PAI-1 Regulation of TGF-β1-induced ATII Cell Senescence, SASP Secretion, and SASP-mediated Activation of Alveolar Macrophages

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ABSTRACT

Senescence of alveolar type II (ATII) cells, progenitors of the alveolar epithelium, is a pathological feature and contributes importantly to the pathogenesis of idiopathic pulmonary fibrosis (IPF). Despite recognition of the importance of ATII cell senescence in IPF pathogenesis, how ATII cell senescence is regulated and how senescent ATII cells contribute to lung fibrogenesis remain unclear. In this study, we show that TGF-β1, a most ubiquitous and potent profibrotic cytokine, induces plasminogen activator inhibitor 1 (PAI-1), a cell senescence and fibrosis mediator, and p16 as well as senescence, but not apoptosis, in primary mouse ATII cells. We also found that senescent ATII cells secretes various cytokines and chemokines, including interleukin 4 (IL-4) and 13 (IL-13), which stimulate the expression of genes associated with a pro-fibrotic phenotype in alveolar macrophages. Similar responses were also observed in TGF-β1-treated rat ATII (L2) and rat macrophage NR8383 cells. Deletion of PAI-1 or inhibition of PAI-1 activity with a small molecule PAI-1 inhibitor, on the other hand, blocks TGF-β1-induced senescence as well as senescence associated secretion phenotype (SASP) in ATII and L2 cells and, consequently, the stimulatory effects of the conditional medium from senescent ATII/L2 cells on macrophages. Moreover, we show that silencing p16 ameliorates PAI-1 protein-induced ATII cell senescence and secretion of pro-fibrotic mediators. Our data suggest that PAI-1 mediates TGF-β1-induced ATII cell senescence and secretion of pro-fibrotic mediators through inducing p16 and that senescent ATII cells contribute to lung fibrogenesis in part by activating alveolar macrophages through secreting pro-fibrotic and pro-inflammatory mediators.

Key words: ATII cell senescence, PAI-1, senescence associated secretion phenotype, alveolar macrophage activation, lung fibrosis

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INTRODUCTION

Alveolar type II (ATII) cells are progenitors of the alveolar epithelium and play an essential role in maintaining alveolar structure and function (1-3). There is overwhelming evidence showing that ATII cells undergo senescence in idiopathic pulmonary fibrosis (IPF), an aging-related progressive fatal lung disorder, and in experimental lung fibrosis models (4-9). Despite increasing appreciation of an important role of ATII cell senescence in the pathogenesis of fibrotic lung diseases (10-13), little is known about how ATII senescence is regulated in fibrotic lung and how senescent cells contribute to the pathogenesis of lung fibrosis.

Plasminogen activator inhibitor 1 (PAI-1), a physiological inhibitor of tissue type and urokinase type plasminogen activators (tPA, uPA), has multiple functions and is involved in the regulation of cell adhesion, migration, proliferation and apoptosis. Studies from this lab and from others have shown that PAI-1 plays a pivotal role in the development of lung fibrosis, although the underlying mechanisms are still not entirely understood (14-18). PAI-1 has long been used as a cell senescence marker. Emerging evidence suggests that PAI-1 is not merely a marker but also a mediator of cell senescence (19-24). In a recent study, we showed that PAI-1 was highly induced in ATII cells in IPF and in bleomycin-induced lung fibrosis model; ablation of PAI-1 in ATII cells in PAI-1 conditional knockout mice, on the other hand, almost abolished bleomycin-induced ATII cell senescence and lung fibrosis (18). Our data suggest that increased PAI-1 may underlie ATII cell senescence in fibrotic lung and that senescent ATII cells contribute important to lung fibrogenesis. Nonetheless, whether transforming growth factor beta1 (TGF-β1), a most ubiquitous and potent pro-fibrogenic cytokine, induces ATII cell senescence and whether PAI-1 mediates TGF-β1 effect is unknown.

Senescent ATII cells not only lose normal physiological functions and reparative capacity but also secret an array of bioactive molecules, collectively called senescent associated secretory

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phenotype (SASP). These bioactive molecules can modulate the function of secreting cells (autocrine functions) as well as adjacent cells (paracrine function). Alveolar macrophages (AMs) are the major type of cells residing in alveoli under unchallenging condition and function as the first line of defense against inhaled infectious agents and environmental pollutants. Macrophages can be activated by various cytokines, chemokines, and growth factors as well as other biological agents. Macrophages activated by interferon gamma (IFNγ) and/or lipopolysaccharide (LPS) are historically called M1 or classically activated macrophages, whereas macrophages activated by T helper 2 (Th2) cytokines IL-4 and/or IL-13, are often referred to as M2 or alternatively activated macrophage (25-28). It is believed in general that M1 macrophages participate in the eradication of bacterial and viral infection, whereas M2 macrophages play a critical role in host defense against parasite infection, tumor progression, and tissue remodeling (26, 27, 29). Whether ATII SASP is involved in alveolar macrophage activation, M1 and/or M2, in fibrotic lung has not been reported. Moreover, whether ATII PAI-1 modulates ATII SASP components and therefore alveolar macrophage activation is also unknown.

In this study, we addressed two issues: 1) whether TGF-β1, the most potent and ubiquitous pro-fibrogenic cytokine, induced ATII cell senescence and the role of ATII PAI-1 in the process: 2) whether ATII SASP promotes pro-fibrotic polarization of AMs and the effect of ATII PAI-1 on ATII SASP secretion as well as macrophage activation. Both primary cells and cell lines were used to address these issues. The findings reported in this study suggest that PAI-1 mediates TGF-β1-induced ATII cell senescence and SASP secretion through inducing p16 and that ATII SASP promotes pro-fibrotic phenotype of alveolar macrophages.

MATERIALS AND METHODS

Isolation of mouse lung ATII cells and alveolar macrophages. Murine alveolar type II (ATII) epithelial cells were isolated from adult mice (6–12 weeks old) following the protocol as we have

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described previously (18). Briefly, mouse lungs were digested with protease solution (300 U/ml collagenase Type I, 4 U/ml elastase, 5 U/ml dispase, and 100 μg/ml DNase I in HBSS) at 37°C for 25 min and then with 0.1% Trypsin-EDTA and 100 μg/ml DNase I at 37 °C for 20 min. Following tissue dissociation, single cell suspensions were prepared by passing the lung digestions through a 40 μm-mesh size cell strainer. Red blood cells were lysed and then macrophages and lymphocytes removed by incubation with biotinylated rat anti-mouse CD45 and rat anti-mouse CD16/32 (BD Biosciences). The cells were then cultured with DMEM/F-12 medium containing 10% FBS in 100 mm culture dishes at 37°C overnight to remove fibroblasts. The suspended cells were harvested for further culture and treatment. Immunostaining with anti-SPC antibody confirms that >90% isolated cells are ATII cells (18). Alveolar macrophages (AMs) were isolated from mice by bronchoalveolar lavage and cultured with RPMI medium containing 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin ,and 100 μg/ml streptomycin. The purity of isolated cells was assessed by flow cytometry analysis after immunostaining with CD64 antibody (CD64-PE-Vio770) or REA isotope control antibody (REA-control-PE-Vio770, BD Biosciences), followed the manufacturer's protocol. The results showed that >90% of isolated cells are macrophages **(sFig 1**). All procedures involving animals were approved by the Institutional Animal Care and Use Committees at the University of Alabama at Birmingham and conducted at the UAB animal facilities under specific pathogen-free conditions.

Cell culture and treatment: Primary ATII cells were cultured with DMEM/F12 medium containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 5% CO_2 and 37°C. Primary rat lung epithelial (L2) cells (American Type Culture Collection, Rockville, MD) that have been stably transduced with nontarget shRNA or PAI-1 shRNA (18) were cultured with Ham's F-12 medium containing 10% FBS and antibiotics. ATII cells and L2 cells were treated with 2 ng/ml of TGF-β1 in serum-free medium for 48 hrs and then cultured in TGF-β1-free and serum-free medium for additional 24 hours. Cells were then harvested for analysis of senescence markers, whereas the

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conditional media (CM) were collected, mixed with RPMI medium (1:1), and used for the treatment of alveolar macrophages or NR8383 cells, a rat macrophage cell line. The treatment conditions are selected based on our preliminary studies, which showed that the secretions of senescent markers interleukin 6 (IL-6) and insulin like growth factor binding protein 3 (IGFBP3) were not increased until 48 hours after TGF-β1 treatment, whereas the activity of caspase3/7, an apoptosis marker increased after 72 hours of TGF-β1 treatment (**sFig 2**).

Determination of the activity of senescence-associated β-galactosidase (SA-β-gal) in ATII and L2 cells: The activity of SA-β-gal was determined using 5-bromo-4-chloro-3-indolyl P3-D-galactoside (X-gal), following the protocol described previously (18). Briefly, cells were fixed in 2% formaldehyde plus 0.2% glutaraldehyde in PBS at room temperature for 10 min, rinsed with PBS, and then incubated SA-β-gal staining solution containing 1 mg/ml X-gal overnight. SA-β gal positive cells (blue color) were counted under microscope and the results were expressed as percentage of total cells.

Western blot analysis: Cells were lysed in RIPA buffer containing protease inhibitor cocktail (Sigma P8340) and phosphatase inhibitor cocktail (Sigma, P5726) and centrifuged at 13,000 x *g,* 4°C, for 10 min. The protein concentrations were determined by BCA assay and Westerns were conducted with 50 μg of proteins from each sample as we have described previously (16, 17). The membranes were probed with the following antibodies at 4°C overnight: PAI-1 (Molecular Innovation, ASMPAI-GF, ASRPAI-GF), p16 (Proteintech 10883-1-AP), p53 (Santa Cruz, SC-6243), pRB (Cell Signaling, CN 8180), Bax (Cell Signaling, CN 2772S), cleaved caspase 3 (Cell Signaling, CN 9661X), GAPDH (Sigma, G9545), and β-actin (Sigma, A5441). The protein bands were visualized using enhanced chemiluminescence detection reagent (Chemiluminescent ECL Substrate) and an Odyssey infrared imager (LI-COR Model: 2800 S/N OFC-0172); the results were normalized by β-actin or GAPDH band intensity.

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ELISAs of cytokines, chemokines, growth factors in the conditional medium: ELISAs were performed to determine the amounts of cytokines/chemokines/growth factors in the conditional medium (CM) from ATII/L2 cells, using ELISA kits from R&D, according to the manufacturer's protocols.

Measurement of caspase 3/7 activity in the conditional medium: The activity of the caspase 3/7 in the cultured medium was assessed using an assay kit from Promega (Caspase-Glo 3/7 Assay), according to the manufacturer's protocol.

Quantitative real-time PCR analysis of mRNAs: Total RNA was isolated from macrophages using Trizol reagent. The purities and concentrations of the isolated RNA were assessed by nanodrop. cDNA was synthesized using high capacity cDNA kit (Invitrogen) and quantitative real-time PCRs (qRT–PCR) were performed using RT-PCR primers (Life Technologies Thermofisher Scientific, CA) as listed in **Supplementary Table 1** (**sTable 1**) and the LightCycler® 480 System (Roche) as described previously (30, 31). Triplicates were analyzed for each of the samples and average numbers were used for calculation. The comparative threshold cycle (Ct) method, also known as the Ct method, was used to calculate relative mRNA levels, according to the equations described previously (30).

Small Interfering RNA transfection: Freshly isolated mouse type II cells were seeded at a density of 2 X 10^5 cells per well in 6-well culture plates with DMEM/F-12 containing 10% serum and antibiotics. Cells were transfected with 25 nM control (non-targeting) siRNA or mouse p16 siRNA (Dharmacon) using Lipofectamine RNAiMAX reagent (LifeTechnologies, Cat 13778030), following the manufacturer's protocol. After 48-hour culture, the media was replaced and cells were treated with active PAI-1 (PAI-A, Molecular Innovation) at a concentration of 1 ug/ml for 48 hrs.

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Statistics: Data are expressed as mean ± standard deviation (SD). T-tests were performed for 2 group comparison whereas one-way ANOVAs were conducted for 4 group comparison. Statistical significance was determined post-hoc by Tukey's test.

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RESULTS

TGF-β1 activated p16-pRb pathway and induced senescence as well as secretion of pro-fibrotic and pro-inflammatory mediators in primary mouse ATII cells

TGF-β1 is a most ubiquitous and potent profibrogenic mediator. Whether TGF-β1 induces ATII cell senescence has not been reported yet. We show, in these experiments, that treatment of primary mouse ATII cells with 2 ng/ml of TGF-β1 increased the activity of senescence associated beta galactosidase (SA-β-gal) (**Fig 1A&B**) as well as the protein levels of PAI-1 and p16 in these cells (**Fig 1C&D**). This was associated with a decrease in retinoblastoma phosphorylation (pRb) (**Fig 1C&D**). TGF-β1, at the treatment conditions, however, had no significant effect on the expression or activity of caspase 3/7, an apoptosis marker (**Fig 1C-1E**). ELISA results further show that TGF-β1 treatment increased the secretion of interleukin 6 (IL-6), insulin like growth factor binding protein 3 (IGFBP3), and PAI-1, three senescence markers (**Fig 1F-1H**). Importantly, TGF-β1 treatment increases the section of IL-4 and IL-13, two T helper 2 (Th2) cell cytokines which induce alternative activation of macrophages (32), as well as platelet derived growth factor (PDGF-BB), a profibrogenic mediator (**Fig 1I-1K**). The amounts of PAI-1 in the CM (ng/ml) was 10-100 fold higher than other SASP components measured, which were in the pg/ml levels (**Fig 1F-1K**). Together, the results suggest that TGF-β1, at the treatment conditions, induces ATII cell senescence but not apoptosis and that senescent ATII cell secret large amount of PAI-1 as well as other pro-fibrotic mediators.

Deletion of PAI-1 blocked TGF-β1-induced ATII cell senescence and secretion of pro-fibrotic mediators

Our previous studies showed that PAI-1 mediated bleomycin-induced ATII cell senescence through activating p53-p21-pRb pathways (18). To determine whether PAI-1 is also involved in

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TGF-β1-induced ATII cell senescence and the potential underlying mechanism, ATII cells isolated from PAI-1 deficient (PAI-$1^{-/-}$) and wild type (PAI-$1^{+/+}$) mice were treated with TGF-β1. The results show that deletion of PAI-1 in ATII cells abolished TGF-β1-stimulated activity of SA-β-gal (**Fig 2A&B**) as well as accumulation of p16 (**Fig 2D&F**). TGF-β1 treatment had no significant effect on the activity of caspase 3/7 (**Fig 2C**) or the expression of p53, Bax, and cleaved caspase 3 protein (**Fig 2D&2G-2I**), although deletion of PAI-1 reduced the basal level of caspase 3/7 activity and Bax expression (**Fig 2C&2D&2G-2I**). Our data also show that, associated with the suppression of ATII cell senescence, deletion of ATII PAI-1 abolished TGF-β1-stimulated secretion of IL-6, IL-4, IL-13, and PDGF (**Fig 3**). These data further suggest that TGF-β1, under the treatment conditions, induces ATII cell senescence but not apoptosis, and that TGF-β1 promotes ATII cell senescence through induction of PAI-1 and thus activation of p16-pRb, but not p53, cell cycle repression pathway.

Deletion of PAI-1 in ATII cells blunted the stimulatory effect of the conditional medium from senescent ATII cells on alveolar macrophage gene expression

To determine whether ATII SASP modulates alveolar macrophage gene expression and the effect of ATII PAI-1, we treated primary alveolar macrophages (AMs) with the conditional medium (CM) from TGF-β1-stimulated senescent, not apoptotic, ATII cells and analyzed gene expression in AMs by qRT-PCR. The results show that incubation of primary AMs with the CM from senescent ATII cells induced an expression profile of selected genes (**Fig 4**), which resembles the gene expression profile of alveolar macrophages in lungs of mice with experimental fibrosis (33). These include found in inflammatory zone 1 (Fizz1, also known as Retnla) and Ym1 (also known as chitinase-like 3), 2 of macrophage alternative activation markers, STAT6, a transcription factor mediating IL-4 and IL-13-induced macrophage alternative activation, as well as inducible nitrate oxide synthase (iNOS), tumor necrosis factor alpha (TNFα), IL-4, IL-6, IL-13, and TGF-β1 (**Fig 4**).

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Importantly, the stimulatory effects of ATII cell SASP on AM gene expression were completely or partially lost when AMs were cultured with the CM from TGF-β1-treated PAI-1$^{-/-}$ ATII cells (**Fig 4**).

Inhibition of PAI-1 activity protected rat lung ATII cells from TGF-β1-induced senescence and suppressed the secretion of profibrotic mediators

Our previous studies showed that administration of TM5275, a small molecule PAI-1 inhibitor, ameliorated lung fibrosis induced by AdTGF-β1$^{223/225}$, an adenovirus expressing constitutively active TGF-β1 (16). To determine whether treatment with TM5275 blocks TGF-β1-induced ATII cell senescence, rat lung ATII (L2) cells were treated with 2 ng/ml TGF-β1 in the presence or absence of TM5275. The results show that treatment of L2 cells with TGF-β1 significantly increased the number of SA-β-gal positive cells (**Fig 5A&B**) but not the activity of caspase 3/7 (**Fig 5C**). Western analysis further show that treatment with TGF-β1 increased the expression of PAI-1 and p16, which was associated with a reduction of Rb phosphorylation (**Fig 5D-5G**), but had no significant effect on the expression of cleaved caspase 3 or Bax, two apoptosis markers (**Fig 5D&5H&5I**). Treatment with TM5275, on the other hand, almost completely blocked TGF-β1's effects on SA-β-gal activity, p16, and Rb phosphorylation (**Fig 5A-5F**). There was no significant effect of TM5275 on the expression of cleaved caspase 3 or Bax, with or without TGF-β1 (**Fig 5D&5H&5I**). Our data also show that TGF-β1 treatment stimulated the secretion of two senescence markers, IGFBP3 and IL-6, as well as IL-4, IL-13, and PDGF from L2 cells (**Fig 6A-5E**). Importantly, our data show that treatment with TM5275 almost completely blocked TGF-β1-induced secretion of senescent markers as well as IL-4, IL-13, and PDGF (**Fig 6 A-5E**). These data further suggest that PAI-1 mediates TGF-β1-induced ATII cell senescence as well as secretion of profibrogenic mediators, probably through inducing p16. Our data also suggest that TM5275, with or without TGF-β1, does not induce L2 cell apoptosis under the treatment conditions.

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Inhibition of PAI-1 activity attenuated the stimulatory effect of L2 cell SASP on macrophage gene expression

To determine whether TM5275 altered L2 cell SASP and therefore the responses of macrophages, rat macrophage NR8383 cells were cultured with the CM from L2 cells that had been treated with TGF-β1 in the presence or absence of TM5275. The results show that incubation of NR8383 cells with the CM from TGF-β1 alone treated L2 cells increased the expression of arginase 1, fizz1, and ym1, three M2 macrophage markers, iNOS and TNFα, two M1 macrophage markers, as well as TGF-β1, although it has no significant effect on the expression of stat1 or stat6 (**Fig 7**). The CM from L2 cells that had been treated with TGF-β1 in the presence of TM5275, however, had significantly reduced stimulatory effects on NR8383 cells (**Fig 7**). These data further suggest that PAI-1 modulates SASP-stimulated macrophage responses.

Silencing p16 attenuated PAI-1 protein-induced ATII cell senescence and secretion of pro-fibrotic mediators

To explore the potential mechanism whereby PAI-1 mediates TGF-β1-induced ATII cell senescence p16 expression was silenced in these cells with p16 siRNA. The results show that treatment of primary ATII cells with active PAI-1 protein significantly increased the number of SA-β-gal positive cells as well as p16 expression. Silencing p16, on the other hand, completely blocked PAI-1 protein-induced SA-β-gal activity and restored Rb phosphorylation, although it had no significant effect on PAI-1 protein expression (**Fig 8A-8F**). Silencing p16 also blocked PAI-1 protein induced secretion of IL-4, IL-6, IL-13, and PDGF (**Fig 8F-8J**). These data suggest that PAI-1 mediates TGF-β1-induced ATII cell senescence and secretion of profiprotic mediators through inducing p16.

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Discussion:

Idiopathic pulmonary fibrosis (IPF) is an aging-related progressive fatal lung disorder with no known etiology and very limited therapeutic options. One of the pathological features of IPF lung is ATII cell senescence (4-9). Although increasing evidence indicates important roles of ATII cell senescence in lung fibrogenesis (10-12, 18), how ATII senescence is regulated in fibrotic lung and how senescent ATII cells contribute to lung fibrogenesis remains poorly understood. In this study, we showed that TGF-β1, a most ubiquitous and potent pro-fibrogenic cytokine, induced ATII cell senescence and that senescent ATII cells secreted an array of pro-fibrotic and pro-inflammatory mediators, which activated alveolar macrophages. We also found that deletion ATII PAI-1 or inhibition of PAI-1 activity almost completely blocked TGF-β1-induced ATII cell senescence, the secretion of pro-fibrotic mediators, and the stimulatory effects of ATII SASP on macrophages. As PAI-1 expression is increased in ATII cells in IPF (10, 18), our results suggest that increased PAI-1 may underlie ATII cell senescence in IPF lung and that senescent ATII cells promotes lung fibrosis in part by activating macrophages through secreting pro-fibrotic mediators.

Previous studies from this lab and from other have shown that PAI-1 is not only a marker but also a key mediator of cell senescence (18-24). Although the mechanisms underlying PAI-1 promotion of cell senescence remain elusive, several hypotheses have been proposed, including inhibition of proteolytic activity and thus degradation of a senescence mediator IGFBP3 and suppression of cyclin D1 and thus Rb phosphorylation (20-22, 24, 34). In a recent study, we showed that PAI-1 expression was increased in ATII cells in IPF lung and in bleomycin-induced lung fibrosis model; ablation of PAI-1 specifically in ATII cells in PAI-1 condition knockout mice, on the other hand, attenuated bleomycin-induced ATII cell senescence and lung fibrosis in mice (18). Mechanistically, we found that ablation of ATII PAI-1 attenuated bleomycin-induced ATII cell senescence through down-regulation of p53, a master regulator of cell proliferation and death, because PAI-1 induced p53 in ATII cells; deletion or silencing PAI-1 reduced bleomycin-

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stimulated increase in p53 protein, whereas silencing p53 abolished PAI-1 protein-induced L2 cell senescence (18). In this study, we further showed that TGF-β1 induced ATII cell senescence, which was associated with induction of p16, but not p53. Importantly, we found that deletion of PAI-1 or inhibition of PAI-1 activity almost completely abolished TGF-β1-induced p16 expression and ATII cell senescence, whereas silencing p16 abrogated PAI-1 protein-induced ATII cell senescence. Our data suggest that PAI-1 mediated TGF-β1-induced ATII cell senescence by inducing p16, although the molecular mechanism underlying PAI-1 induction of p16 is unknown at moment. As PAI-1, p53, and p16 are all upregulated in ATII cells in IPF lung (10, 18, 35), our results suggest that PAI-1 plays a pivotal role in ATII cell senescence in fibrotic lung through activating different cell cycle repression pathways, including p53 and p16, upon stimulation by different stimuli.

Despite the recognition of the important role of ATII cell senescence in IPF pathogenesis, how ATII cell senescence is linked to lung fibrosis is not well understood. Besides loss of normal function and reparative capacity, senescent cells secret an array of bioactive molecules including pro-fibrotic and pro-inflammatory cytokines, chemokines, and proteases, collectively called senescence associated secretory phenotype (SASP). These bioactive molecules can modulate the function of secreting cells as well as adjacent cells. Nonetheless, whether senescent ATII cells secret pro-fibrotic mediators, whether ATII PAI-1 modulates the secretion profile, and whether ATII SASP promotes pro-fibrotic phenotype in alveolar macrophages are unknown. In this study, we showed that senescent ATII cells secreted various pro-inflammatory and pro-fibrotic mediators, including IL-4 and IL-13, two T helper 2 (Th2) cell cytokines involved in M2 activation of macrophages. We also showed that ATII PAI-1 promoted the secretion of pro-fibrotic mediators as deletion of PAI-1 or inhibition of PAI-1 activity abolished TGF-β1-induced secretion of IL-4, IL-13, and PDGF in ATII and L2 cells. These data suggest that senescent ATII cells may facilitate

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lung fibrosis by secreting pro-fibrotic mediators. PAI-1, on the other hand, promotes lung fibrosis at least in part by inducing ATII cell senescence and the secretion of pro-fibrotic mediators.

We would like to point out that ATII cells secret both profibrotic and senescent mediators upon TGF-β1 stimulation. Although we did not do a systematic study to dissect the time-courses of the secretions of senescent vs. profibrotic mediators, our preliminary studies showed that the secretions of IL-6 and IGFBP3, two senescence markers, and IL-13, a profibrotic cytokine, were not increased till 48 hours of TGF-β1 treatment (**sFig 2**). Moreover, we found that the activity of caspase 3/7, an apoptosis marker, was increased after 72 hours of TGF-β1 treatment (**sFig 2**). These data suggest that the secretion of senescence and profibrotic mediators follows a similar time course and that there is a small window between cell senescence and apoptosis. It has been well-documented that ATII cells will be transformed into type I cells when they are cultured for more than 5-6 days. This restricted our time-course study to the maximum 3 days as cell isolation and experiment setting up takes 2 days. It should be stressed that the time-courses may be different with different stimuli. Nonetheless, more studies are needed to address these issues, which is beyond the scope of this study.

Alveolar macrophages are the first line of defense in the airways and play a critical role in lung injury and fibrosis. Alveolar macrophages are activated by diverse stimuli including infectious agents and air pollutants. Whether ATII SASP modulates macrophage activation has not been reported. In this study, we showed for the first time that SASP from TGF-β1-induced senescent ATII cells stimulated the expression of many genes in alveolar macrophages, including M1 macrophage markers iNOS, TNF-α, and IL-6 as well as M2 macrophage markers fizz1, ym1, stat6, arginase1, and TGF-β1. Deletion of PAI-1 in ATII cells or inhibition of PAI-1 activity, on the other hand, attenuated the stimulatory effects of ATII SASP on macrophage gene expression. Together, the data suggest that ATII PAI-1 modulates SASP secretion and thus ATII SASP-

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mediated activation of alveolar macrophages. It should be stressed that, although M1 macrophages are believed to be mainly involved in inflammatory response, whereas M2 macrophages play an important role in tissue remodeling and fibrogenesis (29, 33, 36-39), numerous studies have shown that macrophages in fibrotic lungs express both pro-inflammatory and pro-fibrotic phenotypes and that M1 macrophages are also important in pulmonary fibrogenesis (33, 40-42). Importantly, the gene expression profile of alveolar macrophages reported here resembles that demonstrated by macrophages in lungs of mice with experimental fibrosis (33). In summary, our data suggest that senescent ATII cells promote lung fibrosis in part through secreting bioactive mediators, which, in turn, activate alveolar macrophages, and this process is regulated by ATII PAI-1.

Aging is a major risk factor for many chronic diseases including IPF and increased senescent cell burden is believed to contribute importantly to aging process and the pathogenesis of aging-related diseases. A new class of drugs called senolytics have been used recently in pre-clinic and clinical studies to specifically remove senescent cells for the treatment of aging-related diseases (10-13, 43, 44). Although premature, several studies have shown that targeting senescent ATII cells may be an attractive therapeutic option for the treatment of IPF (10-13). PAI-1 expression increases with age and in many aging-related diseases including IPF (17, 18, 45-49); PAI-1 plays a critical role in lung fibrogenesis (14, 16-18, 50). Most importantly, PAI-1 is not only a validated marker but also a critical mediator of cell senescence (18-24). Therefore, targeting PAI-1 may be an effective therapeutic option for the treatment of IPF as well as other aging-related diseases. Our previous studies have shown that inhibition of PAI-1 activity with a small molecule PAI-1 inhibitor TM5275 attenuated TGF-β1 induced lung fibrosis in mice (16) and reduced bleomycin-induced ATII cell senescence (18). In this study, we further show that inhibition of PAI-1 activity with TM5275 blocked TGF-β1-induced ATII cell senescence, the secretion of SASP, and ATII SASP-induced macrophage activation. These data support the

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notion that small molecule PAI-1 inhibitors may have therapeutic potential for the treatment of IPF. More studies are needed to further explore the therapeutic potential of these small molecule PAI-1 inhibitors for the treatment of aging-related diseases including IPF.

In summary, we showed in this study that TGF-β1-induced senescent ATII cells secret various pro-fibrotic mediators through a PAI-1-p16 dependent mechanism. ATII SASP further induces a pro-fibrotic phenotype in alveolar macrophages. These results suggest that PAI-1 promotes lung fibrosis at least in part by inducing ATII cell senescence and thus secretion of pro-fibrotic mediators and, consequently, activation of alveolar macrophages.

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Figure legend

Fig 1. TGF-β1 activated p16-pRb pathway and induced senescence as well as secretion of pro-fibrotic mediators in primary mouse ATII cells. Freshly isolated mouse ATII cells were treated with 2 ng/ml of TGF-β1 in serum-free medium for 48 hours and then incubated with TGF-β1-free and serum-free medium for additional 24 hours. **A&B)** SA-β-gal activity was revealed by X-gal staining. **C&D)** Western analyses of the proteins of interest in isolated ATII cells. **C**) Representative Western blots; **D**) Quantification of the band intensities, normalized by GAPDH **E**) Caspase3/7 activity in the conditional medium was measured with a kit from Promega. **F-K**) The amounts of different cytokines, chemokines, and growth factors in the conditional medium were assessed by ELISAs. *, Significantly different from untreated ATII cells ($p<0.05$, n=3-6).

Fig 2. Deletion of PAI-1 blocked TGF-β1-induced ATII cell senescence. ATII cells isolated from wild type (WT) and PAI-1 knockout (PAI-$1^{-/-}$) mice were treated with TGF-β1 as described in Fig 1. **A&B**) SA-β-gal activity was revealed by X-gal staining. **C)** Caspase3/7 activity in the conditional medium was measured with a kit from Promega. **D-J)** Western analyses of the proteins of interest in ATII cells. **D**) Representative Western blots; **E-I**) Quantification of the band intensities, normalized by β-actin (n=3-4).

Fig 3. Deletion of PAI-1 in ATII cells blocked TGF-β1-induced secretion of pro-fibrotic mediators. ATII cells isolated from WT and PAI-$1^{-/-}$ mice were treated with TGF-β1 and the conditional medium (CM) was collected at day 3 after TGF-β1 treatment as described in Fig 1. The amounts of IL-4, IL-6, IL-13 and PDGF in the CM were measured by ELISA (n=3-6).

Fig 4. Deletion of PAI-1 in ATII cells blunted the stimulatory effect of the conditional medium from senescent ATII cells on alveolar macrophage gene expression. Alveolar macrophages isolated from lungs of wild type mice were incubated with the CM from ATII cells

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isolated from wild type (WT) or PAI-1 deficient (PAI-1$^{-/-}$) mice and treated with saline or TGF-β1 for 24 hours. The amounts of mRNAs were assessed by quantitative real-time PCR as described in the Method section and the results are expressed as fold changes relative to the control. α, significantly different from AMs incubated with the CM from saline-treated WT ATII cells; β, significantly different from AMs incubated with the CM from TGF-β1-treated WT ATII cells ($p<0.05$, n=3-4).

Fig 5. Inhibition of PAI-1 activity protected rat alveolar epithelial (L2) cells from TGF-β1-induced senescence. L2 cells were treated with 2 ng/ml of TGF-β1 in serum-free medium containing vehicle or 25 μM TM5272 for 48 hours and then cultured in TGF-β1-free and TM5275-free medium for additional 24 hours. **A&B**) SA-β-gal activity was revealed by X-gal staining. **C** Caspase3/7 activity in the conditional medium measured with a kit from Promega. **D-I)** Western analyses of the proteins of interest in L2 cells. **D**) Representative Western blots; **E-I**) Quantification of the band intensities, normalized by GAPDH (n=3-4).

Fig 6. Inhibition of PAI-1 activity blocked TGF-β1-induced secretion of pro-fibrotic mediators from L2 cells. L2 cells were treated with TGF-β1 in the presence or absence of TM5275 as described in Fig 5. The amounts of SASP components in the CM were assessed by ELISAs (n=3-4).

Fig 7. Inhibition of PAI-1 activity attenuated the stimulatory effect of L2 cell SASP on macrophage gene expression. Rat macrophage NR8383 cells were incubated with the CM from L2 cells treated with saline or TGF-β1 in the presence or absence of TM5275 as described in Fig 5 for 24 hours. The expression levels of mRNAs were assessed by quantitative real-time PCR as described in the Method section and the results expressed as fold changes relative to NR8383 cells incubated with the CM from saline and solvent treated L2 cells. nd, not detected.

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α, Significantly different from NR8383 cells incubated with the CM from saline and solvent treated L2 cells; β, significantly different from NR8383 cells incubated with the CM from TGF-β1 plus solvent-treated L2 cells ($p<0.05$, n=3-4).

Fig 8. Silencing p16 attenuated PAI-1 protein-induced ATII cell senescence and secretion of pro-fibrotic mediators. Freshly isolated ATII cells were transfected with non-target (NT) or p16 siRNA and then treated with 1 μg/ml of active PAI-1 protein as described in the Method section. **A&B)** SA-β-gal activity was revealed by X-gal staining. **C-F)** Western analyses of p16, PAi-1, and pRb. **F-J**) The amounts of cytokines/chemokines/growth factor in the conditional medium were assessed by ELISA (n=3-6).

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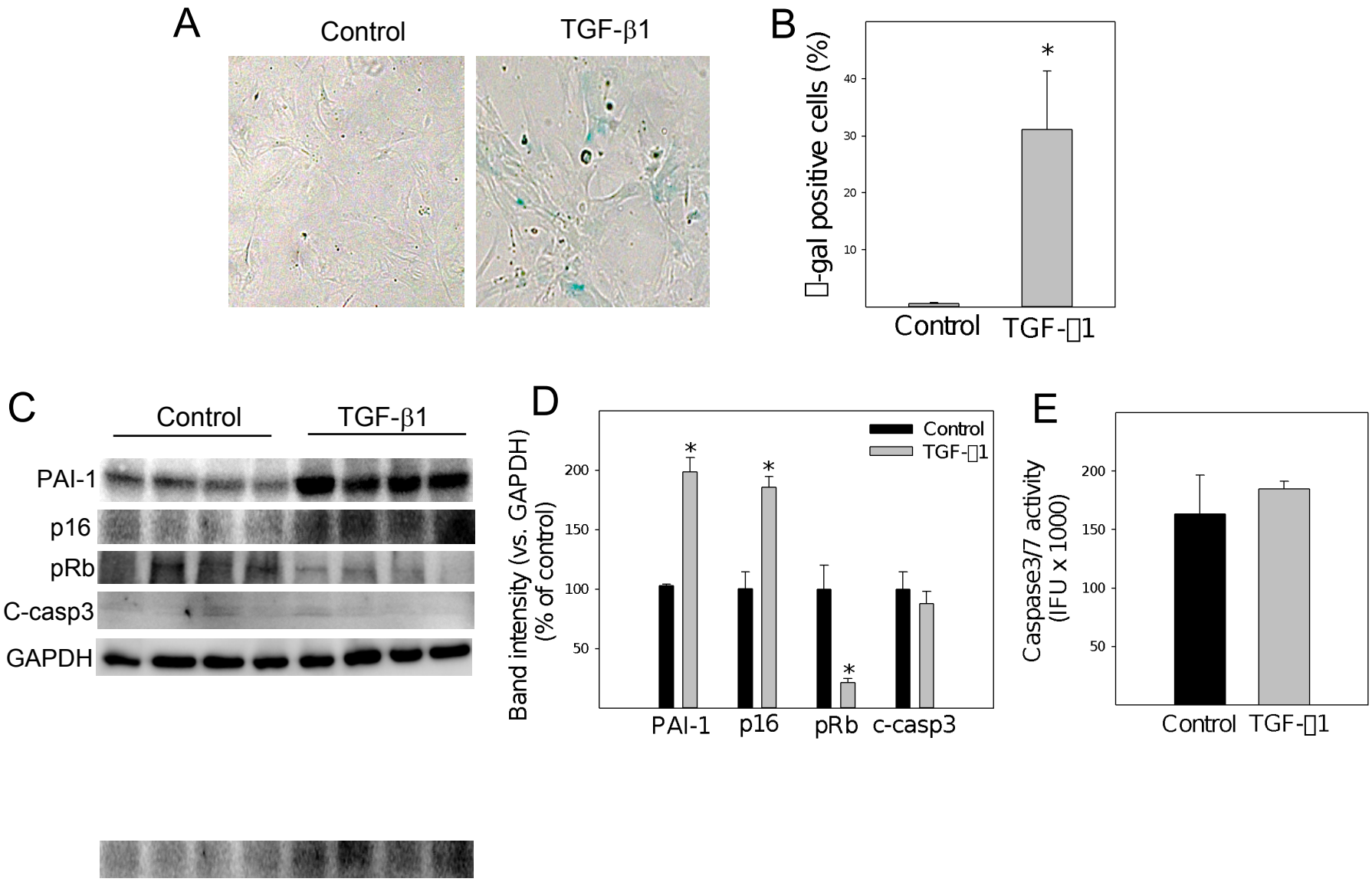

Fig 1

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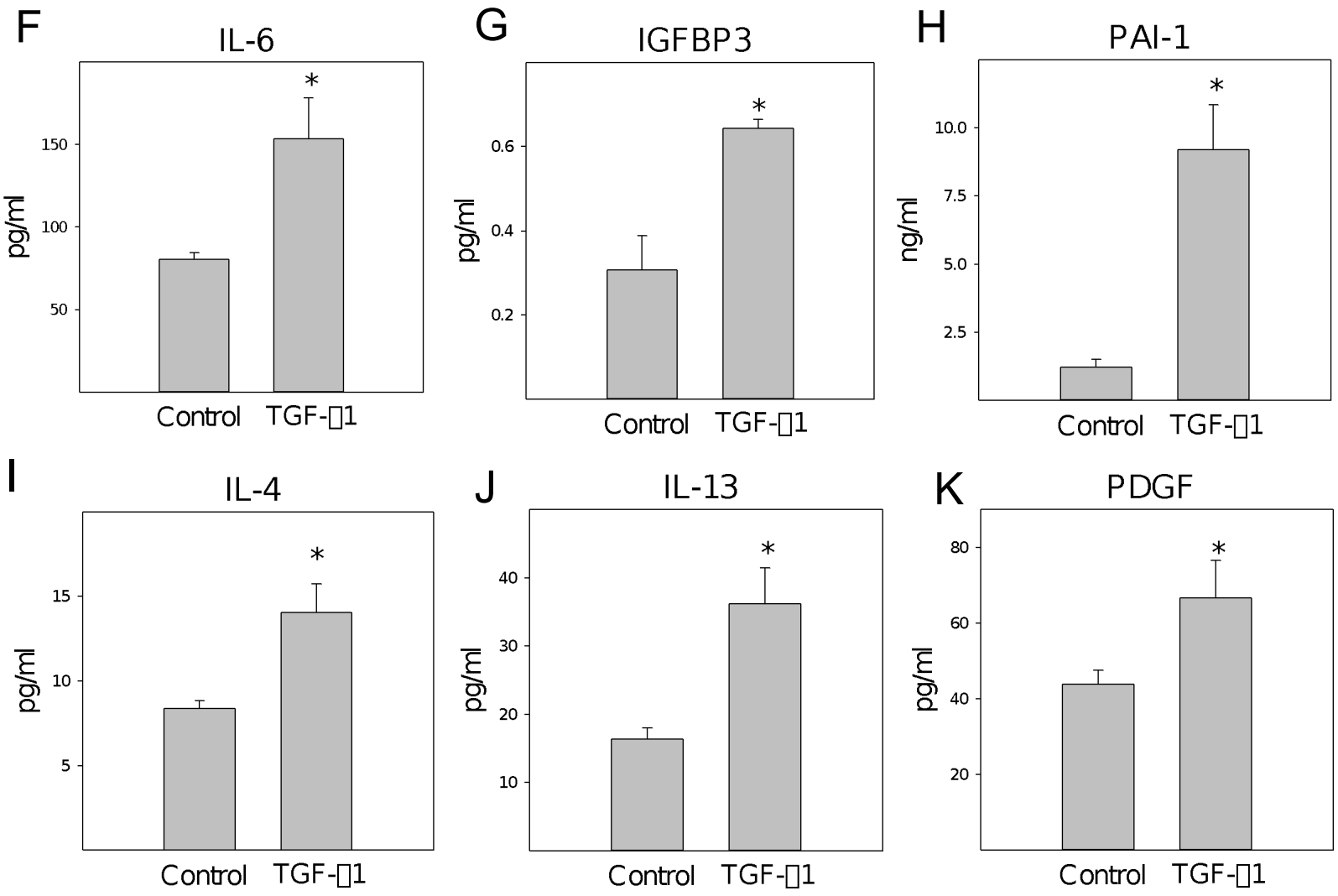

Fig 1

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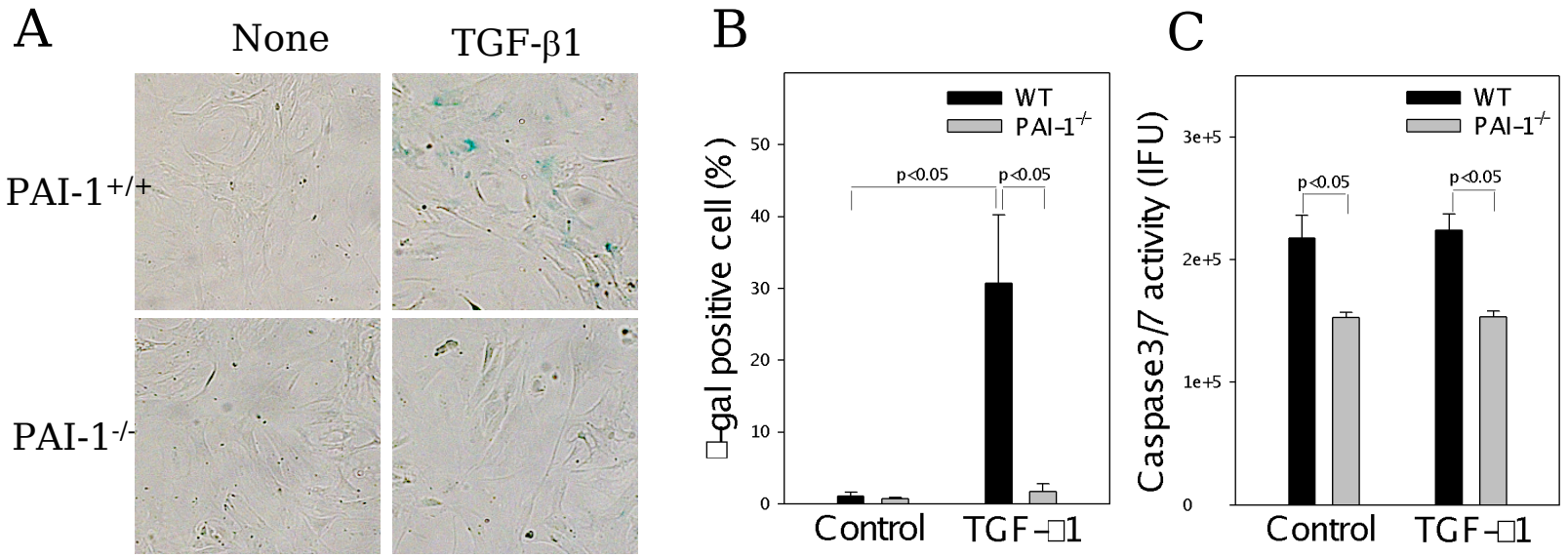

Fig 2

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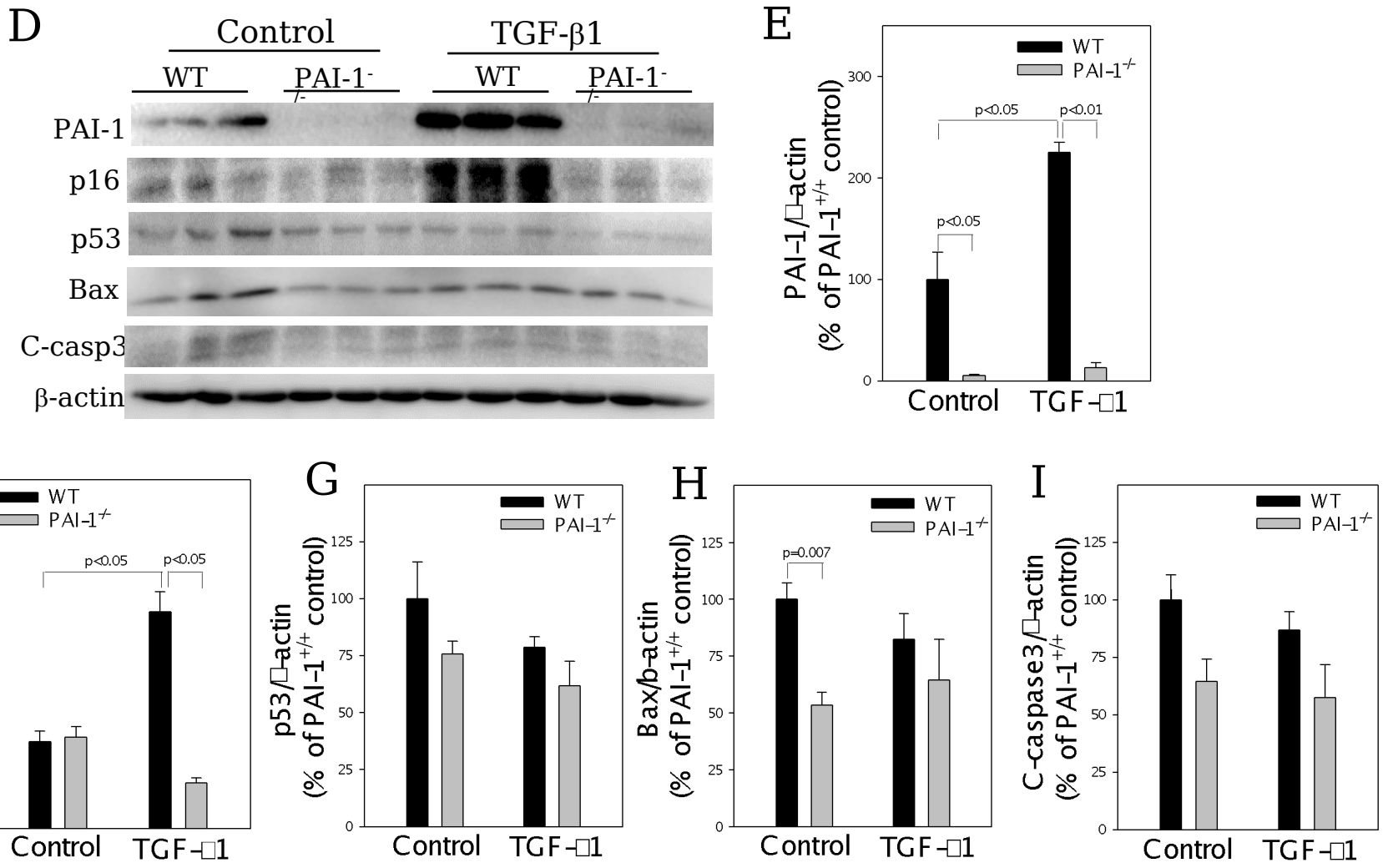

Fig 2

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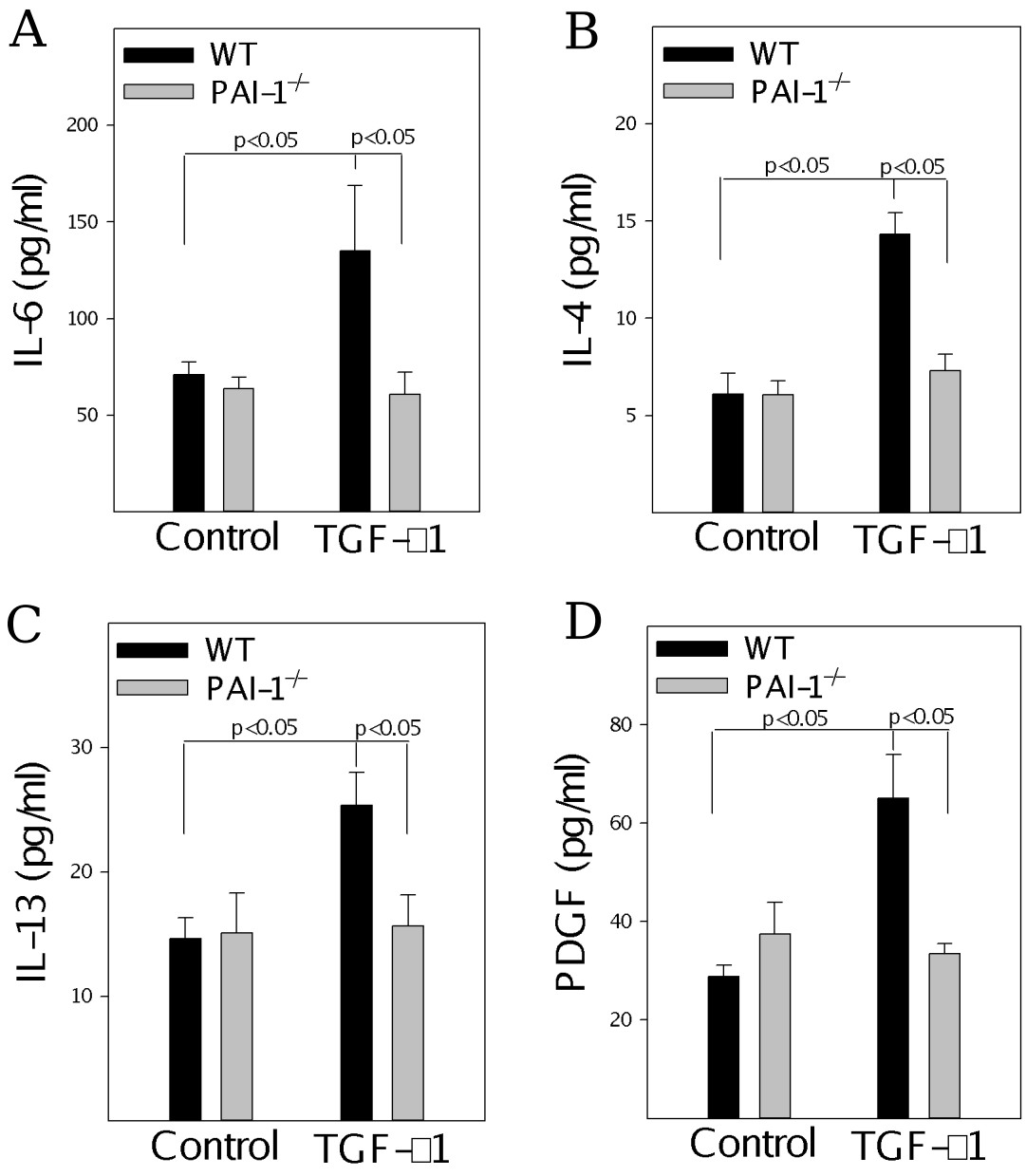

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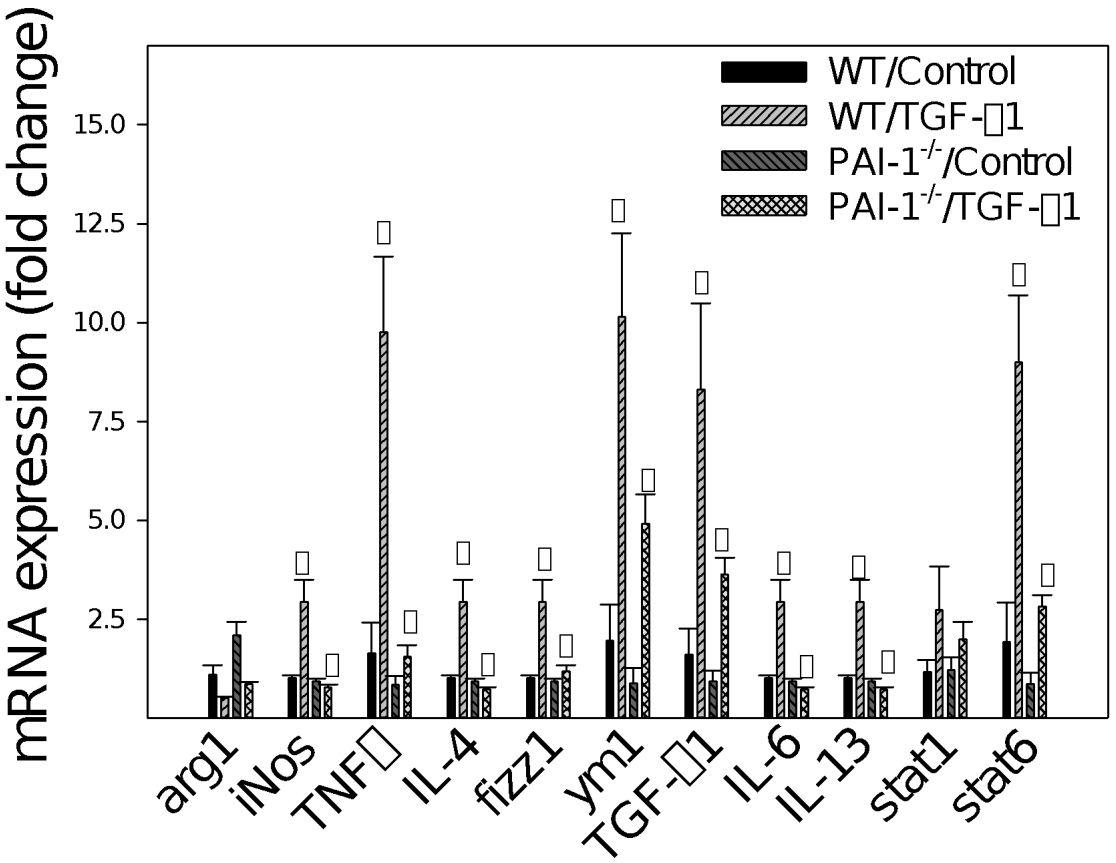

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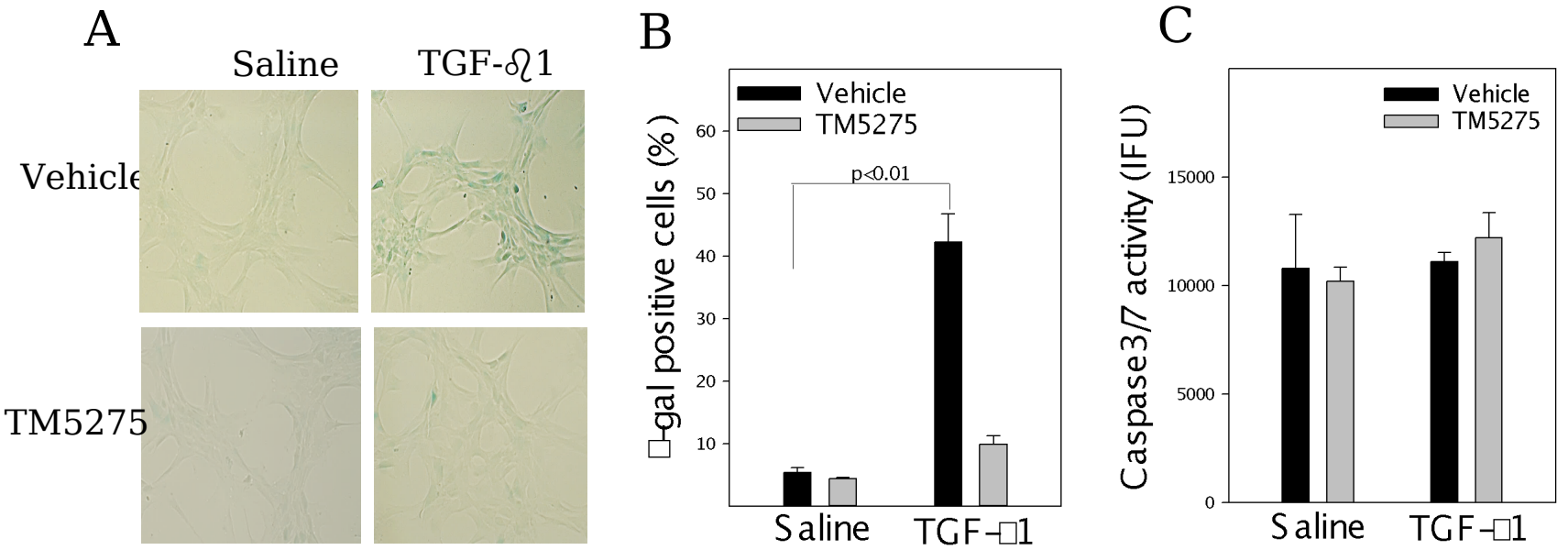

Fig 5

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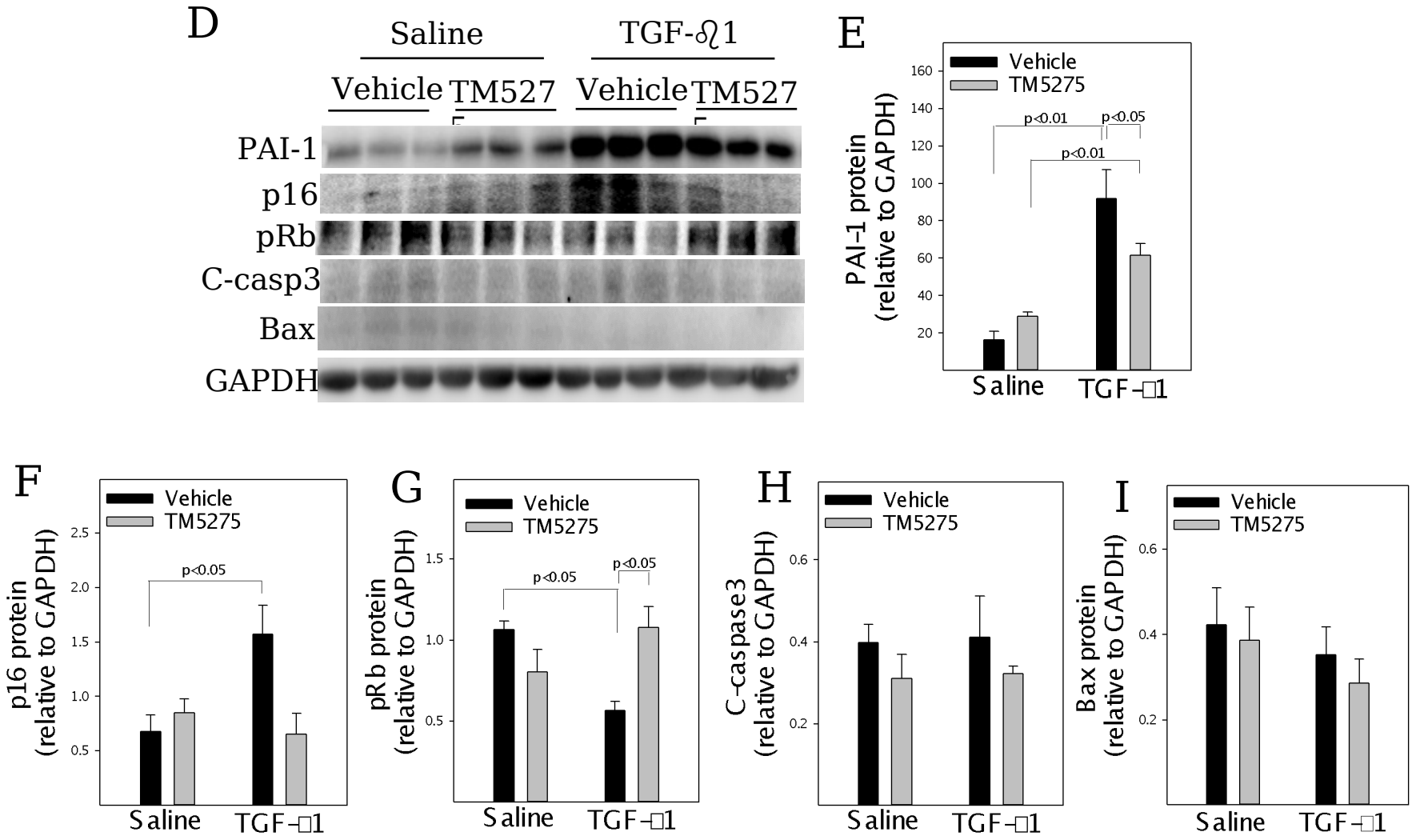

Fig 5

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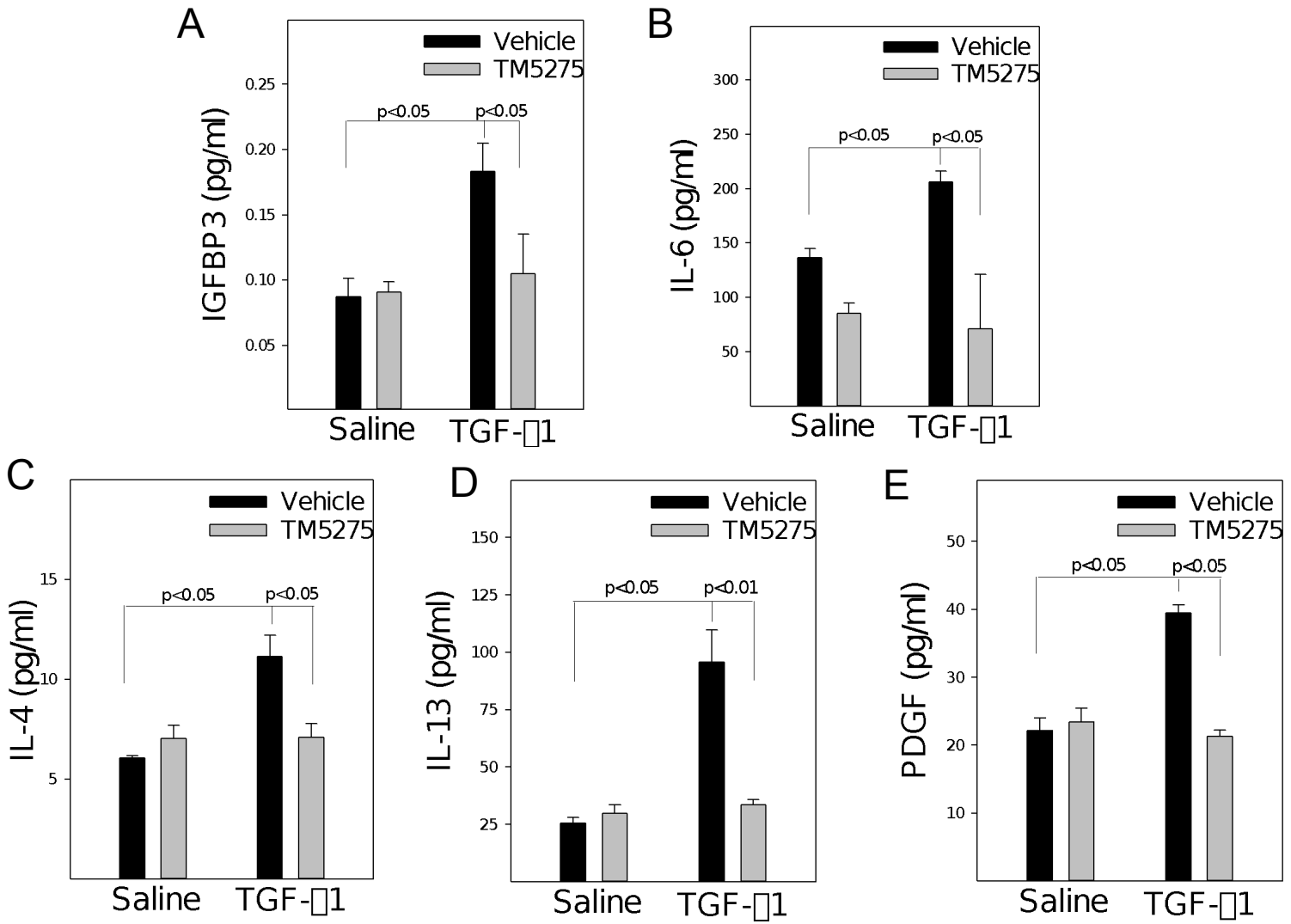

Fig 6

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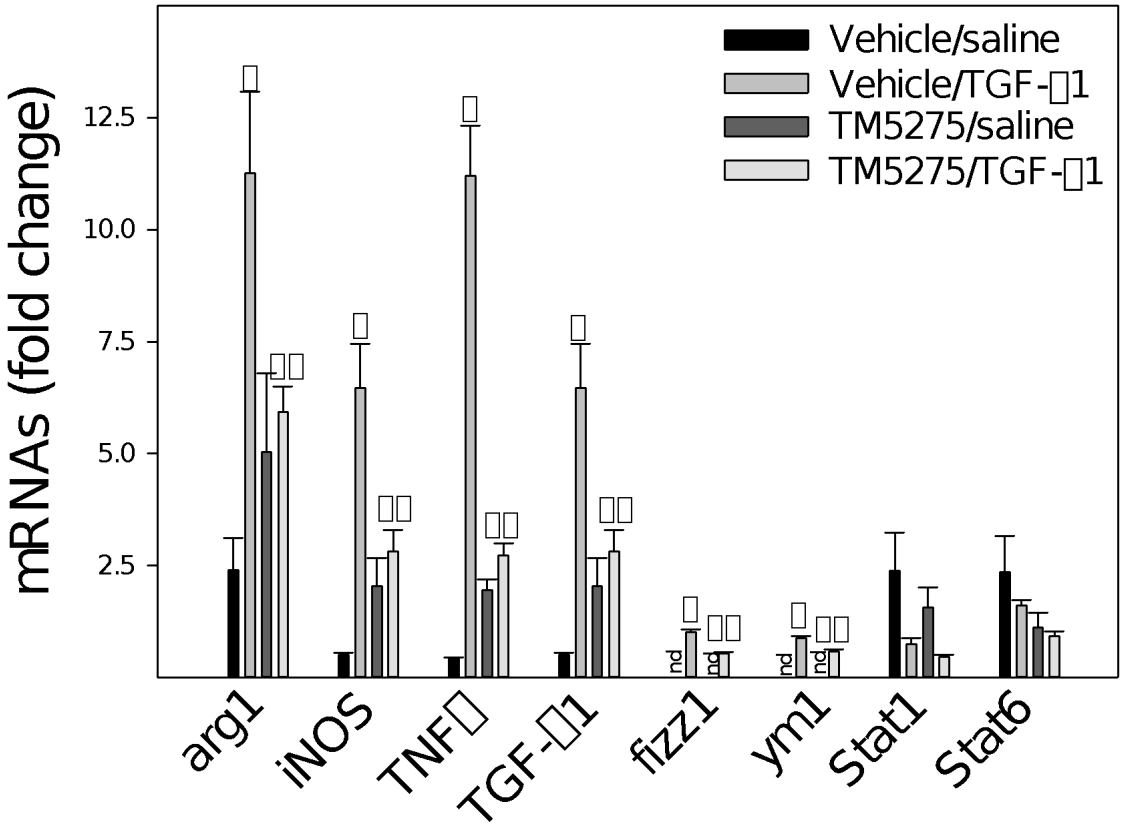

Fig 7

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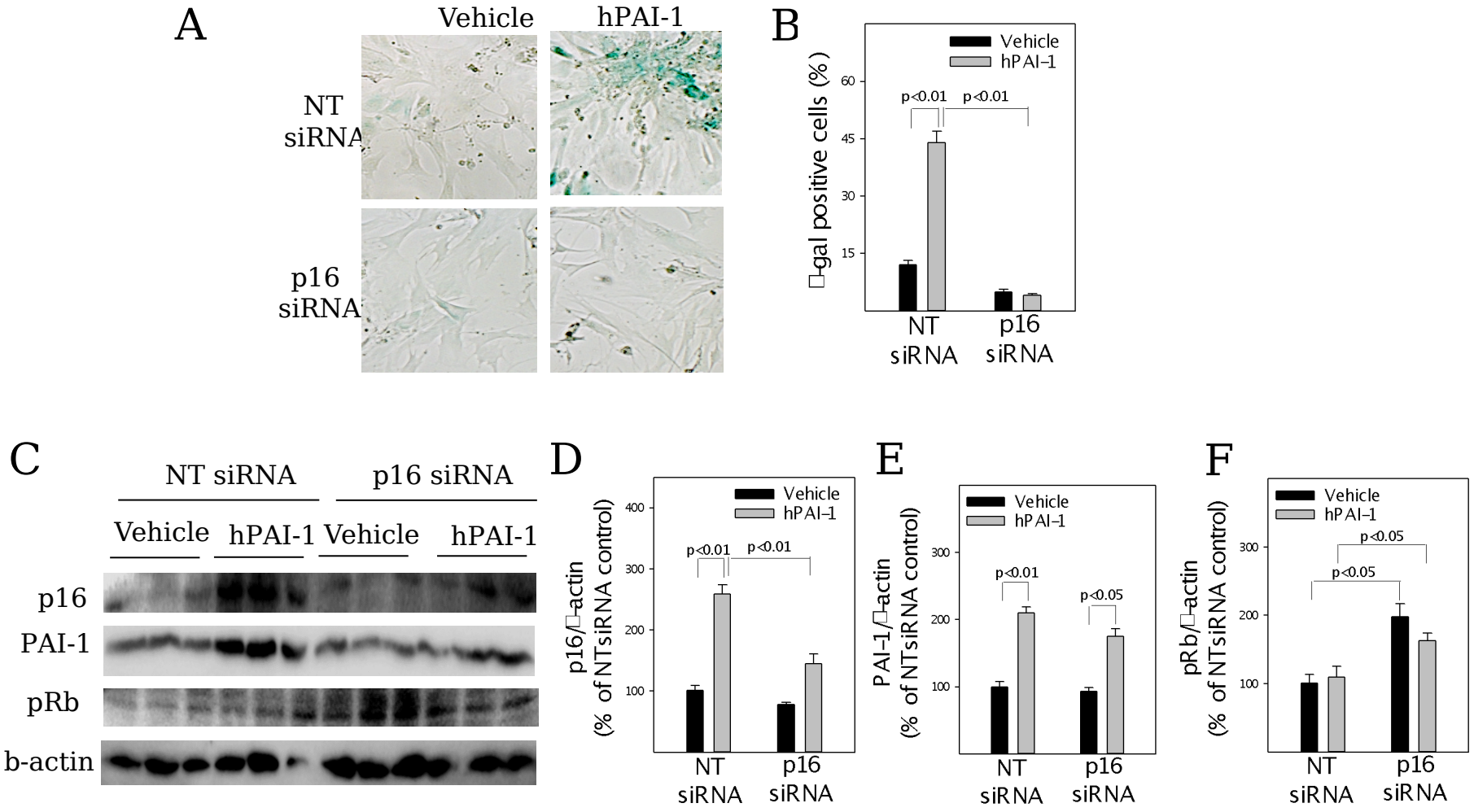

Fig 8

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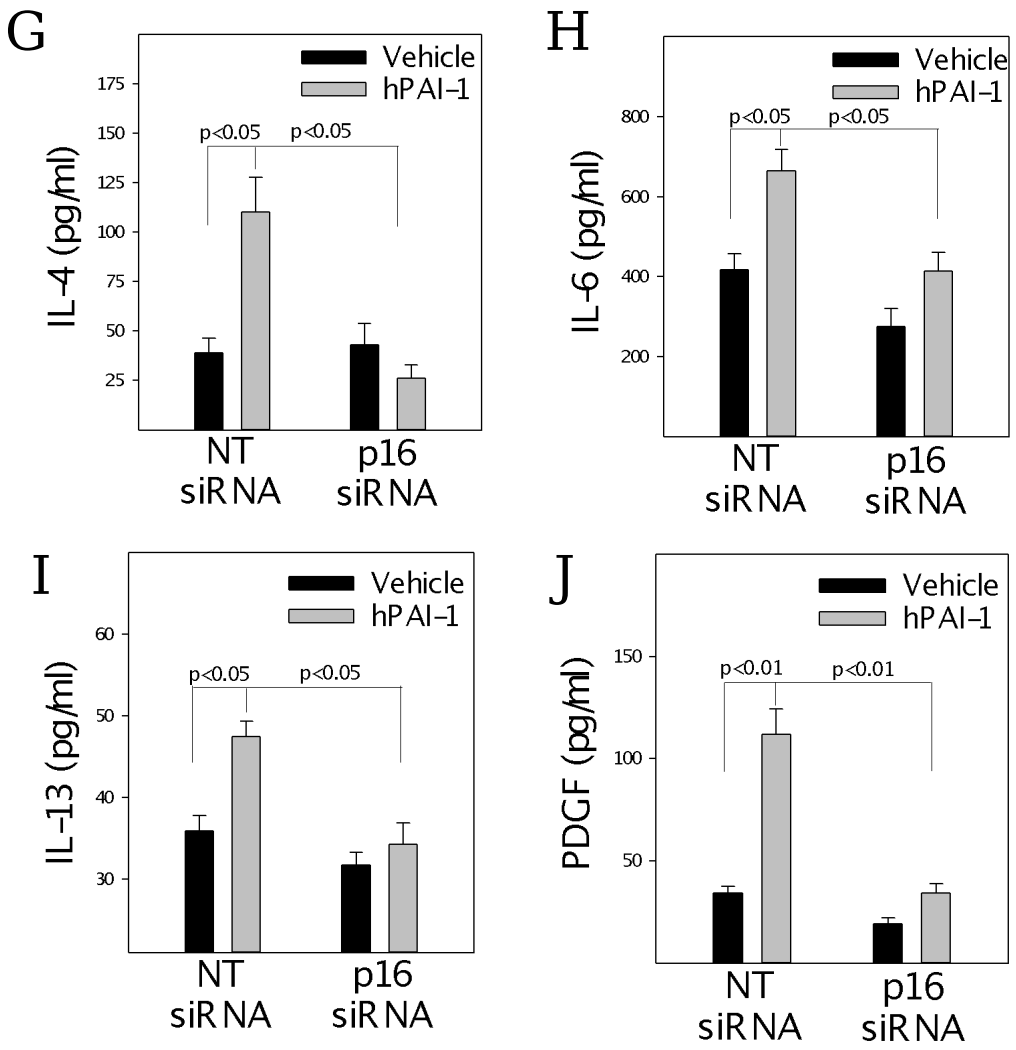

Fig 8

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Online Supplementary Data

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sTable 1. Primers for quantitative real-time PCR

Gene (mouse)	TaqMan Primer ID		Gene (rat)	TaqMan Primer ID
Arginase1	Mm00475988_m1		Arginase1	Rn00691090_m1
iNOS	Mm01309902_m1		iNOS	Rn00561646_m1
TNF-α	Mm00443258_m1		TNF-α	Rn99999017_m1
lL-4	Mm00445259_m1		TGF-β1	Rn00572010_m1
Fizz1	Mm00445109_m1		Fizz1/Retnla	Rn04219584_g1
Ym1	Mm00657889_mH		Ym1/chil3	Rn01523660_g1
TGF-β1	Mm01178820_m1		Stat1	Rn00583505_m1
IL-6	Mm00446190_m1		Stat6	Rn01505881_m1
IL-13	Mm00434204_m1		β-actin	Rn00667869_m1
Stat1	Mm01257286_m1			
Stat6	Mm01160477_m1			
GAPDH	Mm03302249_g1			

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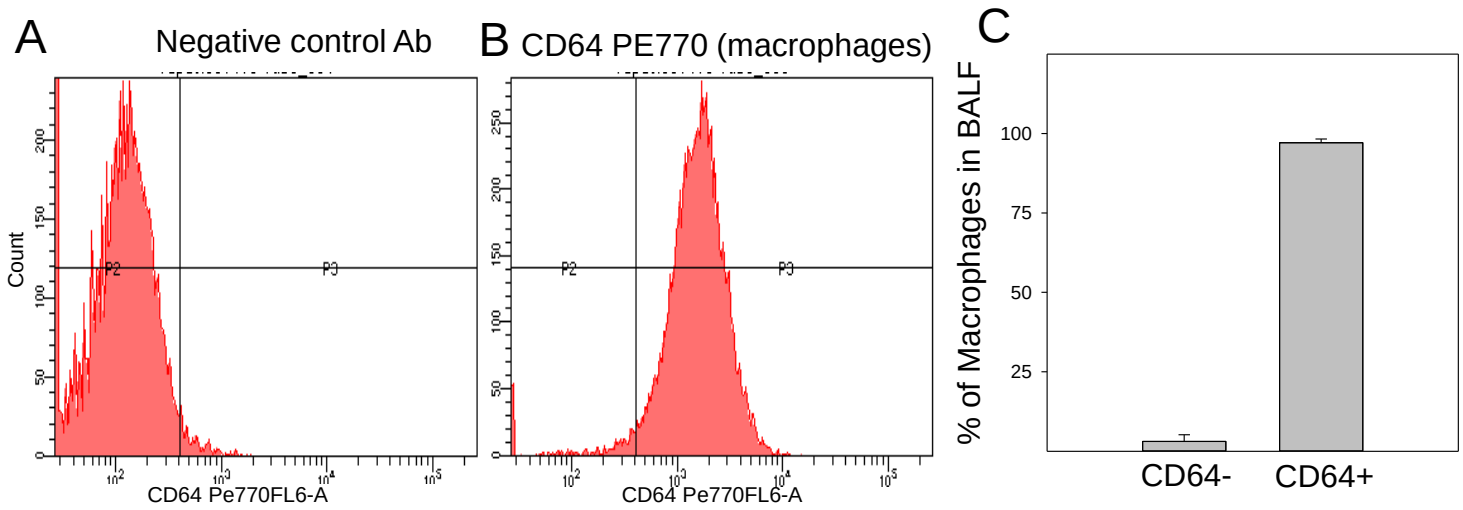

sFig 1. Flow cytometry analysis of isolated alveolar macrophages. Mouse alveolar macrophages isolated by bronchoalveolar lavage were immuno-stained with anti CD64 antibody (macrophage marker) or a negative isotope control antibody and analyzed by flow cytometry as described in the method section. The results were expressed as percentage of total cells (n=3).

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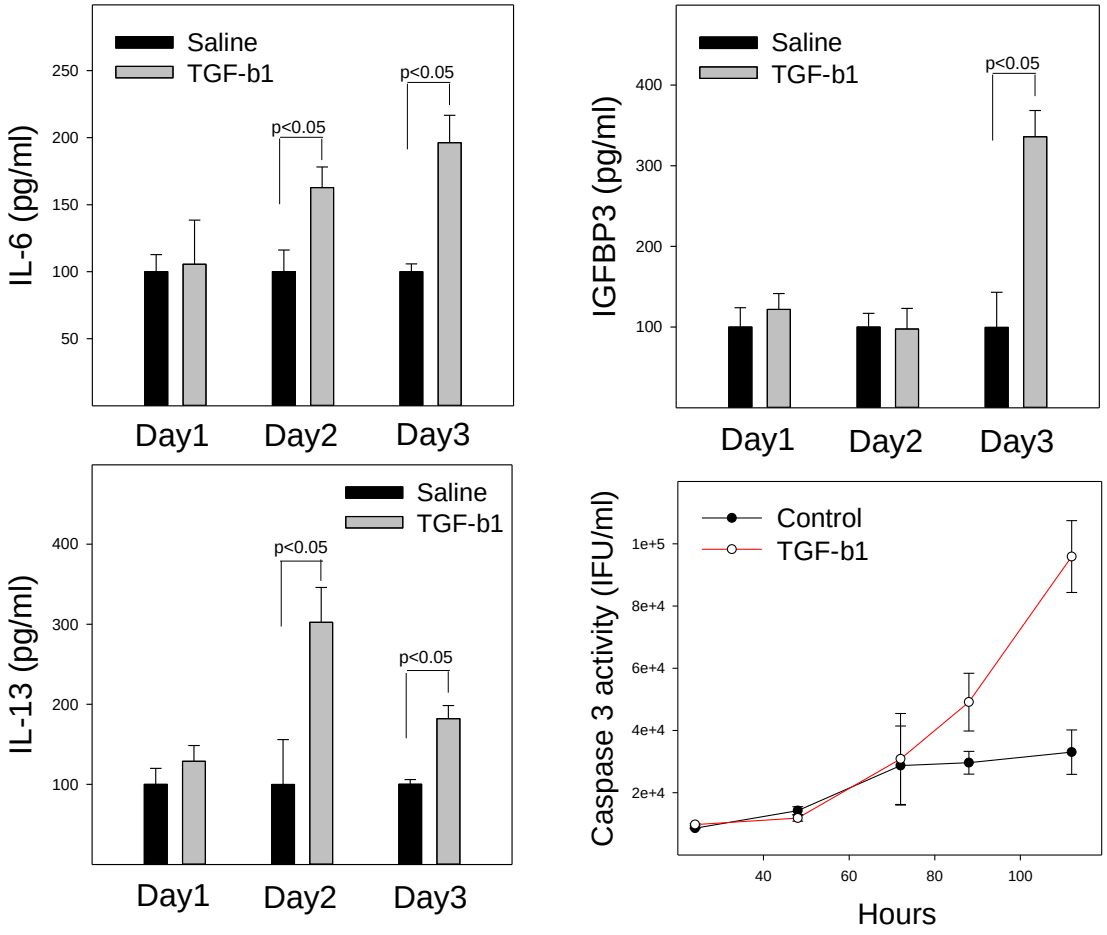

sFig 2. Time-dependent changes in the secretion of senescent and pro-fibrotic mediators as well as caspase 3/7 activity TGF-β1 treated ATII cells. Mouse ATII cells were treated with 2 ng/ml TGF-β1 in the serum-free medium and the conditional medium was collected at times indicated. The amounts of IL-6, IGFBP3, and IL-13 proteins as well as caspase3/7 activity in the medium were assessed by ELISA and a kit from Promega (n=3-6).

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