The American Journal of Pathology, Vol. 180, No. 6, June 2012

http://dx.doi.org/10.1016/j.ajpath.2012.02.010

Cardiovascular, Pulmonary, and Renal Pathology

Mesenchymal-Specific Deletion of C/EBPβ Suppresses Pulmonary Fibrosis

Biao Hu, Zhe Wu, Taku Nakashima, and Sem H. Phan

From the Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan

The CCAAT/enhancer-binding protein β (C/EBPβ) regulates a variety of factors and cellular responses associated with pulmonary fibrosis. To distinguish its role in the mesenchyme from that in other compartments, the effects of mesenchymal-specific deletion of C/EBPβ on pulmonary fibrosis was examined. Crossing of mice with the floxed *C/EBPβ* gene with α2(I) collagen enhancer-CreER(T)-bearing mice successfully generated progeny with a conditional knockout (CKO) of C/EBPβ in collagen I-expressing ("mesenchymal") cells only on treatment with tamoxifen (C/EBPβ CKO). When treated with an endotracheal bleomycin injection, C/EBPβ CKO mice showed significant attenuation of pulmonary fibrosis relative to control C/EBPβ-intact mice. C/EBPβ CKO mice also had reduced myofibroblasts in the lung. However, no significant differences in inflammatory/immune cell influx were noted in the mutant mice relative to the control mice. DNA microarray and real-time PCR analyses identified a series of myofibroblast differentiation regulators as novel target genes of C/EBPβ. Interestingly, C/EBPβ deficiency caused a marked induction of matrix metalloproteinase 12 expression, suggesting its potential role as a repressor, which could account for the noted reduction in fibrosis in the C/EBPβ-deficient mice. Thus, these findings indicate an essential role for C/EBPβ in the mesenchymal compartment in pulmonary fibrosis that is independent of its effects on inflammation or immune cell infiltration. ***(Am J Pathol 2012, 180:2257–2267; http://dx.doi.org/10.1016/j.ajpath.2012.02.010)***

A key element in tissue repair and remodeling or fibrosis is the mesenchymal response that provides the essential extracellular matrix components for the infrastructure necessary for healing on the one hand and chronic progressive fibrosis on the other hand.[1] This response is a result of mesenchymal cell activation, including differentiation into myofibroblasts.[1–5] Myofibroblast differentiation is characterized by induction of α-smooth muscle actin (α-SMA; *ACTA2*) gene expression as well as by increased expression of extracellular matrix components and certain fibrogenic cytokines, such as transforming growth factor β.[1–5] Because deposition of extracellular matrix and release of fibrogenic cytokines are key factors in perpetuating the cycle of injury and fibrosis, studies on the regulation of myofibroblast differentiation may be of value in elucidation of the basic mechanisms in the genesis of chronic progressive fibroproliferative disease.

The essential role of CCAAT/enhancer binding proteins (C/EBPβ) in regulating myofibroblast differentiation and in fibrosis has been reported.[6–9] C/EBPβ is one of the basic region leucine zipper transcription factors[10] and has two main isoforms, the liver-enriched activator protein (LAP) and the N-terminal truncated isoform known as liver-enriched inhibitor protein (LIP).[8,9,11–13] LIP contains the DNA binding domain of LAP but lacks its activation domain.[10] Both LAP and LIP bind to the C/EBPβ binding consensus on the *ACTA2* promoter.[9] However, LAP activates *ACTA2* gene expression, whereas LIP inhibits expression possibly through competition for binding.[9] C/EBPβ plays important roles in fundamental cellular processes, including cell proliferation, cell differentiation, growth arrest, and apoptosis in a manner specific to cell-type.[9,14–17] During bleomycin-induced pulmonary fibrosis, multiple roles of C/EBPβ are identified in studies that used C/EBPβ-null mice.[8] The results show that deficiency of C/EBPβ leads to attenuation of cytokine expression, decrease of myofibroblast differentiation, and increase of fibroblast proliferation.[8] Similar results are

Supported by NIH grants HL28737, HL31963, HL52285, HL77297, and HL91775 and used the Cell and Molecular Biology Core of the Michigan Diabetes Research and Training Center funded by grant DK020572 from the National Institute of Diabetes and Digestive and Kidney Diseases.

Accepted for publication February 13, 2012.

The authors have no commercial affiliations and competing financial interests to be disclosed.

Supplemental material for this article can be found at *http://ajp.amjpathol.org* or at *http://dx.doi.org/10.1016/j.ajpath.2012.02.010*.

Address reprint requests to Sem H. Phan, Ph.D., M.D., Department of Pathology, University of Michigan, 109 Zina Pitcher Pl., Ann Arbor, MI 48109-2200. E-mail: shphan@umich.edu.

obtained in liver fibrosis when C/EBPβ activation by phosphorylation is suppressed.[7] Despite this evidence of an essential role for C/EBPβ in fibrosis, the specific underlying mechanisms remain uncertain, given the plethora of target genes, biological processes, and cell types regulated by this transcription factor. At least one possible mechanism is its importance in regulation of the *ACTA2* gene and thus myofibroblast differentiation.[9] However, as noted above, C/EBPβ deficiency may also affect inflammation,[18] and perhaps other processes, some of which may be related to the acute phase response that is known to be regulated by this transcription factor.[18,19] Moreover, C/EBPβ is shown to be important in regulating gene expression and function of lung epithelial cells.[20] Thus, the relative importance of these potential mechanisms by which C/EBPβ regulates fibrosis requires further elucidation. A possible strategy is to evaluate the effect of selective C/EBPβ deficiency in certain cell types, which have been made amenable by the availability of Cre-lox technology.

Cre-ER(T) is a mutant of Cre recombinase originally isolated from the P1 bacteriophage.[21] It is a fusion protein comprising Cre and a mutated form of the ligand binding domain of the estrogen receptor that renders Cre activity tamoxifen inducible.[21,22] This allows for conditional modification of gene activity in the mammalian cells by administration of tamoxifen because its binding to the Cre recombinase activates its ability to excise DNA fragments flanked by specific LoxP consensus sequences.[22] If driven by a cell-specific promoter, this activity may be localized to express only in that particular cell type. A far-upstream enhancer of the α2(I) collagen gene has been identified and found to be expressed mainly in fibroblasts and other mesenchymal cells.[23] The use of this enhancer and associated promoter to drive the expression of Cre-ER(T) results in selective expression in mesenchymal cells only when transgenic mice harboring this construct are treated with tamoxifen.[24]

In this study, these transgenic Cre-ER(T) mice [*Col1α2-Cre-ER(T)*$^{+/O}$] were used in conjunction with mice (*C/EBPβ*$^{fl/fl}$) harboring the floxed (containing loxP sites) *C/EBPβ* (*CEBPE*) gene[25] to distinguish the role of C/EBPβ in the mesenchymal compartment from that in the inflammatory/immune system and other compartments. Crossing these mice resulted in the generation of *C/EBPβ*$^{fl/fl}$, *Col1α2-Cre-ER(T)*$^{+/O}$ mice, which on treatment with tamoxifen caused a selective deficiency of *C/EBPβ* gene expression only in all type I collagen-expressing cells (ie, mesenchymal cells), and are referred heretofore as C/EBPβ conditional knockout (CKO) mice. The C/EBPβ CKO and control mice were then analyzed for their responsiveness to endotracheal bleomycin injection. The results indicated significantly reduced fibrosis when *C/EBPβ* was selectively knocked out in the mesenchymal compartment, which was accompanied by reduction in myofibroblast differentiation, whereas no significant effects were noted in inflammation or immune cell influx. Further analysis showed that C/EBPβ deficiency in mesenchymal cells affected expression of a series of genes, some of which are known to be regulators of myofibroblast differentiation. These findings indicated that unimpaired expression of C/EBPβ in the mesenchymal compartment was essential for pulmonary fibrosis.

Materials and Methods

Antibodies

The anti-α-SMA (catalog no. A2547) and anti-procollagen I (catalog no. C2456) antibodies were purchased from Sigma-Aldrich Co. (St. Louis, MO) and anti-C/EBPβ antibody (catalog no. SC150) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Fluorescent-labeled antibodies against CD3 (catalog no. 100219), CD4 (catalog no. 130310), CD8 (catalog no. 100711), B220 (catalog no. 103115), F4/80 (catalog no. 122609), Gr-1 (catalog no. 108423), and E-cadherin (catalog no. 324113) for enumeration of T cells, CD4$^+$ and CD8$^+$ T cells, B cells, macrophages, neutrophils, and epithelial cells, respectively, were purchased from Biolegend Inc., San Diego, CA.

Animals

All animal protocols were approved by the University Committee on Use and Care of Animals of the University of Michigan. Mice with *C/EBPβ* coding region flanked with LoxP recombination sites *(C/EBPβ*$^{fl/fl}$) and mice carrying a tamoxifen-inducible Cre-recombinase [Cre-ER(T)] under the control of a regulatory sequence from the α2(I) collagen gene (C57BL/6J-Tg[*Col1α2-Cre-ER(T)*])[24,25] were bred from breeding pairs, which were generous gifts of Dr. Esta Sterneck (Molecular Mechanisms in Development Group, Laboratory of Protein Dynamics and Signaling, National Cancer Institute, Frederick, MD) and Dr. Benoit de Crombrugghe (Department of Genetics, University of Texas M.D. Anderson Cancer Center, Houston, TX), respectively. Both strains were on a C57BL/6 background. To generate mesenchymal cell-specific C/EBPβ-deficient mice, the *C/EBPβ*$^{fl/fl}$ mice were cross bred with *Col1α2-Cre-ER(T)*$^{+/O}$ (heterozygous allele) transgenic mice to generate mice heterozygous for both alleles. The second cross between *C/EBPβ*$^{fl/fl}$ mice and heterozygous mice from the first cross produced *C/EBPβ*$^{fl/fl}$, *Col1α2-Cre-ER(T)*$^{+/O}$ mice, which were used for further experiments.

For treatment of mice, a stock solution of tamoxifen (4-hydroxitamoxifen; Sigma-Aldrich Co.) in ethanol (100 mg/mL) was diluted in corn oil to 10 mg/mL. To selectively delete C/EBPβ in mesenchymal cells, adult *C/EBPβ*$^{fl/fl}$, *Col1α2-Cre-ER(T)*$^{+/O}$ mice (8 weeks old) and the relevant control [*Col1α2-CreER(T)*$^{+/O}$ with wild-type *C/EBPβ*] mice were given daily intraperitoneal injections of the tamoxifen suspension (0.1 mL of diluted stock) for 8 days to induce mesenchymal cell-specific expression of the Cre-ER(T) recombinase and removal of the floxed *C/EBPβ* coding region accordingly. The resultant C/EBPβ-deficient mice were referred to as C/EBPβ CKO mice.

After the tamoxifen treatment regimen, the C/EBPβ CKO and control mice were injected endotracheally with 2 U/kg of body weight bleomycin (Blenoxane; Mead Johnson, Princeton, NJ) dissolved in sterile PBS as before.[8] The con-

trol group received the same volume of sterile PBS only. Daily tamoxifen treatment continued until the mice were sacrificed at the indicated time points after bleomycin treatment. Unless otherwise specified, two to five animals per group were analyzed in each experiment.

All animals used for experiments were genotyped by PCR to detect C/EBPβ and CreER(T) gene sequences as described previously.[24,25] For detection of the floxed C/EBPβ by PCR in genotyping, the primer pairs used were A with sequence (5′-GAGCCACCGCGTCCTCCAGC-3′) and B with sequence (5′-GGTCGGTGCGCGTCATTGCC-3′). For detection of the CreER(T) gene, the following primers were used in genotyping: forward, 5′-TCCAATTTACTGACCGTACACCAA-3′, and reverse, 5′-CCTGATCCTGGCAATTTCGGCTA-3′.

Cell Culture

Mouse lung fibroblasts were isolated as described previously.[9,26] The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% plasma-derived serum (Cocalico Biologicals, Reamstown, PA), 100 U/mL penicillin, 100 U/mL streptomycin, 0.25 U/mL Fungizone, 1% insulin/transferrin/selenium (Sigma-Aldrich Co.), 5 ng/mL platelet-derived growth factor (R&D Systems, Minneapolis, MN), and 10 ng/mL epidermal growth factor (R&D Systems) for 1 day. After removal of the nonadherent cells by washing with PBS, the attached cells were then cultured in complete medium as described for three to five passages before analyses as indicated. T cells from mouse spleens were isolated at >90% purity with the use of the CD3 Microbead Kit (Miltenyi Biotec Inc., Auburn, CA) in accordance with the manufacturer's protocol.

Morphologic Analysis

Morphologic analysis was performed as previous described.[8] Briefly, 21 days after the endotracheal injection of saline or bleomycin, mice were euthanized, and the lungs were thoroughly perfused with saline to remove blood from the lung vascular beds. The lungs were then removed from the thoracic cavity and fixed in formalin, followed by paraffin embedding, sectioning, and staining with H&E.

Hydroxyproline Assay

To assess the extent of fibrosis, the collagen content of whole lung homogenates was assayed by colorimetric assay after acid hydrolysis as described previously.[27]

Western Blot Analysis

Western blot analysis was performed essentially as before.[8] As indicated, homogenized murine lung tissue or fibroblasts were lysed in radioimmunoprecipitation assay protein lysis buffer that contained proteinase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). After determination of protein concentration by bicinchoninic acid assay (Fisher Scientific, Pittsburgh, PA), equal amounts of total protein were electrophoresed through 12% SDS-polyacrylamide gels. The separated protein bands were transferred onto Hybond P membranes and blotted against different antibodies as indicated. Equal loading was confirmed by reblotting the membrane with horseradish peroxidase-conjugated anti-glyceraldehyde-3-phosphate dehydrogenase antibody (catalog no. 9482) from Abcam Inc. (Cambridge, MA). When indicated the blots were scanned and digitized, and the band intensities were quantified with the use of Carestream Molecular Imaging software V.5.0.2.30 (Carestream Health, Inc., Rochester, NY).

mRNA Analysis

Determination of mRNA for the indicated genes was performed with the use of real-time PCR as before.[8] With this method, a CT value reflects the cycle number at which DNA amplification is detected.[28] The amount of target, normalized to endogenous reference and relative to a calibrator, is given by $2^{-\Delta\Delta CT}$.[28] Total RNA was extracted from either murine lung tissue or fibroblasts as indicated. For each assay, 200 ng of total RNA was used as template and 18S rRNA was used as internal control to normalize the amount of input RNA. One-step real-time RT-PCR (48°C for 30 minutes, 95°C for 10 seconds, followed by 50 cycles at 95°C for 10 seconds, 60°C for 1 minute) was performed with Taqman One Step RT-PCR Master Mix with the use of a GeneAmp 7500 Sequence Detection System (Applied Biosystems Inc., Foster City, CA). Primers and probes were purchased from Applied Biosystems Inc.

Flow Cytometric Analysis

Whole lung cell suspensions were prepared by digesting the lungs from C/EBPβ CKO mice and wild-type control mice after bleomycin or saline injection at indicated time points with collagenase and DNase digestion mixture [5 mg of collagenase and 1 mg of DNase (Worthington Biochemical Co., Lakewood, NJ) in 10 mL of Hank's Buffered Salt Solution supplemented with 1 mL of plasma-derived serum] for 1.5 hours at 37°C. The suspensions were then filtered with 100-μm and 40-μm mesh cloth and washed with 1× PBS. The cell suspensions were then fixed with 4% paraformaldehyde in 1× PBS and stained with fluorescent-labeled specific primary antibody of interest or corresponding IgG control in 1× PBS only or 1× PBS containing 0.1% saponin (for staining of intracellular proteins) for 1 hour at room temperature. After stringent washing, they were analyzed on a Becton-Dickinson LSR-II machine (BD Biosceinces, Chicago, IL). Data collected were then analyzed with the use of FlowJo flow cytometric analysis software V.7.6.5 (TreeStar Inc., Ashland, OR). For evaluation of inflammatory/immune response, the whole lung cell suspension was stained with fluorescent-labeled antibodies against CD3, CD4, CD8, B220, F4/80, and Gr-1 for enumeration of T cells, $CD4^+$ and $CD8^+$ T cells, B cells, macrophages, and neutrophils, respectively.[29–32] To evaluate the expression of C/EBPβ, the lung cell suspensions were stained with

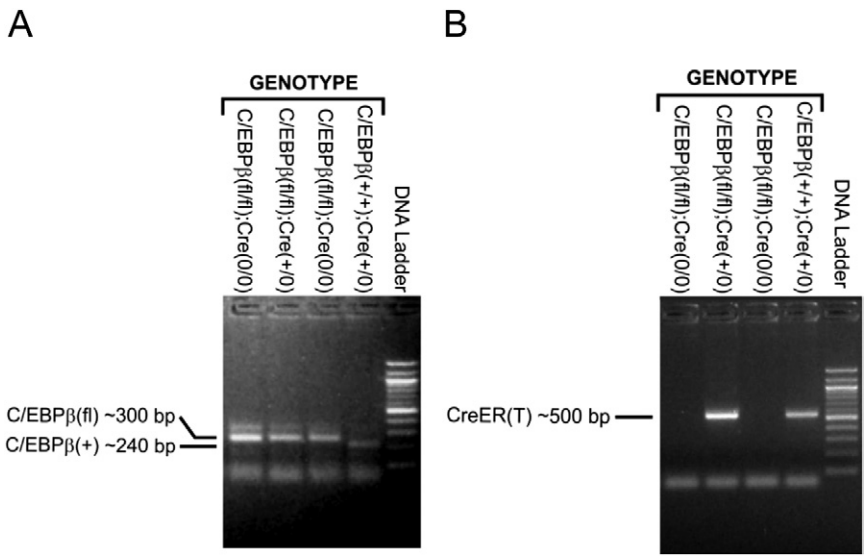

Figure 1. Mouse genotype analysis. **A:** PCR amplification of the *C/EBPβ* gene. Mouse genomic DNA was extracted and mixed with PCR primers specific for the *C/EBPβ* gene. The PCR products and 100-bp ladder (NEB) were separated in a 1.3% agarose gel and photographed. The ~240-bp DNA fragment expected for the C/EBP^{+} allele and an ~300-bp DNA fragment expected for the *C/EBPβ*^{fl} allele are indicated. **B:** The same genomic DNA samples were used with PCR primers specific for the *CreER(T)* gene. The PCR products were separated by agarose gel electrophoresis as above. The ~500-bp amplified *CreER(T)* gene fragment is indicated.

surface marker first, fixed with 4% formaldehyde for 10 minutes, and washed twice with 1× PBS. They were further stained with anti-C/EBPβ antibody and analyzed by flow cytometry.

cDNA Microarray Analysis

cDNA microarray analysis was performed essentially as previously described.[33] Mouse lung fibroblasts were isolated from either C/EBPβ CKO mice ($N = 3$) or the corresponding control mice ($N = 3$) treated with either bleomycin or saline. The total RNAs from each group were then extracted with Tryzol reagent (Invitrogen Co., Carlsbad, CA) and labeled with fluorescent dyes. The total RNAs were hybridized to GeneChip mouse genome 430A 2.0 (part no. 900499; Affymetric, Inc., Santa Clara, CA) at 65°C overnight. Fluorescent images were obtained with the use of a GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA) and analyzed with the GenePix software package v.7 (Axon Instruments, Foster City, CA). Briefly, some quality control plots were checked to confirm the overall quality of the raw data. The potential differences between samples caused by mRNA degradation or *in vitro* translation were precluded. The distribution of each chip was analyzed in a way that all of the data come from the same distribution with the only differences being the location and scale. The expression values for each gene were then calculated with the use of a robust multiarray average method developed by Irizarry et al,[34] which is a modeling strategy that converts the probe values into $\log_2$-transformed expression value for each gene. Genes that show greater than twofold difference between the C/EBPβ CKO cell and control cell samples were shown in a heat map generated with the Heatmap builder program.[35] The gene ontology analysis was done with the DAVID Functional Annotation Bioinformatics Microarray Analysis online (*http://david.abcc.ncifcrf.gov*) according to their instruction.

Statistical Analysis

Statistical analysis was performed as before with the use of analysis of variance, followed, when appropriate, by post hoc testing with Scheffé's test.[8] A P value < 0.05 was used as a criterion for statistical significance in comparisons between any two groups.

Results

Generation of Mice with Selective Deficiency of C/EBPβ in the Mesenchymal Compartment (C/EBPβ CKO mice)

C/EBPβ homozygous floxed mice (*C/EBPβ*$^{fl/fl}$) with C/EBPβ coding region flanked with LoxP recombination sites were cross bred with mice harboring a *CreER(T)* recombinase gene located downstream of a α2(I) collagen promoter/enhancer and then back crossed with the *C/EBPβ*$^{fl/fl}$ mice to obtain mice homozygous for the floxed *C/EBPβ* allele and hemizygous for the *Col1α2-Cre-ER(T)* allele with genotype [*C/EBPβ*$^{fl/fl}$, *Col1α2-Cre-ER(T)*$^{+/0}$]. These mice were verified for genotype with PCR analysis of genomic DNA from tail tissue samples, which showed the correct expression of either wild-type or floxed C/EBPβ (Figure 1A), and CreER(T) (Figure 1B). After verification, the appropriate mice were then treated with tamoxifen as described in *Materials and Methods* to delete the *C/EBPβ* gene in the mesenchymal compartment only and were referred to as C/EBPβ CKO mice. The loss of C/EBPβ expression in the mesenchymal compartment of C/EBPβ CKO mice was evaluated in lung fibroblasts isolated from both saline- or bleomycin-treated C/EBPβ CKO mice and control mice [*C/EBPβ*$^{+/+}$, *Col1α2-Cre-ER(T)*$^{+/0}$], which bore the same *CreER(T)* gene but the wild-type *C/EBPβ* gene. Western blot analysis of protein samples from these cells found the expected C/EBPβ protein isoforms in lung fibroblast samples from control mice, but which were virtually totally absent in those from

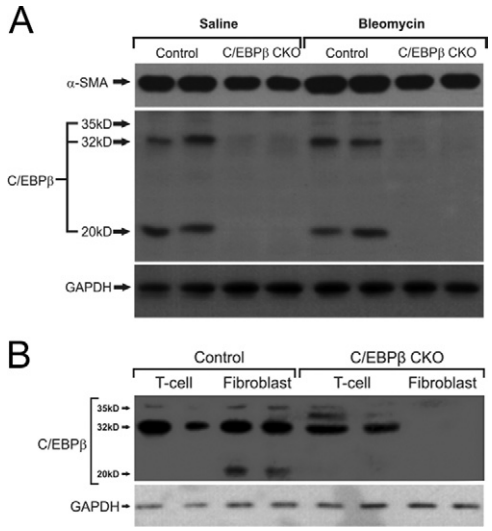

Figure 2. Confirmation of C/EBPβ deficiency in the mesenchymal compartment. C/EBPβ CKO mice with genotype [*C/EBPβ*$^{fl/fl}$, *Col1α2-Cre-ER(T)*$^{+/0}$] and control mice with genotype [*C/EBPβ*$^{+/+}$, *Col1α2-Cre-ER(T)*$^{+/0}$] were pretreated for 8 days with tamoxifen and then separated into two groups for each genotype. One group was injected endotracheally with bleomycin and the other group with PBS. Tamoxifen treatment was continued daily after the bleomycin/PBS injection until the mice were sacrificed 14 days after the bleomycin injection. **A:** Lung fibroblasts from these mice were analyzed for α-SMA and C/EBPβ proteins by Western blot analysis. Mice ($N = 4$) were tested, and a representative blot from two mice is shown. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. **B:** Protein extracts of splenic T cells and lung fibroblasts from tamoxifen-treated C/EBPβ CKO and control mice were analyzed for C/EBPβ protein isoforms by Western blot analysis. Mice ($N = 4$) were tested, and a representative blot is shown with GAPDH used for loading control.

C/EBPβ CKO mice (Figure 2A). However, in these CKO mice C/EBPβ expression was intact in T cells (Figure 2B) and other cells not expressing type I collagen (see Supplemental Figure S1 at *http://ajp.amjpathol.org*), thus confirming the selective deficiency of C/EBPβ expression only in the mesenchymal compartment. Deficiency of C/EBPβ in the CKO mice was accompanied by some reduction in α-SMA expression in lung fibroblast samples from animals treated with saline and bleomycin (Figure 2A). The reduced α-SMA protein levels in the C/EBPβ-deficient cells were significantly less in both cases ($P < 0.05$) relative to their respective saline controls when the blots were digitized and quantitated.

Reduced Fibrotic Response in C/EBPβ CKO Mice

Although C/EBPβ is known to be essential for fibrosis,[6–9] its precise role is uncertain, given its pleiotropic effects in diverse cell types and biological processes.[8,19,20] To narrow the possibilities would require examination of its role in specific cell types involved in the fibrotic process. Thus, the feasibility of producing C/EBPβ CKO mice was exploited to study the effect of selective deficiency of C/EBPβ in the mesenchymal compartment on the bleomycin model of pulmonary fibrosis. Because the C/EBPβ CKO mice had the floxed *C/EBPβ* gene and the Col1α2-CreER(T) transgene, as well as being generated by injection of tamoxifen, several options for control mice had to be considered. Myofibroblast differentiation as measured by α-SMA expression was selected for evaluation of the suitable control mice because it is a known C/EBPβ target gene in the mesenchymal compartment.[8,9] Thus, lung tissue extracts from mice with the genotypes of *C/EBPβ*$^{fl/fl}$, [*C/EBPβ*$^{fl/fl}$, *Col1α2-Cre-ER(T)*$^{+/0}$], and *Col1α2-Cre-ER(T)*$^{+/0}$ were analyzed for α-SMA protein by Western blot analysis to see if any of these genotypic modifications would affect myofibroblast differentiation. Tamoxifen treatment alone was noted to reduce lung tissue α-SMA protein levels in all murine strains (Figure 3). However, among the tamoxifen-treated groups lung α-SMA expression was not significantly different between the mice with the indicated three different genotypes. Thus, the mice with genotype ([*C/EBPβ*$^{+/+}$, *Col1α2-Cre-ER(T)*$^{+/0}$], injected with tamoxifen) was selected as the control mice for the C/EBPβ CKO mice to obtain a genotype as similar as possible to the CKO mice.

After tamoxifen treatment to generate the C/EBPβ CKO and control mice, the mice were then analyzed for responsiveness to bleomycin-induced pulmonary fibrosis. Morphologically the lungs from saline-treated control and C/EBPβ CKO mice did not exhibit any significant pathology and were indistinguishable (Figure 4, A and E versus C and G, respectively). In response to bleomycin treatment, the expected pattern of fibrosis was seen in the control mice with extensive distortion of the normal alveolar architecture characterized by increased thickness of alveolar septa, inflammatory cell infiltration, increased number of fibroblasts, and interstitial collagen deposition (Figure 4, B and F). However, lungs of bleomycin-treated C/EBPβ CKO mice exhibited much smaller areas of fibrosis that were sparsely distributed (Figure 4, D and H) relative to those seen in the control mice and exhibited more normal areas of intact lung. Thus, histopathologically evidence was seen of reduced pulmonary fibrosis in the mice with selective deficiency of C/EBPβ in the mesenchymal compartment.

To further characterize this effect quantitatively, total lung tissue hydroxyproline content was analyzed as a measure of lung collagen deposition on day 21 after bleomycin or saline treatment. In saline-treated mice, C/EBPβ deficiency did not significantly affect lung hydroxyproline content (Figure 5A) or type I collagen protein levels (Figure 5B), but it did cause a significant reduction in lung type I collagen mRNA (Figure 5C). The results also showed that bleomycin treatment caused the expected significant increase in lung hydroxyproline content in control mice (Figure 5A), but which was significantly reduced by >50% in the C/EBPβ CKO mice (69%

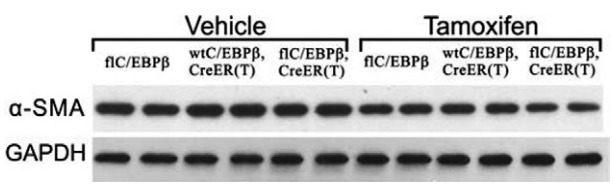

Figure 3. Effects of Cre and tamoxifen on α-SMA expression. Mice with the indicated genotypes were injected with tamoxifen or vehicle only as indicated. The lungs were then removed and homogenized to obtain protein extracts for analysis of α-SMA protein by Western blot analysis. Mice ($N = 4$) were tested, and a representative blot is shown with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the loading control.

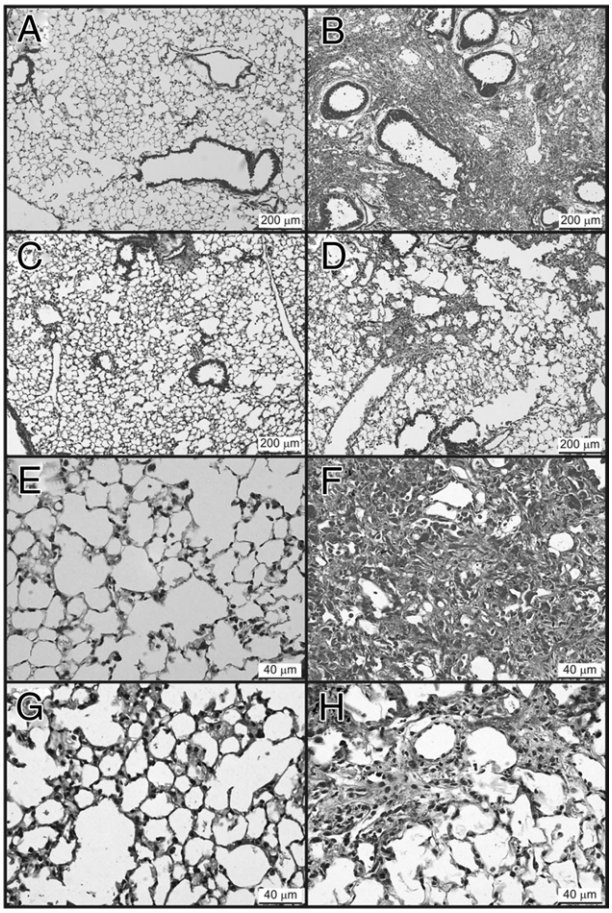

Figure 4. Effect of mesenchymal C/EBPβ deficiency on bleomycin-induced lung histopathology. Tamoxifen-treated C/EBPβ CKO mice were given endotracheal injections of either bleomycin or saline, and 21 days later lung tissue sections were stained with H&E. Representative sections from control mice treated with saline (**A** and **E**) or bleomycin (**B** and **F**) and from C/EBPβ CKO mice treated with saline (**C** and **G**) or bleomycin (**D** and **H**) are shown. Original magnification: ×10 (**A**–**D**); ×40 (**E**–**H**). Scale bars are included in each panel.

versus 33% increase over saline-treated controls, respectively). This significant reduction in the bleomycin-induced increase in lung collagen deposition in C/EBPβ CKO mice was also reflected in lung type I collagen expression analyzed at the protein and mRNA levels. Thus, Western blot analysis of lung protein extracts found a much reduced bleomycin-induced increase in type I collagen protein levels in these mice compared with the vigorous response in control mice (Figure 5B). This reduction in responsiveness to bleomycin treatment was similarly noted for lung α-SMA protein levels. Analysis of lung type I collagen mRNA found similar differences between these two strains of mice (Figure 5C). Thus, selective loss of C/EBPβ expression in the mesenchymal compartment caused a significant reduction in pulmonary fibrosis in this animal model.

Reduction of Myofibroblasts in C/EBPβ CKO Mice

Myofibroblast differentiation is regulated by C/EBPβ with *ACTA2* being one of its target genes.[8,9] Thus, the noted reduction in bleomycin-induced increase in C/EBPβ CKO lung α-SMA protein content (Figure 5B) would be consistent with those prior observations. To confirm the protein analysis, the number of lung α-SMA-expressing cells was analyzed by flow cytometry after dissociation of the lung tissue samples into single-cell suspensions. The results showed a greater than threefold bleomycin-induced increase in lung α-SMA-positive cells in control mice (Figure 6A). This bleomycin-induced effect was significantly blunted in C/EBPβ CKO mice, which exhibited a less than twofold increase. Further analysis of lung α-SMA mRNA by real-time PCR found small but statistically insignificant reductions in lung tissue and fibroblast levels (Figure 6, B and D, respectively) in samples from saline-treated C/EBPβ CKO mice relative to those in saline-treated control mice. However, significant reduction in bleomycin-

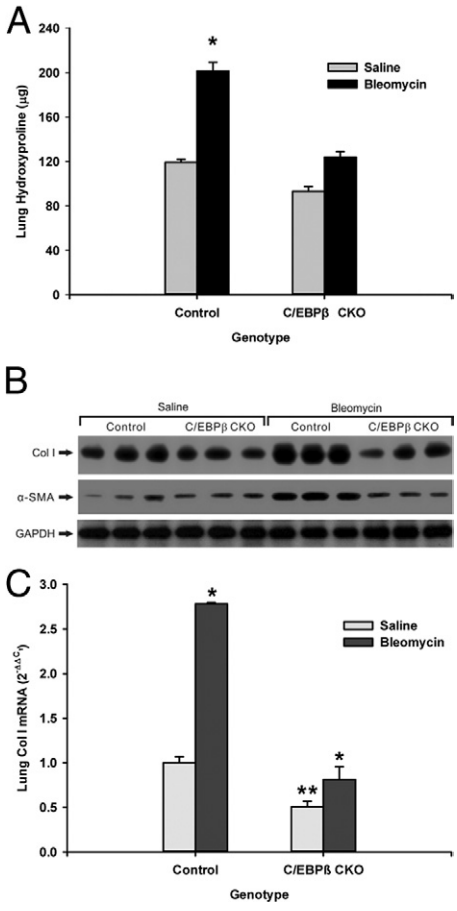

Figure 5. Effect of mesenchymal C/EBPβ deficiency on bleomycin-induced lung collagen deposition. Mice ($N = 5$ per group) were treated with saline or bleomycin after tamoxifen injection for 7 (real-time PCR analysis) or 21 (hydroxyproline assay and Western blot analysis) days. The lungs were harvested and analyzed for hydroxyproline content (**A**), type I collagen and α-SMA protein levels (**B**), and type I collagen mRNA levels (**C**). **A:** Results are expressed as a percentage of the respective saline control values and shown as mean ± SE ($N = 5$). $^*P < 0.05$. **B:** Each lane represents a single animal. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control after stripping the membrane. Only samples from three of the five animals are shown. Similar results were obtained from the remaining two animals (not shown). **C:** α1(I) procollagen mRNA was detected by real-time PCR, and the results are expressed as $2^{-\Delta\Delta CT}$, with 18S rRNA used as the reference and the level in saline-treated wild-type mice used as calibrator. Data are shown as mean ± SE from triplicate samples. $^*P < 0.05$ bleomycin-treated group compared with saline control; $^{**}P < 0.05$ relative to the saline-treated mice with control genotype.

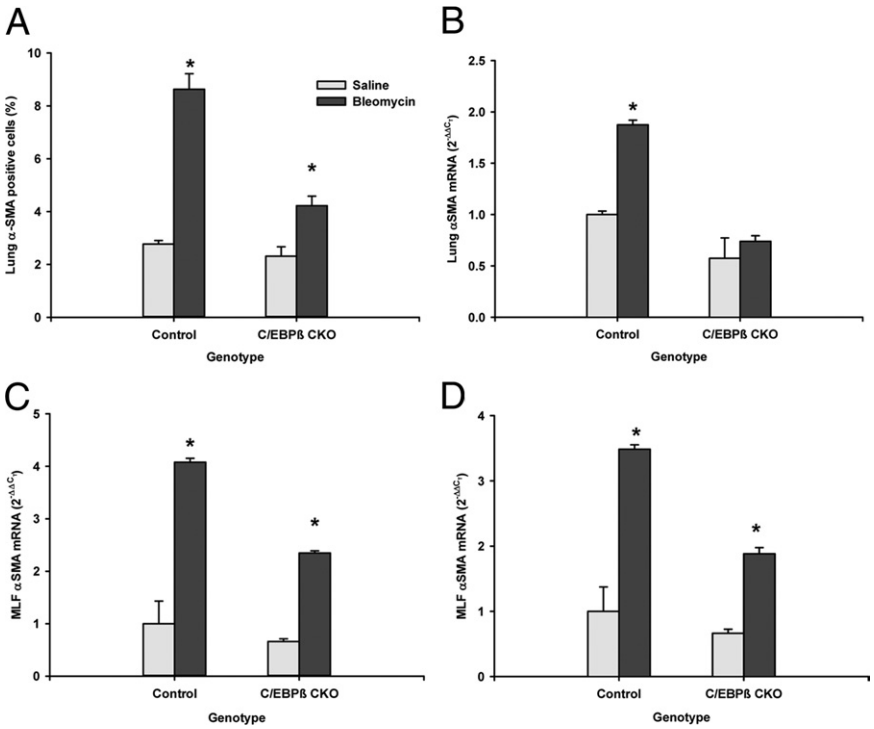

Figure 6. Effect of mesenchymal C/EBPβ deficiency on bleomycin-induced lung myofibroblast differentiation. Mice were treated with saline or bleomycin after tamoxifen injection for 7 (real-time PCR analysis) or 21 (flow cytometry and Western blot analysis) days, and the lung samples were harvested for analysis of myofibroblast differentiation. **A:** Single-cell suspensions were obtained from lung tissue by enzymatic digestion and analyzed for α-SMA-positive cells by flow cytometry. The results were expressed as the percentage of α-SMA-positive cells in the total lung cell suspension. **B:** Lung tissue RNA samples were analyzed for α-SMA mRNA by real-time PCR. **C:** Similar analyses of α-SMA mRNA were performed for lung fibroblasts isolated from lungs of mice treated as in A and B. **D:** Mouse lung fibroblasts were isolated from either C/EBPβ CKO or wild-type mice. They were treated with 4 ng/mL transforming growth factor (TGF)β for 48 hours, and the mRNAs were analyzed by real-time PCR. The mRNA results were expressed as $2^{-\Delta\Delta CT}$, with 18S18S rRNA used as the reference and the level in saline-treated control mice (**A**–**C**) or buffer-treated wild-type cells (**D**) used as calibrator. Data are shown as mean ± SE from triplicate samples. $^{*}P < 0.05$ versus saline-treated controls (**A**–**C**) and TGFβ-treated wild-type cells (**D**).

induced increase in the C/EBPβ CKO mice relative to that in control mice was noted for lung tissue α-SMA mRNA (Figure 6B). Moreover, when lung fibroblasts were isolated from these mice, their α-SMA mRNA levels exhibited similar changes. Thus, lung fibroblasts from bleomycin-treated control mice showed a fourfold increase in α-SMA mRNA compared with those from saline-treated control mice, whereas the comparable cells from the C/EBPβ CKO mice exhibited only a twofold increase (Figure 6C). When these fibroblasts were treated *in vitro* with transforming growth factor β, a known inducer of myofibroblast differentiation, the expected induction of α-SMA expression was significantly reduced in the cells from C/EBPβ CKO mice relative to that seen in cells from wild-type mice (Figure 6D). Thus, attenuated fibrosis was associated with reduction in lung myofibroblast differentiation in mice selectively deficient in C/EBPβ expression in the mesenchymal compartment.

Loss of C/EBPβ in the Mesenchymal Compartment Does Not Affect Inflammation

Endotracheal injection of bleomycin incites acute and chronic inflammation.[36] In view of the role of C/EBPβ in regulation of the inflammatory and acute phase responses, the effect of C/EBPβ deficiency in the mesenchymal compartment on the inflammatory response to bleomycin was also evaluated. Analysis of whole lung cell suspensions by flow cytometry found the expected bleomycin-induced increases in the number of inflammatory and immune cells at day 7 after bleomycin treatment (Figure 7). At this time point the increase in neutrophils was beginning to decline, whereas the increase in mononuclear cells, including macrophages and T lymphocytes, were significant in the control mice. These bleomycin-induced increases in cells were not significantly different in the C/EBPβ CKO mice, whereas the numbers of B lymphocytes were not significantly affected by bleomycin treatment at this time point. Thus, selective deficiency of C/EBPβ in the mesenchymal compartment had no significant effect on the inflammatory response in response to bleomycin-induced lung injury.

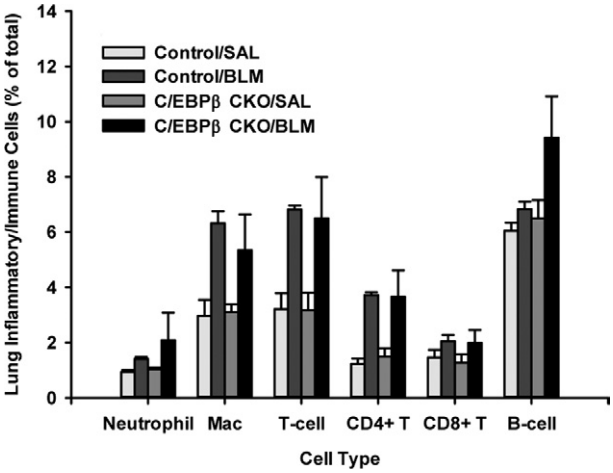

Figure 7. Effect of mesenchymal C/EBPβ deficiency on bleomycin-induced lung inflammation. Single-cell suspensions were prepared from the lungs of tamoxifen-treated C/EBPβ CKO and control mice receiving endotracheal injection of either bleomycin or saline as indicated. Seven days later, the lung samples were analyzed for the indicated inflammatory/immune cells by flow cytometry, and data were expressed as the percentage of total cells in the suspension. Data are shown as mean ± SE from five mice in each group.

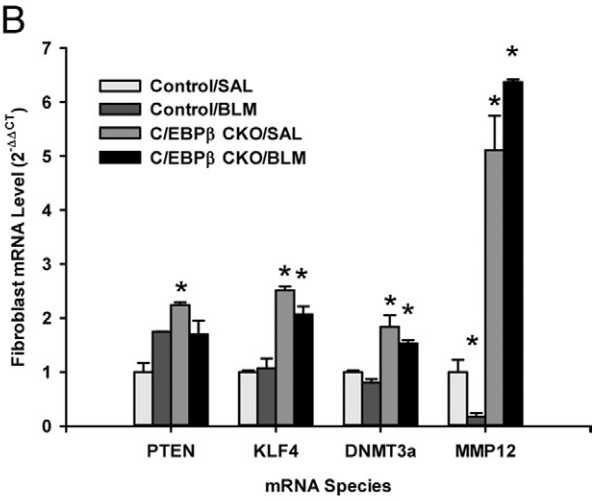

Figure 8. Effect of mesenchymal C/EBPβ deficiency on lung fibroblast gene expression. Lung fibroblasts were isolated from C/EBPβ CKO and control mice treated with either bleomycin or saline for 7 days as indicated after the tamoxifen treatment regimen. Extracts of RNA were then subjected to DNA microarray analysis (**A**) or real-time PCR analysis (**B**). **A:** The expression values for each gene were calculated with a robust multiarray average method that converted the probe values into $\log_2$-transformed expression value for each gene. Genes that showed a greater than twofold difference in DNA microarray analysis between the C/EBPβ CKO cell versus control cell samples ($N = 1$) are shown as a heat map in order of diminishing differences toward the center (vertically). Color intensity is scaled within each row so that the highest expression value corresponds to bright red and the lowest to bright green. The sample identifiers are listed on the top of each column, and the gene names are listed to the right. **B:** Total RNA samples from the cells were analyzed for the indicated mRNA species by real-time PCR. The 18S rRNA was used as reference, and the saline-treated wild-type control was used as calibrator for each gene in calculation of $2^{-\Delta\Delta CT}$. Data are shown as mean ± SE, with $N = 3$. $^*P < 0.05$ versus wild-type control.

C/EBPβ Effects on Myofibroblast Differentiation and Gene Expression

C/EBPβ binding to its binding consensus in the *ACTA2* gene promoter regulates *ACTA2* gene expression and myofibroblast differentiation.[9] To investigate its role in expression of other genes that affect myofibroblast function and differentiation, the effect of this selective C/EBPβ deficiency on lung fibroblast gene expression was evaluated with the use of microarray analysis. The results showed ~100 genes whose expression was altered greater than twofold in fibroblasts as a consequence of the loss of C/EBPβ gene expression (Figure 8A; see also Supplemental Table S1 at *http://ajp.amjpathol.org*). A subset of genes was identified with well-established roles in the regulation of myofibroblast differentiation and function, such as matrix metalloproteinase 12 (MMP12), phosphatase and tensin homolog deleted on chromosome 10 (PTEN), DNA methyltransferase 3a (DNMT3a), and (Kruppel-like factor 4 (KLF4), whose expression was upregulated by C/EBPβ deficiency. The increased expression in these genes was confirmed by real-time PCR, which found that, among these genes, the marked induction of MMP12 (greater than fivefold increase relative to cells from control mice) in cells from CKO mice was highest in amplitude (Figure 8B). In addition, the relative (to cells from saline-treated mice) increase in PTEN and decrease in MMP12 expression in cells from bleomycin-treated control mice was not seen in the comparable cells from CKO mice.

Discussion

C/EBPβ is a multifaceted *trans*-acting factor that can play diverse roles in regulation of cell proliferation, differentiation, and apoptosis.[10] In bleomycin-induced pulmonary fibrosis, C/EBPβ activity is implicated in the regulation of lung cytokine expression, myofibroblast differentiation, and collagen deposition according to studies that used C/EBPβ knockout mice.[8] However, phenotypes of germline knockouts can be complicated by pleiotropic effects of the gene of interest in various tissues and organs through multiple mechanisms. Thus, the use of such knockout mice would not allow distinction between the various possible mechanisms or roles of the targeted gene. In the specific instance of C/EBPβ, it is impossible to distinguish effects on the fibroblasts versus epithelial or inflammatory cells with the use of such knockout mice. To begin to address this issue, in this study the objective was to evaluate the effect of selective deficiency in the mesenchymal compartment on pulmonary fibrosis to distinguish from effects on the epithelial or inflammatory compartment. This was achieved by conditional expression of the Cre recombinase in collagen I-expressing cells on treatment with tamoxifen to knockout C/EBPβ only in those cells, while leaving it intact in epithelial and inflammatory cells that do not express collagen I. These conditional collagen I-driven Cre expressing mice have been previously used in mesenchymal cell-specific knockout of other target genes of interest and showed no significant effects of Cre expression in these cells.[37,38]

Comparison of lung tissue and cells from the C/EBPβ CKO mice with those from control mice found slight decreases in the expressions of type I collagen (*COL1A*) and α-SMA, (*ACTA2*) both of which are known C/EBPβ target genes.[8,9,39] Under these "basal" conditions, C/EBPβ would not be expected to be induced, which could explain the noted slight decrease in target gene expression because of deficiency of this transcription factor. No significant differences were observed for lung immune/inflammatory cell composition as a result of selective mesenchymal cell C/EBPβ deficiency in saline-treated mice. However, notable significant increases in a number of genes (PTEN, KLF4, DNMT3a, and MMP12) were detected in the deficient mice, which may reflect a novel repressor role for C/EBPβ on these genes. Overall, mesenchymal cell C/EBPβ deficiency caused relatively minor changes in the basal expression of type I collagen and α-SMA genes, which are known to be activated by this transcription factor. This selective deficiency, however, caused a greater effect basally on genes that appeared to be repressed by C/EBPβ. However, developmentally and with respect to lung structure, this cell-selective deficiency did not cause any detectable abnormalities.

However, when these mesenchymal-specific conditional C/EBPβ knockout mice were studied with the use of the bleomycin model, wherein C/EBPβ is induced, the results indicated that mesenchymal-specific loss of C/EBPβ resulted in significant reduction of bleomycin-induced lung myofibroblast differentiation. This would be consistent with previous studies in which full-length C/EBPβ is shown to enhance myofibroblast differentiation.[8,9] Because myofibroblasts are the main source of fibrogenic cytokine and extracellular matrix gene expression,[2,3] loss of the myofibroblast in the C/EBPβ CKO mice would be expected to result in reduced fibrosis. This was indeed the case as evidenced by both histopathologic evaluation as well as quantitative biochemical analysis of lung collagen deposition. Further evidence was obtained by analysis of collagen gene expression, which found significantly reduced levels of collagen I mRNA.

In addition to these effects of C/EBPβ on mesenchymal cells, it is also known to be a regulator of the inflammatory response, including the acute phase response,[18,19] which may also affect pulmonary fibrosis via indirect mechanisms (in contrast to directly affecting matrix-producing mesenchymal cells). The C/EBPβ CKO mice exhibited normal levels of C/EBPβ expression in inflammatory/immune as well as lung epithelial cells, thus confirming specific knockout only in the mesenchymal compartment. Moreover, bleomycin-induced lung inflammatory/immune cell recruitment was essentially unimpaired in these CKO mice. Thus, impairment of pulmonary fibrosis in mice lacking C/EBPβ expression in the mesenchymal compartment only was not due to effects on the inflammatory/immune cell recruitment. Moreover, it was also independent of C/EBPβ-regulated genes in the epithelial compartment. The evidence thus supports the conclusion that the observed effects of specifically elim-

inating C/EBPβ in the mesenchymal compartment are mainly mediated through the regulation of myofibroblast differentiation and perhaps other C/EBPβ target genes in lung fibroblasts.

In the search for other potentially relevant C/EBPβ target genes, microarray analysis was performed, which found multiple gene candidates whose expression was significantly altered by C/EBPβ deficiency. These genes included components of Wnt, IL-1β, and IL-13 signaling pathways; cell differentiation; and proliferation regulators, as well as extracellular cell matrices and chemokine receptors. Transcription factors with known regulatory activity on the *ACTA2* promoter affected by C/EBPβ deficiency were also identified. For example, C/EBPβ deficiency increased expression of KLF4, a repressor of myofibroblast differentiation through binding to the TGFβ control element in the *ACTA2* gene promoter and through interaction with Smad3.[40–42] Similar stimulatory effects on expression of PTEN and DNMT3a were also evident in the absence of C/EBPβ. Both of those genes are known to be negative regulators of myofibroblast differentiation.[43,44] In the case of PTEN, its expression is significantly diminished in idiopathic pulmonary fibrosis lung myofibroblasts within fibroblastic foci relative to control lung fibroblasts.[44] DNMT3a catalyzes *de novo* DNA methylation of cytosine phosphate guanine (CpG) islands and was shown to directly affect methylation of *ACTA2* gene sequences that resulted in repression of *ACTA2* gene expression.[43] Thus, in addition to direct negative effects on *ACTA2* gene expression, C/EBPβ deficiency could potentially suppress fibrosis by enhancing expression of these two known suppressors of myofibroblast differentiation. Another potential candidate target gene of interest is MMP12, which was markedly induced in the absence of C/EBPβ. Some evidence suggests a role for MMP12 in fibrosis.[45–47] However, the potential mechanism here would be different and probably independent of effects on myofibroblast differentiation from the standpoint of *ACTA2* gene expression. In view of its matrix-degrading activity, its overexpression would be expected to result in reduced fibrosis and matrix deposition, which was indeed the case in the bleomycin model from this study, as well as in a previous report in IL-13-dependent emphysema.[48] Other potential candidate genes remain to be analyzed and will be the subject of future studies. Thus, in addition to directly affecting the *ACTA2* promoter activity,[9] C/EBPβ may also regulate myofibroblast differentiation indirectly through mediation by other regulators of *ACTA2* gene expression and/or MMP12 expression.

In conclusion, the findings in this study for the first time show the specific importance of C/EBPβ expression in the mesenchymal compartment in pulmonary fibrosis. The mechanism likely involved multiple C/EBPβ target genes with effects on diverse pathways of regulatory importance to myofibroblast differentiation, extracellular matrix production, deposition, and degradation. Although the results confirmed the importance of mesenchymal C/EBPβ expression on myofibroblast differentiation *in vivo*, they did not rule out the contribution of the inflammatory/immune and epithelial compartments. These contributions are suggested by the incomplete suppression of fibrosis by the absence of C/EBPβ in the mesenchymal compartment. Additional future studies are warranted to more precisely examine this contribution by the nonmesenchymal compartments.

Acknowledgments

We thank Matthew Ullenbruch for his technical assistance for the animal studies and Tianju Liu for helpful discussions.

References

1. Bonner JC: Mesenchymal cell survival in airway and interstitial pulmonary fibrosis. Fibrogenesis Tissue Repair 2010, 3:15
2. Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat ML, Gabbiani G: The myofibroblast: one function, multiple origins. Am J Pathol 2007, 170:1807–1816
3. Phan SH: The myofibroblast in pulmonary fibrosis. Chest 2002, 122: 286S–289S
4. Skalli O, Schurch W, Seemayer T, Lagace R, Montandon D, Pittet B, Gabbiani G: Myofibroblasts from diverse pathologic settings are heterogeneous in their content of actin isoforms and intermediate filament proteins. Lab Invest 1989, 60:275–285
5. Darby I, Skalli O, Gabbiani G: Alpha-smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing. Lab Invest 1990, 63:21–29
6. Buck M, Chojkier M: A ribosomal S-6 kinase-mediated signal to C/EBP-beta is critical for the development of liver fibrosis. PloS One 2007, 2:e1372
7. Buck M, Chojkier M: C/EBPbeta associates with caspase 8 complex proteins and modulates apoptosis in hepatic stellate cells. J Clin Gastroenterol 2007, 41(Suppl 3):S295–S299
8. Hu B, Ullenbruch MR, Jin H, Gharaee-Kermani M, Phan SH: An essential role for CCAAT/enhancer binding protein beta in bleomycin-induced pulmonary fibrosis. J Pathol 2007, 211:455–462
9. Hu B, Wu Z, Jin H, Hashimoto N, Liu T, Phan SH: CCAAT/enhancer-binding protein beta isoforms and the regulation of alpha-smooth muscle actin gene expression by IL-1 beta. J Immunol 2004, 173: 4661–4668
10. Ramji DP, Foka P: CCAAT/enhancer-binding proteins: structure, function and regulation. Biochem J 2002, 365:561–575
11. Dearth LR, Hutt J, Sattler A, Gigliotti A, DeWille J: Expression and function of CCAAT/enhancer binding proteinbeta (C/EBPbeta) LAP and LIP isoforms in mouse mammary gland, tumors and cultured mammary epithelial cells. J Cell Biochem 2001, 82:357–370
12. Descombes P, Schibler U: A liver-enriched transcriptional activator protein. LAP, and a transcriptional inhibitory protein LIP, are translated from the same mRNA. Cell 1991, 67:569–579
13. Ossipow V, Descombes P, Schibler U: CCAAT/enhancer-binding protein mRNA is translated into multiple proteins with different transcription activation potentials. Proc Natl Acad Sci U S A 1993, 90:8219–8223
14. Buck M, Poli V, van der Geer P, Chojkier M, Hunter T: Phosphorylation of rat serine 105 or mouse threonine 217 in C/EBP beta is required for hepatocyte proliferation induced by TGF alpha. Mol Cell 1999, 4:1087–1092
15. Buck M, Turler H, Chojkier M: LAP (NF-IL-6), a tissue-specific transcriptional activator, is an inhibitor of hepatoma cell proliferation. EMBO J 1994, 13:851–860
16. Cortes-Canteli M, Pignatelli M, Santos A, Perez-Castillo A: CCAAT/enhancer-binding protein beta plays a regulatory role in differentiation and apoptosis of neuroblastoma cells. J Biol Chem 2002, 277: 5460–5467
17. Hadaschik D, Hinterkeuser K, Oldak M, Pfister HJ, Smola-Hess S: The Papillomavirus E2 protein binds to and synergizes with C/EBP factors involved in keratinocyte differentiation. J Virol 2003, 77:5253–5265
18. Screpanti I, Romani L, Musiani P, Modesti A, Fattori E, Lazzaro D, Sellitto C, Scarpa S, Bellavia D, Lattanzio G, Bistoni F, Frati1 L, Cortese R, Gulino A, Ciliberto G, Costantini F, Poli V: Lymphoprolif-

erative disorder and imbalanced T-helper response in C/EBP beta-deficient mice. EMBO J 1995, 14:1932–1941
19. Burgess-Beusse BL, Darlington GJ: C/EBPalpha is critical for the neonatal acute-phase response to inflammation. Mol Cell Biol 1998, 18:7269–7277
20. Cassel TN, Nord M: C/EBP transcription factors in the lung epithelium. Am J Physiol 2003, 285:L773–L781
21. Feil R, Brocard J, Mascrez B, LeMeur M, Metzger D, Chambon P: Ligand-activated site-specific recombination in mice. Proc Natl Acad Sci U S A 1996, 93:10887–10890
22. Hayashi S, McMahon AP: Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. Dev Biol 2002, 244:305–318
23. Bou-Gharios G, Garrett LA, Rossert J, Niederreither K, Eberspaecher H, Smith C, Black C, Crombrugghe B: A potent far-upstream enhancer in the mouse pro alpha 2(I) collagen gene regulates expression of reporter genes in transgenic mice. J Cell Biol 1996, 134:1333–1344
24. Zheng B, Zhang Z, Black CM, de Crombrugghe B, Denton CP: Ligand-dependent genetic recombination in fibroblasts: a potentially powerful technique for investigating gene function in fibrosis. Am J Pathol 2002, 160:1609–1617
25. Sterneck E, Zhu S, Ramirez A, Jorcano JL, Smart RC: Conditional ablation of C/EBP beta demonstrates its keratinocyte-specific requirement for cell survival and mouse skin tumorigenesis. Oncogene 2006, 25:1272–1276
26. Hu B, Wu Z, Phan SH: Smad3 mediates transforming growth factor-beta-induced alpha-smooth muscle actin expression. Am J Respir Cell Mol Biol 2003, 29:397–404
27. Reddy GK, Enwemeka CS: A simplified method for the analysis of hydroxyproline in biological tissues. Clin Biochem 1996, 29:225–229
28. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001, 25:402–408
29. Austyn JM, Gordon S: F4/80, a monoclonal antibody directed specifically against the mouse macrophage. Eur J Immunol 1981, 11:805–815
30. Coffman RL, Weissman IL: B220: a B cell-specific member of the T200 glycoprotein family. Nature 1981, 289:681–683
31. Exley M, Terhorst C, Wileman T: Structure, assembly and intracellular transport of the T cell receptor for antigen. Semin Immunol 1991, 3:283–297
32. Hestdal K, Ruscetti FW, Ihle JN, Jacobsen SE, Dubois CM, Kopp WC, Longo DL, Keller JR: Characterization and regulation of RB6-8C5 antigen expression on murine bone marrow cells. J Immunol 1991, 147:22–28
33. Liu T, Dhanasekaran S, Jin H, Hu B, Tomlins S, Chinnaiyan A, Phan S: FIZZ1 stimulation of myofibroblast differentiation. Am J Pathol 2004, 164:1315–1326
34. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP: Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 2003, 4:249–264
35. King JY, Ferrara R, Tabibiazar R, Spin JM, Chen MM, Kuchinsky A, Vailaya A, Kincaid R, Tsalenko A, Deng DX, Connolly A, Zhang P, Yang E, Watt C, Yakhini Z, Ben-Dor A, Adler A, Bruhn L, Tsao P, Quertermous T, Ashley EA: Pathway analysis of coronary atherosclerosis. Physiol Genomics 2005, 23:103–118
36. Moeller A, Ask K, Warburton D, Gauldie J, Kolb M: The bleomycin animal model: a useful tool to investigate treatment options for idiopathic pulmonary fibrosis? Int J Biochem Cell Biol 2008, 40:362–382
37. Denton CP, Khan K, Hoyles RK, Shiwen X, Leoni P, Chen Y, Eastwood M, Abraham DJ: Inducible lineage-specific deletion of TbetaRII in fibroblasts defines a pivotal regulatory role during adult skin wound healing. J Invest Dermatol 2009, 129:194–204
38. Hoyles RK, Derrett-Smith EC, Khan K, Shiwen X, Howat SL, Wells AU, Abraham DJ, Denton CP: An essential role for resident fibroblasts in experimental lung fibrosis is defined by lineage-specific deletion of high-affinity type II transforming growth factor beta receptor. Am J Respir Crit Care Med 2011, 183:249–261
39. Houglum K, Buck M, Adir V, Chojkier M: LAP (NF-IL6) transactivates the collagen alpha 1(I) gene from a 5′ regulatory region. J Clin Invest 1994, 94:808–814
40. King KE, Iyemere VP, Weissberg PL, Shanahan CM: Kruppel-like factor 4 (KLF4/GKLF) is a target of bone morphogenetic proteins and transforming growth factor beta 1 in the regulation of vascular smooth muscle cell phenotype. J Biol Chem 2003, 278:11661–11669
41. Adam PJ, Regan CP, Hautmann MB, Owens GK: Positive- and negative-acting Kruppel-like transcription factors bind a transforming growth factor beta control element required for expression of the smooth muscle cell differentiation marker SM22alpha in vivo. J Biol Chem 2000, 275:37798–37806
42. Hu B, Wu Z, Liu T, Ullenbruch MR, Jin H, Phan SH: Gut-enriched Kruppel-like factor interaction with Smad3 inhibits myofibroblast differentiation. Am J Respir Cell Mol Biol 2007, 36:78–84
43. Hu B, Gharaee-Kermani M, Wu Z, Phan SH: Epigenetic regulation of myofibroblast differentiation by DNA methylation. Am J Pathol 2010, 177:21–28
44. White ES, Atrasz RG, Hu B, Phan SH, Stambolic V, Mak TW, Hogaboam CM, Flaherty KR, Martinez FJ, Kontos CD, Toews GB: Negative regulation of myofibroblast differentiation by PTEN (Phosphatase and Tensin Homolog Deleted on chromosome 10). Am J Respir Crit Care Med 2006, 173:112–121
45. Nenan S, Boichot E, Lagente V, Bertrand CP: Macrophage elastase (MMP-12): a pro-inflammatory mediator? Mem Inst Oswaldo Cruz 2005, 100(Suppl 1):167–172
46. England KA, Price AP, Tram KV, Shapiro SD, Blazar BR, Panoskaltsis-Mortari A: Evidence for early fibrosis and increased airway resistance in bone marrow transplant recipient mice deficient in MMP12. Am J Physiol 2011, 301:L519–L526
47. Matute-Bello G, Wurfel MM, Lee JS, Park DR, Frevert CW, Madtes DK, Shapiro SD, Martin TR: Essential role of MMP-12 in Fas-induced lung fibrosis. Am J Respir Cell Mol Biol 2007, 37:210–221
48. Lanone S, Zheng T, Zhu Z, Liu W, Lee CG, Ma B, Chen Q, Homer RJ, Wang J, Rabach LA, Rabach ME, Shipley JM, Shapiro SD, Senior RM, Elias JA: Overlapping and enzyme-specific contributions of matrix metalloproteinases-9 and -12 in IL-13-induced inflammation and remodeling. J Clin Invest 2002, 110:463–474