AJRCMB Articles in Press. Published on 29-June-2015 as 10.1165/rcmb.2014-0461OC

Contribution of fetal but not adult pulmonary mesothelium to mesenchymal lineages in lung homeostasis and fibrosis

running title: PMCDCs in lung development and fibrosis

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Conception and design: AVG, BZ, WTP; Data collection: AVG, SMS, LBH, JHO, CG, BZ; Analysis and interpretation: AVG, SMS, BZ, WTP; Drafting the manuscript for important intellectual content: AVG, WTP

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[†]WTP was supported by funding from the National Institutes of Health (R01HL094683 and U01HL100401).

Abstract

The lung is enveloped by a layer of specialized epithelium, the pulmonary mesothelium. In other organs, mesothelial cells undergo EMT and contribute to organ stromal cells. The contribution of pulmonary mesothelial cells (PMCs) to the developing lung has been evaluated with differing conclusions. PMCs have also been indirectly implicated in lung fibrosis in the progressive, fatal lung disease idiopathic pulmonary fibrosis (IPF). We used fetal or postnatal genetic pulse-labeling of PMCs to assess their fate in murine development, normal lung homeostasis, and models of pulmonary fibrosis. We found that most fetal PMC-derived mesenchymal cells (PMCDCs) expressed markers of pericycytes and fibroblasts, only a small minority expressed smooth muscle markers, and none expressed endothelial cell markers. Postnatal PMCs did not contribute to lung mesenchyme during normal lung homeostasis or in models of lung fibrosis. However, fetal PMCDCs were abundant and actively proliferating within fibrotic regions in lung fibrosis models, suggesting that they actively participate in the fibrotic process. These data clarify the role of fetal and postnatal PMCDCs in lung development and disease.

Key words

Idiopathic pulmonary fibrosis; Pulmonary mesothelium; Epithelial to mesenchymal transition.

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Introduction

Fibrosis is a common component of disease processes of many organs. Fibroblast expansion and activation to form myofibroblasts leads to increased deposition of extracellular matrix, which distorts normal organ architecture and function. In the lung, obliterative fibrosis is the hallmark of interstitial lung diseases, the most common of which is idiopathic pulmonary fibrosis (IPF), a progressive, fatal lung disease that affects about 0.04% of the population (1). The origin of fibroblasts and myofibroblasts that secrete extracellular matrix proteins in interstitial lung diseases such as IPF is incompletely defined. Because the origin of these lineages cannot be definitively evaluated in human IPF, investigators have turned to animal models, which mimic some but not all features of the human disease (2).

Among the proposed sources has been the mesothelial lining of the lung (3). The mesothelium is a polarized epithelial sheet that covers the surface of most visceral organs and is required for their normal development. During organ development, the mesothelium undergoes epithelial to mesenchymal transition (EMT) to form mesenchymal cells that contribute to organogenesis (4,5). Some of these mesenchymal cells retain their plasticity and become resident mesenchymal stem cells (6), while others differentiate into stromal cell types such as fibroblasts, smooth muscle cells (SMCs), and endothelial cells (ECs) (4,5,7). This EMT process becomes quiescent at the completion of organogenesis, but can be reactivated by disease processes. For example, myocardial infarction partially reactivates EMT and causes thickening of the cardiac mesothelium (known as the epicardium), resulting in formation of fibroblasts, myofibroblasts, and SMCs that remain within the thickened epicardium and contribute to the myocardial injury response (8).

The developing lung is also enveloped within a mesothelial lining, the visceral pleura, composed of pulmonary mesothelial cells (PMCs). These cells, like other mesothelial cells, express the transcription factor *Wt1*, and *Wt1*-driven expression of constitutive Cre or inducible CreERT2 alleles has been used to trace the fate of PMCs during lung development, postnatal lung homeostasis, and lung disease, with conflicting results (3,9-11). During lung development, one study using a constitutive Wt1-Cre transgene reported that PMCs contribute to vascular SMCs (11). A second study using a different constitutive Wt1-Cre transgene showed that PMCs contribute to a significant

fraction of pulmonary endothelial cells, $CD34^+$ fibroblasts, bronchial SMCs, and tracheal and bronchial cartilage, as well as vascular SMCs (9). A third study used pulse mesothelial labeling of the fetal lung mesothelium to show that its differentiation is restricted to subpopulations of bronchial and vascular SMCs and $desmin^+$ fibroblasts, while differentiation into ECs was not reported (10). Thus while several studies concur that PMCs form mesenchymal cells during lung development via EMT, the fate of these mesenchymal cells is uncertain.

PMCs have also been proposed to contribute to a subset of myofibroblasts in IPF (3). In the early stages of IPF, fibrosis is localized to distal subpleural regions and progresses proximally. Cultured PMCs exposed to TGFβ1 (12), a growth factor highly upregulated in IPF, undergo phenotypic changes consistent with EMT (13). PMCs labeled in adulthood with $Wt1^{CreERT2}$ appeared to migrate into the sub-mesothelial region in the four hours immediately after TGFβ1 stimulation (3). However, these studies did not determine the extent to which PMC-derived fibroblasts or myofibroblasts contribute to lung fibrosis. In this study we assessed the fate of PMC-derived cells during lung development, homeostasis, and lung fibrosis in murine lung fibrosis models.

Materials and methods

Please see the Supplmentary Materials and Methods for additional details.

Mice

Studies were performed under protocols approved by the Boston Children's Hospital Institutional Animal Care and Use Committee. $Wt1^{CreERT2}$, $Wt1^{GFPCre}$, $Rosa26^{mTmG}$, and $Rosa26^{fsTdTomato}$ mouse lines (Jackson labs) were described previously (5,14,15). Tamoxifen was administered by gavage at 0.12 mg/g body weight (fetal labeling at E10.5) or 0.1 mg/g body weight (postnatal labeling, on postnatal days 4, 5, and 6)

Models of lung fibrosis

We induced pulmonary fibrosis by chronic intranasal bleomycin (modified from ref. 16) or intratracheally administered activated TGFβ1 adenovirus (12). We obtained adenovirus expressing activated TGFβ1 from J. Gauldie (17).

Histology

Lungs were instilled with 4% PFA, fixed overnight, and embedded in OCT. Cryosections were stained with picrosirius-red and fast green. Immunostaining was performed with antibodies listed in the Supplementary Materials and Methods and

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imaged using a confocal microscope. At least 3 mice were observed per group, and for each mouse we analyzed at least 10 sections from each of 2 macroscopically affected lobes.

Results

Fate of PMC-derived cells in the developing lung

We used Cre-loxP lineage tracing technology to study the fate of PMC-derived cells in the developing lung (Supplementary Fig. 1A). Consistent with prior reports (3,9-11), lung WT1 and *Wt1*-driven Cre expression was confined to the mesothelium (Fig. 1A and Supplementary Fig. 1B). Pulse activation of $Wt1^{CreERT2/+}$ by administration of tamoxifen at E10.5 selectively and indelibly recombined Cre-activated reporter alleles, labeling lung mesothelium and its derivatives (Fig. 1B and Supplementary Fig. 1C). We used the highest tamoxifen dose that was compatible with survival of embryos to term, and labeled the large majority (81 ± 1.7%) of mesothelial cells. Pulmonary mesothelium-derived cells (PMCDCs) were found within the lung mesenchyme (Fig. 1B), consistent with having undergone an epithelial to mesenchymal transition as reported previously (9-11). The entry of fetal PMCs into lung mesenchyme was confirmed using cultured fetal lung explants, where mesothelium was selectively labeled using the cell tracking dye CMFDA (Supplementary Fig. 2). In the heart, mesothelial EMT required *Wt1* (18). Interestingly, PMC-derived mesenchymal cells in the lung formed in the absence of *Wt1* (Fig. 1B, right panel), suggesting that regulation of this process differs between heart and lung.

Several studies have examined the fates of PMCDCs, with divergent conclusions (3,9-11). To re-evaluate this question, we pulse-labeled pulmonary mesothelium by administering tamoxifen at E10.5 to $Wt1^{CreERT2/+}$::$R26^{mTmG}$ embryos. We raised embryo to adulthood and analyzed lungs using confocal microscopy (Fig. 1C and Supplementary Fig. 3). Labeled PMCDCs were distributed throughout the mesenchyme of the adult lung, but not in the lung epithelia. PMCDCs, marked by the mGFP lineage tracer, were quantitatively analyzed for co-expression of differentiation markers of ECs (PECAM1), SMCs (SM-MHC; SMA; Calponin), and lung epithelium (SPC; AQP5; Fig. 1C-D; Supplementary Fig. 4A). A minority of cells were classified as "ambiguous" because we could not resolve if the PMCDC was expressing the marker or adjacent to the expressing cell (Supplementary Fig. 4B). Lung epithelium did not express the mGFP

lineage tracer, although a small fraction of PMCDCs (2.3%) were ambiguous for expression of SPC. Although a small fraction of ECs in heart and gut derive from mesothelium, we did not observe this in the lung. A small fraction (~15%) of $mGFP^+$ cells were intimately related to bronchial or vascular SMCs. In some cases the PMCDCs clearly expressed the SMC lineage marker, but a greater fraction were ambiguous as we could not resolve co-expression versus adjacency (Supplementary Fig. 4B and Fig. 1D). Thus, whereas prior studies reported that pulmonary mesothelium differentiates into subsets of lung endothelial and SMCs, our study shows that the large majority of PMCDCs do not acquire these fates during normal lung development and homeostasis.

We investigated other potential PMC fates using this lineage tracing system. Most $mGFP^+$ cells co-expressed PDGFRβ or PDGFRα (Fig. 1C-D). PDGFRα is expressed by fibroblasts, and in the heart the fetal mesothelium (epicardium) is a major source of fibroblasts (7). PDGFRβ is a marker of pericyctes, and many $mGFP^+$ cells also co-expressed the pericycte marker NG2. PMCDC differentiation into NG2-expressing cells was confirmed with an alternate Cre reporter line, $R26^{fsTdTomato}$, which expresses RFP after Cre recombination. Consistent with our observations using $R26^{mTmG}$ reporter, most PMCDCs, marked by RFP, co-expressed pericyte marker NG2, whereas few co-expressed SMA and none co-expressed PECAM (Supplementary Fig. 5).

Together, our data show that the majority of fetal PMCDCs differentiate into mesenchymal cells that express PDGFRα or PDGFRβ/NG2 in the adult lung, suggestive of their differentiation into fibroblasts and pericytes, respectively.

PMCDCs do not contribute to lung parenchyma in the normal postnatal lung

Next, we asked if postnatal PMCs undergo EMT and make cellular contributions to the homeostasis of the postnatal lung. To address this question, we labeled lung mesothelium in newborn $Wt1^{CreERT2/+}$::$Rosa26^{mTmG}$ mice by administering tamoxifen intragastrically at P4-P6. This selectively and efficiently labeled PMCs (Supplementary Fig. 1C; 88.6 ± 6 % of mesothelial cells labeled). We then analyzed the mice's normal lungs at 8 to 20 weeks. Descendants of neonatal PMCs, genetically labeled by GFP expression, remained confined to the mesothelium (Fig. 2). GFP^+ cells were not observed within the lung parenchyma, indicating that EMT of PMCs ceases during postnatal life and is not involved in normal lung homeostasis. However, the

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mesothelium may still be an important signaling center that regulates postnatal lung maturation and function through paracrine factors secretion, by analogy epicardium in heart development (8,18).

Postnatal PMCDCs do not contribute to lung parenchyma in lung fibrosis models

To examine the contribution of adult PMCDCs to pathological lung fibrosis, we used two independent models of lung injury, which recapitulate some features of IPF. First, we used chronic instillation of bleomycin (16). Following bleomycin treatment, we observed gross distortion of lung morphology from atelectasis and scarring (Fig. 3A), consistent with substantial lung injury. Histological sections revealed "honeycombing" characteristic of pulmonary fibrosis, along with cellular infiltration and collagen deposition (Fig. 3B).

Second, we used intratracheal activated TGFβ1 adenovirus administration (12,17). TGFβ1-induced fibrosis may be important for IPF pathogenesis (19). Moreover, TGFβ1 induced EMT of PMCs in culture (13) and stimulated PMCDCs to migrate into the lung in vivo (3). Intratracheal TGFβ1 adenovirus administration robustly stimulated pulmonary fibrosis, scarring, and collagen deposition in TGFβ1 adenovirus-treated lungs (Fig. 3).

To evaluate the cellular contribution of postnatal PMCs to lung fibrosis in these models, we labeled PMCs prior to induction of lung injury by treating P4 $Wt1^{CreERT2/+}$::$Rosa26^{mTmG}$ mice with tamoxifen. When the mice reached adulthood, we established pulmonary fibrosis by bleomycin treatment, and examined the contribution of PMCDCs to the cellular infiltrate found within the parenchyma of the fibrotic lung. Bleomycin-treated lung mesothelium had thickened foci that were multiple cell layers thick (Fig. 4A, columns 3-4), reminiscent of, but less pronounced than, epicardial thickening observed after myocardial infarction (8). PMCDCs, marked by GFP expression, were not found within the lung parenchyma, even in areas with extensive infiltration by $vimentin^+$ or SMA^+ fibroblasts/myofibroblasts (Fig. 4A). In 18 sections examined from lungs of 3 mice, GFP^+ cells had detached from the lung mesothelium and migrated into the parenchyma. Thus, in this fibrotic injury model, cells from the postnatal pulmonary mesothelium do not make a significant contribution to the fibroblast/myofibroblast lineage.

We evaluated the cellular contribution of postnatal PMCs to lung fibrosis in the

independent TGFβ1-induced fibrosis model. As in the bleomycin injury model, postnatal PMCs did not make a cellular contribution to lung fibrosis induced by TGFβ1, including regions with dense infiltration by vimentin$^+$ or SMA$^+$ fibroblasts/myofibroblasts (Fig. 4B). Interestingly, TGFβ1 did not induce mesothelial thickening that was observed in the bleomycin injury model (Fig. 4B, column 3).

Together our data indicate that postnatal PMCDCs do not contribute to the cells engaged in pulmonary fibrosis in adult lung injury by bleomycin or TGFβ1.

Fetal PMCDCs participate in adult lung fibrosis

Our data suggested that fetal PMCs differentiate into adult lung mesenchymal cells that express PDGFRα or PDGFRβ. Therefore we hypothesized that fetal PMCDCs contribute to adult lung fibrosis. We tested this hypothesis by generating adult mice in which fetal PMCDCs were genetically labeled and then subjecting them to bleomycin- or TGFβ1-induced lung injury. We found fetal PMCDCs within areas of lung fibrosis in both injury models, and these PMCDCs expressed vimentin, SMA, and collagen, markers of fibroblasts and myofibroblasts (Fig. 5A-B and Supplementary Fig. 6). This result was independent of the Cre-activated lineage tracing allele used, as we observed it using both the Rosa26^{mTmG} (Fig. 5 and Supplementary Fig. 6) and Rosa26^{fsTdTomato} reporters (Supplementary Fig. 7). In control experiments, we established that cells are not labeled during normal lung development or after lung injury in the absence of tamoxifen treatment (Supplementary Fig. 8), excluding the possibility that lineage traced cells arise from "leakiness" of the genetic lineage tracing system.

We asked if lung injury stimulates expansion of PMCDCs. Following fetal pulse labeling of PMCs and adult lung injury by either bleomycin or TGFβ1, we marked proliferating cells by weekly injection of the S-phase marker EdU. We then measured PMCDC proliferation as the fraction of these cells that incorporated EdU into their DNA. Both bleomycin- and TGFβ1-induced lung fibrosis increased the cell cycle activity of PMCDCs (Fig. 6).

Together these observations show activation and expansion of fetal PMCDCs in pulmonary fibrosis in these models.

Discussion

This study defined the contribution of PMCs and their derivatives to lung mesenchymal cells in development, homeostasis, and fibrotic disease. During lung

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development, PMCs actively formed mesenchymal cells, consistent with prior studies. However, our lineage tracing data indicates that the predominant fate of PMCDCs differs from that reported previously (9-11). We show that this process becomes quiescent postnatally and is not reactivated in two different lung injury and fibrosis models. Our observations exclude a substantial role for PMCs in the formation of lung fibroblasts or myofibroblasts in these disease models, unlike recent reports that reached the contrary conclusion using less definitive lineage tracing approaches (3,13).

In the developing lung, PMCs form mesenchymal cells that migrate into the lung parenchyma. *Wt1* was dispensable for formation and migration of PMCDCs within the lung, whereas this EMT process required *Wt1* in the heart. Sonic hedgehog signaling was previously shown to be required for EMT of PMCs but not epicardium (10). Together, these studies indicate that mesothelial EMT is regulated through organ-specific mechanisms.

Several studies have come to different conclusions regarding the fate of PMCDCs in the lung (9-11). We evaluated this question using an inducible lineage tracing system similar to the one used by Dixit and colleagues (10). A common finding of our study and the prior reports is that a minor subset of pulmonary vascular SMCs were labeled by *Wt1*-driven Cre, indicating that they arise from PMCs. However, PMCs form only a small fraction of pulmonary vascular SMCs, as most of these cells were not labeled by $Wt1^{CreERT2}$. This situation is unlike the heart, where most vascular SMCs originate from the epicardium (4). Although Que and colleagues did not observe *Wt1*-driven Cre labeling of bronchial SMCs (11), we observed that a small subset of bronchial SMCs descended from PMCs, as did Cano et al. (9) and Dixit et al. (10). Whether these PMC-derived SMCs have distinct properties is an interesting area for future study. In addition to SMCs, PMCs also contributed to 5-15% of ECs in the heart and gut (4). Cano et al. found that a constitutive *Wt1*-Cre transgene labeled 15-25% of ECs in the lung (9). However, using the inducible $Wt1^{CreERT2}$ labeling system, we did not observe significant contribution of fetal PMCDCs to the endothelial lineage. This difference might be due to incomplete labeling of a subset of PMCs that contributes to the endothelial lineage, or it might reflect capture of non-mesothelial (and even endothelial) populations by the constitutive Cre, as discussed (8).

In our study, most fetal PMC-derived mesenchymal cells did not express SMC or EC

markers, even when we chased these cells into the adult lung. Rather, a majority of PMCDCs expressed PDGFRβ/NG2 or PDGFRα. PDGFRβ and NG2 are both expressed by pericytes, suggesting that a subset of PMCDCs differentiate into pericytes. However, neither PDGFRβ nor NG2 are specific pericyte markers, and additional experiments will be needed to further define the characteristics of this fetal PMCDC population. PDGFRα is expressed by fibroblasts, and recent studies show that fetal epicardial cells become PDGFRα^{+} fibroblasts in the adult heart (7). Indeed, we observed that fetal PMCDCs contributed to fibrotic regions during adult pulmonary fibrosis, consistent with their contribution to the resident pulmonary fibroblast lineage. Additional PMCDCs may represent a pulmonary progenitor lineage, akin to mesothelium-derived mesenchymal stem cells in the heart (6).

In the postnatal lung, we did not detect significant ongoing contribution of PMCs to the lung parenchyma. Similarly, epicardium does not significantly form derivatives that migrate within the postnatal myocardium, in either heart homeostasis or injury (7,8). However, in the heart manipulation of paracrine signaling pathways promotes amplification, mobilization, and guided differentiation of epicardial progenitors so that they more robustly participate in cardiac repair and regeneration (20). In the future it will be interesting to determine whether similar interventions could be used to drive PMCs to therapeutically participate in lung injury responses.

It has been proposed that postnatal PMCs contribute to the pathogenesis of pulmonary fibrosis by undergoing EMT to form fibroblasts or myofibroblasts (21). Evidence to support this hypothesis has come largely from analysis of cultured PMCs (13,22) and from analyses of marker gene expression in histological sections of human IPF samples and murine models (22). More recently, studies pointed to migration of postnatal PMC-derived cells into the lung parenchyma in vivo during a four hour observation period after TGFβ1 stimulation (3). However, the extent or long term contribution of postnatal PMCDCs to the fibroblast/myofibroblast lineages was not evaluated. Here, we show that postnatal PMCDCs make little to no long term contribution to these lineages in two independent murine lung fibrosis models. This result does not exclude a role of PMCs to participate in the lung injury response through paracrine signaling, as we observed for the epicardium in cardiac injury models (8).

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To the extent that the mouse model recapitulates some features of human IPF, our data suggest that EMT of postnatal PMCs is unlikely to significantly contribute to IPF pathogenesis. However, a limitation of our study is that the mouse models of IPF are imperfect and results cannot be extrapolated with certainty to the human disease.

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Figures

Fig. 1. Fetal PMCs in the developing lung. A. $WT1^{CreERT2}$ was selectively expressed in PMCs at E10.5. ESR is the estrogen hormone receptor, which is fused to Cre in CreERT2. L, lung. **B.** Genetic lineage tracing of PMCs and PMCDCs, GFP-labeled by $Wt1^{CreERT2}$ activated by tamoxifen at E10.5. PMCDCs were observed within lung mesenchyme at E13.5. $Wt1^{CreERT2/CreERT2}$ PMCDCs, which lack functional WT1, continued to enter lung mesenchyme. **C.** Fate of fetal PMCDCs in the adult lung. PMCDCs were labeled by tamoxifen administration at E10.5. Mice were raised to adulthood, and lungs were examined by immunostaining with markers characteristic of major lung lineages. **D.** Quantitation of the fraction of fetally labeled PMCDCs that co-expressed the indicated lineage markers in the adult lung. Bar = 100 μm (A-B), 10 μm (C).

Fig. 2. PMCs in postnatal lung homeostasis. A. PMCs were labeled by tamoxifen administration at postnatal day (P) 4. Lungs, analyzed at P13, showed selective labeling of PMCs. **B.** P4-labeled lungs were analyzed at 30 weeks of life. The PMC label was retained in the mesothelium. Labeled cells were not found within the lung mesenchyme. Bar = 100 μm.

Fig. 3. Models of interstitial lung fibrosis. Chronic bleomycin or TGFβ1 adenovirus efficiently induced pulmonary fibrosis. **A.** Whole mount images showing atelectasis and scarring in bleomycin-treated lungs, compared to saline-treated controls. LL, left lobe. CrL, cranial lobe. CaL, caudal lobe. **B-C.** Picrosirius-red staining revealed collagen deposition, cell infiltration, and honeycombing after bleomycin (B) or TGFβ1 (C) treatment. Bar = 1 mm (A), 100 μm (B-C).

Fig. 4. Bleomycin induced infiltration with myofibroblasts but did not stimulate formation of mesenchymal cells from postnatal PMCs. Neonatal administration of tamoxifen labeled postnatal PMCs with GFP. Pulmonary fibrosis was then induced with bleomycin (A) or TGFβ (B). Infiltrating myofibroblasts strongly expressing vimentin or SMA did not co-express GFP. Boxed areas are magnified in adjacent columns. In

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bleomycin treatment, the mesothelium became focally thickened (boxed areas, panel A column 3). Red lines (panel A column 4) indicate multiple layers of mesothelial cells. Focal mesothelial thickening was not observed with TGFβ1 treatment (panel B column 3). Bar = 100 μm.

Fig. 5.Fetal PMCDCs participate in adult lung fibrosis. Fetal PMCDCs were labeled by treatment of $Wt1^{CreERT2/+}$::$Rosa26^{mTmG}$ with tamoxifen at E10.5. Adult mice were then treated with bleomycin (A) or TGFβ1 (B) to induce lung fibrosis. Lung cryosections were imaged to identify GFP-labeled PMCDCs that co-expressed markers of fibroblasts and myofibrobasts (SMA, collagen II, and vimentin). Fibrotic regions contained PMCDCs that co-expressed these fibrosis markers. Bar = 100 μm (white), 10 μm (teal).

Fig. 6. Fibrotic injury stimulated fetal PMCDC proliferation in the adult lung . $Wt1^{CreERT2/+}$::$Rosa26^{mTmG}$ (A-B) or $Wt1^{CreERT2/+}$::$Rosa26^{fsTdTomato}$ mice (C-D) were treated with tamoxifen at E10.5. After reaching adulthood, the mice were treated with bleomycin (A,C) or TGFβ1 (B,D) to induce lung fibrosis. Proliferating cells were labeled with EdU, injected IP weekly for 8 (bleomycin; A, C) or 4 weeks (TGFß1; B, D) respectively. Yellow arrowheads highlight co-localization of EdU in genetically labeled PMCDCs (GFP^+ in A-B; RFP^+ in C-D). Bar = 100 μm (white), 10 μm (teal).

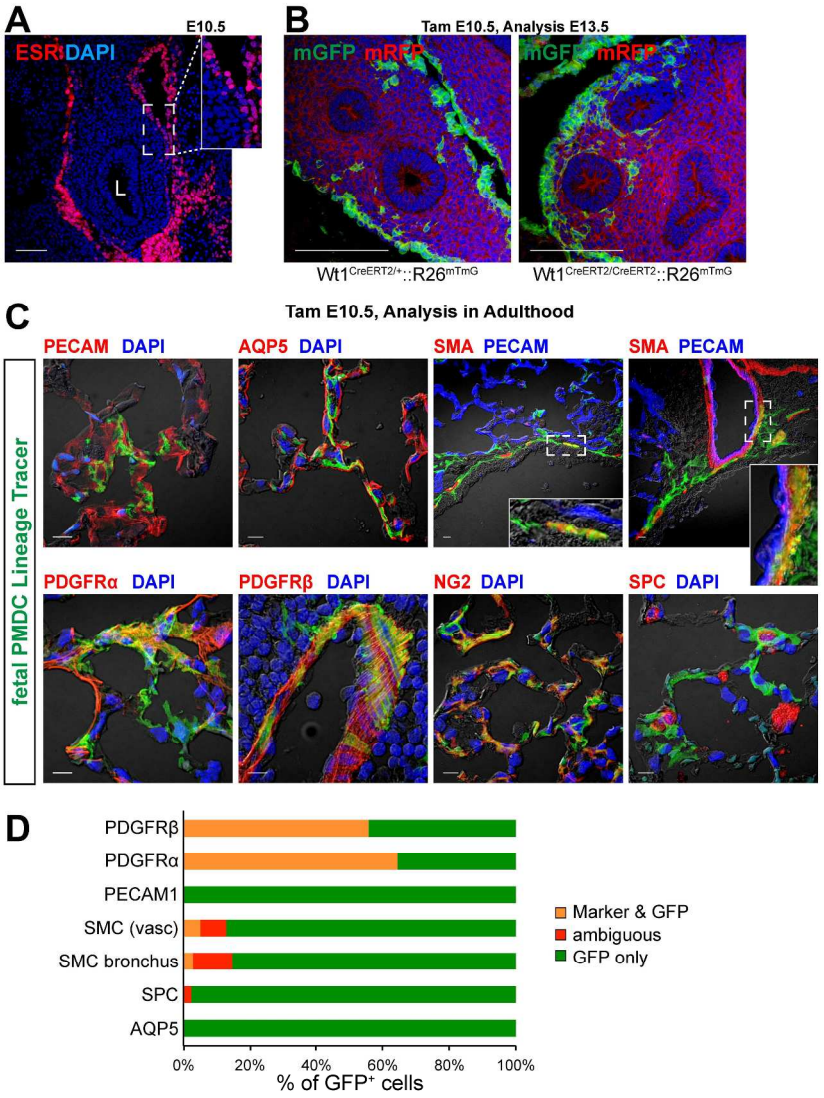

Fig. 1, von Gise et al.

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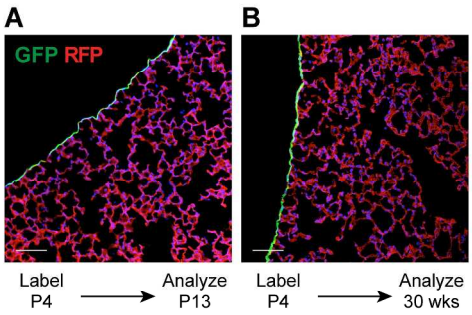

Fig. 2, von Gise et al.

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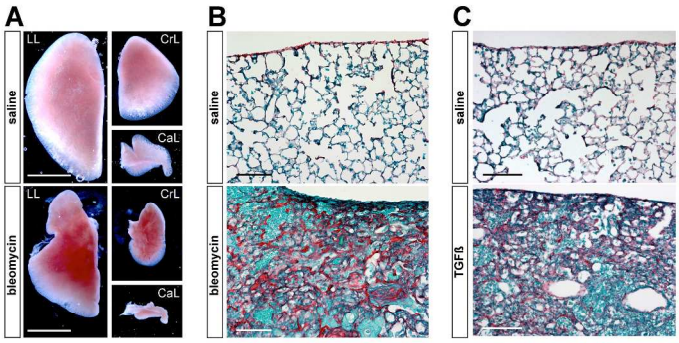

Fig. 3. von Gise et al.

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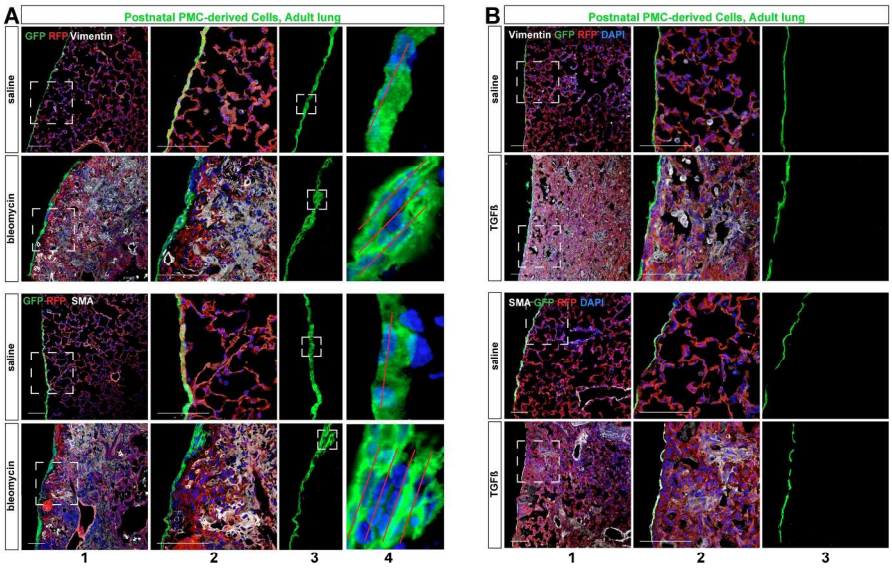

Fig. 4, von Gise et al.

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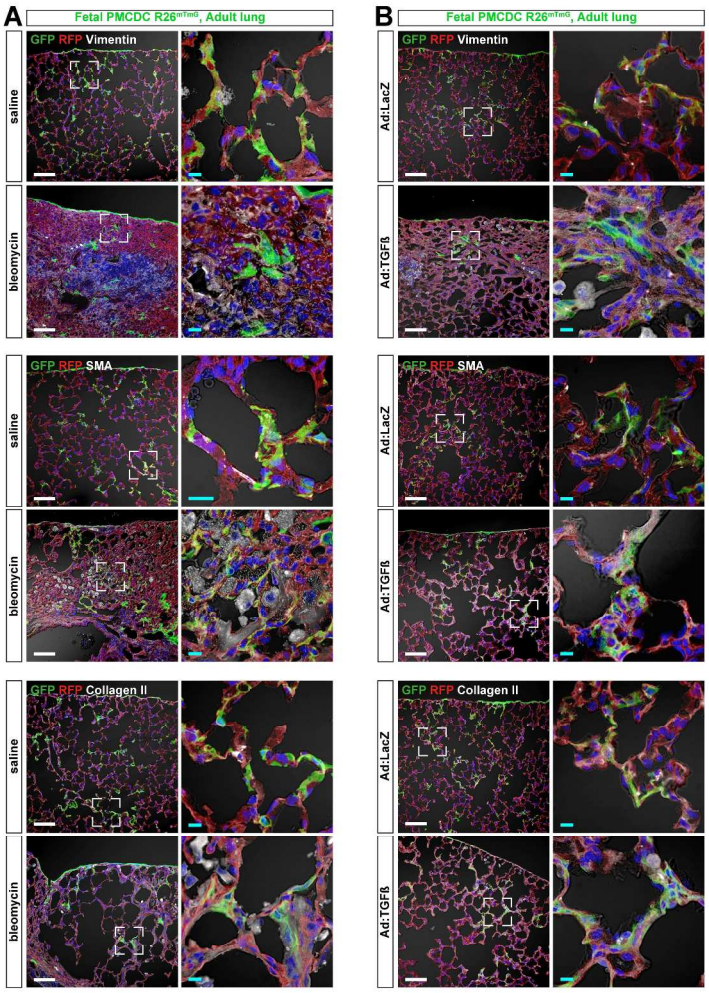

Fig. 5. von Gise et al.

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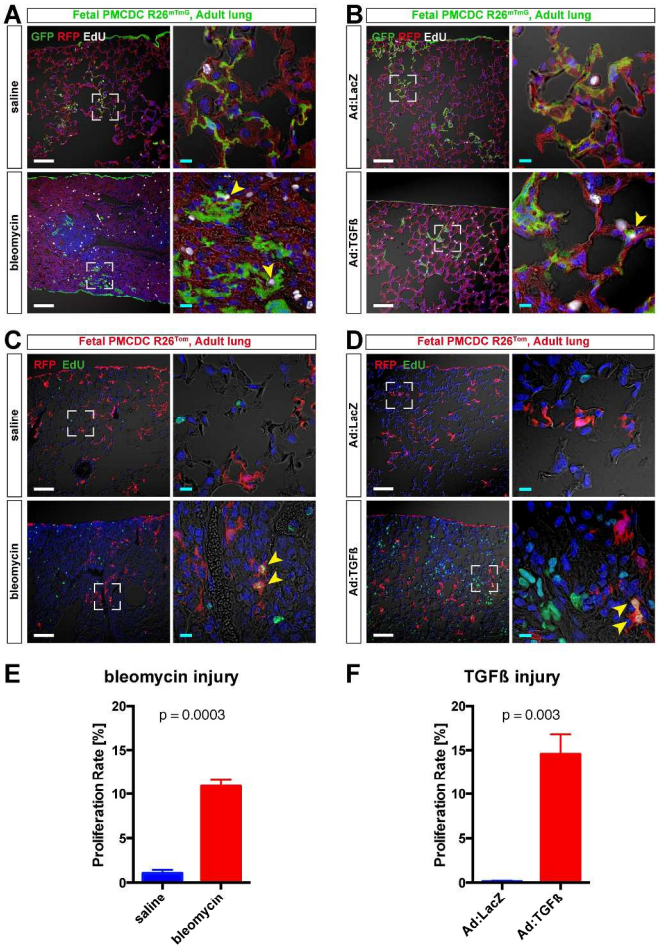

Figure 6, von Gise et al.

297x420mm (300 x 300 DPI)

Supplementary Materials and Methods

Mice

For postnatal analysis of fetuses labeled with tamoxifen, embryos were delivered by cesarian section on E18 and given to foster dams, due to tamoxifen-induced dystocia.

Lung explant culture

Lungs were dissected from E11.5 embryos and incubated for 30 seconds at 37 °C with 1 μM CMFDA in DMEM/F12 without serum. Lungs were washed twice in DMEM/F12, placed on 0.4 μm Corning Transwell inserts, and cultured in wells containing DMEM/F12 supplemented with 10% FBS and antibiotic-antimycotic (Life Technologies). Explants were cultured at 37 °C, 5% CO2 for 48 hours.

Models of lung fibrosis

For chronic bleomycin injury, 20-30 week old mice were anesthetized with isoflorane. Bleomycin (1.5 U/kg BW) was applied to the nostrils for spontaneous aspiration. Lungs were harvested 2 weeks after an eighth biweekly dose.

Activated TGFβ1-expressing adenovirus was administered to induce pulmonary fibrosis. 20 week old mice were anesthetized with isoflorane, intubated, and treated intratracheally with 2.5 x 10^7 pfu of Ad:TGFβ1 or Ad:LacZ control. Lungs were collected 4 to 8 weeks after adenovirus application.

Tissue fixation and Immunohistochemistry

Lungs were perfused with PBS via the right ventricle, followed by intubation and instillation of 4% PFA at 20 mbar. The trachea was clipped, the lungs were recovered from the chest and fixed overnight in 4% PFA at 4°C, and then washed in PBS and embedded in OCT. Immunohistochemistry was performed using the following primary antibodies: Vimentin (R&D Systems, Cat# MAB2105), PECAM1 (Santa Cruz Biotechnology, Cat# sc-17320), AQP5 (Abcam, Cat# ab78486), SMA (Sigma, Cat# A5228), PDGFRa (Biolegend, Cat# 135909), PDGFRβ (Biolegend, Cat# 136009), SPC (Santa Cruz Biotechnology, Cat# sc-7006), FLK1 (R&D Systems, Cat# BAF644), EPHB4 (R&D Systems, Cat# AF446), SOX2 (Santa Cruz Biotechnology, Cat# sc-17320), and NG2 (Millipore, Cat# AB5320). EdU labeling (200 μg subcutaneously) was started 5 days after adenovirus or 7 days after bleomycin application and continued weekly until sacrifice. EdU was detected with the Click-iT® EdU Imaging Kit (Life

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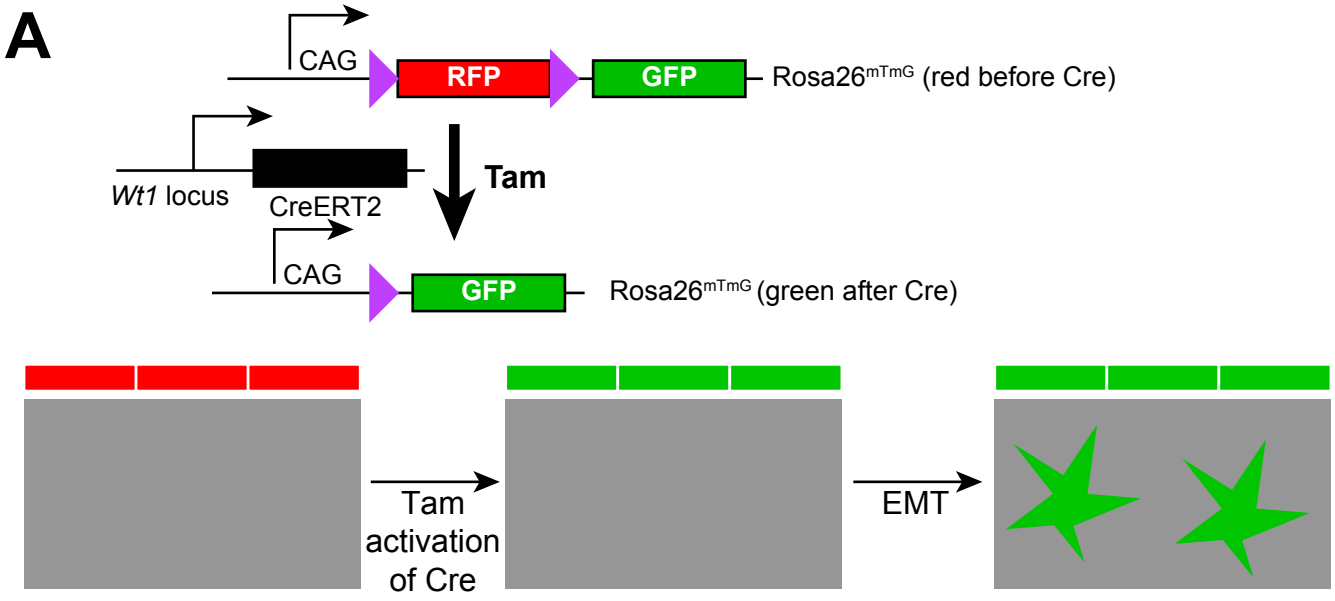
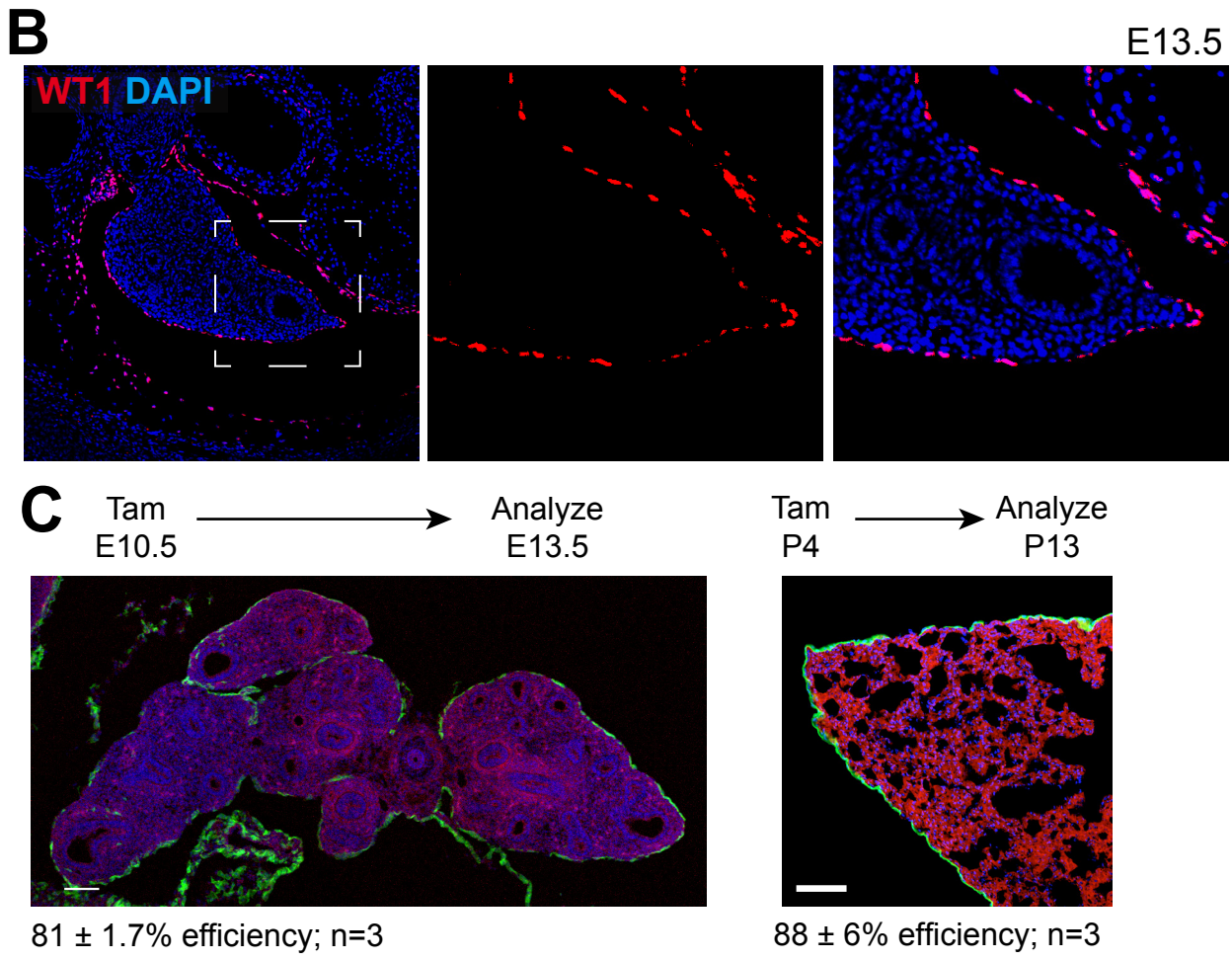
Techologies). Immunostained sections were imaged using an FV1000 confocal microscope (Olympus).





Supplementary Fig. 1. Genetic fate mapping of PMCDC fate. A. Schematic of genetic lineage tracing strategy. Cre confined to the mesothelium irreversibly labels PMC cells. Later in development, labeled cells with mesenchymal morphology are found within the lung, indicative of their origin from PMCs by EMT. **B.** Expression of Wt1 is confined to the lung mesothelium. **C.** Pulse-labeling with tamoxifen efficiently labeled PMCs. Left, fetal labeling. Right, postnatal labeling. Bar = 100 μm.

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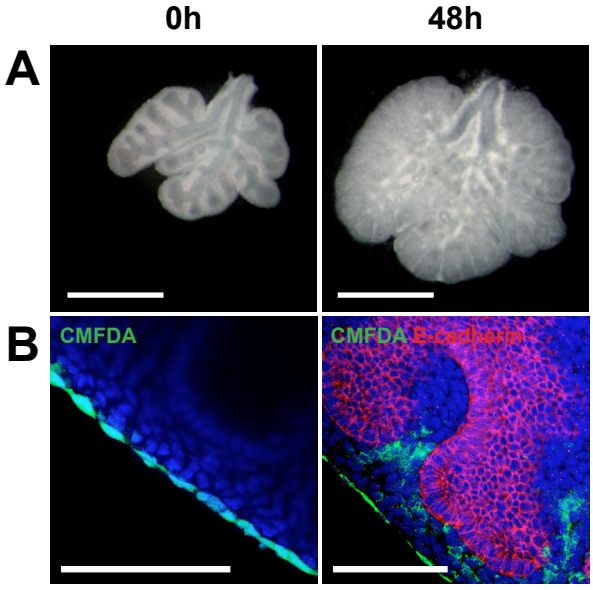
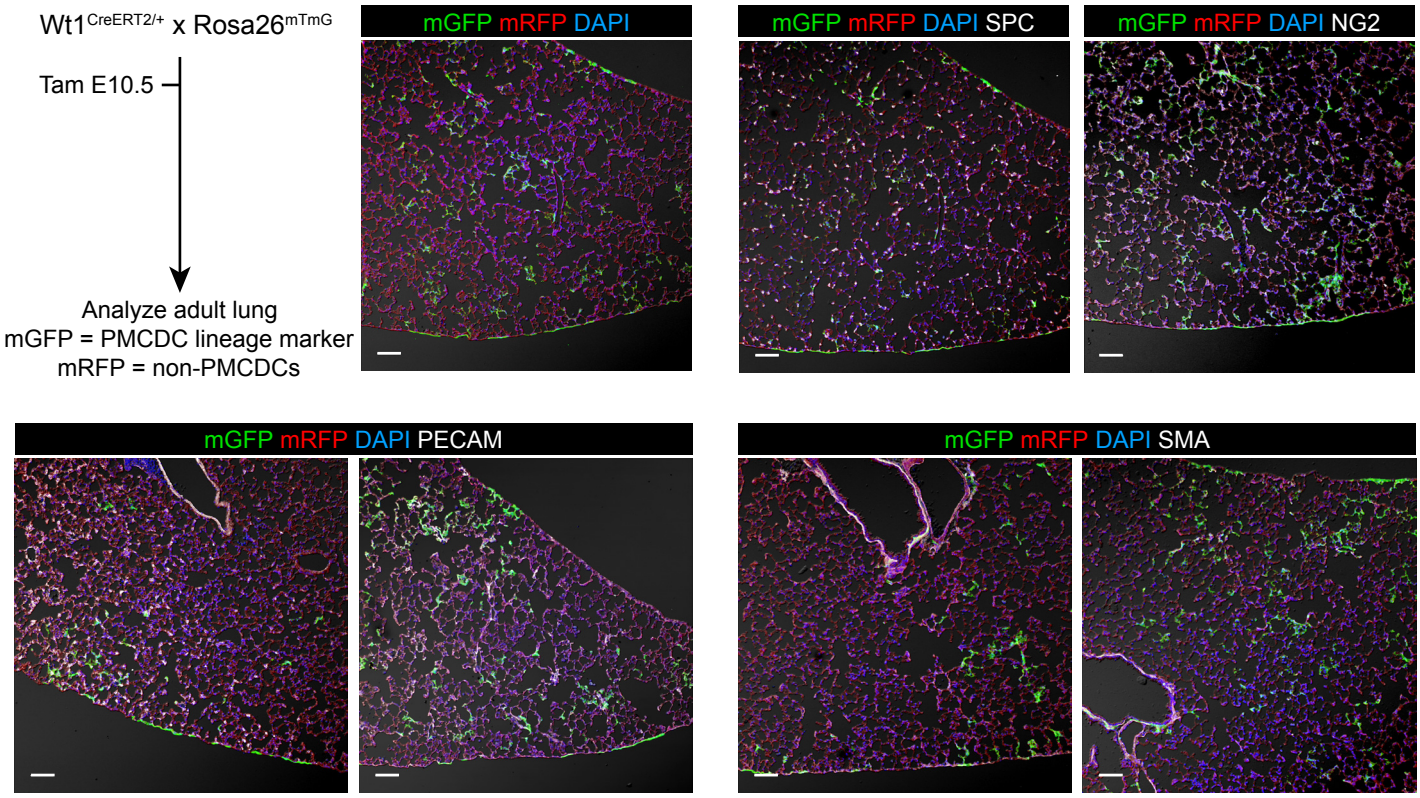


Supplementary Figure 2. PMC EMT detected in lung explant culture. A. Whole mount images of lung explants from E11.5 embryos at 0 and 48 hours of in vitro culture. **B.** Selective labeling by transient exposure of lung explants to the cell tracking dye CMFDA. Left panel shows the explant immediately after labeling, and the right panel shows the explant after 48 hours in culture, followed by fixation and staining for E-cadherin. Bar = 1 mm (A), 100 μm (B).



Supplementary Fig. 3. Panoramic views of the distribution of fetally labeled PMCDCs in the mesenchyme of the adult lung. PMCDCs were pulse labeled by tamoxifen treatment of E10.5 Wt1$^{CreERT2/+}$::Rosa26^{mTmG} embryos. The labeled PMCDCs expressed mGFP, while the non-Cre labeled cells expressed mRFP. Lineage markers SPC, NG2, PECAM, and SMA were co-stained to provide additional anatomical landmarks. Bar = 100 μm.

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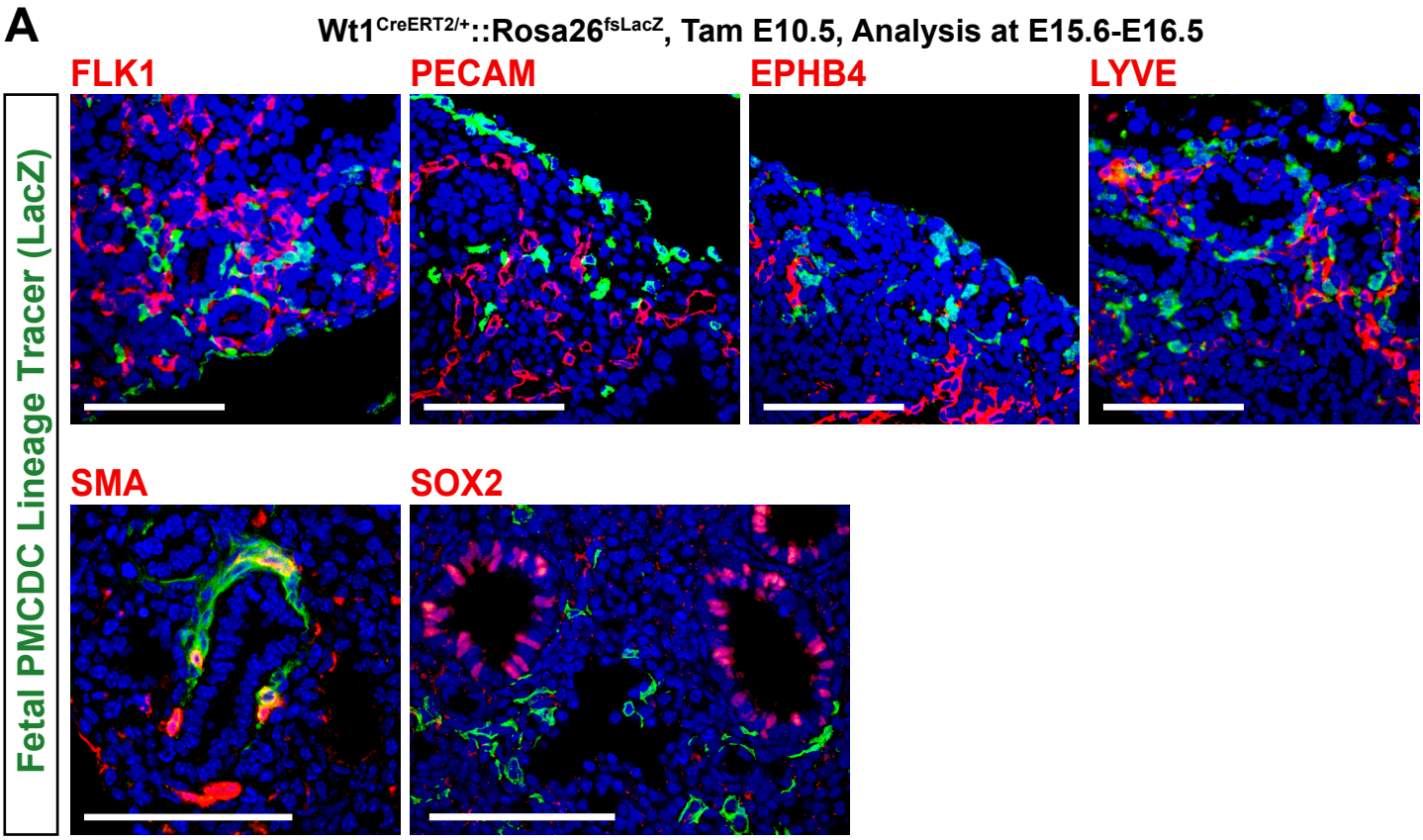
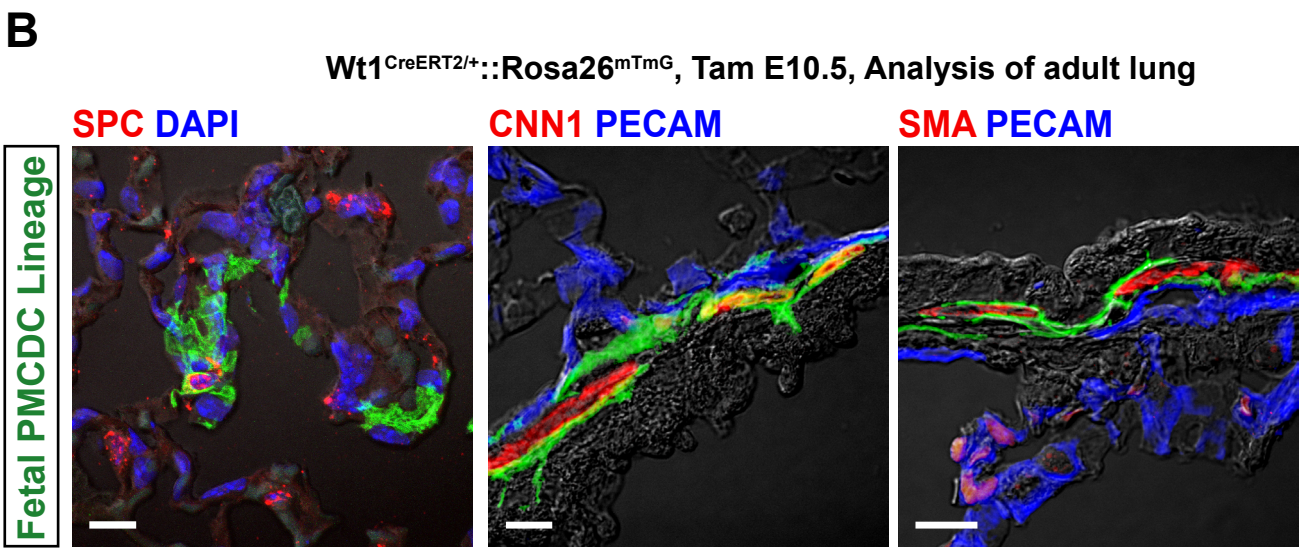
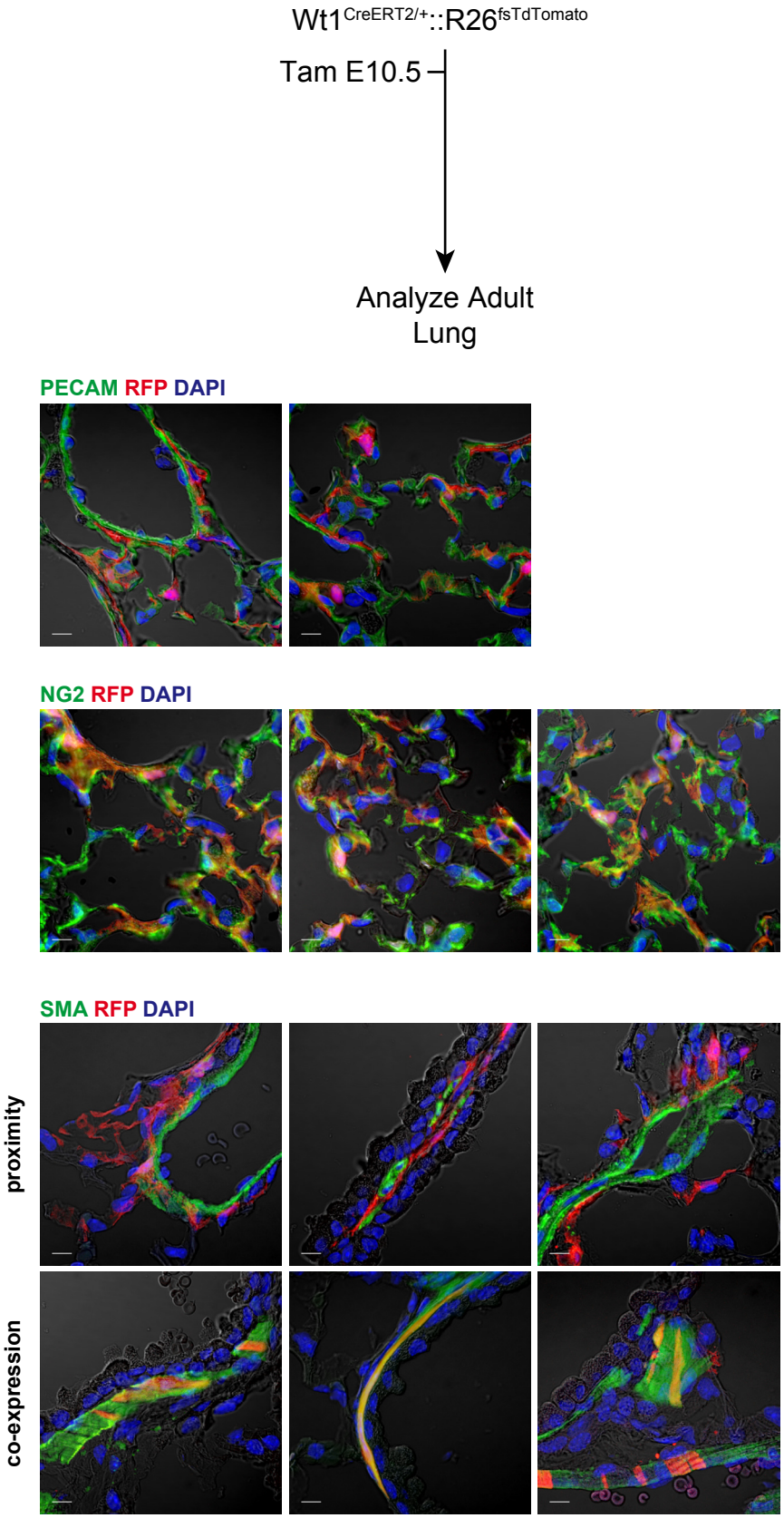




Supplemental Fig. 4. Fetal PMCDC expression of lineage markers. Fetal PMCDCs were labeled by tamoxifen administration at E10.5. **A.** Rosa26^{fsLacZ} was used as the Cre-activated lineage tracer. Cre-induced expression of LacZ was detected by immunostaining. Lungs were analyzed at E15.5-E16.5. Markers of lung lineages were immunostained in red. **B.** Examples of PMCDCs in which we could not determine with certainty whether the same cell or adjacent cells expressed the co-stained lineage marker. Rosa26^{mTmG} was used as the Cre-activated lineage tracer, so that lineage traced cells are GFP^{+}. Adult lungs were analyzed. Scale bar = 100 μm.



Supplementary Fig. 5. Lineage tracing of fetal PMCDCs using the R26^{fsTdTomato} reporter allele. Fetal PMCs were labeled by treatment with Tam at E10.5. Adult lung PMCDCs (RFP^{+}) were then analyzed for co-expression of PECAM, NG2, or SMA. Bar = 10 μm.

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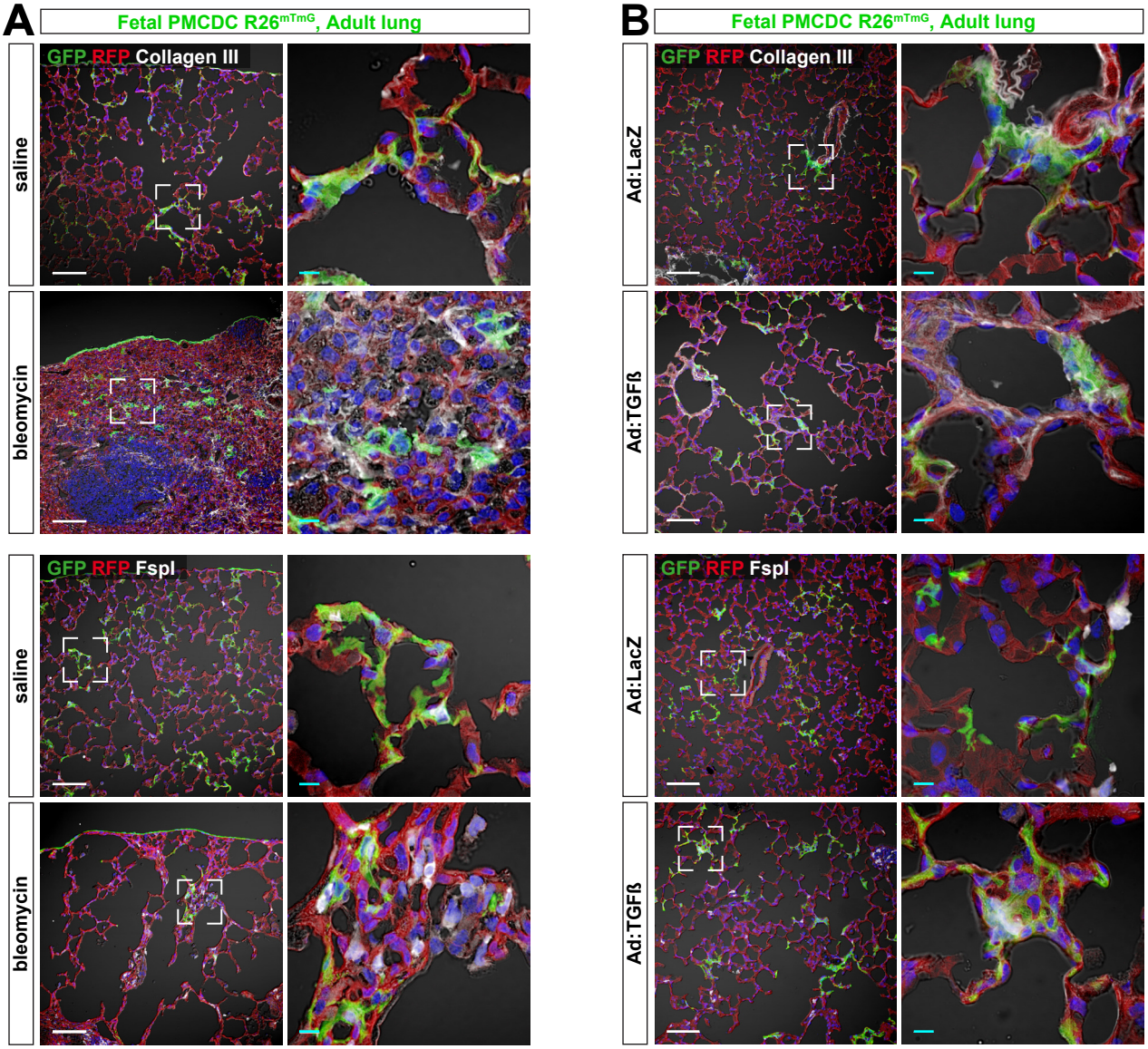
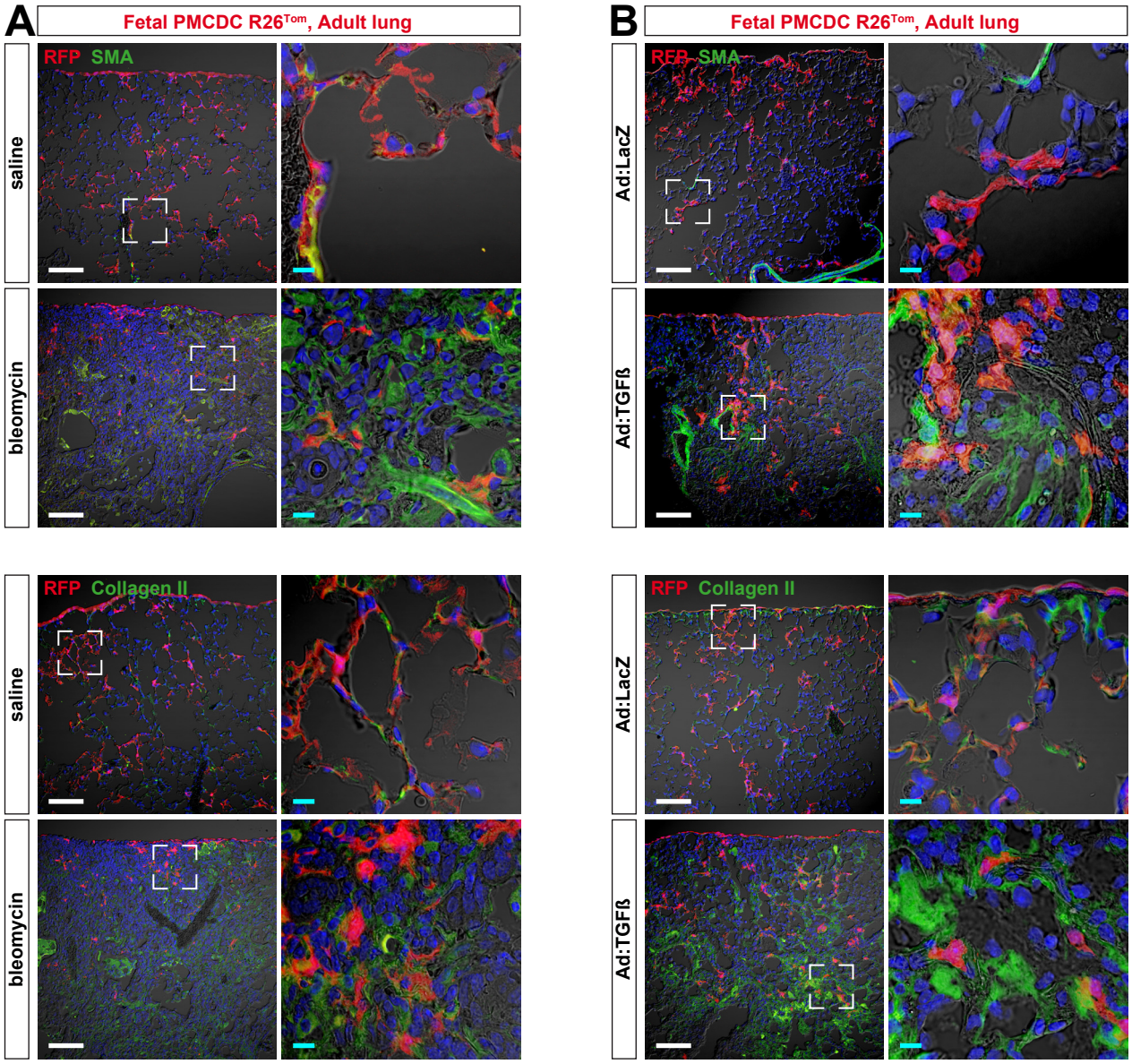


Supplementary Fig. 6. Fetal PMCDCs participate in adult lung fibrosis. Wt1$^{CreERT2/+}$::Rosa26^{mTmG} mice were treated at E10.5 with tamoxifen to pulse label PMCs and their derivatives. The mice were then treated with bleomycin (A) or TGFβ1 adenovirus (B) or controls. Lungs were cryosectioned and stained for Fsp1 or Collagen III, markers of fibroblasts and myofibroblasts. PMCDCs were present in fibrotic areas and co-expressed these fibrotic cell markers. Boxed regions in panels are magnified to the right. Bar = 100 μm (white), 10 μm (teal).



Supplementary Fig. 7. Fetal PCDMCs participate in adult lung fibrosis. Wt1$^{CreERT2/+}$::Rosa26^{fsTdTomato} embryos were pulse labeled with tamoxifen at E10.5. Adult lungs were injured with bleomycin (A) or Ad:TGFβ1 (B) treatment and analyzed by immunohistochemistry. Boxed regions are magnified to the right. Bar = 100 μm (white), 10 μm (teal).

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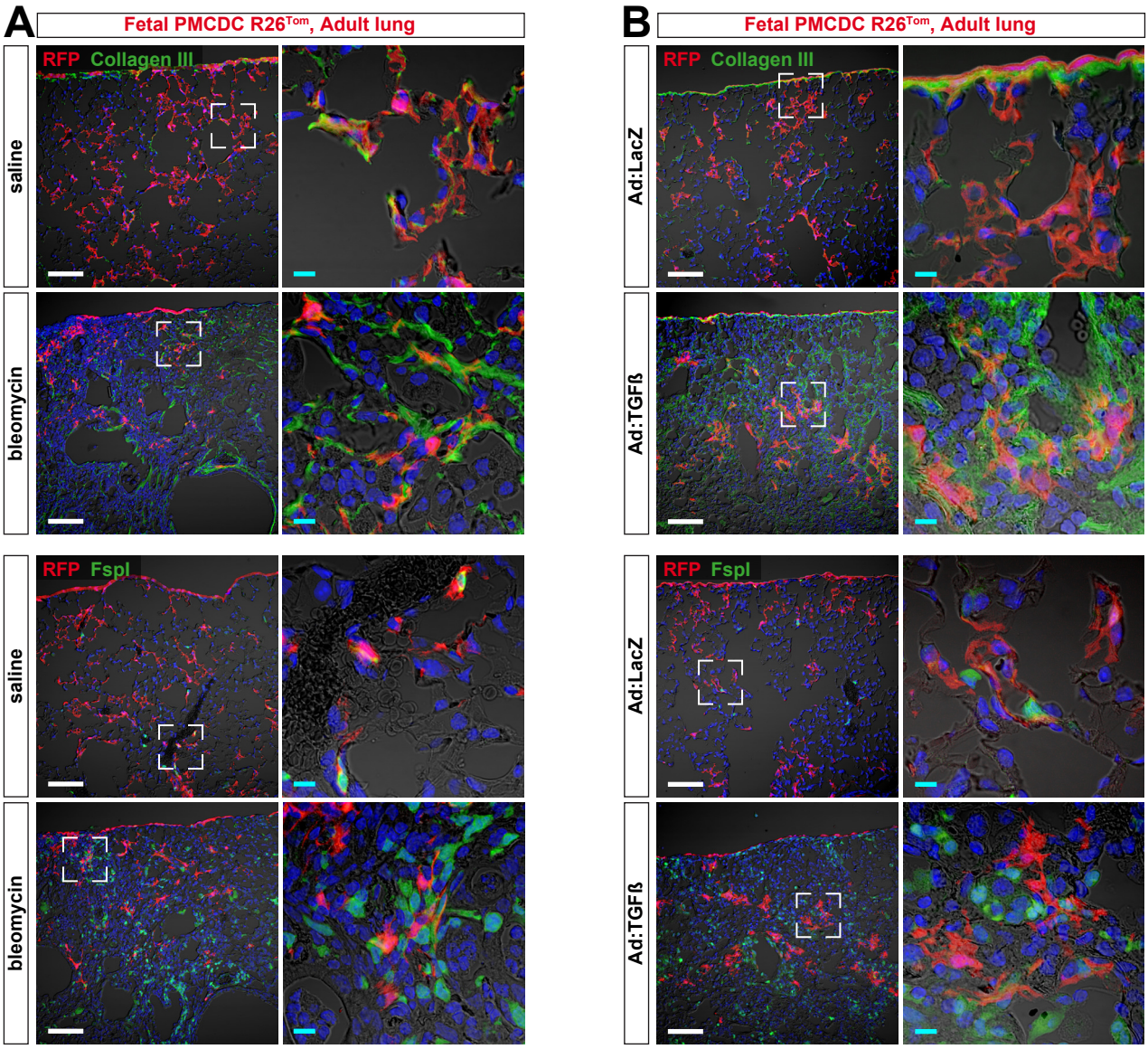
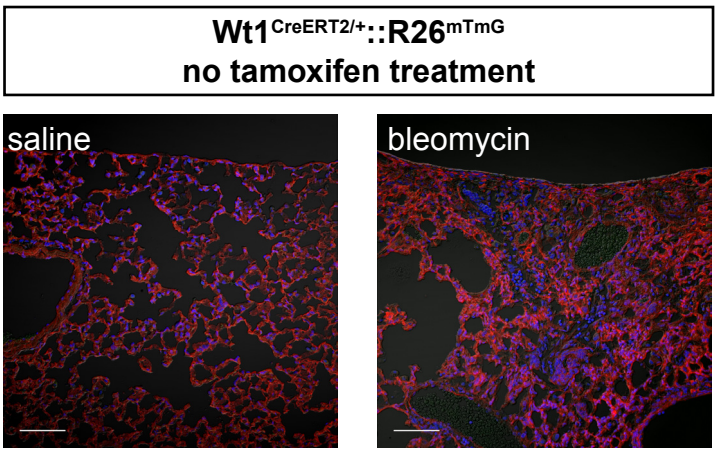


Supplementary Fig. 7, continued.



Supplementary Fig. 8. No labeling by Wt1^{CreERT2} in the absence of tamoxifen treatment. Lung injury by bleomycin did not activate the Cre-dependent reporter in the absence of tamoxifen treatment. Red = no Cre activation. Green = Cre activation. Nuclei are stained blue. Bar = 100 μm.