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Pleural Mesothelial Cell Differentiation and Invasion in Fibrogenic Lung Injury

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The origin of the myofibroblast in fibrotic lung disease is uncertain, and no effective medical therapy for fibrosis exists. We have previously demonstrated that transforming growth factor-β1 (TGF-β1) induces pleural mesothelial cell (PMC) transformation into myofibroblasts and haptotactic migration *in vitro*. Whether PMC differentiation and migration occurs *in vivo*, and whether this response can be modulated for therapeutic benefit, is unknown. Here, using mice recombinant for green fluorescent protein (GFP) driven by the Wilms tumor-1 (WT-1) promoter, we demonstrate PMC trafficking into the lung and differentiation into myofibroblasts. Carbon monoxide or the induction of heme oxygenase-1 (HO-1) inhibited the expression of myofibroblast markers, contractility, and haptotaxis in PMCs treated with TGF-β1. Intrapleural HO-1 induction inhibited PMC migration after intratracheal fibrogenic injury. PMCs from patients with idiopathic pulmonary fibrosis (IPF) exhibited increased expression of myofibroblast markers and enhanced contractility and haptotaxis, compared with normal PMCs. Carbon monoxide reversed this IPF PMC profibrotic phenotype. WT-1—expressing cells were present within fibrotic regions of the lungs in IPF subjects, supporting a role for PMC differentiation and trafficking as contributors to the myofibroblast population in lung fibrosis. Our findings also support a potential role for pleural-based therapies to modulate pleural mesothelial activation and parenchymal fibrosis progression. *(Am J Pathol 2013, 182: 1239—1247; http://dx.doi.org/10.1016/j.ajpath.2012.12.030)*

Idiopathic pulmonary fibrosis (IPF), the most common idiopathic interstitial pneumonia, is characterized by cellular and structural changes in the parenchyma associated with the proliferation of myofibroblasts and deposition of extracellular matrix components.[1] IPF begins in the subpleural region and extends centrally, resulting in a progressive decline in lung function. The origin of the pathogenic myofibroblast is uncertain. The hallmark lesions of IPF, the fibroblastic foci seen on two-dimensional histopathological slides, were thought of as discrete sites of epithelial injury and repair.[2] Cool et al[3] used three-dimensional reconstruction to demonstrate that these foci are part of a complex, highly interconnected reticulum and suggested the leading edge of the fibroblastic invasion extends from the pleura to the underlying parenchyma like a wave of fibrosis.

The pleura is a metabolically active monolayer of mesothelial cells that intimately approximates the lung parenchyma. The close proximity of PMCs to the underlying lung ideally positions them to respond to cytokines, chemokines, and growth factors released during parenchymal stress, injury, infection, or inflammation. The cytokine transforming growth factor-β1 (TGF-β1) is a crucial mediator of epithelial—mesenchymal transition (EMT) and acts as a master switch for induction of fibrosis in many organs, including the lung.[4–6] PMC transformation into myofibroblasts and haptotactic migration occur *in vitro* in response to TGF-β1.[7] The concept of pleural mesothelial—mesenchymal transition (MMT) was investigated in a recent study demonstrating PMC migration into the lung and expression of myofibroblast phenotypic markers. This study demonstrated the presence

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of PMCs in the lung parenchyma of patients with IPF and a correlation with disease severity and the degree of fibrosis.[8] PMC differentiation into myofibroblasts and subsequent migration may play a crucial role in the development of fibrotic lung disease.

Heme oxygenase-1 (HO-1) is the inducible form of the rate-limiting enzyme involved in the degradation of heme with the generation of equimolar quantities of carbon monoxide (CO), iron, and biliverdin.[9] HO-1 induction with subsequent CO production is highly sensitive to numerous stimuli and cellular insults that cause oxidative stress.[10] Such induction represents a beneficial response to injurious stimuli in diverse diseases, including atherosclerosis, sepsis, and fibrosis. The protective effects of HO-1 and CO are due to anti-inflammatory, antiapoptotic, antioxidant, and antiproliferative properties.[11] Indeed, HO-1 deficiency has been associated with increased fibrosis, tubular TGF-β1 expression, inflammation, and enhanced EMT in a model of obstructive kidney disease.[12] Up-regulation of HO-1 provides protection against renal injury after unilateral ureteral obstruction and suppression of tubulointerstitial fibrosis via anti-apoptotic pathway modulation.[13] Additionally, adenoviral transfer of the HO-1 gene, as well as administration of CO and bilirubin, has been associated with suppression of fibrosis in animal models of fibrotic lung disease.[14,15] Here, we present definitive evidence for the presence of PMCs in the lung parenchyma of patients with IPF and show that the fibrotic disposition of these cells can be reversed by CO and HO-1 pathway modulation *in vitro*. Furthermore, using animal models of lung injury, we demonstrate PMC differentiation into myofibroblasts and parenchymal invasion that is inhibited by intrapleural HO-1 induction.

Materials and Methods

Cell Culture

Mouse wild-type PMCs of passages between 15 and 35 were used. Cells were grown in F12K medium (catalog no. 11765-054; Life Technologies—Invitrogen, Carlsbad, CA) supplemented with heat-inactivated fetal bovine serum (10%) or in serum-free conditions before (16 hours) and during TGF-β1 treatments. Human PMCs were derived from lung explants from patients diagnosed with IPF undergoing lung transplantation. Cells were cultured in Medium 199 (catalog no. 11150-059; Life Technologies—Invitrogen) supplemented with epidermal growth factor (EGF) and 10% fetal bovine serum or in serum-free conditions without EGF before and during TGF-β1 exposure.

Cytokine Treatments

Cells were serum-starved for 16 hours and then were treated with 5 ng/mL TGF-β1 (catalog no. 101-B1; R&D Systems, Minneapolis, MN) activated in 4 mmol/L HCl, 0.5% bovine serum albumin.

Induction of HO-1

Cells were treated with 5 μmol/L hemin in dimethyl sulfoxide (catalog no. 51280-1G; Sigma-Aldrich, St. Louis, MO) in either serum-free conditions or in 10% fetal bovine serum medium, depending on the experimental design. Cells were treated with 2-cyano-3,12-dioxooleana-1,9-dien-28-imidazolide (CDDO-Im) at doses of 25 nmol/L or 50 nmol/L, depending on the experiment. The CDDO-Im was provided by Michael Sporn and Karen Liby of Dartmouth Medical School.

CO Treatment

Tricarbonyldichlororuthenium (II) (CORM-2) was purchased from Sigma-Aldrich (catalog no. 288144-500 MG). Cells were exposed to CORM-2 dissolved in dimethyl sulfoxide (10 μmol/L), which served as a source of CO.

Collagen Gel Contraction

Transformed normal pleural mesothelial cells (Met5A) or PMCs from patients with IPF were seeded at a density of 1×10^6 cells/mL. Cells and serum medium were mixed with rat tail collagen reconstituted in 0.1 mol/L acetic acid (catalog no. C7661-50 MG; Sigma-Aldrich) in a 3:1 proportion and seeded in individual wells in a 24-well culture dish. Cells were allowed to acclimatize for 16 hours and then were pretreated with hemin, CDDO-Im, or CORM-2 for 30 minutes, followed by addition of 5 ng/mL TGF-β1; cells were allowed to contract for 48 hours.

Western Blotting

All protein isolates were prepared with radioimmunoprecipitation assay buffer. Samples were sonicated at 4°C, followed by centrifugation to remove insoluble material, and then were subjected to protein assay using a Micro BCA kit (Pierce; Thermo Fisher Scientific, Rockford, IL). All protein levels were adjusted to identical concentrations, followed by reducing with 0.1 mol/L dithiothreitol and resolved on 4 to 20% NuPAGE minigels (catalog no. NP0320; Life Technologies—Invitrogen) using a morpholine propane sulfonic acid (NP0001) buffer system; samples were then transferred to nitrocellulose membrane (catalog no. 162-0115; Bio-Rad Laboratories, Hercules, CA), followed by blocking in 5% nonfat dry milk reconstituted in Tris-buffered saline/Tween (pH 7.4) (TBST). The following antibodies were used for immunoblotting: α-SMA mouse monoclonal (1:1000; catalog no. 03-61001; American Research Products, Belmont, MA), β-actin mouse monoclonal (1:2000; catalog no. A5441; Sigma-Aldrich), HO-1 rabbit polyclonal (1:1000; catalog no. ADI-SPA-895; Enzo Life Sciences International, Plymouth Meeting, PA), horseradish peroxidase conjugated α-rabbit IGG (1:5000; catalog no. 31462; Thermo Fisher Scientific), α-mouse IGM (1:5000; catalog no. 31432; Thermo Fisher Scientific), and NOX-4 (catalog no. NB110-58849;

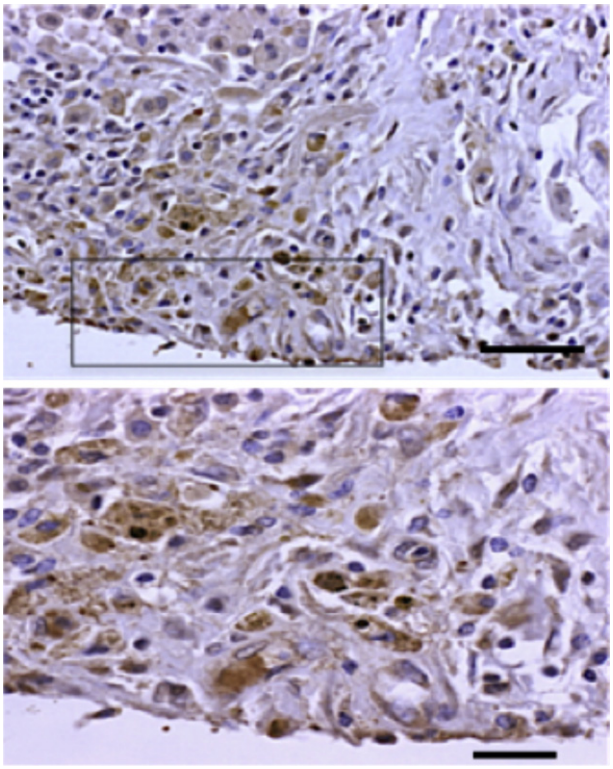

Figure 1 PMCs are present in the explanted lungs from patients with IPF. Sixteen explanted lungs from 16 patients with IPF were preserved after lung transplantation and specimens were stained (brown) for WT-1 on standard microscope slides. Immunohistochemistry revealed abnormal presence of WT-1^{+} cells within the parenchyma of an explanted lung (representative of 16 explanted lungs). The **boxed area** in the **top panel** is presented at higher magnification in the **bottom panel**. Scale bars: 100 μm (**top panel**); 40 μm (**bottom panel**).

Novus Biologicals). All primary antibodies were prepared in 5% nonfat dry milk reconstituted in TBST (pH 7.4) and probed overnight at 4°C, followed by three washes and then incubation with appropriate horseradish peroxidase conjugated secondary antibody at room temperature for 2 hours. The immunoblots were then washed five times in TBST, followed by developing with chemiluminescent substrate application (Pierce ECL, catalog no. 32106; Thermo Fisher Scientific).

Mouse Models

To investigate the effects of HO-1 during bleomycin injury, we used a pleural delivery model in wild-type and *Hmox*$^{-/-}$ mice on a C57BL/6J background. To track PMCs, we used a transgenic mouse model that constitutively expresses GFP in Wt1-expressing cells (*Wt1*$^{tm1(EGFP/cre)Wtp}$/J).

Cell Migration Assays

Haptotaxis assays were performed in Costar Transwell migration chambers (Corning Life Sciences, Corning, NY) as described previously, with modifications.[7] In brief, the lower sides of the filters were coated with 1 mg/mL bovine serum albumin, or TGF-β1, or were left untreated and kept overnight at 37°C in humidified air in the presence of 5% CO_2. The filters were removed, washed with PBS, and air-dried. Filters were placed into 48-well plates. The lower portion of the chamber was filled with Medium 199 (Life Technologies—Invitrogen) with 1% fetal bovine serum. PMCs (1×10^5 cells) treated with TGF-β1 alone, with TGF-β1 and either CDDO-Im, CORM-2, or hemin, or with no treatment (control) were seeded into the upper chamber and incubated for 6 hours at 37°C. At the end of incubation, medium from the upper well was discarded. The upper sides of the filters were scraped to remove adherent cells. The filters were removed, and cells were fixed in formalin and stained with 20% Giemsa stain (catalog no. 48900, Fluka; Sigma-Aldrich). The number of cells that migrated was quantified by counting the number of cells on the distal surface of the filter under an optical microscope. The results are expressed as the haptotactic index [ie, the number of cells visualized per 20 high-power (×40) fields]. Coating the filters with TGF-β1 acts as an attractant for the PMCs.

Explanted Lungs

Sixteen lungs from 16 patients with IPF, eight lungs from eight patients with COPD, two lungs from two patients with cystic fibrosis, and one lung from one patient with pulmonary arterial hypertension, were preserved after lung transplantation at the University of Alabama at Birmingham (Birmingham, AL),

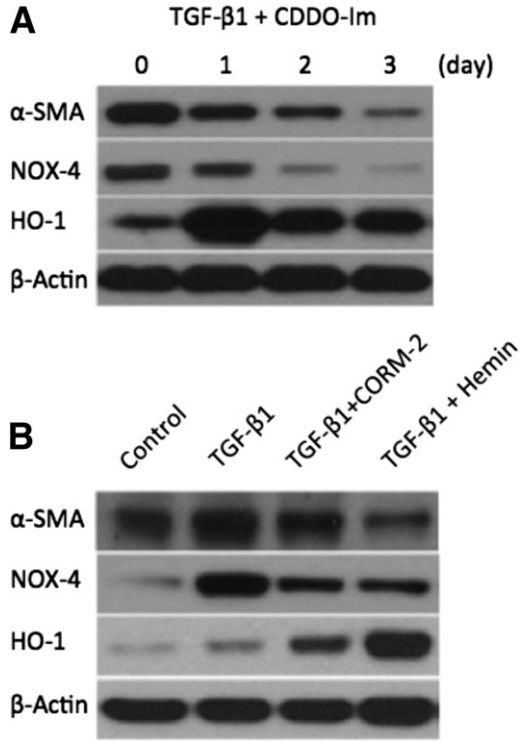

Figure 2 HO-1 induction or treatment with the CO-releasing molecule CORM-2 inhibits PMC differentiation into myofibroblasts. **A:** Wild-type mouse PMCs were treated with CDDO-Im (50 nmol/L) after treatment with TGF-β1 (5 ng/mL). **B:** Wild-type mouse PMCs were left untreated (control) or were treated with TGF-β1 alone for 48 hours, or with both TGF-β1 and either hemin (5 μmol/L) or CORM-2 (10 μmol/L) for 48 hours. Representative Western blots demonstrate expression of α-SMA, NOX-4, HO-1, and β-actin.

with informed consent. PMCs from explanted lungs were obtained by surface scraping of the visceral pleura and used in passages 2 to 4. PMCs expressed calretinin, mesothelin, and WT-1. Lung specimens were fixed in formalin, embedded in paraffin, and sectioned with a microtome before immunohistochemical staining on standard microscope slides.

Imaging

For fluorescence imaging, an Olympus BX51 microscope was used (40× lens, numerical aperture of 1.3; Olympus America, Center Valley, PA). The fluorochromes used were as follows: for green fluorescent protein (GFP), Alexa Fluor 488 anti-chicken; for smooth muscle actin, Alexa Fluor 594 anti-mouse (Life Technologies—Invitrogen). DAPI was used as a nuclear counterstain. A Retiga camera was used (12-bit color; QImaging, Surrey, BC, Canada) with light microscopy and Bioquant Osteo 2009 (Bioquant Image Analysis, Nashville, TN). For immunohistochemistry, an Axiovert 200M light microscope was used (40× lens, numerical aperture 1.3; Carl Zeiss, Göttingen, Germany), at room temperature, with glass slides and bright-field fluorochromes. Images were captured using a QImaging QIClick camera and Q Capture Pro version 7 software (QImaging, Surrey, BC, Canada).

All human and animal studies were approved by the Institutional Review Board at the University of Alabama at Birmingham. Written informed consent was obtained from the participants before inclusion in the study.

Results

PMCs Are Present in Lung Explants from Patients with IPF

WT-1 is a zinc-finger protein that regulates many properties of the developing mesothelium. It is expressed in mesoderm-derived tissue, including normal pleural mesothelium, but is absent in normal lung, making it an ideal marker of PMCs. WT-1—expressing PMCs have been shown to migrate into the lung parenchyma to form smooth muscle cells of the vascular wall and other cells of the lung mesenchyme during mouse lung development.[16] The major role of WT-1 in mesenchymal differentiation and development has implications for lung pathology, including pulmonary fibrosis. In the present study, using immunohistochemistry, we have demonstrated the abnormal presence of WT-1^+ cells within the parenchyma of an explanted lung from a representative patient with IPF, one of the 16 explanted lungs that underwent immunohistochemistry for WT-1 (Figure 1). WT-1^+ cells were absent from the explanted lungs of patients with non-IPF interstitial lung disease, cystic fibrosis, and chronic obstructive pulmonary disease (data not shown).

HO-1 Induction or Treatment with a CO-Releasing Molecule Inhibits PMC Differentiation into Myofibroblasts in Response to TGF-β1 in Mouse PMCs

Treatment of mouse PMCs with TGF-β1 increased expression of the myofibroblast marker α-smooth muscle actin (α-SMA) and NADPH oxidase-4 (NOX-4). NOX-4 has been shown to mediate myofibroblast activation and fibrogenic responses to lung injury. NOX-4—dependent generation of hydrogen peroxide is required for TGF-β1—induced myofibroblast differentiation, extracellular matrix production, and contractility.[17] CDDO-Im, a synthetic triterpenoid, is a multifunctional molecule with antiproliferative, antidifferentiating, and anti-inflammatory activities that at nanomolar concentrations induces the expression of HO-1.[18] To investigate the effects of HO-1 induction on PMC differentiation and fibrosis, we treated mouse PMCs with TGF-β1 with and without

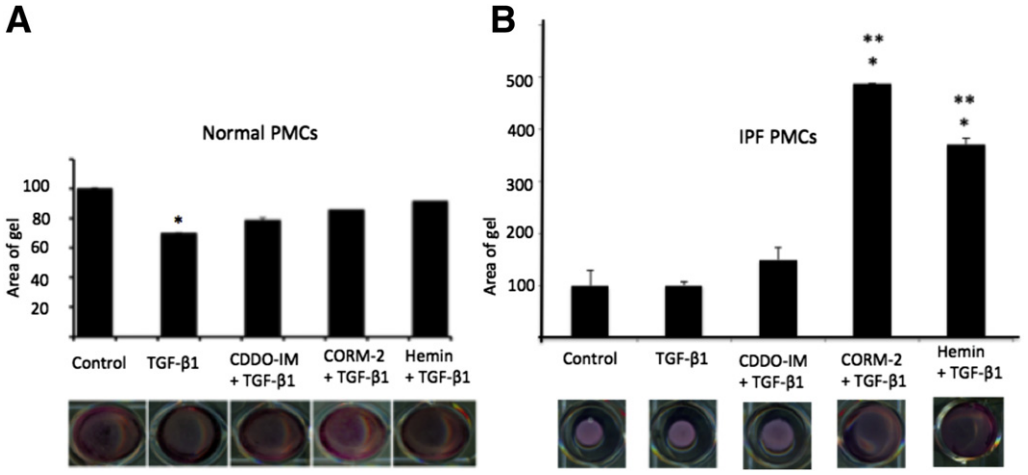

Figure 3 HO-1 modulates PMC contractility. Normal PMCs (**A**) and PMCs from patients with IPF (**B**) were seeded on collagen wells and were treated with TGF-β1 (5 ng/mL) alone or were pretreated with hemin (5 μmol/L), CDDO-Im (25 nmol/L), or CORM-2 (10 μmol/L) for 30 minutes before TGF-β1; the PMCs were allowed to contract for 48 hours. Gel area was subsequently measured as a surrogate for PMC contractility. Data are expressed as means ± SD. $*P < 0.05$ versus control and cotreated samples (**A**) or versus control (**B**); $**P < 0.05$ versus TGF-β1 alone and CDDO-Im + TGF-β1 (**B**).

administration of CDDO-Im. CDDO-Im administration at the time of PMC treatment with TGF-β1 caused an increase in HO-1 expression and a decrease in NOX-4 and α-SMA expression. Treatment with ferriprotoporphyrin IX chloride (hemin), a physiological HO-1 inducer, increased HO-1 and decreased expression of NOX-4 and α-SMA in PMCs treated with TGF-β1. Treatment with CORM-2, a CO-releasing molecule, similarly inhibited NOX-4 and α-SMA expression (Figure 2). Taken together, these results demonstrate a role for HO-1 in the prevention of PMC MMT into myofibroblast-like cells.

HO-1 Induction and CO Inhibit PMC Contractility

We performed functional gel contraction assays to investigate the effects of HO-1 induction and CO on the response to TGF-β1 of PMCs from both healthy subjects and patients with IPF. PMCs were treated with TGF-β1 alone or were treated with either hemin, CDDO-Im, or CORM-2 before TGF-β1. In normal human PMCs, gel contraction was significantly decreased in control and in all three cotreatments, compared with TGF-β1 alone (Figure 3A). The PMCs from patients with IPF exhibited inherent contractility, even without TGF-β1 treatment, with no significant difference in gel area (a surrogate for PMC contractility) between control PMCs and TGF-β1—treated PMCs from patients with IPF. Cotreatment with hemin and TGF-β1, but not with CDDO-Im and TGF-β1, caused a significant increase in gel area; the addition of CORM-2 to TGF-β1—treated IPF PMCs likewise caused a significant increase in gel area. These findings suggest reversion to a more normal PMC phenotype (Figure 3B). The data demonstrate a role for HO-1 induction or CO in preventing the contractile phenotype of normal PMCs treated with TGF-β1 and for reversing contractility in PMCs from patients with IPF.

HO-1 Induction and CO Prevent PMC Haptotaxis in Normal PMCs and PMCs from Patients with IPF

Haptotaxis assays were performed in Transwell migration chambers. Haptotaxis (ie, cell movement in response to gradients immobilized on a substratum) is distinct from chemotaxis, which is defined as cell migration along a concentration gradient of solubilized molecules. Haptotaxis more closely represents cell movement in the extracellular matrix. Normal PMCs (1×10^5 cells) were treated with TGF-β1 alone, or with CDDO-Im, CORM-2, or hemin treatment followed by TGF-β1; untreated cells were used as control. The number of migrated cells was quantified, and data were expressed as the haptotactic index. Treatment with TGF-β1 caused significant haptotaxis, which was inhibited by CDDO-Im, CORM-2, and hemin (Figure 4A). Haptotaxis assays with IPF PMCs, performed as described above, demonstrated increased baseline haptotaxis, along with increased haptotaxis after treatment with TGF-β1 that was significantly inhibited by hemin or CORM-2 cotreatment, but not by CDDO-Im (Figure 4B).

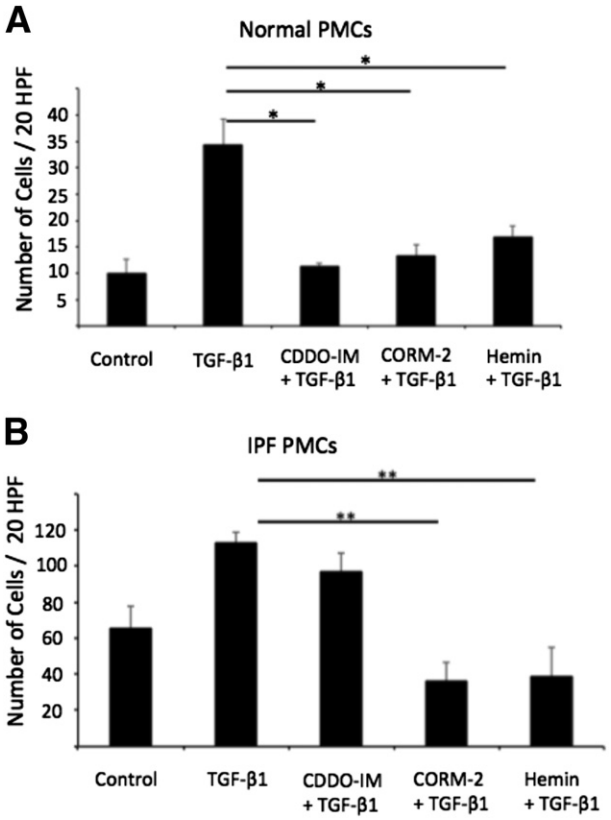

Figure 4 HO-1 induction and the CO-releasing molecule CORM-2 modulate PMC migration. Haptotaxis assays in normal PMCs (**A**) and in PMCs from patients with IPF (**B**) were performed in Transwell migration chambers. PMCs (1×10^5 cells) treated with TGF-β1 alone (5 ng/mL), with the same TGF-β1 and either CDDO-Im (25 nmol/L), CORM-2 (10 μmol/L), or hemin (5 μmol/L , or with no treatment (control) were seeded into the upper chamber and incubated for 6 hours at 37°C. The haptotactic index was determined as the number of migrated cells visualized per 20 high-power fields (HPF). Data are expressed as means ± SD. $^{**}P < 0.05$.

HO-1 Deficiency Potentiates Pleural and Subpleural Fibrosis in Mice

To further investigate the role of HO-1 *in vivo* in pleural and parenchymal fibrosis, we administered intrapleural bleomycin to $Hmox^{-/-}$ and $Hmox^{+/+}$ mice. Trichrome staining for collagen and H&E staining demonstrated a more robust pleural fibrotic response in HO-1—deficient mice, compared with wild-type mice (Figure 5).

Mouse PMCs Undergo MMT and Migrate into the Lung Parenchyma after Intratracheal TGF-β1

To track PMC migration, we used heterozygotic mice recombinant for GFP driven by the *WT-1* promoter. At 24 hours after intratracheal TGF-β1 treatment, we observed GFP^+ cells inside the lung parenchyma (Figure 6A). The

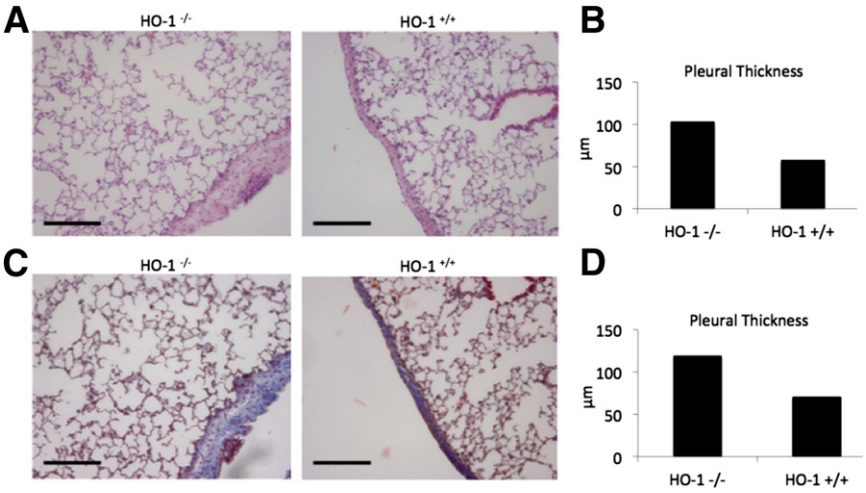

Figure 5 HO-1 deficiency predisposes mice to pleural and subpleural fibrosis. Intrapleural bleomycin (4 U/kg) was administered to $Hmox^{+/+}$ and $Hmox^{-/-}$ mice; the fibrotic response was evaluated at 21 days. **A** and **B**: H&E staining (**A**), with pleural thickness (**B**) measured in the same mice. **C** and **D**: Trichrome staining for collagen (**C**), with pleural thickness (**D**) measured in the same mice. Images are representative of six $Hmox^{+/+}$ and *six* $Hmox^{-/-}$ mice. Scale bars: 152 μm (**A** and **C**, **left panels**); 303 μm (**A** and **C**, **right panels**).

GFP^+ cells costain for α-SMA, further supporting the concept that PMCs have not only migrated into the lung in response to inhaled stimuli, but have also undergone differentiation to express a myofibroblast phenotype (Figure 6B).

Intrapleural HO-1 Induction with CDDO-Im Prevents PMC Migration after Inhaled Fibrogenic Stimulus in Mice

To test the effects of pleural HO-1 induction on parenchymal PMC migration and fibrosis, we treated six wild-type mice with either intratracheal bleomycin alone or intratracheal bleomycin combined with intrapleural CDDO-Im. Control mice treated with intratracheal bleomycin alone exhibited $WT\text{-}1^+$ cells along the pleura and in the parenchyma at 21 days after bleomycin treatment. Mice treated with intrapleural CDDO-Im at day 3 after bleomycin treatment exhibited WT-1 staining exclusively along the pleura at 21 days after bleomycin treatment (Figure 7). Intrapleural HO-1 induction thus prevented parenchymal PMC migration in response to an inhaled fibrotic stimulus.

Hemin, CORM-2, and CDDO-Im Inhibit Myofibroblast Marker Expression in PMCs from Patients with IPF

We measured expression of α-SMA, NOX-4, and HO-1 in PMCs from patients with IPF, both before and after treatment with TGF-β1, with or without hemin, CORM-2, or CDDO-Im treatment. PMCs from IPF patients exhibited a predisposition to robustly express NOX-4 and α-SMA at baseline and also after TGF-β1. Treatment with hemin, CORM-2, or CDDO-Im decreased expression of myofibroblast markers (Figure 8). Interestingly, the induction of HO-1 in IPF PMCs appeared less robust than in similar

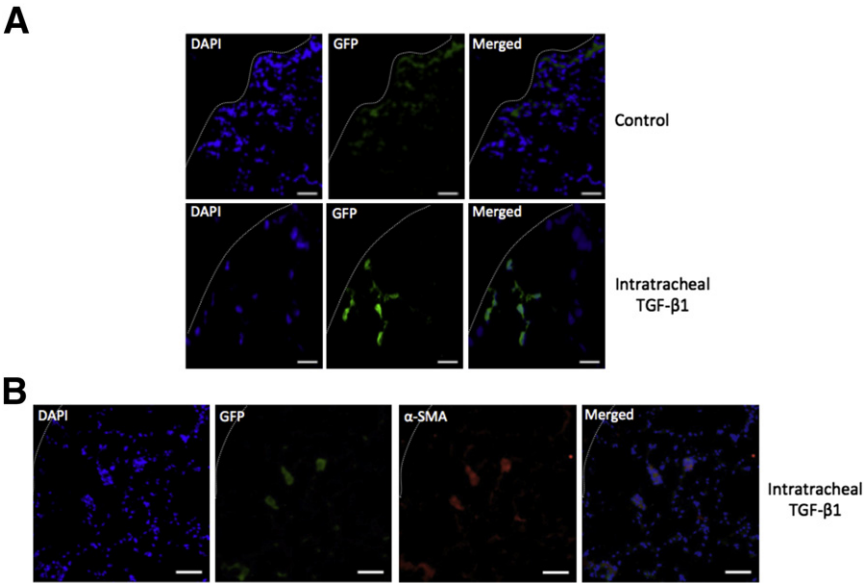

Figure 6 Mouse PMCs undergo MMT transition and parenchymal invasion in response to intratracheal TGF-β1. **A**: Heterozygotic mice recombinant for GFP driven by the WT-1 promoter were treated with intratracheal PBS as control or intratracheal TGF-β1 to track PMCs. Immunofluorescence staining was performed for GFP (green) at 24 hours after intratracheal TGF-β1 treatment. DAPI was used as a nuclear counterstain (blue). **B**: Merged images demonstrate colocalization of GFP and the myofibroblast marker α-SMA (red). A **white dotted line** approximates the location of the pleura. Scale bar = 100 μm.

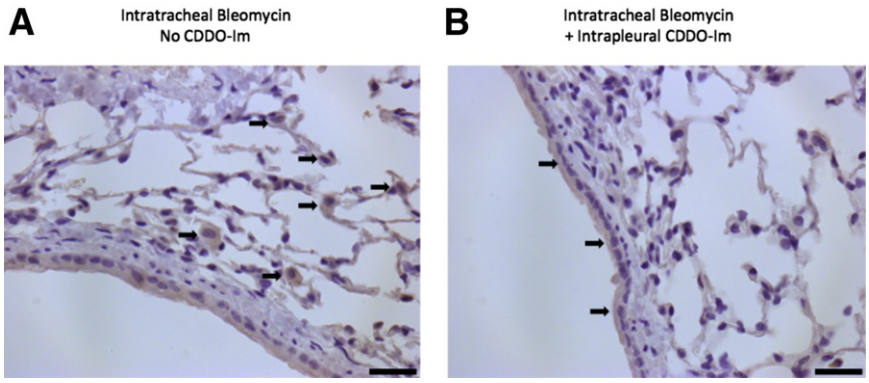

Figure 7 PMC parenchymal migration is inhibited by intrapleural HO-1 induction. Wild-type mice were treated with intratracheal bleomycin (50 mU/mL) alone (**A**) or intratracheal bleomycin followed by intrapleural CDDO-Im (25 nmol/L) at day 3 after bleomycin treatment (**B**), to test the effects of pleural HO-1 induction on PMC migration. Mice were sacrificed at 21 days after bleomycin treatment, and immunohistochemistry was performed for WT-1. PMCs expressing WT-1 are marked with **arrows**. Images are representative of six mice. Scale bar = 40 μm.

experiments performed in normal human PMCs (data not shown).

Discussion

The subpleural distribution of fibrosis in IPF is unexplained. Morphometric analysis of histological sections of usual interstitial pneumonia from patients with IPF using three-dimensional reconstruction shows the fibroblastic foci of usual interstitial pneumonia at the leading edge of a reticulum that extends from the pleura to the underlying parenchyma.[3] In another study, carbon particles administered to the chest cavity of mice along with intratracheal bleomycin resulted in severe pleural fibrosis.[19] This was associated with progressive subpleural fibrosis, similar to IPF. The authors concluded that the PMCs had acquired myofibroblast characteristics and that the matrix accumulation within the subpleural area had evolved through mesothelial–fibroblastoid transformation.

The origin of the myofibroblast in IPF is uncertain. These pathogenic cells have been variously proposed to be derived from local mesenchymal cells, circulating fibroblasts, and epithelial cells that have undergone EMT.[20] In experimental models of pulmonary fibrosis, both EMT and bone marrow progenitors contribute to the fibroblast population. However, neither is a principal contributor to lung myofibroblasts.[21] The role of EMT and MMT in development and disease pathogenesis is established in other organs. HO-1 deficiency is associated with increased fibrosis, tubular TGF-β1 expression, inflammation, and enhanced EMT in obstructive kidney disease.[12] Recently, our research group reported PMC transformation into myofibroblasts and haptotactic migration in response to TGF-β1 *in vitro*.[7] Subsequently, our research group reported that PMCs migrate into the lung and display myofibroblast phenotypic markers; importantly, their presence correlated with the severity of fibrosis in IPF.[8]

Serosal surfaces play an integral role in organogenesis and development. The mesothelial covering of the embryonic heart is a major source of cells to the coronary system.[22–25] Wilm et al[26] used *WT-1–Cre* genetic lineage marking to demonstrate that serosal mesothelial cells differentiate into smooth muscle of all major blood vessels in the mesenteries and gut. The ability of PMCs to undergo transformation and migration plays a crucial role in the developing lung. A recent study of mouse lung development demonstrated that the mesothelial cells covering the lung surface contribute to a variety of cell types in the lung, including vascular smooth

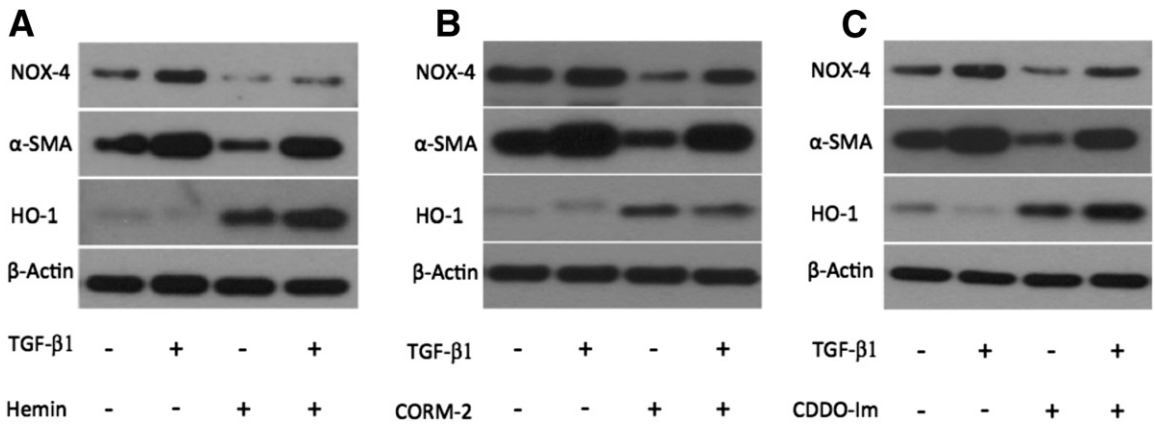

Figure 8 The fibrotic disposition of PMCs from patients with IPF can be overcome with treatment. Western blotting was used to assess in expression of α-SMA, NOX-4, and HO-1 in PMCs from patients with IPF in response to treatment with TGF-β1 (5 ng/mL) with and without pretreatment for 48 hours with (**A**) hemin (5 μmol/L), (**B**) the CO-releasing molecule CORM-2 (10 μmol/L), or (**C**) CDDO-Im (25 nmol/L).

muscle cells and possibly also alveolar myofibroblasts and interstitial fibroblasts.[16] The authors suggested a common mechanism linking the development of coelomic organs with the maturation of the internal vasculature and postulated that adult PMCs are multipotent and possibly able to give rise to mesenchymal cells during injury, repair, or pathology. A subsequent study provided definitive evidence of the multipotent capacity of PMCs by demonstrating differentiation into osteoblast and adipocyte-like cells when exposed to an appropriate growth medium; the authors concluded that mesothelial cell differentiation is a potential source of the different tissue types found in malignant mesothelioma and other serosal pathologies, suggesting potential roles in regenerative therapies.[27] Is it noteworthy that WT-1−expressing cells have a capacity to switch between mesenchymal and epithelial states.[28] This may indicate that WT-1 expression is necessary, but not sufficient, to permit the transition between mesothelial and mesenchymal phenotypes.

The pleura is a metabolically active monolayer of mesothelial cells that intimately approximates the lung parenchyma. This proximity of PMCs to the underlying lung ideally positions them to respond to signals released during parenchymal stress, whether mechanical, inflammatory, or infectious. In fact, a recent magnetic resonance imaging study in rats demonstrated the appearance of a marked inflammatory signal arising from the pleura 6 hours after inhaled antigen or endotoxin; the inflammatory signal correlated with a histological analysis revealing severe pleural cavity edema.[29] The reciprocal communication that occurs between the pleura and parenchyma in the developing lung may therefore continue in the adult and contribute to disease.

With the present study, we have demonstrated *in vitro* PMC differentiation and acquisition of a myofibroblast phenotype, as evidenced by expression of mesenchymal markers and functional assays, including haptotaxis and gel contraction, in response to TGF-β1 treatment. Furthermore, induction of HO-1 or treatment with CO abrogated PMC differentiation and/or MMT and parenchymal migration. HO-1−deficient mice also experienced greater pleural fibrosis in response to intrapleural bleomycin treatment, compared with wild-type mice.

WT-1^+ cells were present in lung explants from patients with IPF, but not in explants from patients with cystic fibrosis or chronic obstructive pulmonary disease (data not shown). Importantly, the present study is the first to demonstrate significant phenotypic and functional differences in PMCs from patients with IPF, compared with normal PMCs. Strikingly, IPF PMCs displayed a propensity to express the myofibroblast-specific marker α-SMA, as well as NOX-4, a known driver of myofibroblast activation and fibrotic responses to lung injury.[17] Not only did IPF PMCs express myofibroblast markers, but they also exhibited increased contractility and propensity for migration. Inducibility of HO-1 expression in PMCs from patients with IPF was lower, compared with normal PMCs, suggesting an inherent or acquired defect. Abnormalities or deficiencies in HO-1 expression or activity in IPF may predispose these patients to fibroblast proliferation and progressive disease.

There is a potential to use HO-1 modulation and CO as therapies for fibrotic lung disease. Studies in other organ systems have shown, using mouse models, that HO-1 induction opposes pathological postinfarction left ventricular remodeling[30] and prevents the progression of liver fibrosis.[31] CO also suppresses bleomycin-induced lung fibrosis in mice.[32] Pioneering work by Ryter and Choi[33] has opened the exciting prospect of translational applications for HO-1 research. In fact, a phase 2 study is recruiting participants (Study of Inhaled Carbon Monoxide to Treat Idiopathic Pulmonary Fibrosis; *http://www.clinicaltrials.gov/ct2/show/NCT01214187*, last accessed February 9, 2013).

In the present study, treatment of PMCs with CO in the form of CORM-2 or hemin not only prevented or reversed the expression of α-SMA and NOX-4, but also significantly attenuated the contractile phenotype of IPF PMCs and PMC haptotaxis. It is important to note that, although hemin and CORM-2 reversed the increased gel contraction and haptotaxis seen in TGF-β1−treated PMCs, CDDO-Im was unable to rescue the TGF-β1−treated IPF PMCs. This suggests that HO-1 induction alone may be insufficient to protect against fibrosis in IPF. Perhaps an inherent or acquired HO-1 substrate deficiency also predisposes to IPF disease. The downstream products of the heme oxygenase reaction may hold more promise for modulating PMC fibrotic phenotypic expression. The present findings raise the possibility that intrapleural modulation of HO-1 or delivery of CO to the pleural space could contribute to novel strategies in the treatment of IPF by inhibiting PMC differentiation and parenchymal trafficking.

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References

1. Raghu G, Weycker D, Edelsberg J, Bradford WZ, Oster G: Incidence and prevalence of idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 2006, 174:810−816
2. Gross TJ, Hunninghake GW: Idiopathic pulmonary fibrosis. N Engl J Med 2001, 345:517−525
3. Cool CD, Groshong SD, Rai PR, Henson PM, Stewart JS, Brown KK: Fibroblast foci are not discrete sites of lung injury or repair: the fibroblast reticulum. Am J Respir Crit Care Med 2006, 174:654−658
4. Hills CE, Squires PE: TGF-beta1-induced epithelial-to-mesenchymal transition and therapeutic intervention in diabetic nephropathy. Am J Nephrol 2010, 31:68−74
5. Meindl-Beinker NM, Dooley S: Transforming growth factor-beta and hepatocyte transdifferentiation in liver fibrogenesis. J Gastroenterol Hepatol 2008, 23(Suppl 1):S122−S127
6. Yáñez-Mó M, Lara-Pezzi E, Selgas R, Ramírez-Huesca M, Domínguez-Jiménez C, Jiménez-Heffernan JA, Aguilera A, Sánchez-Tomero JA, Bajo MA, Alvarez V, Castro MA, del Peso G, Cirujeda A,

Gamallo C, Sánchez-Madrid F, López-Cabrera M: Peritoneal dialysis and epithelial-to-mesenchymal transition of mesothelial cells [Erratum appeared in N Engl J Med 2005, 353:2827]. N Engl J Med 2003, 348: 403–413
7. Nasreen N, Mohammed KA, Mubarak KK, Baz MA, Akindipe OA, Fernandez-Bussy S, Antony VB: Pleural mesothelial cell transformation into myofibroblasts and haptotactic migration in response to TGF-beta1 in vitro. Am J Physiol Lung Cell Mol Physiol 2009, 297: L115–L124
8. Mubarak KK, Montes-Worboys A, Regev D, Nasreen N, Mohammed KA, Faruqi I, Hensel E, Baz MA, Akindipe OA, Fernandez-Bussy S, Nathan S, Antony V: Parenchymal trafficking of pleural mesothelial cells in idiopathic pulmonary fibrosis. Eur Respir J 2012, 39:133–140
9. Tenhunen R, Marver HS, Schmid R: The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. Proc Natl Acad Sci USA 1968, 61:748–755
10. Keyse SM, Tyrrell RM: Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. Proc Natl Acad Sci USA 1989, 86: 99–103
11. Jarmi T, Agarwal A: Heme oxygenase and renal disease. Curr Hypertens Rep 2009, 11:56–62
12. Kie JH, Kapturczak MH, Traylor A, Agarwal A, Hill-Kapturczak N: Heme oxygenase-1 deficiency promotes epithelial-mesenchymal transition and renal fibrosis. J Am Soc Nephrol 2008, 19:1681–1691
13. Kim JH, Yang JI, Jung MH, Hwa JS, Kang KR, Park DJ, Roh GS, Cho GJ, Choi WS, Chang SH: Heme oxygenase-1 protects rat kidney from ureteral obstruction via an antiapoptotic pathway. J Am Soc Nephrol 2006, 17:1373–1381
14. Tsuburai T, Suzuki M, Nagashima Y, Suzuki S, Inoue S, Hasiba T, Ueda A, Ikehara K, Matsuse T, Ishigatsubo Y: Adenovirus-mediated transfer and overexpression of heme oxygenase 1 cDNA in lung prevents bleomycin-induced pulmonary fibrosis via a Fas-Fas ligand-independent pathway. Hum Gene Ther 2002, 13:1945–1960
15. Wang HD, Yamaya M, Okinaga S, Jia YX, Kamanaka M, Takahashi H, Guo LY, Ohrui T, Sasaki H: Bilirubin ameliorates bleomycin-induced pulmonary fibrosis in rats. Am J Respir Crit Care Med 2002, 165:406–411
16. Que J, Wilm B, Hasegawa H, Wang F, Bader D, Hogan BL: Mesothelium contributes to vascular smooth muscle and mesenchyme during lung development. Proc Natl Acad Sci USA 2008, 105: 16626–16630
17. Hecker L, Vittal R, Jones T, Jagirdar R, Luckhardt TR, Horowitz JC, Pennathur S, Martinez FJ, Thannickal VJ: NADPH oxidase-4 mediates myofibroblast activation and fibrogenic responses to lung injury. Nat Med 2009, 15:1077–1081
18. Liby K, Hock T, Yore MM, Suh N, Place AE, Risingsong R, Williams CR, Royce DB, Honda T, Honda Y, Gribble GW, Hill-Kapturczak N, Agarwal A, Sporn MB: The synthetic triterpenoids, CDDO and CDDO-imidazolide, are potent inducers of heme oxygenase-1 and Nrf2/ARE signaling. Cancer Res 2005, 65: 4789–4798
19. Decologne N, Wettstein G, Kolb M, Margetts P, Garrido C, Camus P, Bonniaud P: Bleomycin induces pleural and subpleural fibrosis in the presence of carbon particles. Eur Respir J 2010, 35:176–185
20. King TE Jr, Pardo A, Selman M: Idiopathic pulmonary fibrosis. Lancet 2011, 378:1949–1961
21. Tanjore H, Xu XC, Polosukhin VV, Degryse AL, Li B, Han W, Sherrill TP, Plieth D, Neilson EG, Blackwell TS, Lawson WE: Contribution of epithelial-derived fibroblasts to bleomycin-induced lung fibrosis. Am J Respir Crit Care Med 2009, 180:657–665
22. Dettman RW, Denetclaw W Jr, Ordahl CP, Bristow J: Common epicardial origin of coronary vascular smooth muscle, perivascular fibroblasts, and intermyocardial fibroblasts in the avian heart. Dev Biol 1998, 193: 169–181
23. Männer J: Does the subepicardial mesenchyme contribute myocardioblasts to the myocardium of the chick embryo heart? A quail-chick chimera study tracing the fate of the epicardial primordium. Anat Rec 1999, 255:212–226
24. Mikawa T, Borisov A, Brown AM, Fischman DA: Clonal analysis of cardiac morphogenesis in the chicken embryo using a replication-defective retrovirus: I. Formation of the ventricular myocardium. Dev Dyn 1992, 193:11–23
25. Reese DE, Mikawa T, Bader DM: Development of the coronary vessel system. Circ Res 2002, 91:761–768
26. Wilm B, Ipenberg A, Hastie ND, Burch JB, Bader DM: The serosal mesothelium is a major source of smooth muscle cells of the gut vasculature. Development 2005, 132:5317–5328
27. Lansley SM, Searles RG, Hoi A, Thomas C, Moneta H, Herrick SE, Thompson PJ, Newman M, Sterrett GF, Prêle CM, Mutsaers SE: Mesothelial cell differentiation into osteoblast- and adipocyte-like cells. J Cell Mol Med 2011, 15:2095–2105
28. Scholz H, Kirschner KM: Oxygen-dependent gene expression in development and cancer: lessons learned from the Wilms' tumor gene, WT1. Front Mol Neurosci 2011, 4:4
29. Quintana HK, Cannet C, Schaeublin E, Zurbruegg S, Sugar R, Mazzoni L, Page CP, Fozard JR, Beckmann N: Identification with MRI of the pleura as a major site of the acute inflammatory effects induced by ovalbumin and endotoxin challenge in the airways of the rat. Am J Physiol Lung Cell Mol Physiol 2006, 291:L651–L657
30. Wang G, Hamid T, Keith RJ, Zhou G, Partridge CR, Xiang X, Kingery JR, Lewis RK, Li Q, Rokosh DG, Ford R, Spinale FG, Riggs DW, Srivastava S, Bhatnagar A, Bolli R, Prabhu SD: Cardioprotective and antiapoptotic effects of heme oxygenase-1 in the failing heart. Circulation 2010, 121:1912–1925
31. Barikbin R, Neureiter D, Wirth J, Erhardt A, Schwinge D, Kluwe J, Schramm C, Tiegs G, Sass G: Induction of heme oxygenase 1 prevents progression of liver fibrosis in Mdr2 knockout mice. Hepatology 2012, 55:553–562
32. Zhou Z, Song R, Fattman CL, Greenhill S, Alber S, Oury TD, Choi AM, Morse D: Carbon monoxide suppresses bleomycin-induced lung fibrosis. Am J Pathol 2005, 166:27–37
33. Ryter SW, Choi AM: Heme oxygenase-1/carbon monoxide: from metabolism to molecular therapy. Am J Respir Cell Mol Biol 2009, 41: 251–260