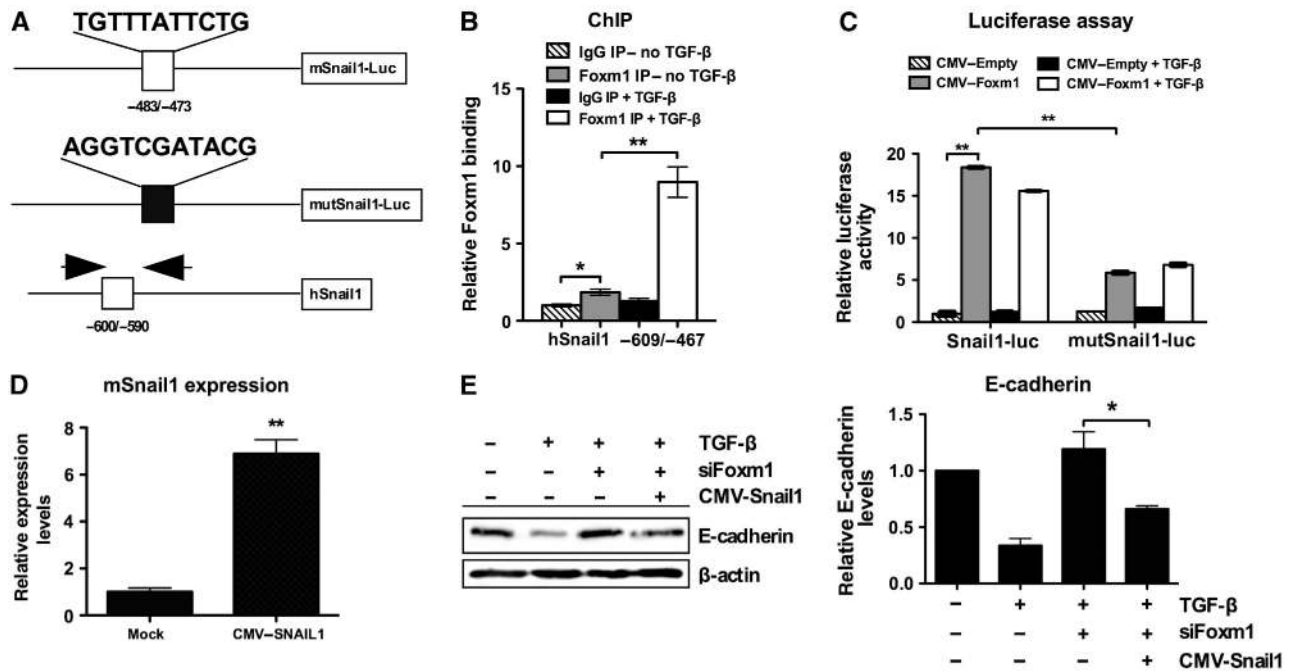


Figure 7 *Snail1* is a direct transcriptional target of Foxm1. (A) A schematic drawings of promoter region of the human *Snail1* gene (hSnail1) and mouse *Snail1* gene (mSnail1). Location of a potential Foxm1 DNA-binding site is indicated (box). Site-directed mutagenesis was used to mutate the Foxm1-binding site in mouse *Snail1* promoter (from TGTTTATTCTG to AGGTCGATACG, -483/-473, mutSnail1-luc) (Littler *et al*, 2010). (B) Foxm1 directly binds to the *Snail1* promoter region after TGF- β treatment. A549 cells were incubated with TGF- β for 24 h and protein/DNA complexes were immunoprecipitated using antibodies specific for Foxm1. Addition of TGF- β to culture media significantly increased Foxm1 binding to the *Snail1* promoter. Foxm1 binding was normalized to DNA samples immunoprecipitated with isotype control antibodies. Data represent one of two independent experiments. (C) In A549 cells, transcriptional activity of the -720bp mouse *Snail1* promoter was increased by CMV-Foxm1b transfection, but not by TGF- β alone. Activity of *mutSnail1-Luc* was lower compared to *Snail1-Luc*. Data represent one of two independent experiments. (D) Increased *Snail1* mRNA after transfection of A549 cells with *CMV-Snail1* shown by qRT-PCR. (E) *CMV-Snail1* restored downregulation of E-cadherin protein in Foxm1-deficient cells after TGF- β 1 treatment. A549 cells were co-transfected with *CMV-Snail1* and siFoxm1 for 48 h and then treated with TGF- β for 24 h. Western blot is shown in the left panel and quantitative densitometry analysis in the right panel. Data represent one of three independent experiments. A *P*-value <0.05 is shown with (*) and *P*-value <0.01 is shown with (**). Source data for this figure is available on the online supplementary information page.

but decreased *E-cadherin* mRNA, when compared to control GFP-negative type II cells from control mice (Figure 8E). Thus, expression of Foxm1- Δ N mutant in type II cells increased the contribution of EMT to pulmonary fibrosis after radiation-induced and bleomycin-induced lung injury. Finally, to demonstrate that Foxm1-expressing type II cells express both epithelial and mesenchymal markers in human fibrotic disease, colocalization experiments were performed using lung sections from IPF patients. These studies clearly identified the population of proSPC-positive type II epithelial cells (blue) that were also positive for vimentin (green) and Foxm1 (red) (Figure 8F). Altogether, our data from mouse and human lungs indicate that Foxm1 induces EMT and contributes to progression of pulmonary fibrosis.

Discussion

Pulmonary fibrosis is refractory to treatment, carries a high mortality rate and the only effective treatment currently available is lung transplantation (Wynn, 2011). Radiation-induced pulmonary fibrosis is a severe and life-threatening complication of radiotherapy in cancer patients. Since the survival rates for cancer patients are increasing, physicians will increasingly care for long-term complications of radiation therapy. Existing treatments for pulmonary fibrosis have not

significantly improved survival, leading to a critical need for new approaches. In the present study, we identified the Foxm1 transcription factor as a critical regulator of pulmonary fibrosis and inflammation. Foxm1 exacerbates fibrosis after thoracic irradiation by inducing EMT, increasing inflammation and inducing proliferation of fibroblasts (Figure 9). Our findings provide the foundation for the development of new therapeutic approaches based on the inhibition of Foxm1 and directed on the prevention of pulmonary fibrosis and interstitial pneumonitis after radiation treatment.

Alveolar epithelial injury and pulmonary fibrosis

AECs play an important role in the pathogenesis of pulmonary fibrosis by producing and responding to fibrotic mediators (Sisson *et al*, 2010). Apoptosis of alveolar type II cells is sufficient to cause pulmonary fibrosis, directly demonstrating that injury to the respiratory epithelium results in lung fibrosis (Sisson *et al*, 2010). Likewise, repeated injury to bronchiolar epithelium due to either expression of diphtheria toxin in Clara cells or due to a directed alloimmune response in graft-versus-host reaction after hematopoietic stem cell transplant resulted in pulmonary fibrosis (Panoskaltsis-Mortari *et al*, 2007; Perl *et al*, 2011). The patients with mutations in the gene encoding surfactant-associated protein C (*SFTPC*), a type II cell-specific gene,

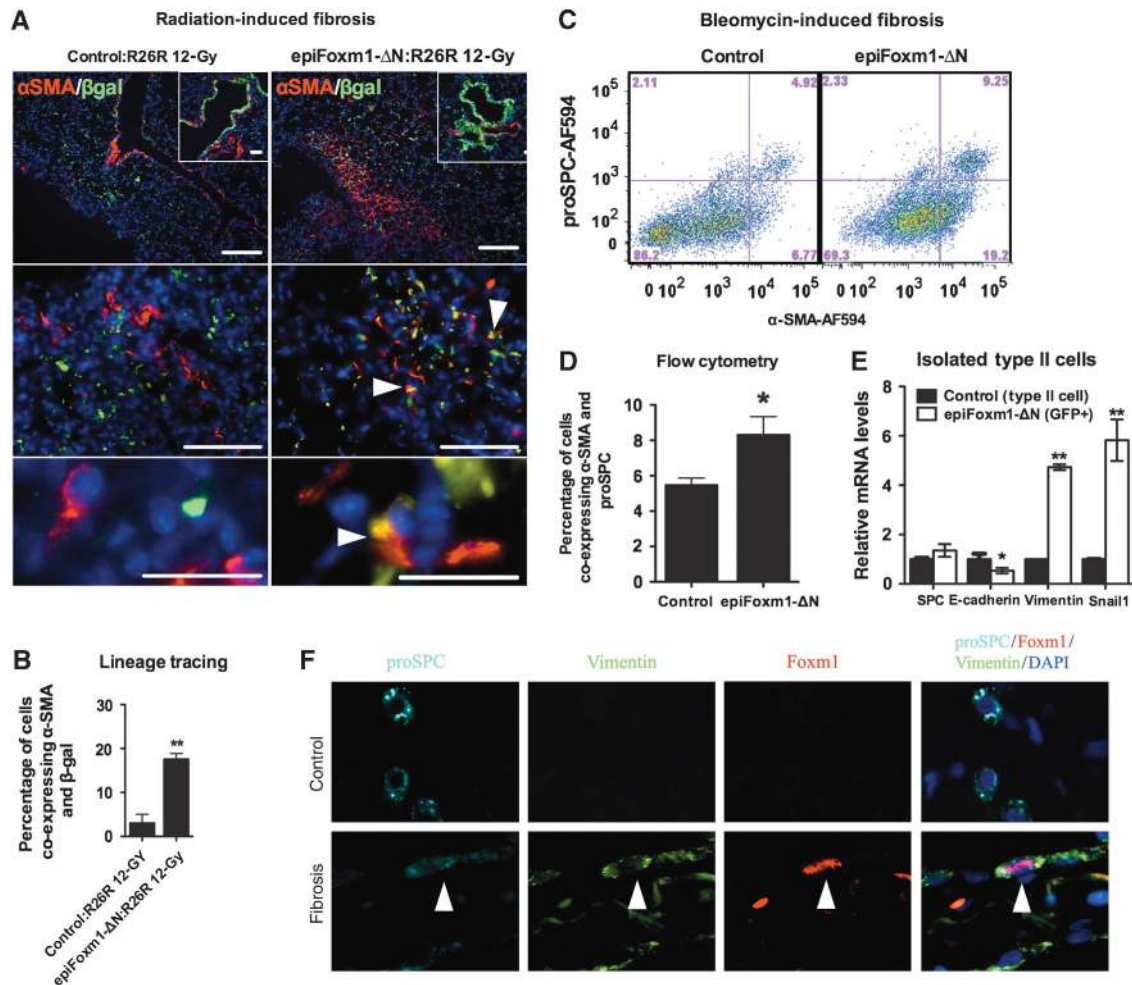


Figure 8 Lineage-tracing experiments demonstrated that Foxm1 promotes EMT during radiation-induced fibrosis. (A) Colocalization studies identified cells (arrows) positive for both β -gal (green) and α -SMA (red) in irradiated *epiFoxm1-ΔN/R26R* lungs. (B) Counting of double-positive α -SMA + β -gal + cells were performed using a confocal microscope. Images and counts were obtained from five random fields in each of six individual mice per group and scored blindly. Data represent mean \pm s.d. (C) Over-expression of Foxm1 in type II epithelial cells increased the number of proSPC + / α -SMA + cells in bleomycin-treated *epiFoxm1-ΔN* lungs as demonstrated by flow cytometry. (D) Percentage of proSPC + / α -SMA + cells in bleomycin-treated control ($n = 3$ mice) and *epiFoxm1-ΔN* lungs ($n = 3$ mice) were determined by flow cytometry and presented as mean \pm s.d. (E) Increased *Snail1* and *Vimentin*, but decreased *E-cadherin*, mRNAs are shown by qRT-PCR in isolated type II cells from bleomycin-treated *epiFoxm1-ΔN* lungs. Primary type II cells were purified from control and *epiFoxm1-ΔN* lungs followed by flow cytometry-based cell sorting for GFP-positive type II cells. β -actin mRNA was used for normalization. (F). Colocalization studies using lung sections from the patients with IPF identified proSPC-positive (blue) epithelial cells that were also positive for vimentin (green) and Foxm1 (red). Representative sections from three IPF and three normal control samples are shown. Magnification is $\times 2000$. A P -value < 0.05 is shown with (*) and P -value < 0.01 is shown with (**).

suffer from interstitial lung disease and pulmonary fibrosis that is likely mediated by alveolar cell injury (Nogee *et al*, 2001; Thomas *et al*, 2002; Mulugeta *et al*, 2005). *SFTPC* mutations affect pro-SP-C folding in the endoplasmic reticulum (ER), leading to ER stress and activation of the unfolded protein response (UPR) (Mulugeta *et al*, 2005). UPR activation in AECs contributes to lung fibrosis by decreasing cell survival and impairing the ability to repair alveoli after injury. Thoracic irradiation causes diffuse alveolar damage and replacement of normal lung parenchyma with fibrotic tissue that is influenced by inflammation, the proliferation of fibroblasts and tissue remodelling (Johnston *et al*, 1998; Kwa *et al*, 1998; Kong *et al*, 2008). The present study utilized radiation exposure as a source of epithelial injury and demonstrated that aberrant expression of Foxm1 exacerbated pulmonary inflammation and EMT leading to aggravated fibrosis.

Foxm1 induces EMT during pulmonary fibrosis

During fibrotic lung remodelling, fibroblasts produce excessive amounts of ECM proteins. Current evidence supports at least three different cellular origins of fibroblasts contributing to fibrotic lesions. These include proliferation of resident lung fibroblasts, recruitment of circulating progenitor fibrocytes from bone marrow and differentiation of epithelial cells into fibroblasts through EMT (Quan *et al*, 2006; Andersson-Sjoland *et al*, 2008; Andersson-Sjoland *et al*, 2011; Wynn, 2011). There is increasing evidence that EMT contributes to pulmonary fibrosis. Epithelial marker proteins (TTF-1, SP-B and SP-C) and mesenchymal markers (α -SMA and N-cadherin) are colocalized in hyperplastic epithelial cells within fibrotic foci in lung biopsies of IPF patients (Willis *et al*, 2005; Kim *et al*, 2006). In mice, TGF- β - and bleomycin-induced lung injury was associated with increased vimentin and α -SMA staining in cells co-expressing the epithelial cell

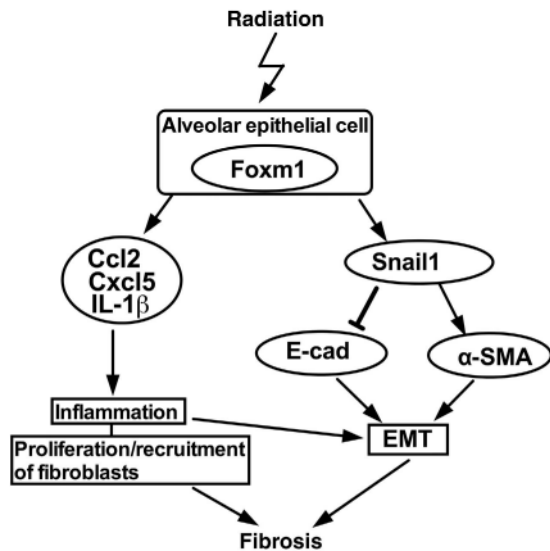


Figure 9 Role of Foxm1 in respiratory epithelial cells during radiation-induced pulmonary fibrosis. Foxm1 transcriptionally activates *Snail1*, a key regulator of EMT. Foxm1 increases *Ccl2*, *Cxcl5* and *IL-1 β* , which promote pulmonary inflammation and proliferation of myofibroblasts. Increased lung inflammation, elevated proliferation of myofibroblasts and EMT of type II cells contribute to lung fibrosis after irradiation.

markers, E-cadherin and proSP-C (Kim *et al*, 2006; Wu *et al*, 2007). The importance of EMT to the pathogenesis is presently controversial. The numbers of cells undergoing EMT during pulmonary fibrosis was estimated to be between 5 and 20% depending on the mouse model or human samples (Kim *et al*, 2006; Kim *et al*, 2009; Tanjore *et al*, 2009; Marmai *et al*, 2011); while other studies did not detect epithelial cells undergoing EMT (Yamada *et al*, 2008; Rock *et al*, 2011). TGF- β induces EMT in A549 pulmonary adenocarcinoma cells and in primary type II epithelial cells *in vitro* (Kasai *et al*, 2005). Epithelial-derived tumour cells undergo EMT *in vitro* and *in vivo*, resulting in increased migration and invasiveness of tumour cells and contributing to tumour metastasis (Kalluri and Weinberg, 2009). In the present manuscript, lineage-tracing studies demonstrated a minor, but consistent, contribution of EMT to pulmonary fibrosis following after thoracic irradiation or bleomycin injury, findings consistent with previous studies (Kim *et al*, 2006; Kim *et al*, 2009; Tanjore *et al*, 2009; Marmai *et al*, 2011). The percentage of cells undergoing EMT was increased in *epiFoxm1- Δ N* mice, demonstrating the profibrotic and cell-autonomous role of Foxm1 in type II epithelial cells during fibrosis. Considering that the SPC-rtTA transgene used in these studies caused mosaic expression of Foxm1- Δ N and did not target all of type II epithelial cells in the lung (Perl *et al*, 2005), the contribution of EMT to fibrosis in *epiFoxm1- Δ N* mice may be underestimated. Since SPC-rtTA does not target integrin α 6 β 4 epithelial progenitor cells (Chapman *et al*, 2011), the potential contribution of these cells to EMT is not clear in our model of radiation-induced pulmonary fibrosis.

Foxm1 is a transcriptional activator of *Snail1*

Loss of E-cadherin is a hallmark of EMT. In our studies, expression of activated Foxm1- Δ N-mutant in type II cells resulted in the loss of E-cadherin after thoracic radiation.

Previous studies demonstrated that the loss of E-cadherin was mediated by the inhibitor activation of the E-box sequences within the E-cadherin promoter (Hennig *et al*, 1996). Snail1 represses E-cadherin expression through direct binding to these E-boxes (Batlle *et al*, 2000; Cano *et al*, 2000). Snail1 was sufficient to induce EMT and the expression of EMT-associated genes, suggesting that Snail1 may act as a switch to promote EMT programme in epithelial cells (Peinado *et al*, 2007). Recent studies demonstrated that ectopic expression of Snail1 promoted EMT in epithelial tumour cell lines (Batlle *et al*, 2000; Cano *et al*, 2000). Consistent with the important role of Snail1 in EMT, the present studies demonstrated that Snail1 was induced during radiation-induced fibrosis. Moreover, the transgenic expression of activated Foxm1- Δ N in pulmonary epithelial cells further increased Snail1 levels in irradiated lungs, implicating Foxm1 in the regulation of Snail1. Conditional deletion of Foxm1 from alveolar type II cells and siRNA-mediated depletion of Foxm1 from cultured A459 cells decreased Snail1 mRNA and protein. Foxm1 was capable of inducing transcriptional activity of the -720 bp Snail1 promoter region in co-transfection experiments. In the context of endogenous *Snail1* promoter, TGF- β increased Foxm1 binding to the Snail promoter DNA as demonstrated by ChIP assay, suggesting that there is a crosstalk between TGF- β signalling and Foxm1 in the regulation of *Snail1* promoter. Interestingly, despite high levels of Foxm1 in A549 cells, they do not express Snail1 protein or undergo EMT in the absence of TGF- β . These data suggest that the endogenous *Snail1* promoter is repressed at basal conditions and that addition of TGF- β alleviates this repression mechanism, allowing Foxm1 to function as transcriptional activator. Published studies demonstrated that MTA2/Mi-2/NuRD repressor complex was bound to the -500 bp *Snail1* promoter region (Fujita *et al*, 2003; Dhasarathy *et al*, 2007). Activation of TGF- β pathway eliminated this repression mechanism (Dhasarathy *et al*, 2007). It is possible that there is a crosstalk between Foxm1 and MTA2/Mi-2/NuRD repressor complex in the regulation of *Snail1* transcription by TGF β (Fujita *et al*, 2003). The finding that Foxm1 directly bound to and increased activity of Snail1 promoter demonstrates that *Snail1* is a direct transcriptional target of Foxm1 providing a mechanism by which Foxm1 induces EMT and potentially contributed to radiation-induced fibrosis. Our results are consistent with previous studies showing an increase in Snail1 in hepatocellular carcinomas expressing Foxm1 in the absence of *p19ARF* gene (Park *et al*, 2011).

While *Zeb2* was reduced in Foxm1-deficient mice and A549 cells, the transcriptional activity of *Zeb2* promoter was not affected by Foxm1 in our studies, suggesting that Foxm1 regulates *Zeb2* indirectly. Alternatively, DNA regulatory sequences that were not included in the *Zeb2* promoter construct may contain Foxm1-responsive elements. Altogether, the direct transcriptional activation of Snail1 by Foxm1 may contribute to the increased severity of pulmonary fibrosis in *epiFoxm1- Δ N* mice after thoracic irradiation (Figure 9).

Foxm1 induces pulmonary inflammation after radiation exposure

Epithelial injury leads to release of cytokines and growth factors that recruit and activate inflammatory cells, including macrophages, neutrophils, lymphocytes and stromal cells. Inflammatory mediators induce fibroblast proliferation, and

play a role in both the initiation and progression of pulmonary fibrosis (Wynn, 2011). An important role of Foxm1 in myeloid inflammatory cells was recently demonstrated for CCL4-mediated liver injury (Ren *et al*, 2010) and BHT-mediated lung injury (Balli *et al*, 2011a). In the present study, we found that expression of Foxm1-ΔN mutant in type II epithelial cells increased radiation-induced lung inflammation, indicated by elevated levels of several inflammatory mediators, including CCL2, CCL5 and IL-1β. CCL2 is a potent mononuclear cell chemoattractant (Rose *et al*, 2003). Therefore, the increased numbers of macrophages and lymphocytes in fibrotic lungs of *epiFoxm1-ΔN* mice may be mediated by the increased production of CCL2. CCL2 was also implicated in fibrosis by its ability to recruit circulating fibrocytes following lung injury (Moore *et al*, 2005). CCL2 expression was increased in the lungs of patients with IPF (Antoniades *et al*, 1992). Increased expression of IL-1β was associated with airway inflammation and remodelling in mice (Kolb *et al*, 2001; Lappalainen *et al*, 2005; Wilson *et al*, 2010), as well as increased levels of CXCL5 was implicated in pulmonary remodelling (Strieter *et al*, 2007). Altogether, the elevated levels of CCL2, IL-1β and CXCL5 could induce lung inflammation and fibroblast proliferation, exacerbating pulmonary fibrosis in *epiFoxm1-ΔN* mice (Figure 9).

In summary, the present findings provide new molecular insights into the pathogenesis of pulmonary fibrosis. Foxm1 staining was increased in AECs within fibrotic lesions in human and mouse lungs. The finding that increased activity of Foxm1 in type II AECs enhanced radiation-induced pulmonary fibrosis in mice and ablation of Foxm1 from type II cells prevented lung fibrosis after thoracic irradiation support the important role of Foxm1 and the respiratory epithelium in the pathogenesis of fibrotic remodelling. We have demonstrated that one of the roles of Foxm1 during lung fibrosis is to induce EMT through direct transcriptional activation of *Snail1* and promote pulmonary inflammation through increased expression of inflammatory mediators (Figure 9).

Materials and methods

Patient samples and transgenic mice

Lung samples from patients with IPF ($n=20$) and control organ donors ($n=6$) were obtained anonymously following lung transplantation at the University of Vienna and the University of Giessen Lung Centre (Plantier *et al*, 2011). Diagnosis was done according to the American Thoracic Society/European Respiratory Society (ATS/ERS) criteria for IPF. The generation of transgenic *SP-C-rtTA^{tg/-}/tetO-Foxm1-ΔN^{tg/-}* (*epiFoxm1-ΔN*) mice and *SP-C-rtTA^{tg/-}/TetO-Cre^{tg/-}/Foxm1^{fl/fl}* (*SP-C-rtTA^{tg/-}/TetO-Cre^{tg/-}/Foxm1^{fl/fl}*) (*SP-C-rtTA^{tg/-}/TetO-Cre^{tg/-}/Foxm1^{fl/fl}*) or *epiFoxm1 KO* mice have been described previously (Kalin *et al*, 2008b; Wang *et al*, 2010). The *epiFoxm1-ΔN* mice were maintained in FVBN genetic background. The *epiFoxm1 KO* mice were maintained in C57BL/6 genetic background. To induce Foxm1-ΔN or Cre expression, mice were given Dox in food chow beginning at 6 weeks of age and kept on Dox for the remainder of study. Dox-treated *tetO-Foxm1-ΔN* littermates lacking the *Spc-rtTA* transgene were used as control for *epiFoxm1-ΔN* transgenic mice. Dox-treated *Foxm1^{fl/fl}* littermates lacking the *SP-C-rtTA*, the *TetO-Cre* or both transgenes were used as controls for *epiFoxm1 KO* mice. Further controls included *SP-C-rtTA^{tg/-}/tetO-Foxm1-ΔN^{tg/-}* or *SP-C-rtTA^{tg/-}/TetO-Cre^{tg/-}/Foxm1^{fl/fl}* mice without Dox treatment. For lineage-tracing experiments, *epiFoxm1-ΔN* mice (*Spc-rtTA/tetO-Foxm1-ΔN*) were crossed with *tetO-Cre/Rosa26-LSL-LacZ* mice to generate quadruple transgenic mice (*epiFoxm1-ΔN:R26R*). Control mice

used were *Spc-rtTA/tetO-Cre/Rosa26-LSL-LacZ* triple transgenic littermates. Animal studies were reviewed and approved by the Animal Care and Use Committee of Cincinnati Children's Hospital Research Foundation.

Pulmonary fibrosis models

For radiation-induced fibrosis studies, the thorax was irradiated with a single dose of 12 Gy (gain-of-function model) or 18 Gy (loss-of-function model) 2 weeks after Dox induction using the (¹³⁷Cs) Mark I-68A irradiator (JL Shepard and Associates, San Fernando, CA) at a dose rate of 0.725 Gy per minute (Chiang *et al*, 2005). Lead shielding was used to protect all parts of the body except the thoracic region. Lungs were harvested 3 and 6 months post radiation. Lungs were isolated, fixed and embedded into paraffin blocks or were used to prepare total lung RNA with RNA-stat-60 (Tel-Test 'B' Inc.). BAL samples were taken as described (Kalin *et al*, 2008a). For bleomycin-induced fibrosis studies, mice were anesthetized with 3% isofluorene, and 0.1 U of bleomycin was instilled intratracheally (Lawson *et al*, 2005). Tissue was harvested 21 days after bleomycin administration. Quantification of lung collagen content was performed using the Sircoll collagen assay following manufacturer's protocol (Biocolor, Carrickfergus, UK).

Immunohistochemistry and immunofluorescence

Paraffin sections (5 μm) were cut and stained with hematoxylin and eosin (H&E) for gross morphology. Immunostaining and immunofluorescence with following antibodies: F4/80 (Caltag Laboratories, Burlingame, CA), pro-SPC (Seven Hills Bioreagents, Cincinnati, OH), α-SMA, β-galactosidase (Abcam, Cambridge, MA), CD3, Vimentin C20 and Foxm1 K19 and H300 (all from Santa Cruz Biotechnology, Santa Cruz, CA), were done as previously described (Balli *et al*; Wang *et al*, 2009). Fibrosis was identified using Masson's trichrome staining (Siemens, following the manufacturers' protocol). Z-stacks of optical sections (0.5 μm each in depth) were captured on a Zeiss Axioplan2 Apotome microscope (Carl Zeiss Inc., Peabody, MA, USA) using an Apotome slider. The images were analysed with the 3D function in the Axiovision software, which projects the images in both the horizontal and vertical orientation. Each optical section was examined for colocalization or overlap of β-gal and vimentin.

TGF-β-induced EMT

A549 cells were cultured in DMEM medium supplemented with 10% FBS. Cells were transfected with Foxm1-specific siRNA using lipofectamine 2000 (Invitrogen, Grand Island, NY) (Wang *et al*, 2009). Forty-eight hours after transfection, 10 ng/ml of rTGF-β (R&D systems, Minneapolis, MN) was added to culture media for an additional 24 h. Protein lysates were isolated for western blot analysis. EMT rescue experiments were performed by co-transfecting CMV-Snail1 (MC201419, Origene, Rockville, MD) with Foxm1 siRNA 48 h prior to TGF-β treatment.

Western blot

Protein extracts were prepared from A549 cells at 48 h following siRNA transfection or mock transfection using RIPA buffer. Western blot analysis was done as described previously (Kalin *et al*, 2006). Primary antibodies were incubated overnight at 4°C in 1% nonfat, dry milk. β-Actin was used as loading control. The following antibodies were used: Foxm1, SMAD2, phospho-SMAD2, ZEB2 (Santa Cruz Biotechnology), phospho-AKT, AKT, JNK1/2, c-JUN, phospho-c-JUN, ZEB1, SNAIL1 (all from Cell Signaling, Danvers, MA), E-CADHERIN, FIBRONECTIN (BD Transduction, San Jose, CA), β-ACTIN and α-SMOOTH MUSCLE ACTIN (Sigma Aldrich, St. Louis, MO). The signals from the primary antibody were amplified by HRP-conjugated anti-mouse IgG (Bio-Rad, Hercules, CA) and detected with Enhanced Chemiluminescence Plus (Amersham Pharmacia Biotech, Piscataway, NJ) followed by autoradiography.

Quantitative real-time RT-PCR

Total lung RNA was isolated from experimental mice at 3 and 6 months post radiation exposure. RNA was analysed 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 combined with the following inven-

toried Taqman gene expression assays: *Ccl2* (Mm99999056_m1), *Cxcl5* (Mm00436451_g1), *IL-1 β* (Mm01336189_m1), *Snail1* (Mm00441533_g1), *Snail2* (Mm00441531_m1), *Twist1* (Mm00442036_m1), *Twist2* (Mm00492147_m1), *Zeb1* (Mm00495564_m1), *Zeb2* (Mm00497193_m1), *Foxf1* (Mm00487497_m1), *E-cadherin* (Mm00486906_m1), *Jnk1* (Mm00489514_m1), mouse *Foxm1* (Mm00514924_m1), human *Foxm1* (Hs00153543_m1), *collagen 1 α 1* (Mm00801666_g1), *collagen 3 α 1* (Mm00802331_m1) and α -smooth muscle actin (Mm00725412_s1). Data are representative of three separate experiments.

Flow cytometry, type II cell isolation and GFP+ sorting

Flow cytometry experiments were conducted as previously described (Ren et al, 2010). Two weeks after bleomycin treatment, lungs were perfused with saline, removed and minced in RPMI 1640 medium containing Liberase Blendzyme 3 (Roche Diagnostics) and DNase 1 (Sigma) for 1 h at 37°C. Cell suspension was passed through 100- μ m and 40- μ m cell strainers. Remaining red blood cells were lysed with ACK lysis buffer (Invitrogen). Cells were permeabilized and stained with fluorescence-labelled antibodies specific for α -SMA and pro-SPC. Staining was performed at 4°C after incubation with FcBlock (eBiosciences) for 30 min. All flow cytometry data were acquired using FACSCanto II (BD Bioscience).

Primary type II lung epithelial cells were isolated as described previously (Rice et al, 2002). GFP-positive (Foxm1- Δ N-expressing) type II cells and GFP-negative control cells were isolated by cell sorting using a 5-laser FACSARIA II flow cytometer (BD Bioscience). RNA was extracted from cells using RNeasy kit following the manufacturer's protocol (Qiagen).

Cotransfection studies and ChIP assay

Seven hundred and twenty base pairs (-686/+61) of the mouse *Snail1* promoter (NC_000068.6) was PCR amplified and cloned into the pGL2-Basic luciferase vector using the following primers: 5'-TCTTACCCCGGGCTTTCCCTCG-3' and 5'-CCGCTCGAGTGGCAGAGCGACATAG-3'. 1.7 kb (-1660/+51) of the mouse *Zeb2* promoter (NC_000068.6) was cloned using the following primers: 5'-TCCCCGGGTGTGTAACCAACTGTGAAAAT-3' and 5'-CCGCTCGAGATTATACCTTGAAGTCTCCGCA-3'. 1.3 kb (-71/-1389) of the mouse *Ccl2* promoter (NC_000077.5) was cloned using the following primers: 5'-CCCGGGTCTGTGTAAGCCATACAATGC-3' and 5'-CCGCTCGAGAAGGAGTAGCATCACCTGGATAAG-3'. 1.14 kb (-1078/+63) of the mouse *Cxcl5* promoter (NC_000071.5) was cloned using the following primers: 5'-CGACGCGTCGCCACTGTCAAATCCCTATCTGG-3' and 5'-CCGCTCGAGATGGTGTCTCAAACCACTGC-3'. SRE-luciferase construct was previously de-

scribed (Lange et al, 2009). A549 or U2OS cells were transfected with CMV-Foxm1 (Kim et al, 2005) or CMV-empty plasmids as well as with luciferase constructs. CMV-Renilla was used as an internal control. Dual luciferase assay (Promega, Madison, Wisconsin) was performed 24 h after transfection. Site-directed mutagenesis kit was used to mutate the Foxm1-binding site in *Snail1* promoter following the manufacturers' protocol (Invitrogen). Mutation of predicted Foxm1-binding sites was done using the following primers: 5'-TAGTATTCGAATCCTCAGGTCGATACGTCGTCTCT-3' and 5'-GAGAGA GACAGACGTATCGACCTGAGGATTCGAATACTA-3' and contained the random sequence AGGTCGATACG (Littler et al, 2010). ChIP was performed as described previously (Balli et al, 2011b). Briefly, A549 cells were treated with TGF- β for 24 h. Cells were crosslinked by addition of formaldehyde and sonicated to create DNA fragments between 500–1000 bp in size. Protein/DNA complexes were used for immunoprecipitation with Foxm1 antibodies (C20, Santa Cruz Biotechnology) or control rabbit serum. Reverse crosslinked ChIP DNA samples were subjected to real-time PCR, using primers specific to human *Snail1* 5'-TTCAACGAACTCTAACCAAGTCC-3' and 5'-TGAGGGAGACAGACGAAGTAAACAG-3'.

Statistical analysis

We used Microsoft Excel Program to calculate s.d. and statistically significant differences between samples using the Student *t*-test. *P*-values <0.05 were considered statistically significant.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: DB, VU, YZ, I-CW and AJM performed experiments. TVK, DB, JAW and VVK designed and analysed experiments. TVK, DB, JAW and VVK wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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