Matrix Metalloproteinase 12 Overexpression in Lung Epithelial Cells Plays a Key Role in Emphysema to Lung Bronchioalveolar Adenocarcinoma Transition

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Abstract

Chronic obstructive pulmonary disease (COPD) and lung cancer are two diseases that are related to smoking in humans. The molecular mechanism linking these two diseases is poorly understood. Matrix metalloproteinase 12 (MMP12) is a member of the MMP family, which can be induced by smoking. Because MMP12 overexpression in epithelial cells has been reported in inflammation-triggered lung remodeling, a murine CCSP-rtTA/(tetO)7-MMP12 bitransgenic model was created. In this model, MMP12-Flag fusion protein overexpression and its increased enzymatic activity were observed in the lung in an inducible manner, which led to inflammatory cell infiltration and increased epithelial growth. In sequential events, spontaneous emphysema and bronchioalveolar adenocarcinoma were developed as a result of MMP12 overexpression. During this process, the concentration of interleukin-6 was steadily increased in bronchioalveolar lavage fluid, which activated the oncopgenic signal transducer and activator of transcription 3 (Stat3) in alveolar type II epithelial cells. Expression of Stat3 downstream genes that are known to stimulate inflammation and tumor formation was significantly increased in the lung. When tested in humans, MMP12 up-regulation was highly associated with COPD and lung cancer in patients. Together, these studies support that MMP12 is a potent proinflammatory and oncopgenic molecule. MMP12 up-regulation plays a critical role in emphysema to lung cancer transition that is facilitated by inflammation.

Introduction

Smoking leads to chronic obstructive pulmonary disease (COPD; the major phenotype is emphysema) and lung cancer, which are associated with pulmonary inflammation. Human COPD patients (especially with smoking history) are a high-risk population for developing lung cancer. Even after having given up smoking, lung inflammation persists and progresses in humans with COPD (1). The molecular mechanism that links COPD and lung cancer is poorly understood.

Matrix metalloproteinases (MMPs) are a family of >20 secreted or transmembrane proteins that are capable of degrading extracellular matrix (ECM) and basement membrane components under physiologic conditions. MMPs play very important roles in normal connective tissue turnover during morphogenesis, tissue development, wound healing, and reproduction. MMPs also act as modulators of inflammation and innate immunity by activating, deactivating, or modifying the activity of signaling cytokines, chemokines, and receptors (2, 3). In oncology, MMPs have long been considered as molecules necessary to promote tumor invasion and metastasis through the degradation of the ECM (4, 5). Nevertheless, their roles in directly initiating and inducing tumor have never been reported. MMP12 is a 22-kDa metal-dependent protease that was first detected by Werb and Gordon in 1975 (6). It can degrade elastin and other substrates, such as type IV collagen, fibronectin, laminin, gelatin, vitronectin, entactin, heparin, and chondroitin sulfates (7). In the lung, MMP12 is identified in alveolar macrophages of cigarette smokers as an elastolytic MMP (8). Inactivation of the MMP12 gene in MMP12 knockout mice suggests that MMP12 plays a critical role in smoking-induced COPD (9). The clinical relevance of MMP12 in non–small cell lung cancer (NSCLC) had been studied, in which MMP12 correlates with early cancer-related deaths in NSCLC, especially for those associated with tobacco cigarette smoke exposure (10). It has been reported that the MMP1-MMP3-MMP12 gene cluster plays important roles in lung cancer development and progression (11). Studies using comparative genomic hybridization analysis obtained a high-resolution map of frequent chromosomal gains and losses associated with lung cancer. An amplified MMP cluster region (11q22) with overexpressed MMP1, MMP12, and MMP13 was identified (12). Although these studies showed association of MMP12 overexpression with lung cancer, the role of MMP12 up-regulation in lung cancer as a causative factor remains to be defined. In addition to macrophages, MMP12 is overexpressed in lung epithelial cells (13, 14). During lysosomal acid lipase deficiency in the lung, blockage of cholesteryl esters and triglycerides to free cholesterol and free fatty acids triggered pulmonary inflammation, emphysema, and hypercellularity (14–18). MMP12 was highly overexpressed (100-fold) in the lal−/− lung as determined by the Affymetrix GeneChip microarray analysis. Expression of the MMP12 gene is down-regulated by lipid mediators and anti-inflammatory peroxisome proliferator-activated receptor γ (PPARγ; ref. 14).

The roles of inflammation, tumor microenvironment, and ECM remodeling during tumorigenesis are complex, as multiple cell types are involved in intricate cross-talk that is difficult to recapitulate in vitro. Thus, to understand the role of MMP12 in the process of emphysema to tumor transition and tumor-associated inflammation, conditional overexpression of MMP12 in lung epithelial cells was created by using the reverse tetracycline transactivator (rtTA) that is under the control of the Clara cell secretory protein (CCSP) promoter.
Materials and Methods

Animal care. The animal care was the same as previously described (19).

Generation of doxycycline-controlled MMP12 transgenic mice. To generate the (tetO)-CMV-MMP12 transgenic mouse line, MMP12 cDNA was amplified by PCR using a downstream primer (5′-AAGGAAAAA-AGCCGCCGCTGATCACTTGTGACGTCGTTGACAGATTTC-3′) and an upstream primer (5′-AGGCAGTCCGCGCACTGAAATTTCTCATGATG-3′) that contains a Kozak sequence and a NotI site. The PCR product was digested with NotI and subcloned downstream of the (tetO)-CMV minimal promoter linked to seven TetA doxycycline-controlled bitransgenic mouse model was generated from cross-breeding of CCSP-rtTA transgenic mice and (tetO)7-CMV-MMP12 transgenic mice. The expression cassette, containing the CMV minimal promoter, the MMP12 cDNA, and the human globin polyadenylation signaling sequence, was dissected out and purified for microinjection into fertilized eggs of FVB/N mice by the Transgenic Core Facility at the University of Cincinnati College of Medicine. Founder lines were identified by a pair of primers corresponding to a pTRE2 plasmid sequence (5′-CCATC-CCAGCGTTTTGACC-3′) and a MMP12 cDNA coding region sequence (5′-GACTTGAGTTGCTCACTTGGCC-3′). The CCSP-rtTA transgenic mice were genotyped with an upstream primer corresponding to the CCSP promoter sequence (5′-ACTGCCCATGCCCAAAACAC-3′) and a downstream primer corresponding to the rtTA coding region sequence (5′-AAAAAT- CTTGCCAAGTTTCCCCC-3′). CCSP-rtTA/(tetO)-CMV-MMP12 double transgenic mice were generated from cross-breeding of CCSP-rtTA transgenic mice and (tetO)-CMV-MMP12 transgenic mice.

MMP12 activity. The MMP12-specific activity from bronchioalveolar lavage fluid (BALF) was measured by the SensolYTE 490 MMP-12 Assay kit (AnaSpec).

Histology, tumor incidence, and MetaMorph quantification of emphysema. Histology, tumor incidence, and MetaMorph quantification of emphysema were the same as described previously (20, 21).

Fluorescence-activated cell sorting analysis. Fluorescence-activated cell sorting (FACS) analysis was the same as previously described (19).

Reverse transcription and real-time PCR. Reverse transcription and real-time PCR were the same as previously described (22, 23). Human MMP12 immunohistochemical staining. MMP12 immunohistochemical staining was the same as previously described (13).

Results

Generation of CCSP-rtTA/(tetO)-CMV-MMP12 bitransgenic mice. A doxycycline-controlled bitransgenic mouse model was generated to specifically direct MMP12 expression in lung epithelial cells. In this system, a previously established CCSP-rtTA transgenic mouse line (25) was crossbred with a newly generated (tetO)-CMV-MMP12 transgenic mouse line. In this founder line, a Flag sequence was added at the COOH terminus of the MMP12 cDNA to distinguish it from the endogenous MMP12 molecule. CCSP-rtTA/(tetO)-CMV-MMP12 bitransgenic mice were identified by PCR using specific primers and purified mouse tail DNA (Supplementary Fig. S1). Induction of MMP12 mRNA expression in AT II epithelial cells was achieved by doxycycline treatment as monitored by real-time PCR assay. WT and MMP12 single transgenic mice were also treated as controls with no MMP12 mRNA induction observed (Fig. 1A). By FACS analysis, expression of the MMP12-Flag fusion protein was also significantly induced in AT II epithelial cells as costained with Flag antibody and SP-C antibody (AT II cell marker) in doxycycline-treated bitransgenic mice (Fig. 1B). No MMP12-Flag fusion protein was detected in Clara cells and alveolar macrophages as costained with Flag antibody and CCSP (Clara cell marker) or CD11b antibody (macrophage marker) regardless of doxycycline treatment. To confirm that overexpressed MMP12-Flag fusion protein was secreted into alveolar lumen and had the enzymatic activity, BALF from doxycycline-treated (n = 3) and untreated (n = 4) bitransgenic mice was collected and incubated with a fluorescence-quenched MMP12 substrate. Compared with the doxycycline-untreated BALF samples (230 ± 26 ng/μL), the enzymatic product of MMP12 was 10 times higher in the doxycycline-treated BALF samples (2,270 ± 120 ng/μL; Fig. 1C). The MMP12 enzymatic activity remained lower in other controls as well, including doxycycline-treated (tetO)-CMV-MMP12 single transgenic mice (210 ± 55 ng/μL; n = 3) and WT mice (135 ± 30 ng/μL; n = 4). Immunohistochemical staining revealed MMP12 overexpression on the alveolar epithelial wall and hyperplasia cells in the doxycycline-treated bitransgenic lung (Fig. 1D).

Emphysema and bronchioalveolar adenocarcinoma in the lung of bitransgenic mice. To assess the pathophysiologic consequence of MMP12 overexpression in lung epithelial cells, bitransgenic mice were treated with doxycycline for various time lengths. Histopathologic analyses revealed lung abnormalities in bitransgenic mice beginning at 6 weeks of doxycycline treatment. At this stage, marked inflammatory cell infiltration and emphysema were readily detectable (Fig. 2A, +Dox 6W). Quantitative characterization revealed decreased alveolar number and increased alveolar perimeter, alveolar radius, alveolar mean cord length, alveolar surface area, and alveolar volume in doxycycline-treated bitransgenic lungs compared with those in doxycycline-untreated bitransgenic lungs (Supplementary Table S1). These resulted in significant reduction of the overall alveolar surface area (69.16%) for gas exchange in doxycycline-treated bitransgenic mice. After 10 to 15 weeks of doxycycline treatment, the bitransgenic mice began to develop adenomatoid hyperplasia in both parenchyma and small conducting airways (Fig. 2A, +Dox 10W), which resembles to the histopathologic feature of dysplasia in clinical lesions. Bronchioalveolar adenocarcinomas were observed in the lungs of bitransgenic mice after doxycycline treatment as early as 16 weeks (Fig. 2A, +Dox 16W). To determine the tumor incidence rate, bitransgenic mice were euthanized at 9 to 11 months after doxycycline treatment for histopathologic analyses (Fig. 2B). Of 40 doxycycline-untreated bitransgenic mice, only 1 had lung bronchioalveolar adenocarcinoma (2.5%) and 2 others developed pulmonary dysplasia without cancer. In marked contrast, 16 of 47 doxycycline-treated bitransgenic mice in the same age group had lung bronchioalveolar adenocarcinoma (34.04%). Five of them developed both pulmonary dysplasia and bronchioalveolar adenocarcinoma, and three mice...
had dysplasia without bronchioalveolar adenocarcinoma. In most tumor incidences, one tumor per lung was observed (Fig. 2A, +Dox 40W). A few mice showed tumors on both sides of lung lobes (lobectomy). None of WT and single transgenic mice developed bronchioalveolar adenocarcinoma regardless of doxycycline treatment \((n = 10, \text{respectively})\). In the soft agar assay, tumor cell colonies were observed from lung cells that were isolated from doxycycline-treated bitransgenic mice. No tumor cell colony was observed from those of untreated mice (Fig. 2C). This observation suggests that MMP12 overexpression facilitates the neoplasia process. Finally, a doxycycline on/off experiment was performed. First, bitransgenic mice were treated with doxycycline for 2 months to allow emphysema formation. One group of mice was terminated of doxycycline treatment, whereas another group of mice continued to be treated. Interestingly, both groups of mice developed hyperplasia 2 months later (Fig. 2D). This observation suggests that a MMP12-initiated pathogenic mechanism is irreversible to induce emphysema to hyperplasia transition.

**Proliferation and apoptosis of alveolar epithelial cells in bitransgenic mice.** Lung adenocarcinoma formation depends on both uncontrolled epithelial cell growth and apoptotic inhibition. To evaluate if MMP12 overexpression stimulates epithelial cell proliferation during tumor initiation and progression, bromodeoxyuridine (BrUrd) was injected into 3-, 6-, and 9-month doxycycline-treated or untreated bitransgenic mice \((n = 3)\). After 72 hours, the BrUrd-labeled cells were analyzed by FACS after double staining of whole lung cells with fluorochrome-conjugated...
anti-BrdUrd and anti–SP-C antibodies. As shown in Fig. 3A, there was a steady increase of BrdUrd pulse-labeled AT II epithelial cells in the doxycycline-treated bitransgenic mice compared with untreated littermates. The increase of MMP12-induced cell proliferation was time dependent. To evaluate if MMP12 over-expression alters apoptotic activity in lung epithelial cells during tumor initiation and progression, whole lung cells were labeled with Annexin V and fluorochrome-conjugated anti–SP-C antibodies (Fig. 3B). FACS analysis showed a more than 5-fold decrease of Annexin V–labeled AT II epithelial cells in 9-month doxycycline-treated (DOX(+)) or untreated (DOX(-)) bitransgenic mice at 9 to 11 mo of age (n > 40). C, soft agar assay for tumor cell colony formation. Lung single cells from doxycycline-treated (with tumors) or untreated bitransgenic mice were grown on soft agarose gel. D, doxycycline on/off study. Bitransgenic mice were treated with doxycycline for ~2 mo. One group of mice was terminated of treatment and another group of mice continued to be treated. Two months later, H&E staining was performed on doxycycline-untreated mice (~Dox), doxycycline-treated mice (+Dox), and doxycycline-treated and off-treated mice (+Dox on/off).

Activation of oncogenic pathways in the lung of bitransgenic mice. In searching for molecular mechanism by which MMP12 overexpression stimulates emphysema to bronchioalveolar adenocarcinoma transition, the IL-6/signal transducer and activator of transcription 3 (Stat3) pathway was studied. This is because IL-6 up-regulation causes emphysema (26). More importantly, persistent activation of IL-6 downstream Stat3 in AT II epithelial cells directly induced lung inflammation and bronchioalveolar adenocarcinoma (22). To determine secreted IL-6 in BALF, bitransgenic mice at 1 month old were treated with doxycycline for 0, 1, 3, 6, and 9 months. Protein concentrations of secreted IL-6 in BALF were measured by ELISA. As shown in Fig. 4A, IL-6 concentration in BALF of doxycycline-treated mice was steadily
increased compared with those in untreated bitransgenic mice in a time-dependent fashion, correlated well with the process from emphysema to bronchioalveolar adenocarcinoma transition. Increase of the IL-6 concentration can potentially activate downstream Stat3 (phosphorylation) in AT II epithelial cells to induce bronchioalveolar adenocarcinoma. To test this assumption, cells from whole lungs of 9-month doxycycline-treated or untreated bitransgenic mice were isolated and double labeled with fluorescence-conjugated anti–phospho-Stat3Y705 and SP-C (AT II cell marker) antibodies. Labeled cells were analyzed by flow cytometry. In gated SP-C–positive cells, percentage (32.46% versus 2.07%), total positive cells, and mean fluorescence intensity (MFI) of phospho-Stat3Y705 were dramatically increased in doxycycline-treated bitransgenic mice compared with untreated bitransgenic mice.

**Figure 3.** Proliferation and apoptosis of alveolar epithelial cells in bitransgenic mice. **A,** whole lung cells from BrdUrd-injected 3-, 6-, and 9-mo doxycycline-treated [DOX(+)] or untreated [DOX(-)] bitransgenic mice or WT mice were double stained with SP-C and BrdUrd antibodies for FACS analysis. Example of histogram analysis of 9-mo–treated bitransgenic mice. Isotypic antibody (shaded area) served as control. The right two panels showed total BrdUrd-positive SP-C cells and MFI of 3-, 6-, and 9-mo doxycycline-treated and untreated mice or WT mice (n = 3) by FACS analysis. *, P < 0.05; **, P < 0.01. **B,** whole lung cells from 3-, 6-, and 9-mo doxycycline-treated or untreated bitransgenic mice were doubly stained with SP-C and Annexin V antibodies for FACS analysis. Top, example of dot plot analysis of 9-mo–treated bitransgenic mice. Isotypic antibody served as control (data not shown). Bottom, summary studies of 3-, 6-, and 9-mo doxycycline-treated or untreated bitransgenic mice were doubly stained with SP-C and Annexin V antibodies for FACS analysis. Top, example of dot plot analysis of 9-mo–treated bitransgenic mice. Isotypic antibody served as control (data not shown). Bottom, summary studies of 3-, 6-, and 9-mo doxycycline-treated or untreated bitransgenic mice were doubly stained with SP-C and Annexin V antibodies for FACS analysis. **C,** total RNAs were purified from AT II epithelial cells of 3-mo doxycycline-treated or untreated bitransgenic mice. The expression levels of proapoptotic molecules were measured by real-time PCR. Data represent three independent (n = 3) studies.
mice and WT mice (Fig. 4B). At the gene transcriptional level, both IL-6 and Stat3 mRNA levels were up-regulated in response to MMP12 overexpression (Fig. 4C). Other intracellular oncogenic molecules were also analyzed (Supplementary Fig. S2). NFκB, extracellular signal-regulated kinase (ERK), p38, and AKT were all significantly activated in doxycycline-treated bitransgenic mice compared with untreated bitransgenic mice.

We identified multiple Stat3 downstream cytokines and chemokines in AT II epithelial cells that play important roles in initiating inflammation-triggered bronchioalveolar adenocarcinoma in vivo (22). To further establish the connection between the IL-6/Stat3 pathway and MMP12-induced tumorigenesis, expression levels of these molecules were measured in bitransgenic mice before tumor formation. Total RNAs were purified from AT II epithelial cells after 1 month of doxycycline treatment. The expression levels of Stat3 downstream genes and MMP genes were normalized by GAPDH mRNA expression.

Figure 4. Activation of the IL-6/Stat3 pathway in AT II epithelial cells of bitransgenic mice. A, bitransgenic mice were treated [DOX(+)] or untreated [DOX(-)] with doxycycline for 0, 1, 3, 6, and 9 mo. BALF from the lungs was analyzed by IL-6 ELISA kit. P < 0.05 (n = 3–4). B, whole lung single cells from 9-mo doxycycline-treated (red line) or untreated (blue line) bitransgenic mice or WT mice (green line) were double labeled with anti-phospho-Stat3Y705 and SP-C antibodies and analyzed by flow cytometry. Isotypic antibody (shaded area) served as control. In gated SP-C–positive cells, the percentage numbers of phospho-Stat3Y705–positive cells were presented in histograms. Total phospho-Stat3Y705–positive cells and MFI of phospho-Stat3Y705 in gated SP-C–positive cells were also determined. The study represents five independent experiments (n = 5). C, total RNA was purified from AT II epithelial cells of bitransgenic mice that were treated or untreated with doxycycline for 9 mo. Real-time PCR was used to quantify mRNA expression levels of IL-6, LIF, and Stat3 and normalized by GAPDH mRNA expression. P < 0.05 (n = 3–4). D, total RNA was purified from AT II epithelial cells of bitransgenic mice (Bi Tg), WT mice, and MMP12 single transgenic mice that were treated or untreated with doxycycline for 1 mo. Real-time PCR was used to quantify mRNA expression levels of Stat3 downstream genes and MMP genes and normalized by GAPDH mRNA expression.

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VEGF to 160.3-fold in doxycycline-treated bitransgenic mice compared with those untreated mice (Fig. 4D). CSF-1 remained unchanged. In addition, Stat3-induced downstream developmental gene HNF4α was up-regulated to 78.6-fold, Foxa3 to 9.3-fold, and SHH to 3.3-fold (Fig. 4D). None of these molecules was induced in WT and MMP12 single transgenic mice regardless of doxycycline treatment. Interestingly, increased expression of several other MMPs was also observed in doxycycline-treated bitransgenic mice compared with untreated mice.

**Inflammatory cell infiltration in the lung of bitransgenic mice.** One major manifestation in Stat3-induced bronchoalveolar adenocarcinoma is inflammatory cell infiltration in the lung (22). Because persistent activation of the IL-6/Stat3 pathway was observed by MMP12 overexpression, it is necessary to assess if

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**Figure 5.** Inflammatory cell infiltration in bitransgenic mice. **A,** whole lung cells were isolated from the lungs of 3-, 6-, and 9-mo doxycycline-treated [DOX(+)]) or untreated [DOX(−)] bitransgenic mice and age-matched WT mice. A representative of 3-mo–treated and untreated bitransgenic mice and age-matched WT mice is presented. **B,** the time-dependent study of total Gr-1^+CD11b^+ cell numbers in the lung of 3-, 6-, and 9-mo doxycycline-treated and untreated bitransgenic mice and age-matched WT mice. Columns, mean of at least four independent animals (n > 4); bars, SD. *, P < 0.05. **C,** CFSE-labeled normal CD4^+ T cells were stimulated with anti-CD3 mAb plus anti-CD28 mAb in the presence or absence of MDSCs isolated from doxycycline-treated bitransgenic mice. The ratios of MDSC:T cell were 0:1, 1:25, 1:5, and 1:1, respectively. Proliferation of CD4^+ T cells was evaluated as CFSE dilution by flow cytometry. **D,** inhibition of IL-2 secretion in supernatants of stimulated CD4^+ T cell and MDSC cocultured cells measured by ELISA.
MMP12 overexpression induces inflammatory cell infiltration during the course of pathogenesis in the lung of bitransgenic mice. Whole lung cells were isolated from the lungs of 3-, 6-, and 9-month doxycycline-treated or untreated bitransgenic mice and WT mice for staining with fluorochrome-conjugated anti-mouse CD11b (for monocytes) or Gr-1 (for neutrophils) antibody in FACS analysis. Compared with untreated bitransgenic mice, the percentage numbers of CD11b+ (2.59% versus 11.84%) and Gr-1+ (0.64% versus 5.87%) inflammatory cells were significantly increased in the lung of 3-month doxycycline-treated bitransgenic mice (Fig. 5A). Importantly, the percentage number of CD11b/Gr-1 double-positive cells (2.91% versus 15.58%) was significantly increased in the lung of doxycycline-treated mice (Fig. 5A). CD11b/Gr-1 double-positive cells are MDSCs. Constant influx of these cells suppresses adaptive immunity and supports the angiogenesis and stroma remodeling for tumor growth (27). With disease progression, the total number of this cell population was gradually increased in the lungs of 3-, 6-, and 9-month doxycycline-treated bitransgenic mice (Fig. 5B). MDSCs isolated from doxycycline-treated bitransgenic mice were able to inhibit T-cell proliferation, in which CFSE-labeled CD4+ T cells were stimulated with anti-CD3 mAb plus anti-CD28 mAb. The inhibition was MDSC concentration dependent (Fig. 5C). IL-2 secretion from stimulated CD4+ T cells was also inhibited by MDSCs in the cocultured study as measured by ELISA, an indication of functional impairment of these cells (Fig. 5D). These results indicate that inflammatory cell infiltration and immunosuppression plays a critical role in emphysema by bronchioalveolar adenocarcinoma transition.

**MMP12 up-regulation in human adenocarcinoma, squamous cell carcinoma, and COPD.** Because MMP12 overexpression caused lung tumor formation in the CCSP-rtTA/(tetO)\textsubscript{7}-CMV-MMP12 bitransgenic mouse model, it is important to determine if up-regulation of MMP12 is associated with lung cancers and COPD in humans. The expression levels of MMP12 mRNA in human adenocarcinomas (n = 24), squamous cell carcinomas (n = 22), COPD without smoking (n = 21), and COPD with smoking (n = 25) versus normal samples (n = 12) were examined by quantitative real-time PCR (Fig. 6). In comparison with normal human lungs, the average of MMP12 mRNA expression levels was 10.06-fold higher in adenocarcinomas, 5.31-fold higher in squamous cell carcinomas, 2.23-fold higher in lung tissues with COPD from nonsmokers, and 2.92-fold higher in lung tissues with COPD from smokers. Therefore, up-regulation of MMP12 is associated with emphysema and lung cancer in humans. Stat3 up-regulation has been observed in the same human samples as we previously described (23). Co-up-regulation of MMP12 and Stat3 in human patients provides additional evidence supporting that both molecules are connected and contribute to lung cancer formation.

**Discussion**

To understand the pathophysiologic roles of MMP12 in the lung, a conditional bitransgenic mouse model was generated to overexpress MMP12 in lung epithelial cells. In this bitransgenic model, doxycycline treatment induced MMP12-Flag fusion protein expression at both mRNA and protein levels (Fig. 1A and B). In BALF of bitransgenic mice after doxycycline treatment, the MMP12 enzymatic activity was significantly increased (Fig. 1C). Together, these results suggest that CCSP-rtTA/(tetO)\textsubscript{7}-CMV-MMP12 bitransgenic mice are able to synthesize and secrete the active form of the MMP12-Flag fusion protein in the alveolar lumen.

As a pathologic consequence, persistent overexpression of the MMP12-Flag fusion protein in AT II cells caused emphysema 6 weeks after doxycycline treatment in bitransgenic mice (Fig. 2A). This is probably due to degradation of ECM that weakens the interstitial alveolar structure. With longer MMP12 exposure, dysplasia (10–15 weeks of doxycycline treatment) and bronchioalveolar adenocarcinoma (16–40 weeks of doxycycline treatment) were observed. This sequential emphysema to lung cancer transition resembles the clinical situation that COPD is highly likely to develop lung cancer in humans. It seems that MMP12 serves as a trigger. In the doxycycline on/off study (Fig. 2D), in which doxycycline treatment was terminated once emphysema was established but before hyperplasia, the mice still developed hyperplasia 2 months later. This may be explained by that inflammation (e.g., inflammatory cell infiltration and cytokine/chemokine up-regulation) had already been induced, which triggered irreversible pathologic cascades toward tumorigenesis. In a similar clinical situation, some smokers develop lung cancer even after quitting smoking. Before and during tumor formation,
AT II epithelial cells underwent both increased cell proliferation as determined by BrdUrd pulse-labeling analysis and decreased apoptosis as determined by Annexin V labeling analysis under MMP12 overexpression (Fig. 3).

It has been proposed previously that degraded fragments from ECM by MMPs serve as chemoattractants for inflammatory cell infiltration in the lung (28). This seems to be supported by our observation that inflammatory cells are readily detectable in the alveolar region before and after tumor formation in AT II bitransgenic lungs after doxycycline treatment (Fig. 5). Especially, tumor-promoting MDSCs were significantly increased in the lung of bitransgenic mice. As we reported recently, increased concentration of MDSCs alone by apoptosis inhibition is sufficient to cause bronchioalveolar adenocarcinoma in vivo (19). Infiltration of innate and adaptive immune cells into lung changed the local microenvironment and hijacked the immune surveillance system to favor tumor growth. These cells change the microenvironment to assist tumorigenesis to influence angiogenesis, remodel ECM, and increase mutational rate that leads to genetic or epigenetic instabilities.

One obvious microenvironment change in BALF of bitransgenic mice is the steady increase of IL-6 concentration (Fig. 4A). Interestingly and importantly, IL-6 up-regulation has been reported to cause emphysema and to play a role in lung adenocarcinoma (22, 26). Phosphorylation of IL-6 downstream effector Stat3 in AT II epithelial cells was significantly increased as a result of IL-6 surge in BALF of doxycycline-treated bitransgenic mice (Fig. 4B). Simultaneously, IL-6 and Stat3 gene transcription was activated in AT II epithelial cells after doxycycline treatment (Fig. 4C). We reported previously that persistent activation of Stat3 in AT II epithelial cells directly induced lung bronchioalveolar adenocarcinoma (22). Consequently, Stat3 downstream genes in AT II epithelial cells were activated in bitransgenic mice (Fig. 4D). These genes are known to promote inflammation (e.g., IL-6, CSF-2, TNFα, CCL5, CCL8, CXCR2, and VEGF) and to stimulate epithelial growth (e.g., HNF-3α, Foxa3, and SHH). It is conceivable that up-regulation of these Stat3 downstream genes along with other MMP genes exacerbates inflammation and induces epithelial cell transformation into bronchioalveolar adenocarcinoma in the lung of bitransgenic mice. Therefore, activation of the IL-6/Stat3 pathway is responsible, at least partially, for MMP12-induced emphysema and tumorigenesis. To formally prove this concept, disruption of the IL-6/Stat3 pathway in CCSP-rtTA/(tetO)-CMV-MMP12 bitransgenic mice by crossbreeding with IL-6 and Stat3 knockout mice should be performed in the future. As we reported recently, Stat3 and its downstream genes serve as biomarkers for lung adenocarcinoma and COPD diagnosis in humans (23). Importantly, up-regulation of MMP12 is associated with human COPD, adenocarcinomas, and squamous cell carcinomas (6). Other oncogenic molecules (e.g., ERK1/2, AKT, p38, and NFκB) were also activated in doxycycline-treated bitransgenic mice compared with untreated bitransgenic mice (Supplementary Fig. S2), suggesting that MMP12-induced tumorigenesis is a complex process.

In summary, sequential appearance of emphysema, epithelial dysplasia, and bronchioalveolar adenocarcinoma in the CCSP-rtTA/(tetO)-CMV-MMP12 bitransgenic mouse model supports that MMP12 is a key player that controls emphysema to tumor transition in the lung. This seems achieved by activation of the proinflammatory IL-6/Stat3 pathway (at least partially) and inflammatory cell infiltration, therefore providing additional evidence showing that chronic inflammation facilitates formation of emphysema and lung cancer. As we showed previously, MMP12 is under the control of anti-inflammatory PPARγ and its lipid hormonal ligands (14). Activation of PPARγ inhibits the proliferation of lung carcinoma cells (29). Therefore, our study supports a concept that the balance and antagonism between anti-inflammatory PPARγ and proinflammatory IL-6/Stat3 determine lung cancer formation, similar to that observed in myeloma (30).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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