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TGF-β receptor II in Epithelia Versus Mesenchyme Plays Distinct Role in Developing Lung

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Running head: TGF-β signaling regulates lung development

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Abstract

TGF-β signaling plays important roles in regulating lung development. However, the specific regulatory functions of TGF-β signaling in developing lung epithelial versus mesenchymal cells are still unknown.

By immunostaining, we first determined the expression pattern of TGF-β type II receptor (TβRII) in developing mouse lung. The functions of TβRII in developing lung were then determined by conditionally knocking out TβRII in lung epithelium of floxed-TβRII/SPC-rtTA/TetO-Cre mice versus mesenchyme of floxed-TβRII/Dermo1-Cre mice, respectively.

TβRII was expressed only in distal airway epithelium at early gestation (E11.5), but in both airway epithelium and mesenchyme from mid-gestation (E14.5) to postnatal day 14. Abrogation of TβRII in mouse lung epithelium resulted in retardation of postnatal lung alveolarization with markedly decreased type I alveolar epithelial cells, while no abnormality in prenatal lung development was observed. In contrast, blockade of TβRII in mesoderm-derived tissues including lung mesenchyme resulted in mildly abnormal lung branching and reduced cell proliferation after mid gestation, accompanied by multiple defects in other organs including diaphragmatic hernia. The primary lung branching defect was verified in embryonic lung explant culture.

Our novel findings suggest that TβRII-mediated TGF-β signaling plays distinct roles in lung epithelium versus mesenchyme to differentially control specific stages of lung development.

Key Words: TGF-β, TGF-β receptor II, lung alveolarization, lung branching morphogenesis

Introduction

Lung development is initiated by the formation of a pair of primary epithelial buds that evaginate from the laryngo-tracheal groove in the ventral surface of the primitive foregut endoderm into the surrounding splanchnic mesenchyme [1;2]. The respiratory tree then develops by branching morphogenesis, in which reiterated outgrowth, elongation and subdivision of epithelial buds occurs, followed later on by alveolarization to form a large gas-exchange surface [3;4]. Disruption of normal lung developmental processes can result in neonatal respiratory failure or distress if lung formation is severely affected, or susceptibility to lung diseases during later life if milder changes occur in the developing lung [5].

Since the lung developmental process is quite well conserved, mouse lung development is an ideal model for studying the mechanism of lung organogenesis and congenital respiratory diseases in man. In mouse, lung development begins at embryonic day (E) 9.5, and is divided histologically into pseudoglandular stage (E9.5-E16.5), canalicular stage (E16.6-E17.4), saccular stage (E17.5 to postnatal day 5 or P5), and alveolar stage (P5-P30) [1]. During lung development, epithelial-mesenchymal interaction plays a critical role in guiding early lung branching morphogenesis and later on alveogenesis, which is regulated by many growth factors including members of the transforming growth factor-β (TGF-β) superfamily [6].

TGF-β1, 2, 3 ligands are closely related members of the TGF-β superfamily, which have differential expression patterns *in vivo* and biological activities *in vitro*. TGF-β ligands bind to heteromeric complexes of TGF-β serine/threonine kinase type I and type II receptors (TβRI and TβRII) [7;8]. Upon ligand-induced aggregation of the receptors, constitutively activated TβRII kinase phosphorylates and activates the TβRI, which subsequently recognizes and phosphorylates receptor-bound TGF-β specific Smad proteins (Smad2 and Smad3) on the

carboxyl terminal SSXS motif. These phosphorylated Smads dissociate from the receptors, form complexes with a common partner Smad4, translocate into the nucleus, directly or indirectly bind to the TGF-β responsive element and act as transcriptional co-modulators to induce or repress TGF-β-target gene expression [9]. In addition, Smad-independent signaling pathways are also activated through the same receptors [10]. TβRII is the only type II receptor specific for TGF-β.

TGF-β signaling plays a key role in normal lung development. Null mutation of TGF-βs results in either abnormal fetal lung development or excessive inflammation in the postnatal lung [11-14], indicating that TGF-β signaling is essential for normal lung formation and function. In addition, abnormal lung branching morphogenesis was observed in an intact embryonic lung explant culture system when exogenous TGF-β1 was added into the culture medium [15]. Furthermore, overexpression of *TGF-β1* driven by a 3.7 kb human *SP-C* promoter in lung epithelium of transgenic mice exhibited a hypoplastic lung phenotype [16], suggesting that appropriate TGF-β signaling at the right place and right time is essential for normal lung organogenesis. Moreover, changes in endogenous TGF-β signaling has been speculated to mediate delay in male fetal lung maturation caused by elevated androgens [17;18]. However, the lung is a complex organ so that global alteration of TGF-β ligand level may affect TGF-β signaling activities differently in either lung epithelium or mesenchyme, or indeed both by changing autocrine and/or paracrine signaling activities, which may be difficult to distinguish. Conventional knockout of the critical *TβRII* results in early embryonic lethality, due to defects in hematopoiesis and vasculogenesis prior to lung formation [19]. Herein, we have selectively abrogated endogenous TβRII-mediated TGF-β signaling in either lung epithelial cells or mesenchymal cells of the developing mouse lung using Cre/*loxP* conditional knockout

approaches, and found that TGF-β signaling plays important and distinct roles in lung epithelial versus mesenchymal cells to differentially control normal mouse lung development at different developmental stages.

Materials and Methods

Mouse strains and breeding. Floxed *TβRII* (*TβRII*$^{fx/fx}$) mice were generated in Dr. Harold Moses' lab [20]. In *TβRII*$^{fx/fx}$, the exon 2 of *TβRII* gene was flanked with two *loxP* DNA elements. Deletion of exon 2 causes frameshift and eliminates functional TβRII protein expression. Inducible lung epithelial-specific Cre transgenic mice (*SPC-rtTA*/*TetO-Cre*) were generated and provided by Dr. Jeffrey Whitsett [21]. Mesoderm-specific *Dermo1-Cre* heterozygous knockin mice (*Dermo1-Cre*$^{+/-}$) were generated and kindly provided by Dr. David Ornitz [22].

Timed mating between *TβRII*$^{fx/fx}$ and *TβRII*$^{fx/+}$/*SPC-rtTA*/*tetO-Cre* mice generated lung epithelial specific *TβRII* conditional knockout (Ep-CKO) mice (*TβRII*$^{fx/fx}$/*SPC-rtTA*/*TetO-Cre*), heterozygous *TβRII* knockout (HT) mice (*TβRII*$^{fx/+}$/*SPC-rtTA*/*TetO-Cre*), and control mice (*TβRII*$^{fx/fx}$, or *TβRII*$^{fx/+}$, *TβRII*$^{fx/fx}$/*SPC-rtTA*, or *TβRII*$^{fx/fx}$/*TetO-Cre*, or *TβRII*$^{fx/+}$/*SPC-rtTA*, or *TβRII*$^{fx/+}$/*TetO-Cre*) when inducing agent doxycycline was present. Normal lung development in the control mice is the same as in wild-type mice (*TβRII*$^{+/+}$). Administration of doxycycline started from either early gestation stages E6.5 or postnatal day 30 (P30) to the end point of experiment by feeding the pregnant or young mice with doxycycline food (625mg/kg, TestDiet, Richmond, IN) and drinking water (0.5 mg/ml, Sigma, St. Louis, MO).

Timed mating between *TβRII*$^{fx/fx}$ and *TβRII*$^{fx/+}$/Dermo1-Cre^{+} mice generated mesoderm-specific *TβRII* conditional knockout (Me-CKO) mice (*TβRII*$^{fx/fx}$/*Dermo1-Cre*$^{+}$), heterozygous *TβRII*

knockout (HT) mice (*TβRII*$^{fx/+}$/*Dermo1-Cre*$^{+}$), and control mice (*TβRII*$^{fx/+}$, or *TβRII*$^{fx/fx}$). All mice were bred in the C57BL/6 strain background, and genotyped by genomic DNA PCR. Mice used in this study were housed in pathogen-free conditions according to the protocol approved by IACUC at Saban Research Institute of Children Hospital Los Angeles.

Histology and morphometric analysis. Lung was fixed with 4% buffered paraformaldehyde at 4°C overnight, dehydrated and embedded in paraffin. 5μm sections were stained with hematoxylin and eosin (HE), as reported previously [23]. Elastin was stained using Hart's resorcin-fuchsin solution, and counterstained with 0.5% tartrazine. For morphometric analysis, five sections from the same lobes of each sample were randomly chosen at approximate 250 μm intervals, and stained with HE. The mean linear intercept (MLI) was then measured according to established methods [23-25]. Briefly, an image of each section examined was digitally captured at 40x magnification. The horizontal and vertical lines of a rectangle grid at ~0.9-mm intervals were then used to count alveolar surface intersections using ImagePro software. The MLI was then calculated by the equation: the sum of the length of all counting lines divided by the total number of counted intercepts of alveolar septa. Results were analyzed with Students *t*-test to compare the differences between mean values, and considered significant if $P<0.05$. In order to avoid the sex differences in fetal lung maturation [26], this quantitative comparison was performed among the fetuses with the same gender at each time point.

Immunohistochemistry. Antibodies used in these studies: TβRII and aquoporin 5 (AQP5) goat polyclonal antibodies were purchased from Santa Cruz (Santa Cruz, CA). α-smooth muscle actin (SMA) and laminin antibodies were purchased from Sigma (St. Louis, MO). Surfactant protein-C (SP-C) was obtained from Seven Hill (Cincinnati, OH).

Immunohistochemical staining was performed using HistoStain kit from Zymed Laboratories (South San Francisco, CA), according to the manufacturer's instruction. Either 3-amino-9-ethylcarbazole (AEC) or 3,3'-diaminobenzidine (DAB) was used as chromogenic substrate.

Cell proliferation and apoptosis. Cell proliferation was analyzed by PCNA (proliferating cell nuclear antigen) staining using Zymed PCNA staining kit, and apoptosis was evaluated by TUNEL staining using ApopTag kit (Millipore), as published previously [27]

Western blot. Detection of lung tissue proteins has been previously described [28]. Briefly, fresh lung tissues were lysed on ice in RIPA buffer containing 1 mM PMSF, protease inhibitor cocktail (Roches), and 1 mM sodium orthovanadate. Protein concentration was measured by the Bradford method using the reagents purchased from Bio-Rad Laboratories (Hercules, CA). Equal amounts (40 μg) of total tissue lysate proteins were separated in NuPAGE 4-12% gradient SDS-PAGE gels using a MOP buffering system (Invitrogen). After protein was transferred onto PVDF membrane, proteins of interest were detected by specific antibodies. Antibodies for CDK2, β-actin, TβRII were purchased from Santa Cruz, and anti-GAPDH was obtained from RDI (Flanders, NJ).

Data presentation and statistical analysis. At least three pairs of TβRII CKO and normal control littermate mice from different dams were analyzed in each experimental subgroup. All quantitative data were expressed as mean±SD. ANOVA and student's t-test were used for comparison of statistical difference and P values < 0.05 were considered as significant.

Results

Conditional abrogation of TGF-β type II receptor in mouse lung epithelial cells during lung development. Mouse embryos with the conventional *TβRII* null mutation die before E10.5 with defects in hematopoiesis and vasculogenesis before lung development [19]. Thus, the

conventional *TβRII* knockout mouse model is not applicable for studying TβRII function in lung formation, and a lung specific conditional *TβRII* knockout mouse model using a Cre-*loxP* system is required for this *in vivo* study. In order to select cell lineage-specific Cre driver lines to abrogate TβRII function during lung development, TβRII protein expression at different lung developmental stages was first examined using immunohistochemistry (Fig.1). Interestingly, TβRII was specifically expressed in distal lung airway epithelial cells at the early embryonic stage E11.5, with no detectable expression in mesenchymal cells, although both epithelial and mesenchymal cells expressed TβRII protein later on at gestational day E14.5. TβRII was also strongly expressed in both epithelial and mesenchymal cells in the postnatal lung during alveogenesis, with the majority of positively stained cells localized within the alveolar septa.

Therefore, lung epithelium-specific *TβRII* conditional knockout mice were generated first by crossing floxed *TβRII* mice with *SPC-rtTA*/*TetO-Cre* transgenic mice, in which Cre expression was induced in airway epithelial cells of whole lung and distal bronchus by a lung epithelium-specific *SP-C* promoter driven *rtTA* transgene, in combination with the inducing agent doxycycline (Dox) given prior to lung formation (E6.5) [21]. As a result of Cre-mediated *loxP* DNA recombination, floxed-*TβRII* exon 2 deletion in genomic DNA isolated from lung tissues was confirmed by PCR genotyping (Fig.2). Significant reduction of TβRII protein in whole lung tissue lysates of *TβRII* epithelium conditional knockout (Ep-CKO) mice at postnatal day 28 (P28) was also confirmed by western blot (Fig.2).

Abrogation of *TβRII* gene expression in lung epithelia from early mouse lung organogenesis results in retarded postnatal alveogenesis, but not a detectable phenotype in prenatal lung development.

Newborn lung epithelium-specific *TβRII* Ep-CKO mice (*TβRII*$^{fx/fx}$/*SPC-rtTA*/*TetO-Cre*) breathed normally without any signs of respiratory distress. Histological study confirmed normal lung saccular structure formation in P1 *TβRII* Ep-CKO lung compared to normal littermate controls. However, markedly retarded lung alveolarization was detected in *TβRII* Ep-CKO mice during postnatal alveogenesis (Fig.3), which occurs from P5 to P30 in mice. During mouse postnatal lung alveolarization, secondary crests develop and extend to make new secondary septa that further subdivide terminal air sac structures, accompanied by decreased mean alveolar size. Therefore, the larger the alveolar size, the fewer the alveoli, and alveogenesis can thus be quantified by calculating mean linear intercept (MLI). At P7 during early alveolarization, MLI in *TβRII* Ep-CKO lung was slightly higher than normal (Fig.3). As alveogenesis continued, the significant difference in MLI between *TβRII* Ep-CKO and normal control markedly increased at P14, and remained elevated to the end of alveolarization (P28) as well as into early adulthood (2M, Fig.3), suggesting a major maturational arrest of alveolarization. In order to exclude the possibility that the reduced alveolarization in *TβRII* Ep-CKO is caused by non-specific effects of compound transgenic genotypes, lung alveolarization was also compared between the mice with the same genotype of *TβRII* Ep-CKO versus normal control, but without Dox induction. No change in alveolarization, as examined by morphology and morphometric MLI measurement (Fig.3), was detected in the absence of Dox induction. In addition, mice with Dox-induced *TβRII* heterozygous genotype (*TβRII*$^{fx/+}$/*SPC-rtTA*/*TetO-Cre*) had normal lung alveolarization as seen in controls. These data suggest that retarded alveolarization of *TβRII* Ep-CKO mice is specifically due to lack of TβRII function in lung epithelia.

Conditional knockout of TβRII function in lung epithelial cells resulted in abnormal cell proliferation and differentiation during postnatal lung alveogenesis.

Secondary septal formation during alveogenesis is a complicated process requiring fine coordination of outgrowth of epithelial cells, extension and simplification of capillary networks, alveolar myofibroblast involvement, and correct deposition of the elastic interstitial matrix. This is regulated by many factors including TGF-β signaling. Abrogation of TβRII in lung epithelial cells resulted in decreased cell proliferation during the alveolarization stage, as indicated by reduced PCNA protein level and PCNA-positive stained cells in *TβRII* Ep-CKO mouse lungs (P7, Fig.4A-B). Furthermore, decreased cell proliferation was also verified by reduced cyclin dependent kinase 2 (CDK2) expression in P14 and P28 *TβRII* Ep-CKO lungs (Fig.4B). However, apoptosis during lung alveolarization, particularly at the later alveolarization stage of P28, was not increased (Fig.4C). Therefore, reduced cell proliferation in affected cell lineages, rather than increased cell death, may cause fewer alveolar septa to form in the *TβRII* Ep-CKO lung.

Cell differentiation was also evaluated by immunostaining the molecular markers of different cell lineages in the lung at P14, a time at which obvious retardation of alveolarization is seen in the *TβRII* Ep-CKO lung. Surfactant protein C (SP-C) and aquoporin 5 (AQP5) are cell-specific markers for alveolar epithelial type II cells (AECII) and type I cells (AECI), respectively. No significant change in SP-C positive cells was observed in *TβRII* Ep-CKO lung (Fig.4D). However, AQP5 positive cells and the intensity of AQP5 in positively stained cells were markedly reduced when TβRII was abrogated in lung epithelium (Fig.4F), suggesting that reduced AECI lineage differentiation and/or amplification may be an important cellular mechanism underlying retarded alveogenesis in the *TβRII* Ep-CKO mouse lung. In addition, myofibroblasts and related important extracellular proteins were evaluated by detecting α-

smooth muscle actin (SMA), laminin, and elastin fiber deposition in septal structures (Fig.4E, G, H). Laminin, a major protein component in capillary basement membrane, was similarly distributed in the septal tips of both *TβRII* Ep-CKO and the control mouse lungs, suggesting normal capillary outgrowth. A similar pattern of SMA positive signal was detected in smooth muscle cells surrounding bronchioles and large blood vessels, as well as in myofibroblasts within septal structures of both *TβRII* Ep-CKO and normal control lungs. Furthermore, deposition of elastin fibers at the tips of alveolar septal structures of *TβRII* Ep-CKO lung remained similar to normal controls, suggesting that alteration in mesenchymal cell proliferation and differentiation as well as extracellular matrix deposition was not the direct cause of abnormal alveolarization in these mice.

Conditional abrogation of TGF-β type II receptor in mouse lung mesenchymal cells during fetal lung development.

Since TβRII was also found to be expressed in the mesenchyme of mouse embryonic and adult lungs, as shown above (Fig.1), TβRII-mediated TGF-β signaling in mesenchymal cells may play an unique role in regulating embryonic lung formation, particularly branching morphogenesis. By taking advantage of mesoderm-derived tissue-specific expression of Cre in the *Dermo1-Cre* knockin driver mouse line [22], mesenchyme-specific Cre-mediated *loxP* DNA recombination in multiple mouse embryonic organs including lung was achieved (Fig.5A). Floxed *TβRII* mice were then crossed with *Dermo1-Cre* mice to generate *TβRII* lung mesenchymal conditional knockout mice (*TβRII* Me-CKO), as shown by their genotypes (Fig.5B). The floxed-*TβRII*/*dermo1-Cre* generated *TβRII* Me-CKO mice have severe defects in other important developmental processes, including defective secondary ventral body wall formation, congenital

diaphragmatic hernia, with abnormal cardiac development (Fig.5C-5D). These non-pulmonary abnormalities were all due to disrupted TGF-β signaling in other key mesoderm-derived tissues. Early lung bud formation in *TβRII* Me-CKO mice was not noticeably affected at the early stage (E12.5), but obvious deformity of the lung, particularly in the left and right inferior lobes, was observed around E14.5 (Fig.5E), which could be attributed to abnormal positioning of the heart and liver due to defective thoracic wall formation and diaphragmatic closure. This *Dermo1-Cre* driven *TβRII* conditional knockout was lethal around E16.5, possibly due to severe defects in other organs including heart.

Mesenchymal abrogation of TβRII signaling in prenatal lungs disrupts normal branching morphogenesis.

By gross comparison between *TβRII* Me-CKO and control mouse lungs at the early stage of branching morphogenesis (E12.5), no significant changes in early lung branching were found (Fig.7A). However, histological study found slight dilation of peripheral airways accompanied by reduced mesenchymal cell density in *TβRII* Me-CKO lung at E14.5 (Fig.6A). These phenotypic changes were more evident two days later at E16.5. By PCNA immunostaining, the number of proliferating cells in *TβRII* Me-CKO lungs was decreased in both the airway epithelium and surrounding mesenchyme compared to normal littermate controls (Fig.6B). However, differentiation of peripheral airway epithelial cells and mesenchymal myofibroblasts/smooth muscle cells was not changed, as shown by SP-C and SMA immunostaining (Fig.6C-D).

In order to exclude the possibility that abnormal lung branching in *TβRII* Me-CKO is due to physical distortion of the developing lung, whole embryonic lung explant culture was then

performed. Embryonic lung explants were isolated at E12.5, when no branching difference was observed between *TβRII* Me-CKO and normal controls (Fig.7A). After a three-day culturing, the numbers of terminal branches in the growing lung explants were counted and compared between *TβRII* Me-CKO and controls (Fig.7A-B). Significant reduction of terminal branches in *TβRII* Me-CKO lung explants was detected (66% of that in normal controls, $P<0.05$). In contrast, lung branching in three-day cultures of *TβRII* Ep-CKO lung explants was not significantly changed compared to normal control (data not shown), suggesting that TβRII-mediated signaling in embryonic and fetal lung mesenchyme play an essential role during lung branching morphogenesis, whilst in contrast, TβRII-mediated signaling in airway epithelial cells may not be essential to early embryonic lung branching, but rather is critical for postnatal alveolarization.

Discussion

Lung development includes both early airway branching morphogenesis and late peripheral alveolarization. Disruption of either process will result in abnormal lung structure and function, with the consequence of either respiratory failure if early lung formation is severely affected, or susceptibility to lung diseases during later life if mild changes occur in developing lung [5]. Epithelial-mesenchymal interaction plays an important role in regulating normal lung formation, possibly through direct cell-cell contact as well as through indirect regulation by changing growth factor secretion and extracellular matrix protein deposition. TGF-βs are one group of important growth factors involved in regulating lung development.

TGF-β1, β2, β3 have all been detected in murine embryonic lungs [29-32]. Null mutation of TGF-βs results in either abnormal fetal lung development and/or postnatal lung excessive inflammation, indicating that TGF-β signaling is essential for normal lung formation and

function [11-14]. In contrast, overexpression of TGF-β1 driven by a 3.7kb human *SP-C* promoter in lung epithelium of transgenic mice caused a hypoplastic lung phenotype [16], suggesting that appropriate levels of TGF-β signaling at the right place and right time are essential for normal lung organogenesis. However, whole organ alteration of TGF-β ligand level may affect TGF-β signaling activities in both lung epithelial and mesenchymal cells by changing autocrine and/or paracrine signaling pathways, which are difficult to distinguish. In order to study regulatory functions of endogenous TGF-β signaling in lung epithelial versus mesenchymal cells during lung development, we therefore selectively abrogated TGF-β intracellular signaling activity by deleting TβRII functional protein production specifically in either lung epithelium or mesenchyme, using Cre/*loxP* approaches, with *SPC-rtTA*/*TetO-Cre* and *Dermo1-Cre* driver lines, respectively.

Interestingly, blockade of endogenous TβRII function in airway epithelial cells alone (Ep-CKO) failed to elicit any detectable alterations to prenatal mouse lung formation, particularly branching morphogenesis *in vivo*. The neonatal *TβRII* Ep-CKO mice breathed normally and displayed similar saccular organization as seen in control mice at P1. However, secondary alveolar septa formation was severely retarded in *TβRII* Ep-CKO mice during postnatal mouse lung alveogenesis. Alveolarization is a complicated process with coordinated growth of alveolar epithelial cells (both AECI and AECII), capillary endothelial cells, myofibroblasts, and deposition of extracellular matrix, particularly elastin. Cell proliferation and differentiation in each of the above cell lineages contribute to the secondary septa and hence related alveolar surface formation, generating sufficient gas exchange membrane between terminal airspaces and the pulmonary circulation. By measuring cell cycle-specific protein markers PCNA and CDK2, we found that overall cell proliferation in the *TβRII* Ep-CKO lung was reduced, suggesting that

TβRII-mediated TGF-β signaling in lung epithelial cells is essential for directly regulating epithelial cell growth and/or indirectly affecting endothelial cells or myofibroblasts. To further narrow down the affected cell lineages in *TβRII* Ep-CKO lung, we have examined these different types of cells by examining expression of cell-specific markers. No significant changes in myofibroblasts, as stained by SMA, were observed in septal structures of *TβRII* Ep-CKO mouse lung. Consistently, deposition of elastin fibers in the septal extracellular spaces was not changed in *TβRII* Ep-CKO lung. These data suggest that alveogenesis controlled by the PDGF-pathway through promoting myofibroblast growth is not indirectly affected by abrogating epithelial TGF-β signaling activity in this mouse model [33]. Similarly, normal laminin deposition, a major basement membrane component of the capillary network, was detected at the tips of septal structures, indicating normal capillary endothelial development in *TβRII* Ep-CKO mice. Moreover, comparable distribution of SP-C positively stained epithelial cells was observed in both control and *TβRII* Ep-CKO lungs, suggesting that lack of TGF-β signaling activity in lung epithelial cells does not disrupt normal SP-C-positive AECII and/or related progenitor cell differentiation. However, AQP5 positively stained AECI cells in *TβRII* Ep-CKO lung were markedly reduced, indicating that TβRII-mediated TGF-β signaling alone in lung epithelial cells may directly regulate AECI differentiation and expansion *in vivo*. Reduced AECI cell population may thus contribute to reduced secondary septal growth and alveolar surface membrane formation. Consistent with our *in vivo* result, Bhaskaran et al recently reported that, in primary cultured rat lung epithelial cells, abrogation of endogenous TGF-β signaling by adding TGF-β1 neutralization antibody or silencing downstream Smad4 function using RNA interference inhibited AECI cell differentiation [34]. In addition, we have previously shown that mice with conventional knockout of TβRII downstream Smad3 also suffered from retarded lung

alveogenesis [23]. These data together strongly suggest that TGF-β-Smad3 dependent signaling activity in lung epithelial cells plays a critical regulatory role in promoting AECI cell differentiation and lineage expansion, particularly during lung alveogenesis.

Interestingly, in conventional Smad3 knockout mice, subsequent destruction of preformed alveolar structures follows after abnormal alveolarization around P28, resulting in central lobular emphysema-like pathology [23]. Conventional null mutation of latent TGF-β binding protein 4 (*LTBP-4*), which is required for mature TGF-β peptide secretion and activation, also results in abnormal lung alveolarization in neonates and development of emphysema in adult mice [35]. However, no emphysema-like lung tissue destruction was observed herein with the lung epithelial-specific *TβRII* conditional knockout, although retarded alveolarization persisted into adulthood. These data suggest that TGF-β-Smad signaling activity in lung epithelial cells per se is essential for mature lung development, and that disruption of TGF-β-Smad signaling in other lung cells, including myofibroblasts and/or inflammatory cells (macrophage and neutrophil) are further required for proteinase-mediated tissue destruction [23]. Further dissection of related mechanisms *in vivo* will require *TβRII* CKO in other cell lineages such as leukocyte, macrophage, and/or myofibroblasts in combination with *TβRII* mutation in lung epithelial cells.

Previous studies using transgenic and *ex vivo* organ culture approaches indicate that TGF-β signaling plays an important regulatory role in embryonic lung branching morphogenesis. Surprisingly, lung branching morphogenesis was normal when endogenous TβRII function in mouse embryonic lung airway epithelial cells was blocked *in vivo*. Whereas, abrogation of TβRII function in mesoderm-derived lung mesenchymal cells resulted in relatively mild reduction of lung branching morphogenesis, as detected only at E14.5 and afterwards, but not in early gestation (E12.5). This is consistent with our immunostaining data, which showed that TβRII

expression in embryonic lung mesenchyme was only detected at mid-gestation and afterwards. In contrast to *SPC-rtTA*/*TetO-Cre* driven Cre specific expression in lung epithelia, *Dermo1-Cre* expression is not restricted to lung mesenchymal tissue only. Therefore, abrogation of TβRII function using a *Dermo1-Cre* driver mouse line generated *TβRII* conditional knockout in multiple organs, including the ventral body wall, heart, and diaphragm. Thus, multiple defects in *TβRII* Me-CKO mice make it difficult to determine the related and specific mechanisms underlying the lung phenotype. In particular, the diaphragmatic hernia-like defect may also contribute directly to abnormal lung branching morphogenesis at embryonic stages [36;37]. For example, lung hypoplasia was also found at early embryonic stages of mice with loss of Fog2-Gata4 interaction, prior to development of congenital diaphragmatic hernia caused by loss of function mutation in Fog2 or Gata4. This suggests that pulmonary hypoplasia could be an independent developmental defect instead of a secondary consequence of increased intrathoracic pressure due to a dislocation of abdominal organs to chest via the diaphragmatic hernia. In addition, marked physical deformity of the lungs, caused by diaphragmatic hernia and thoracic ventral body wall defects, may also indirectly and adversely affect normal lung growth after E12.5 [38;39]. Nevertheless, by *ex vivo* culture, our studies verified that endogenous TβRII function in lung mesenchyme alone is essential for normal lung branching. But the *TβRII* Me-CKO mouse line generated by crossing floxed *TβRII* and *Dermo1-Cre* mouse may not be an ideal model for further dissection of the mechanisms by which TGF-β signaling in mesenchyme regulates airway epithelial branching, due to the multiple confounding factors discussed above.

In summary, TβRII-mediated TGF-β signaling plays distinct roles in developing mouse lung epithelium versus mesenchyme. The integrated functions of TβRII are very important in embryonic lung branching morphogenesis and postnatal lung alveolarization. The developmental

immaturity of lung structure and function, as a result of loss of function mutations in TGF-β signaling pathway components, may therefore contribute to early postnatal respiratory problems such as bronchopulmonary dysplasia. It may also increase the susceptibility to respiratory diseases including emphysema later in life.

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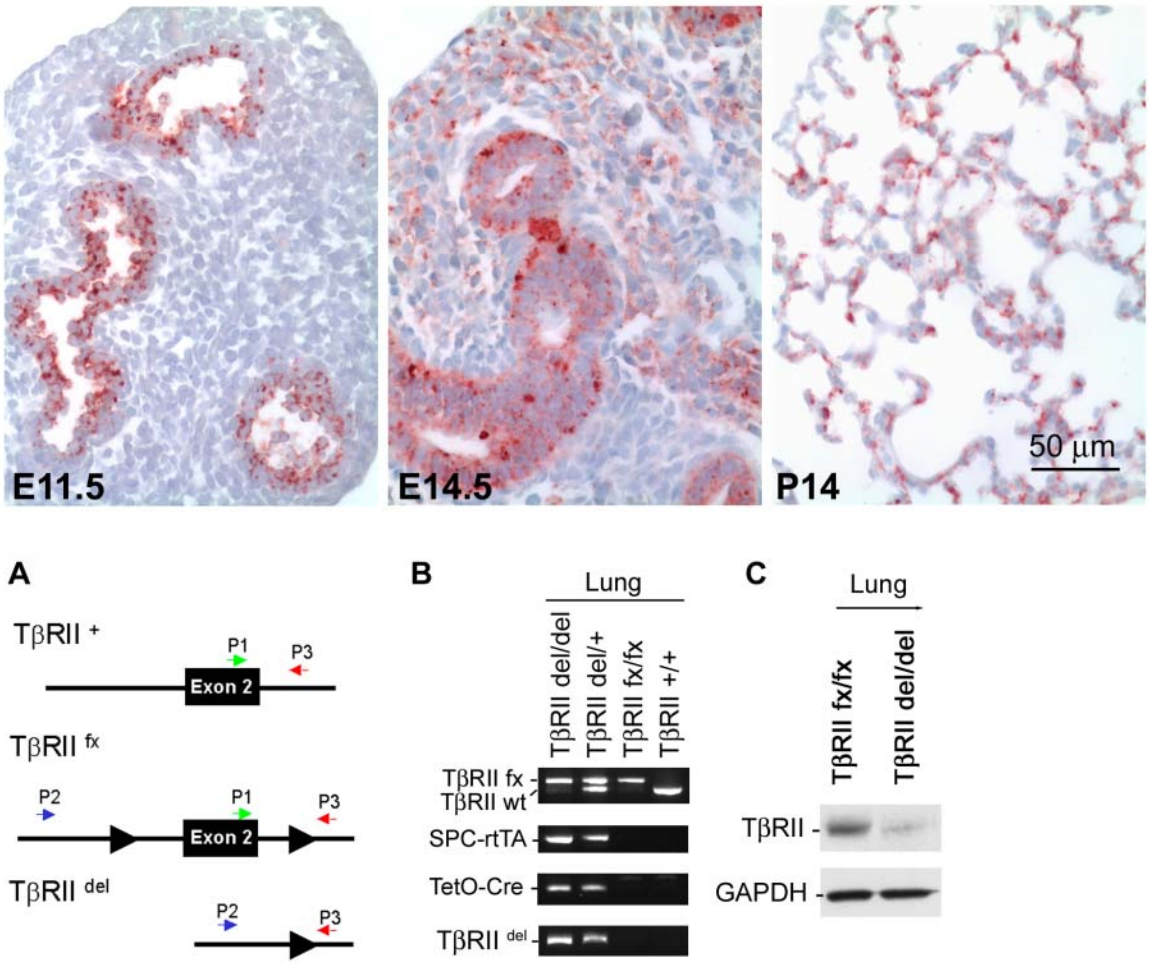
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E14.5
P14
50 µm
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TβRII +
P1
P3
Exon 2
TβRII fx
P2
P1
P3
Exon 2
TβRII del
P2
P3
B
Lung
TβRII del/del
TβRII del/+
TβRII fx/fx
TβRII +/+
TβRII fx
TβRII wt
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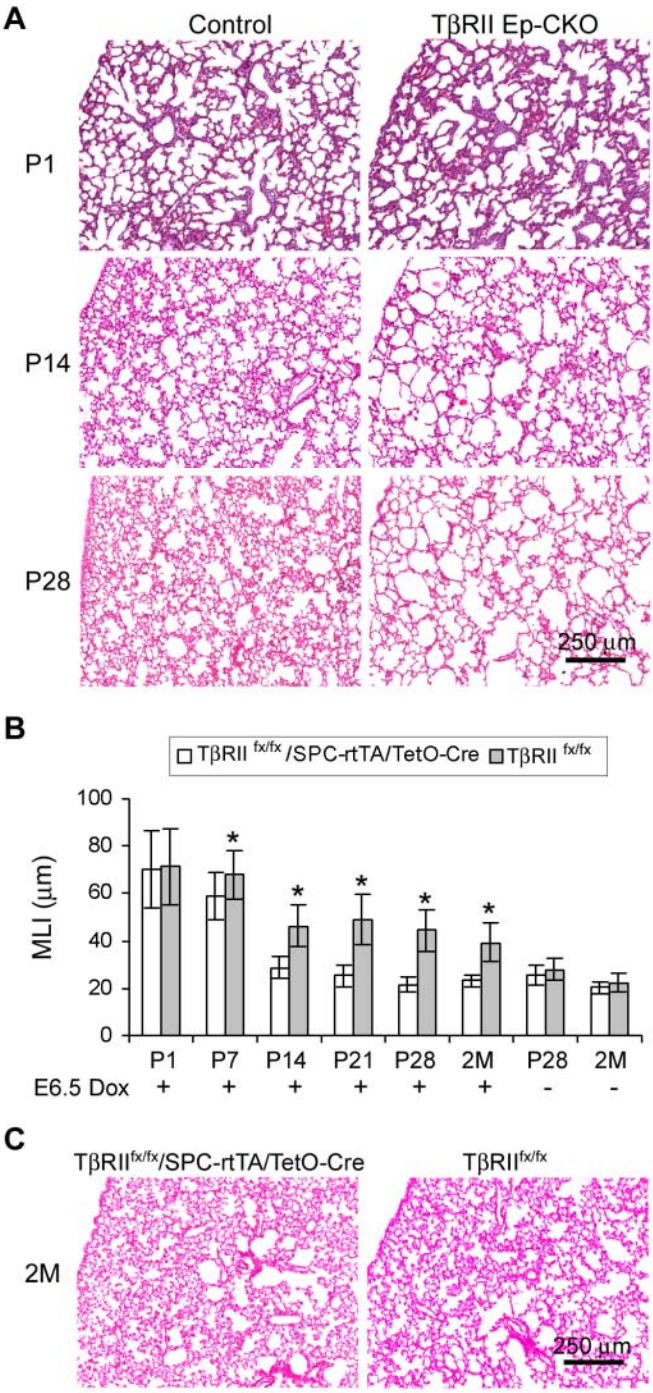
GAPDH


A
Control
TβRII Ep-CKO
P1
P14
P28
250 μm
B
TβRII fx/fx /SPC-rtTA/TetO-Cre
TβRII fx/fx
MLI (μm)
100
80
60
40
20
0
P1
P7
P14
P21
P28
2M
P28
2M
E6.5 Dox
+
+
+
+
+
+
-
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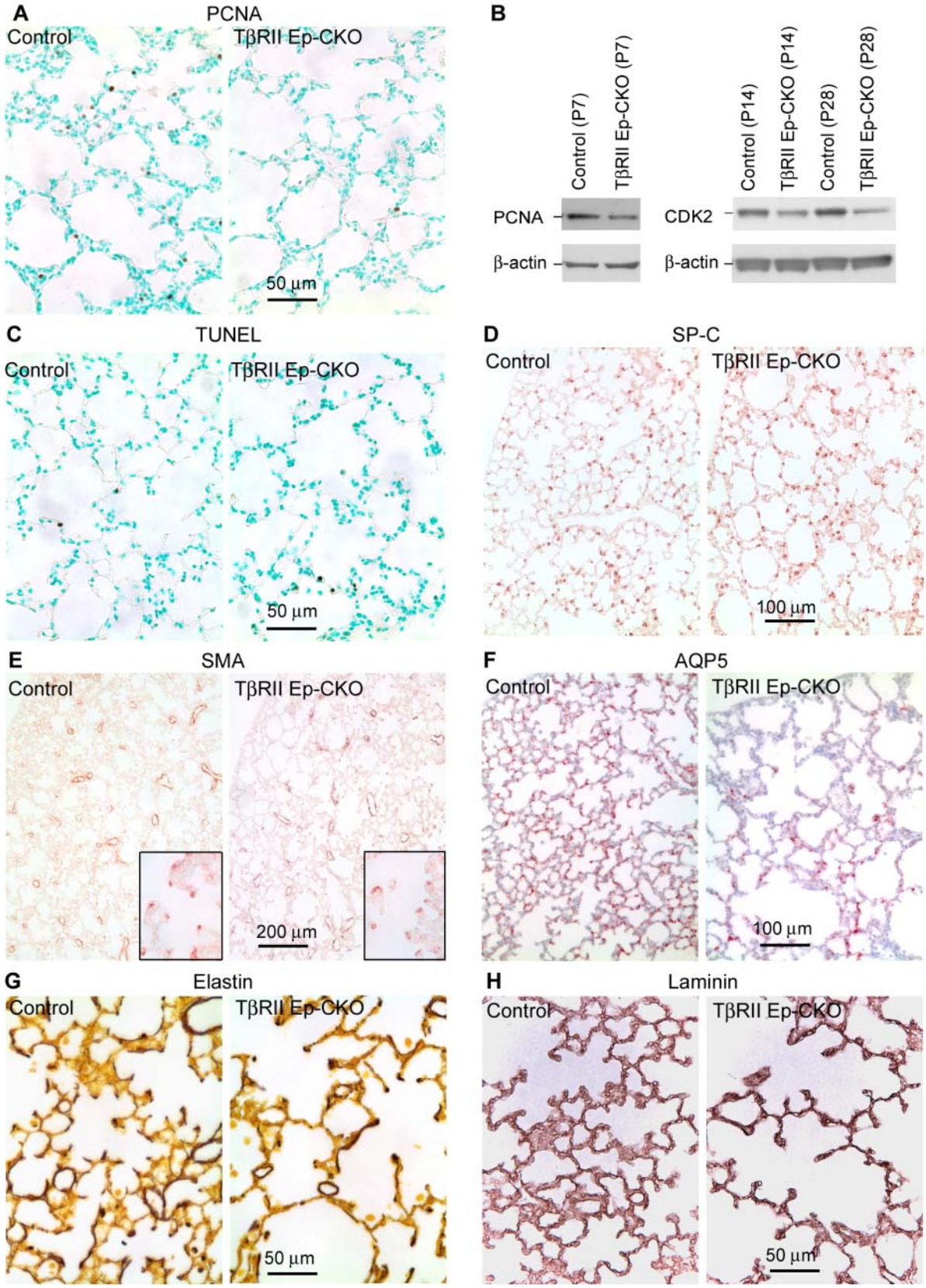
2M
250 μm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B
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Control (P28)
TβRII Ep-CKO (P28)
PCNA
CDK2
β-actin
β-actin
C
TUNEL
Control
TβRII Ep-CKO
50 μm
D
SP-C
Control
TβRII Ep-CKO
100 μm
E
SMA
Control
TβRII Ep-CKO
200 μm
F
AQP5
Control
TβRII Ep-CKO
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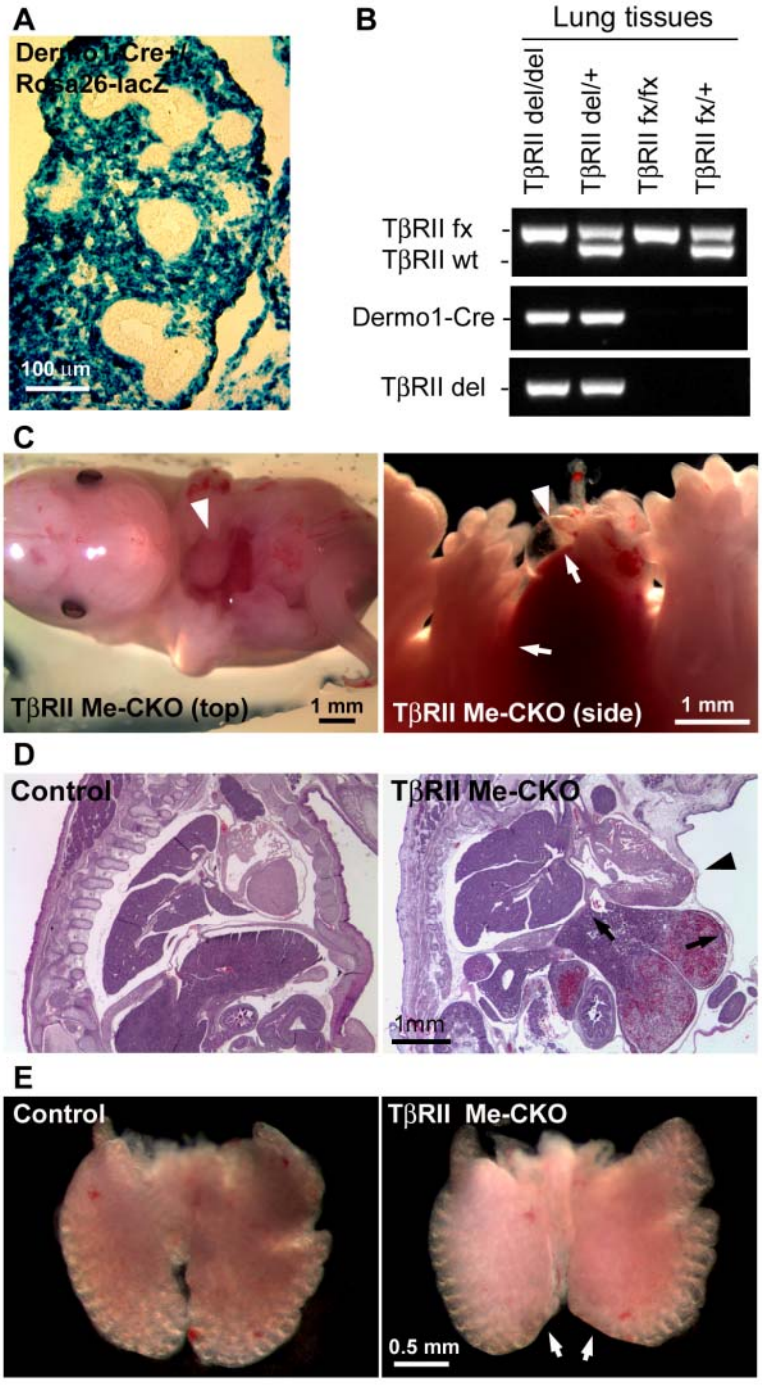
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Dermo1-Cre+/-
Rosa26-lacZ
100 μm
B
Lung tissues
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TβRII fx/+
TβRII fx
TβRII wt
Dermo1-Cre
TβRII del
C
TβRII Me-CKO (top)
1 mm
TβRII Me-CKO (side)
1 mm
D
Control
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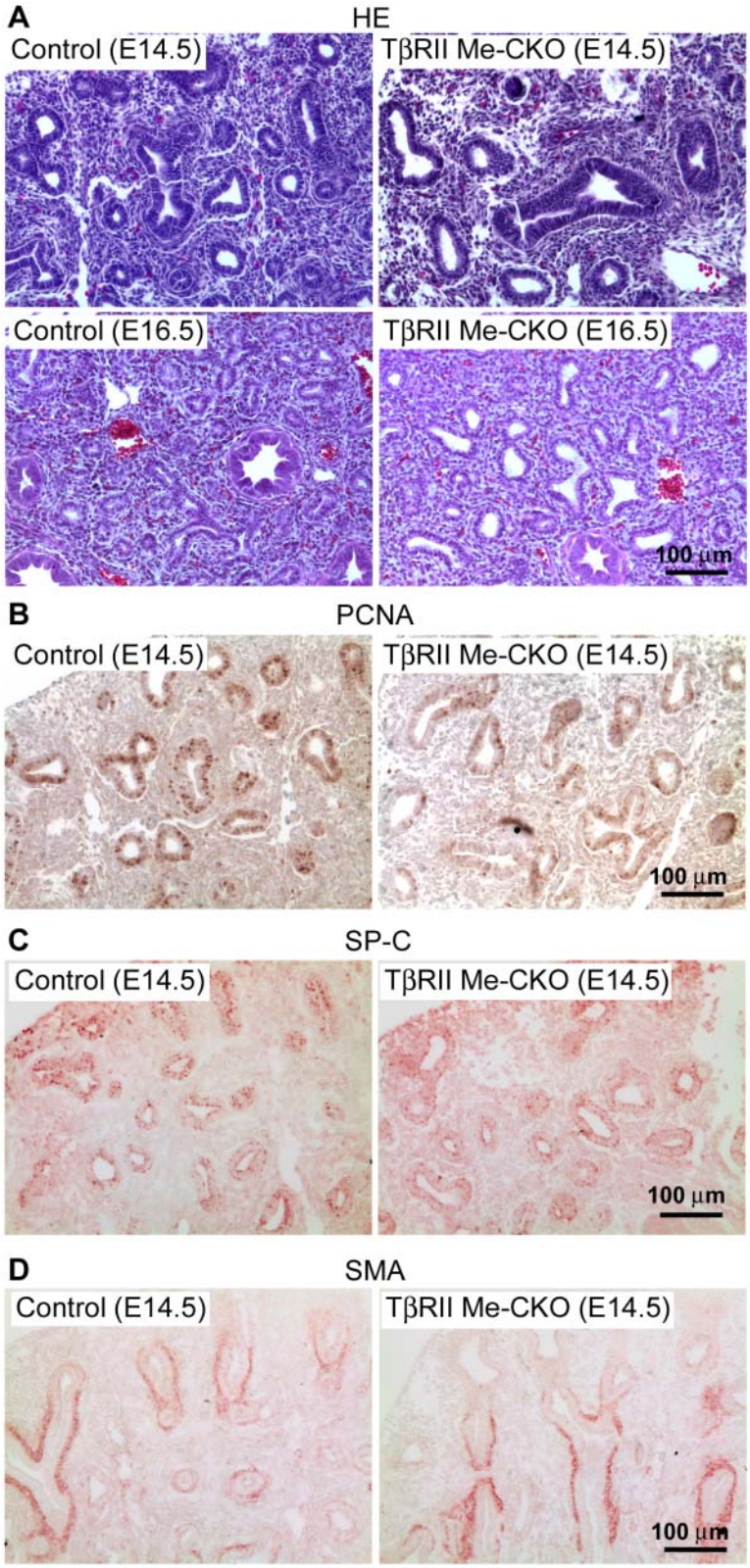
1mm
E
Control
TβRII Me-CKO
0.5 mm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Control (E16.5)
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100 μm
B
PCNA
Control (E14.5)
TβRII Me-CKO (E14.5)
100 μm
C
SP-C
Control (E14.5)
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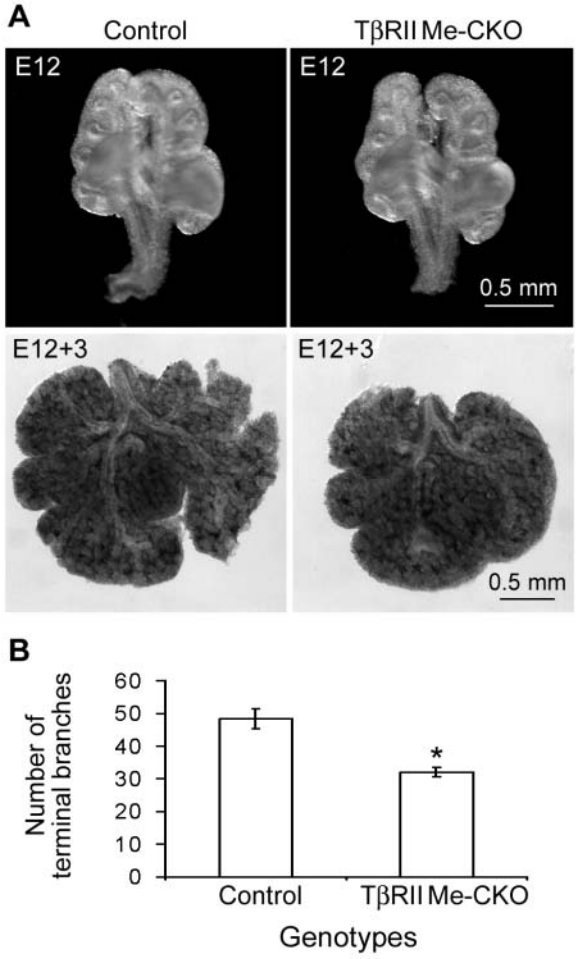
D
SMA
Control (E14.5)
TβRII Me-CKO (E14.5)
100 μm


A
Control
TβRII Me-CKO
E12
E12
0.5 mm
E12+3
E12+3
0.5 mm
B
Number of terminal branches
60
50
40
30
20
10
0
*
Control
TβRII Me-CKO
Genotypes