Early Growth Response-1 Suppresses Epidermal Growth Factor Receptor–Mediated Airway Hyperresponsiveness and Lung Remodeling in Mice

Elizabeth L. Kramer¹, Elizabeth M. Mushaben¹, Patricia A. Pastura¹, Thomas H. Acciani¹, Gail H. Deutsch², Gurjit K. Khurana Hershey^{3,4}, Thomas R. Korfhagen¹, William D. Hardie⁵, Jeffrey A. Whitsett¹, and Timothy D. Le Cras¹

¹Section of Neonatology, Perinatal & Pulmonary Biology, ²Divisions of Pathology, ³Asthma Research, ⁴Allergy and Immunology, and ⁵Pulmonary Medicine, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio

Transforming growth factor (TGF)- α and its receptor, the epidermal growth factor receptor, are induced after lung injury and are associated with remodeling in chronic pulmonary diseases, such as pulmonary fibrosis and asthma. Expression of TGF- α in the lungs of adult mice causes fibrosis, pleural thickening, and pulmonary hypertension, in addition to increased expression of a transcription factor, early growth response-1 (Egr-1). Egr-1 was increased in airway smooth muscle (ASM) and the vascular adventitia in the lungs of mice conditionally expressing TGF- α in airway epithelium (Clara cell secretory protein-rtTA^{+/+}/[tetO]₇-TGF- α ^{+/+}). The goal of this study was to determine the role of Egr-1 in TGF- α -induced lung disease. To accomplish this, TGF- α -transgenic mice were crossed to Egr-1 knockout (Egr-1^{ko/ko}) mice. The lack of Egr-1 markedly increased the severity of TGF- α -induced pulmonary disease, dramatically enhancing airway muscularization, increasing pulmonary fibrosis, and causing greater airway hyperresponsiveness to methacholine. Smooth muscle hyperplasia, not hypertrophy, caused the ASM thickening in the absence of Egr-1. No detectable increases in pulmonary inflammation were found. In addition to the airway remodeling disease, vascular remodeling and pulmonary hypertension were also more severe in Egr-1^{ko/ko} mice. Thus, Egr-1 acts to suppress epidermal growth factor receptor–mediated airway and vascular muscularization, fibrosis, and airway hyperresponsiveness in the absence of inflammation. This provides a unique model to study the processes causing pulmonary fibrosis and ASM thickening without the complicating effects of inflammation.

Keywords: transforming growth factor- α ; pulmonary fibrosis; asthma; pulmonary hypertension; vascular remodeling

Lung remodeling contributes to the chronicity and abnormal lung function that occurs in chronic obstructive pulmonary disease, cystic fibrosis, bronchopulmonary dysplasia, pulmonary fibrosis, and asthma. In asthma, airway remodeling is believed to contribute to the more rapid decline of lung function, and may occur early in the disease (1). Proliferation of fibroblasts, myofibroblasts, and smooth muscle cells, and excessive production of extracellular matrix, contributes to airway and vascular remodeling, and are resistant to current therapies, including corticosteroids (2, 3). Hence, identification of pathways driving the proliferation of these cells and lung remodeling

CLINICAL RELEVANCE

This study demonstrates that loss of early growth response-1 exacerbates transforming growth factor- α -induced lung disease in mice, characterized by dramatic airway smooth muscle (ASM) hyperplasia, hyperresponsiveness, and fibrosis. This noninflammatory model of lung remodeling may facilitate the testing of experimental therapies to inhibit airway and vascular smooth muscle remodeling without the complicating effects of inflammation. This is pertinent, as recent clinical studies have suggested that targeted elimination of ASM improves outcome in asthma.

is critical to identify novel therapeutic targets for treatment of chronic pulmonary disorders.

The epidermal growth factor (EGF) ligands and ErbB receptor family play critical roles in development and tissue repair after injury; however, a number of clinical studies now suggest that dysregulated activation of these pathways may also contribute to remodeling in chronic lung diseases (4, 5). For example, in asthma, EGF receptor (EGFR) expression is increased, and correlates with the severity of airway smooth muscle (ASM) thickening (6). Expression and release of EGFR ligands, including EGF and transforming growth factor (TGF)- α , are increased in ASM and epithelial cells from subjects with asthma and are stimulated by proinflammatory cytokines and allergens (7). Experimental studies provide strong support for the role of EGFR signaling in the pathogenesis of pulmonary fibrosis and allergen-induced airway disease. TGF- α and EGFR are increased in bleomycin-induced pulmonary fibrosis, and the severity of fibrosis is attenuated in TGF- α -null mice compared with wild-type mice treated with bleomycin (8). In the ovalbumin-induced model of asthma in mice and rats, treatment with an EGFR inhibitor reduced inflammation, airway hyperresponsiveness (AHR), ASM hyperplasia, and lung remodeling (9, 10). Monocrotaline-induced vascular remodeling and pulmonary hypertension (PH) were also blocked by treatment with an EGFR inhibitor (11). Collectively, clinical and experimental studies suggest that EGFR signaling pathways contribute to remodeling in these chronic lung diseases (4, 5).

Studies in transgenic mice have shown that increased expression of TGF- α by lung epithelial cells using the surfactant protein C promoter causes extensive fibrosis and remodeling in the absence of inflammation (12–14). Activation of TGF- α expression in the epithelium of the distal and conducting airways of adult mice, using a conditional doxycycline (Dox)-regulated transgene and the Clara cell secretory protein (CCSP) promoter, also caused perivascular and peribronchiolar fibrosis, pleural thickening, increased vascular muscularization, and PH (15–17). Treatment with EGFR inhibitors prevented lung fibrosis and remodeling in these mice, and halted further pro-

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Correspondence and requests for reprints should be addressed to Tim Le Cras, Ph.D., Cincinnati Children's Hospital Medical Center, 3333 Burnet Ave., Cincinnati, OH 45229. E-mail: tim.lecras@cchmc.org

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gression when inhibitor treatment was started after the fibrosis and remodeling were initiated (17). Interestingly, even transient expression of TGF- α in the lungs of neonatal mice caused chronic lung disease that persisted even after TGF- α expression was down-regulated (18).

Microarray studies have now identified genes that were induced during the lung remodeling process in these transgenic models (19, 20). One of the most highly induced genes in the lungs of fetal and neonatal mice expressing TGF- α was the immediate early gene, early growth response (Egr)-1 (20). Studies by Liu and colleagues (21) had previously detected increases in Egr-1 gene expression in the lungs of adult mice after systemic treatment with EGF. Egr-1, a zinc finger transcription factor, also known as NGFI-A, Krox-24, and zif268, is induced by a number of receptors, growth factors, and signaling pathways, and binds to a GC-rich consensus sequence in the promoters of genes (22). Egr-1 can stimulate or repress transcription depending on the gene, the other cofactors present, and the cell type (22). In some tumor cells, Egr-1 has been shown to promote cell growth, whereas in others it acts as a tumor suppressor (23–28).

In the present study, we determined that Egr-1 expression was low in the normal adult lung, but was rapidly induced in remodeling airways and vessels after TGF- α induction. Induction of TGF- α in mice lacking Egr-1 (Egr-1^{ko/ko}) caused a rapid decrease in body weight, as well as severe ASM thickening and AHR. The severity of fibrosis, vascular remodeling, and PH was also increased. There was no detectable inflammation after TGF- α induction in Egr-1 wild-type or null mice. Hence, loss of Egr-1 expression unmasks a noninflammatory, severe reactive airway disease during TGF- α -induced EGFR signaling, indicating a potential role for Egr-1 in suppression of chronic pulmonary remodeling *in vivo*. Some of the results of these studies have been previously reported in the form of an abstract (29).

MATERIALS AND METHODS

Animals

Protocols for animal use were approved by the Animal Care and Use Committee at the Cincinnati Children's Hospital Research Foundation. CCSP-reverse tetracycline-controlled transactivator (rtTA)^{+/+}-(tetracycline operator [tetO])⁺-TGF- α ^{+/+} mice (FVB/N strain) were used to express TGF- α in the lung epithelium under control of Dox (16). No TGF- α transgene expression is detectable in the absence of Dox (18). Adult CCSP-rtTA^{+/+}-(tetO)⁺-TGF- α ^{+/+} mice (6–8 wk of age) were put on Dox-containing chow (625 mg/kg) for 4 days, 10 days, or 3 weeks, and then killed using pentobarbital sodium (65 mg/ml) solution (Fort Dodge Animal Health, Fort Dodge, IA) for assessment of Egr-1 expression. Bitransgenic mice not on Dox were used as control animals.

To determine the role of Egr-1 in TGF- α -induced lung disease, CCSP-rtTA^{+/+} and (tetO)⁺-TGF- α ^{+/+}-transgenic mice were mated to Egr-1 knockout mice (C57Bl/6 strain) obtained from Taconic (Germantown, NY) (30). Pups with both CCSP-rtTA^{+/+} and (tetO)⁺-TGF- α ^{+/+} transgenes were generated that were Egr-1^{+/+} (wild-type), Egr-1^{+/-}, or Egr-1^{-/-} (knockout). Hereafter, these will be referred to as Egr-1^{wt/wt}, Egr-1^{ko/wt}, and Egr-1^{ko/ko} mice.

At 6–8 weeks of age, mice were placed on Dox for 10 days, 3 weeks, or 8 weeks to examine early and chronic responses to TGF- α -induced pulmonary disease. Control mice for these studies were CCSP-rtTA^{+/+}-(tetO)⁺-TGF- α ^{+/+} mice that were either homozygous, heterozygous, or null for Egr-1, and not placed on Dox (no-Dox controls). Body weights were measured weekly after placing mice on Dox. Adult mice were killed at the end of the study period using either 0.2–0.5 ml pentobarbital sodium or 0.2–0.4 ml of a ketamine/xylazine/acepromazine (4:1:1) solution.

Western Blot Analysis

Western blot analysis was performed on lung homogenates from mice on Dox for 10 days, no-Dox controls, and cell lysates, as previously

described (20). Primary antibodies against Egr-1 (1:400 dilution, C19; Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated EGFR (pY1086, 1:1000; Epitomics, Burlingame, CA), total EGFR (1:4,000; Epitomics, Burlingame, CA), and pan-actin (1:20,000; MAB1501, clone C4; Chemicon, Billerica, MA) were used. Secondary antibodies used included goat anti-rabbit or goat anti-mouse (Santa Cruz Biotechnology), and chemiluminescence detection was performed using the ECL Plus system (Amersham Biosciences, Piscataway, NJ). Images of Western blots were obtained using the LAS4000 imaging system (Fujifilm, Valhalla, NY) or X-ray film.

Immunohistochemistry and Immunofluorescence

Immunohistochemistry was performed on the lungs of Egr-1^{wt/wt}, Egr-1^{ko/wt}, and Egr-1^{ko/ko} mice treated with Dox and on no-Dox control animals. Lungs were inflation fixed and stained as previously described (18, 20). Immunohistochemical staining for Egr-1, Ki67, α -smooth muscle actin (α -SMA), and smooth muscle II myosin heavy chain (SM-MHC) was performed on 5- μ m paraffin-embedded sections by incubating slides overnight at 4°C with Egr-1 antibody (1:600 dilution, C19; Santa Cruz Biotechnology), Ki67 (1:500 dilution, Clone TEC-3; Dako, Carpinteria, CA), α -SMA (1:10,000 dilution, Clone 1A4; Sigma, St. Louis, MO) or SM-MHC (1:25 dilution; Biomedical Technologies, Stoughton, MA). Movat's pentachrome staining of paraffin-embedded sections was also performed.

Immunofluorescence was performed using primary antibodies for Egr-1 (1:60 dilution, C19; Santa Cruz Biotechnology) and α -SMA (1:1000 dilution, Clone 1A4; Sigma) incubated on slides overnight at 4°C. Secondary antibodies used were 594 red donkey anti-rabbit (1:200 dilution; Invitrogen, Carlsbad, CA) or 488 green goat anti-mouse labeled IgG (1:200 dilution; Invitrogen). Vecto Shield with 4',6'-diamidino-2-phenylindole (DAPI) was applied to each slide before coverslipping to stain cell nuclei. Digital images of the immunostaining were acquired using a Zeiss Axioplan 2 microscope (Carl Zeiss Microimaging, Thornwood, NY).

In Vitro Studies

Human pulmonary artery smooth muscle cells (HPASMC; Cascade Biologics, Portland, OR) and human lung fibroblasts (CCD-19Lu cells; ATCC, Manassas, VA) were cultured under standard conditions at 37°C and 5% CO₂. HPASMCs were cultured in Medium 231 (Cascade Biologics), 0.2% gentamycin/amphotericin solution (Cascade Biologics), and 5% smooth muscle growth supplements (Cascade Biologics). CCD-19Lu cells were cultured in Eagle's minimal essential medium (EMEM) with nonessential amino acids (Gibco, Grand Island, NY), 1 mM sodium pyruvate (Gibco), 2 mM L-glutamine (Sigma), 1% penicillin-streptomycin solution (Sigma), and 10% FBS (Sigma). Before stimulation with EGF, semiconfluent cells were cultured for 24 hours in low-serum media, defined as normal media plus either 0.05% smooth muscle growth supplement (HPASMCs) or 0.1% FBS (CCD-19Lu). EGF was added to the media to stimulate EGFR signaling (50 ng/ml for CCD-19Lu cells; 100 ng/ml for the HPASMCs; PeproTech, Rocky Hill, NJ). After 1 hour, cells were trypsinized, scraped, lysed, and cell lysates stored at -80°C for Western blot analysis. Unstimulated control cells were also collected after 1 hour.

Sircol Collagen Assay

To assess pulmonary fibrosis, lung collagen was measured using the Sircol collagen assay with the left lung from Egr-1^{wt/wt}, Egr-1^{ko/wt}, and Egr-1^{ko/ko} mice on Dox for either 3 or 8 weeks and no-Dox control animals, as previously described (17).

ASM Morphometry

Immunofluorescent staining for α -SMA was performed on lung sections from Egr-1^{wt/wt} and Egr-1^{ko/ko} mice on Dox for 8 weeks, as well as no-Dox control mice. Internal perimeter (a marker of airway size), ASM area, and the number of α -SMA-positive cells were measured and analyzed using previously described techniques (31–33). ASM morphometry was performed after immunofluorescent staining for α -SMA on lung sections from Egr-1^{wt/wt} and Egr-1^{ko/ko} mice on Dox 8 weeks and no-Dox control mice. The image analyzer was blinded to the mouse genotypes. In each section, all airways cut perpendicularly were used for

imaging, and images of the green (α -SMA) and blue (DAPI) channels were captured using a Zeiss Axioplan 2 microscope. Images were imported into MetaMorph imaging software (v6.2; Universal Imaging/Molecular Devices, Downingtown, PA) for analysis. Internal perimeter (luminal border of airway epithelial cells) was measured. The threshold needed to include all α -SMA staining based upon image intensity was selected. The area of this thresholded section was calculated, and the DAPI-stained cell nuclei within that section were counted using MetaMorph. ASM area was divided by the number of cell nuclei in that area to determine individual ASM cell size, assuming that one nucleus corresponded to one cell. The number of ASM nuclei present around each airway and the square root of ASM area were normalized to internal perimeter, as previously described (33). One-way ANOVA with the Tukey *post hoc* test was used to analyze results.

AHR and Lung Mechanics

Baseline lung mechanics and AHR to methacholine (acetyl- β -methylcholine chloride; Sigma) were assessed in mice on Dox for 3 or 8 weeks and no-Dox control mice using flexiVent, an animal mechanical ventilator system (SCIREQ, Montreal, PQ, Canada) (34). Mice were anesthetized with an intraperitoneal injection of ketamine/xylazine/acepromazine solution, tracheostomized, and connected to the flexiVent. Mechanical ventilation was set at 150 breaths/minute with a tidal volume of 0.16 ml/kg and a positive end-expiratory pressure of 3 cm H₂O. Baseline lung mechanics (airway resistance, airway elastance, compliance, tissue elastance, tissue damping, and hysteresivity) were measured in each mouse after nebulization of PBS (vehicle for methacholine) for 10 seconds using an Aeroneb ultrasonic nebulizer (SCIREQ). After baseline measurements, methacholine challenges were performed. Mice on Dox for 3 weeks were challenged with nebulized methacholine at the following concentrations: 3.125, 6.25, 12.5, 25, and 50 mg/ml. Based on these dose-response data, mice on Dox for 8 weeks were challenged with a single dose of 12.5 mg/ml methacholine. A separate group of mice on Dox for 8 weeks was treated with albuterol (25 mg/ml) (salbutamol hemisulfate salt; Sigma), and lung mechanics were measured as described above.

PH

Right ventricle (RV) and left ventricle plus septum weights were measured as previously described in Egr-1^{wt/wt} and Egr-1^{ko/ko} mice on Dox 8 weeks as well as no-Dox control mice (18). RV hypertrophy (RVH) was assessed by calculating the RV to left ventricle plus septum weight ratio.

Bronchoalveolar Lavage and Inflammatory Cell Counts

Egr-1^{wt/wt} and Egr-1^{ko/ko} mice were placed on Dox for 10 days and 3 weeks, and then bronchoalveolar lavage fluid (BALF) was collected and analyzed as previously described (16).

Data and Statistical Analysis

Data analyses were performed using the Prism 4 software package (GraphPad Software, San Diego, CA). One-way ANOVA with the Tukey *post hoc* test and unpaired *t* tests were used to make statistical comparisons, and *P* < 0.05 was considered statistically significant.

RESULTS

TGF- α and EGFR Signaling Induce Expression of Egr-1

TGF- α -expressing (Egr-1^{wt/wt}) mice were treated with Dox for 10 days to induce TGF- α expression and EGFR signaling. Egr-1 protein was markedly induced by the expression of TGF- α (Figure 1A). In no-Dox control lungs, Egr-1 was barely detectable. TGF- α increased Egr-1 in the nuclei of cells surrounding airways, as well as in the adventitial and medial layer of arteries (Figure 1B). Dual immunofluorescence labeling demonstrated that Egr-1 (red) and α -SMA (green) were coexpressed in cells underlying the airway epithelium (Figure 1C). Egr-1 was also induced in α -SMA-negative cells in the adventitial layer surrounding vessels (Figure 1C, *yellow arrows*). In addition, EGFR activa-

Figure 1. Transforming growth factor (TGF)- α influences the expression and localization of early growth response (Egr)-1. (A) Clara cell secretory protein (CCSP)-rtTA^{+/-(tetO)7}-TGF- α ^{+/-(tetO)7} mice were left untreated or treated for 10 days with doxycycline (Dox). Western blot demonstrated increased Egr-1 in lung homogenates after expression of TGF- α . (B) Egr-1 was detected by immunohistochemistry around airways (A) and vessels (V) in cells in both the peribronchiolar area and the vascular adventitia, and the number of Egr-1-positive cells was increased by expression of TGF- α regulated by Dox treatment for 4 days and 3 weeks. Scale bars = 50 μ m. (C) Dual immunofluorescence demonstrated coexpression of Egr-1 (red) and α -smooth muscle actin (SMA; green) in cells underlying airways (A), as well as Egr-1 induction in α -SMA-negative cells (*yellow arrows*) in the adventitia of vessels (V). Egr-1-positive smooth muscle (SM) cells are shown in a high-power image underlying the airway epithelium (E). Scale bars = 20 μ m.

tion *in vitro* induced Egr-1 protein expression in both human pulmonary artery smooth muscle cells and human lung fibroblasts within one hour (see Figure E1 in the online supplement).

Breeding and Body Weight Changes in Egr-1

Loss-of-Function Studies

To determine the role of Egr-1 in TGF- α -induced lung disease, CCSP-rtTA^{+/+} or (tetO)₇-TGF- α ^{+/+}-transgenic mice were bred to Egr-1 knockout mice (Figure 2A). All experimental animals from these crosses were CCSP-rtTA^{+/+}/(tetO)₇-TGF- α ^{+/+}, and occurred in the expected 1:2:1 ratio (Egr-1^{wt/wt}:Egr-1^{ko/wt}:Egr-1^{ko/ko}). Mice were treated with Dox to induce TGF- α . Weekly body weight measurements showed that both Egr-1^{wt/wt} and Egr-1^{ko/wt} mice initially gained weight; body weight gain leveled off after 4–5 weeks of exposure to Dox (Figure 2B). In Egr-1^{ko/ko} mice, however, weight loss occurred after only 4 weeks on Dox as compared with Egr-1^{wt/wt} mice. Hence, mice were studied after 3 weeks of Dox treatment to examine the early phenotype. Another group of mice was collected after 8 weeks on Dox to examine the chronic effects of TGF- α in Egr-1^{wt/wt} and Egr-1^{ko/ko} backgrounds.

Effects of Egr-1 on Lung Remodeling and Fibrosis after 3-Week TGF- α Induction

To assess changes in lung remodeling and fibrosis in Egr-1^{ko/ko} mice, pentachrome staining was performed on Egr-1^{wt/wt}, Egr-1^{ko/wt}, and Egr-1^{ko/ko} mice treated with Dox for 3 weeks, as well as on no-Dox control mice. As expected, in no-Dox control animals, fibrosis and airway and vascular remodeling were not detected (Figure 3A). Egr-1^{wt/wt} mice on Dox showed developing fibrosis around airways and vessels. Fibroblastic plugs, protruding areas of fibroblast, and extracellular matrix accumulation were present in the airways of Egr-1^{wt/wt} and Egr-1^{ko/ko} mice on Dox (Figure 3A, red arrows). Egr-1^{ko/ko} mice on Dox also had fibrotic remodeling, and showed evidence of prominent smooth muscle thickening around airways and vessels (Figure 3A, yellow arrows). Immunostaining for the cell proliferation marker, Ki67, identified proliferating cells in the fibrotic and remodeling areas in Egr-1^{wt/wt} and Egr-1^{ko/ko} mice on Dox 3 weeks (Figure 3A). Sircol collagen assays demonstrated increased collagen content in lungs of Egr-1^{ko/ko} and Egr-1^{ko/wt} mice on Dox for 3 weeks compared with Egr-1^{wt/wt} mice and no-Dox control mice (Figure 3B).

Figure 2. Breeding protocol and body weights of Egr-1^{wt/wt}, Egr-1^{ko/wt}, and Egr-1^{ko/ko} mice during expression of TGF- α . (A) Egr-1-null mice were bred with CCSP-rtTA^{+/+}-transgenic mice and (tetO)₇-TGF- α ^{+/+}-transgenic mice. The final mating performed is shown, and resulted in litters composed of all CCSP-rtTA^{+/+}/(tetO)₇-TGF- α ^{+/+}-bitransgenic mice that differed in their Egr-1 genotype (Egr-1^{wt/wt}, Egr-1^{ko/wt}, or Egr-1^{ko/ko}). (B) Mice were placed on Dox for 8 weeks and body weights were measured weekly ($n = 6-9$ per group). Egr-1^{ko/ko} mice lost body weight early, after just 4 weeks of Dox. * $P < 0.05$ versus Egr-1^{wt/wt} mice.

Figure 3. TGF-α-induced fibrosis and cell proliferation in Egr-1^{ko/ko} and Egr-1^{wt/wt} mice. (A) Pentachrome staining showed that mice on Dox for 3 weeks developed perivascular and peribronchiolar fibrosis, as well as fibroblastic plugs in airways (red arrows). Egr-1^{ko/ko} mice also had prominent smooth muscle thickening around airways and vessels (yellow arrows). Staining for Ki67 showed increased proliferation in remodeling areas around airways and vessels in Egr-1^{wt/wt} and Egr-1^{ko/ko} mice on Dox. Scale bars = 100 μm. (B) Sircol collagen assays were performed on Egr-1^{wt/wt}, Egr-1^{ko/wt}, and Egr-1^{ko/ko} mice on Dox ($n = 5, 3$, and 11 , respectively), as well as no-Dox control animals ($n = 7$). Egr-1^{ko/ko} and Egr-1^{ko/wt} mice on Dox had higher lung collagen content than no-Dox control mice. After 3 weeks on Dox, Egr-1^{wt/wt} animals did not yet have increased lung collagen compared with no-Dox control mice. * $P < 0.05$ versus Egr-1^{wt/wt} mice.

Effects of Egr-1 on Airway Muscularization and Lung Mechanics after 3-Week TGF-α Induction

Staining for α-SMA showed normal airway muscularization in no-Dox control mice, regardless of Egr-1 genotype (Figure 4A). In Egr-1^{ko/ko} mice, expression of TGF-α for 3 weeks caused dramatic thickening of the smooth muscle around airways and vessels (Figure 4A, black arrows). Immunostaining for SM-MHC showed similar staining patterns to α-SMA, indicating an increase in differentiated airway and vascular smooth muscle cells (Figure 4A, open arrows). Measurement of lung mechanics did not reveal differences in baseline airway resistance between the groups, but lung compliance was significantly decreased in Egr-1^{ko/ko} mice expressing TGF-α. After methacholine challenge, TGF-α-induced airway hypersensitivity and hyperreactivity were increased in Egr-1^{ko/ko} mice (Figure 4B and Figure E2). At 6.25 to 50 mg/ml methacholine, the airway resistance of Egr-1^{ko/ko} mice was increased to a much greater extent than in Egr-1^{wt/wt} mice. TGF-α-induced abnormalities in compliance were significantly increased at all methacholine doses up to 50 mg/ml. At this dose, airway resistance was increased 6.4-fold,

and lung compliance decreased 2.9-fold, in Egr-1^{ko/ko} mice compared with Egr-1^{wt/wt} mice.

Effects of Egr-1 on Fibrosis, Airway Muscularization, and Lung Mechanics after 8-Week TGF-α Induction

To examine the consequences of chronic TGF-α expression in Egr-1^{wt/wt}, Egr-1^{ko/wt}, and Egr-1^{ko/ko} animals, mice were placed on Dox for 8 weeks. Pentachrome staining showed extensive fibrosis around airways and vessels in both Egr-1^{ko/ko} and Egr-1^{wt/wt} mice (Figure 5A). Airway and vascular muscularization were increased in the absence of Egr-1. TGF-α-induced remodeling, fibrosis, and muscularization were more extensive after 8 weeks compared with 3 weeks of Dox treatment. Collagen content was increased in all mice after TGF-α expression (Figure 5B). Collagen content was increased in Egr-1^{ko/ko} and Egr-1^{ko/wt} compared with Egr-1^{wt/wt} mice (1.8-fold greater and 1.3-fold greater, respectively). After 8 weeks of TGF-α expression, lung collagen content was higher in Egr-1^{ko/ko} mice than in Egr-1^{ko/wt} mice, suggesting a dose-dependent effect of Egr-1 loss on the severity of fibrosis (Figure 5B).

Figure 4. Effect of TGF- α on airway muscularization and lung mechanics in Egr-1^{ko/ko}, Egr-1^{ko/wt}, and Egr-1^{wt/wt} mice on Dox for 3 weeks. (A) Immunostaining for α -SMA and smooth muscle II myosin heavy chain (SM-MHC) in lung sections from Egr-1^{wt/wt} and Egr-1^{ko/ko} no-Dox control animals showed normal lung histology. After Dox treatment for 3 weeks, however, Egr-1^{ko/ko} animals had dramatic smooth muscle thickening around airways, and also smooth muscle metaplasia in the adventitia of vessels, as shown by α -SMA (closed arrows) and SM-MHC (open arrows) staining. Scale bar = 100 μ m. (B) Lung mechanics were measured in Egr-1^{wt/wt}, Egr-1^{ko/wt}, and Egr-1^{ko/ko} mice on Dox ($n = 5, 3$, and 11, respectively), as well as no-Dox control mice ($n = 7$). Airway resistance was higher in Egr-1-null mice compared with Egr-1^{wt/wt} mice after inhalation of 6.25 mg/ml methacholine and higher doses. Lung compliance in Egr-1^{ko/ko} animals was lower than Egr-1^{wt/wt} littermates at baseline and all doses of methacholine. Statistical comparisons are only shown for Egr-1^{ko/wt} and Egr-1^{ko/ko} mice. * $P < 0.05$ compared with Egr-1^{wt/wt} animals; # $P < 0.05$ compared with baseline measurements; $\wedge P < 0.05$ compared with Egr-1^{ko/wt} animals.

Next, we examined airway and vascular muscularization in Egr-1^{wt/wt} and Egr-1^{ko/ko} mice expressing TGF- α for 8 weeks. Increased numbers of α -SMA-positive cells were observed around both airways and arteries in Egr-1^{ko/ko} mice after expression of TGF- α (Figure 6A). Immunostaining for α -SMA and SM-MHC on adjacent sections showed that α -SMA-positive cells were also positive for SM-MHC, indicating that the increase in muscularization included differentiated smooth muscle cells in Egr-1^{ko/ko} mice (Figure 6A).

Because Egr-1^{ko/ko} mice after 8 weeks of Dox showed extensive muscularization of airways, we performed morphometric analysis on coimmunofluorescent staining for α -SMA and DAPI. This analysis enabled us to determine whether TGF- α -induced ASM thickening in Egr-1^{ko/ko} mice was due to smooth muscle cell hyperplasia and/or hypertrophy. ASM area, number of ASM nuclei, and internal perimeters were measured in cross-sectioned airways from control no-Dox mice and Egr-1^{wt/wt} and Egr-1^{ko/ko} mice treated with Dox for 8 weeks (Figure E3). The range of internal perimeters assessed was not significantly different across the three groups (data not shown). As expected based on lung histology and immunostaining, Egr-1^{ko/ko} mice had 1.7-fold higher ASM area around airways compared with Egr-1^{wt/wt} mice, and ASM area was twofold higher than in

control animals (Figure 6B). The number of ASM cells seen after TGF- α expression was increased 1.8-fold in Egr-1^{ko/ko} mice compared with Egr-1^{wt/wt} mice. The area per ASM cell was not affected by Dox treatment or genotype (Figure E3). Taken together, these data indicate that smooth muscle cell hyperplasia is the predominant cause of TGF- α -induced ASM thickening in Egr-1^{ko/ko} mice.

Lung mechanics and responses to methacholine challenge were assessed in Egr-1^{wt/wt}, Egr-1^{ko/wt}, and Egr-1^{ko/ko} mice after 8 weeks on Dox (Figure 6C and Figure E4). A challenge with 12.5 mg/ml methacholine was performed based upon the dose-response studies performed after 3 weeks on Dox. Baseline airway resistance was increased in Egr-1^{ko/ko} and Egr-1^{ko/wt} mice compared with Egr-1^{wt/wt} animals, and baseline compliance was decreased in Egr-1^{ko/ko} and Egr-1^{ko/wt} animals as compared with Egr-1^{wt/wt} littermates (Figure 6C). After challenge with 12.5 mg/ml methacholine, the airway resistance of Egr-1^{ko/ko} animals on Dox was 3.7-fold higher than in Egr-1^{wt/wt} mice. Lung compliance in Egr-1^{ko/ko} mice after methacholine challenge was 78.5% less than the lung compliance of Egr-1^{wt/wt} mice and 86.9% less than the lung compliance of no-Dox control animals. Airway resistance and airway compliance of Egr-1^{ko/wt} mice and Egr-1^{wt/wt} mice after methacholine chal-

Figure 5. TGF- α -induced pulmonary fibrosis in Egr-1^{wt/wt}, Egr-1^{ko/wt}, and Egr-1^{ko/ko} mice after 8 weeks of Dox treatment. (A) Pentachrome staining of Egr-1^{wt/wt} and Egr-1^{ko/ko} mice showed increased proteoglycan-rich extracellular matrix around vessels and airways as compared with no-Dox control animals. Egr-1^{ko/ko} mice also showed greater smooth muscle remodeling in these areas. Scale bar = 100 μm . (B) All mice on Dox, regardless of Egr-1 genotype, had higher lung collagen content than no-Dox controls ($n = 5-9$ mice per group). Lung collagen was also higher in Egr-1^{ko/wt} mice than Egr-1^{wt/wt} mice, and Egr-1^{ko/ko} mice had greater lung collagen content than Egr-1^{ko/wt} mice. * $P < 0.05$ compared with no-Dox controls; # $P < 0.05$ compared with Egr-1^{wt/wt} mice; ^ $P < 0.05$ compared with Egr-1^{ko/wt} mice.

lunge were similar; however, the baseline values of these two groups differed (Figure 6C).

Lung mechanics of Egr-1^{wt/wt} and Egr-1^{ko/ko} mice on Dox for 8 weeks were also measured after treatment with the bronchodilator albuterol. Although Egr-1^{ko/ko} mice had higher baseline airway resistance (also seen before methacholine challenge; Figure 6C), treatment with albuterol failed to lower airway resistance (Figure E4). This result indicates that increased baseline airway resistance in these mice is fixed, consistent with the observed airway pathology, which includes severe fibrosis.

Inflammation in Egr-1 Loss-of-Function Studies

To assess whether inflammatory cells might be contributing to airway remodeling, BALF was collected and inflammatory cells counted ($n = 4-8$ mice/group/time point). BALF was collected from Egr-1^{ko/ko} and Egr-1^{wt/wt} animals after 10 days (early in the disease course) and 3 weeks of Dox treatment (when AHR was already present), as well as no-Dox control animals. Total cell counts in no-Dox control mice, Egr-1^{wt/wt} mice, and Egr-1^{ko/ko} were similar (Table E1). Likewise, differential counts revealed no differences between the different genotypes on Dox or no-Dox control animals. Macrophages were the most common inflammatory cell type, whereas

lymphocytes, neutrophils, eosinophils, and basophils made up less than 5% of the remaining cells (Table E1). Measurements of cells in BALF suggest that inflammatory cells were not increased in Egr-1^{ko/ko} mice compared with Egr-1^{wt/wt} mice on Dox. Inflammatory cells were also not observed in lung histology from either Egr-1^{wt/wt} mice or Egr-1^{ko/ko} mice on Dox (Figures 1, 3, 5).

Pulmonary Arterial Hypertension and Vascular Remodeling

In addition to increased TGF- α -induced ASM thickening, extensive muscularization of pulmonary arteries was observed in Egr-1^{ko/ko} mice (Figure 7A). α -SMA and pentachrome staining of serial sections showed severe fibrosis around vessels in both Egr-1^{wt/wt} and Egr-1^{ko/ko} mice treated with Dox for 8 weeks (Figure 7A, black arrows). However, smooth muscle metaplasia in the vascular adventitia was seen only in Egr-1^{ko/ko} mice (Figure 7A, red arrows). Arteries with extreme smooth muscle metaplasia had narrowing of their lumens or were occluded (Figure 7A, yellow arrowhead). Since previous studies demonstrated that PH occurs after elevated expression of TGF- α , we assessed RVH in Egr-1^{ko/ko} and Egr-1^{wt/wt} mice treated with Dox for 8 weeks (17-19). RV weights were increased in Egr-1^{ko/ko} compared with Egr-1^{wt/wt} mice, indicating increased pulmonary arterial hypertension severity consistent

Figure 6. Effect of TGF- α on lung mechanics and airway smooth muscle (ASM) morphometry in Egr-1^{wt/wt}, Egr-1^{ko/wt}, and Egr-1^{ko/ko} mice on Dox for 8 weeks. (A) α -SMA immunostaining showed that airway muscularization was similar in all no-Dox animals regardless of Egr-1 genotype. Egr-1^{ko/ko} mice on Dox had increased airway and vascular adventitial α -SMA staining (arrows) compared with Egr-1^{wt/wt} mice. Staining for SM-MHC and α -SMA on serial sections showed similar increased staining patterns in Egr-1^{ko/ko} mice, indicating that these α -SMA-positive cells were differentiated smooth muscle. Scale bar = 100 μ m. (B) The area of ASM in Egr-1^{ko/ko} mice on Dox normalized to internal perimeter was increased compared with no-Dox control mice as well as Egr-1^{wt/wt} mice on Dox. The number of ASM nuclei was also greater in Egr-1^{ko/ko} mice on Dox. * $P < 0.05$ compared with no-Dox controls; # $P < 0.05$ versus Egr-1^{wt/wt} mice. (C) Airway resistance was increased, whereas compliance was decreased, in Egr-1^{ko/ko} mice both at baseline and after inhalation of 12.5 mg/ml methacholine compared with Egr-1^{wt/wt} mice. Egr-1^{ko/wt} mice had increased baseline airway resistance, but responses after methacholine challenge were similar to Egr-1^{wt/wt} mice on Dox ($n = 5$ –10 mice per group). * $P < 0.05$ compared with Egr-1^{wt/wt} animals at the same dose; # $P < 0.05$ compared with baseline measurements of the respective genotype; ^ $P < 0.05$ compared with Egr-1^{ko/wt} animals at the same dose.

with increased severity of vascular remodeling in these mice (Figure 7B).

DISCUSSION

In this study, TGF- α and EGFR signaling induced expression of Egr-1 in smooth muscle cells and fibroblasts in remodeling

airways and vessels in transgenic mice, as well as in human lung fibroblasts and smooth muscle cells *in vitro*. The severity of TGF- α -induced airway and vascular muscularization was markedly increased in Egr-1^{ko/ko} mice. AHR to methacholine, pulmonary fibrosis, and PH induced by TGF- α were exacerbated by the lack of Egr-1. These changes occurred earlier in Egr-1^{ko/ko} mice, suggesting that loss of Egr-1 enhances the rate, as well as the

Figure 7. TGF- α -induced severe muscularization of pulmonary arteries and right ventricular (RV) hypertrophy in Egr-1^{ko/ko} mice. (A) α -SMA and pentachrome staining of serial sections showed fibrosis around vessels in both Egr-1^{ko/ko} and Egr-1^{wt/wt} mice (black arrows). Egr-1^{ko/ko} mice also displayed extensive smooth muscle metaplasia in the vascular adventitia (red arrows), which was coincident with narrowing or occlusion of the lumen of vessels in many cases (yellow arrow-head). Scale bar = 50 μ m. (B) RV weights were increased in Egr-1^{ko/ko} mice compared with Egr-1^{wt/wt} mice on Dox for 8 weeks ($n = 17$ –22 per group). * $P < 0.05$ compared with no-Dox control mice; # $P < 0.05$ compared with Egr-1^{wt/wt} mice.

severity, of remodeling. Taken together, our data indicate that the induction of Egr-1 in airways and vessels suppresses EGFR-mediated pulmonary remodeling, suggesting an important role in the pathogenesis of TGF- α -induced lung pathology.

The severity of AHR in the Egr-1^{ko/ko} mice was similar to a recently published model of severe inflammatory asthma in which mice were challenged with a combination of ovalbumin and poly-L-lysine (34). However, inflammatory changes were not detected in Egr-1^{ko/ko} mice. The role of Egr-1 has been examined in inflammatory models of lung remodeling and fibrosis induced by TGF- β and IL-13 overexpression (35, 36). These models are characterized by significant epithelial apoptosis, inflammation, and induction of Egr-1. When TGF- β - and IL-13-transgenic mice were crossed to Egr-1^{ko/ko} mice, epithelial apoptosis and inflammation were reduced, and, therefore, the mice developed less fibrosis. In contrast, in our study, we used a noninflammatory model of lung remodeling, and the loss of Egr-1 resulted in more severe fibrosis and AHR. Furthermore, in the TGF- α -transgenic mice, Egr-1 was induced predominantly in smooth muscle cells and fibroblasts, and lung

remodeling downstream of EGFR is primarily a proliferative rather than apoptotic process (16, 20).

Because these Egr-1^{ko/ko} mice developed ASM thickening in the absence of inflammatory changes, the data from this study address several questions recently posed in a report from a National Heart, Lung, and Blood Institute workshop (37). First, the physiologic relevance of increased ASM mass was raised. Most asthma models also involve inflammation, which can increase AHR. Here, our model shows that increased ASM mass alone can cause both severe AHR and hypersensitivity to methacholine. Second, the workshop report noted that the growth factors that control ASM mass are not well characterized. In this study, we show that TGF- α , which activates EGFR signaling, can cause dramatic increases in ASM mass in the absence of Egr-1. The report also suggested that it is not clear what transcriptional pathways regulate ASM mass. Our data suggest that Egr-1 plays a critical role in regulating ASM mass downstream of TGF- α -induced lung remodeling. Whether increased ASM cell number (hyperplasia) or cell size (hypertrophy) is the predominant cause of increased ASM mass in asthmatic

airways and in mouse models of asthma is also controversial (38). ASM hyperplasia has been identified in a number of studies of asthmatic airways (38, 39). There are conflicting reports on the presence of ASM hypertrophy in patients with asthma. Woodruff and colleagues (38) reported ASM hypertrophy in patients with severe asthma, but not in those with milder disease. In Egr-1^{ko/ko} mice during TGF- α expression, we found only evidence of ASM hyperplasia contributing to severe ASM thickening.

In addition to increased airway remodeling, Egr-1^{ko/ko} mice also developed more severe vascular remodeling and RVH than Egr-1^{wt/wt} mice, indicating exacerbation of PH. PH was observed in previous studies of TGF- α -expressing mice, including in adult mice both after chronic TGF- α overexpression and after transient TGF- α expression during the alveolar phase of lung development (16, 18, 40). Vascular remodeling in these models included increased muscularization, but this was confined to the vascular media (40). Increased fibrosis was also seen in the vascular adventitia after chronic TGF- α expression in adult mice (16). In Egr-1^{ko/ko} mice, vascular remodeling also included smooth muscle metaplasia in the vascular adventitia, along with fibrotic changes, resulting in some vessels in which the lumen appeared to be completely occluded. These changes likely contributed to the increased severity of PH in Egr-1^{ko/ko} mice. In another model of PH, Egr-1 expression rapidly increased in the lungs of mice exposed to hypoxia (41, 42). In calves with hypoxia-induced PH, Egr-1 increased in the adventitial layer of remodeling vessels, and small interfering RNA knockdown of Egr-1 inhibited the autonomous growth of these adventitial fibroblasts *in vitro* (43). However, the role of Egr-1 in hypoxia-induced PH *in vivo* remains unclear.

Disparate roles for Egr-1 have been found in different tumors. For example, Egr-1 promotes growth and survival of prostate cancer cells, whereas decreased Egr-1 has been associated with lung, brain, and breast tumors, and Egr-1 suppresses transformation and tumorigenicity in glioma and sarcoma cell lines (23–28). Our study suggests that Egr-1 is acting to suppress airway and vascular muscularization and fibrosis in this noninflammatory model of EGFR-mediated lung remodeling. The mechanisms downstream of Egr-1 are unclear, although it has been shown to regulate tumor suppressor genes, such as PTEN, p53, p21, and p73 (44–48). Whether these genes are altered in Egr-1^{ko/ko} mice in response to EGFR signaling and contribute to the pathogenesis of lung remodeling will be addressed in future studies.

In summary, loss of Egr-1 expression caused severe ASM and vascular smooth muscle thickening in TGF- α -induced lung remodeling in the absence of detectable changes in inflammation. This study suggests that EGFR signaling and Egr-1 may be important regulators of ASM in addition to fibrosis. Because proinflammatory cytokines and allergens induce EGFR signaling, the pathways studied here might act downstream of inflammation. This model may facilitate the testing of experimental therapies to inhibit ASM and vascular smooth muscle remodeling, while avoiding the complicating effects of inflammation. This is pertinent, as recent clinical studies have suggested that targeted elimination of ASM improves outcome in asthma (37, 49, 50).

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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