ORIGINAL RESEARCH

Discoidin Domain Receptor 2 Signaling Regulates Fibroblast Apoptosis through PDK1/Akt

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Abstract

Progressive fibrosis is a complication of many chronic diseases, and collectively, organ fibrosis is the leading cause of death in the United States. Fibrosis is characterized by accumulation of activated fibroblasts and excessive deposition of extracellular matrix proteins, especially type I collagen. Extensive research has supported a role for matrix signaling in propagating fibrosis, but type I collagen itself is often considered an end product of fibrosis rather than an important regulator of continued collagen deposition. Type I collagen can activate several cell surface receptors, including $\alpha_2\beta_1$ integrin and discoidin domain receptor 2 (DDR2). We have previously shown that mice deficient in type I collagen have reduced activation of DDR2 and reduced accumulation of activated myofibroblasts. In the present study, we found that DDR2-null mice are protected from fibrosis. Surprisingly, DDR2-null fibroblasts have a normal and possibly exaggerated activation response to transforming growth factor-β and do not have diminished proliferation compared with wild-type fibroblasts. DDR2-null fibroblasts are significantly more prone to apoptosis, *in vitro* and *in vivo*, than wild-type fibroblasts, supporting a paradigm in which fibroblast resistance to apoptosis is critical for progression of fibrosis. We have identified a novel molecular mechanism by which DDR2 can promote the activation of a PDK1 (3-phosphoinositide dependent protein kinase-1)/Akt survival pathway, and we have found that inhibition of PDK1 can augment fibroblast apoptosis. Furthermore, our studies demonstrate that DDR2 expression is heavily skewed to mesenchymal cells compared with epithelial cells and that idiopathic pulmonary fibrosis cells and tissue demonstrate increased activation of DDR2 and PDK1. Collectively, these findings identify a promising target for fibrosis therapy.

Keywords: fibrosis; collagen; apoptosis; discoidin domain receptor 2

Progressive fibrosis is a devastating consequence of a wide variety insults, affecting nearly every organ system and collectively accounting for over 45% of deaths in the developed world (1, 2). Fibrosis in the lung can result from an acute injury, chronic inflammation, or primary diseases such as idiopathic pulmonary fibrosis (IPF). Regardless of the cause, fibrosis can lead to a devastating clinical course owing to a lack of good therapeutic treatment (3–5).

Pulmonary fibrosis is characterized by accumulation of activated fibroblasts and deposition of fibrotic extracellular matrix (ECM) proteins (3, 4). Progressive fibrosis is believed to be the result of a dysregulated repair mechanism in response to injury or some other inciting event, which leads to excessive and continued deposition of fibrotic matrix proteins (1). Activated fibroblasts are believed to accumulate in the fibrotic tissue through increased proliferation, differentiation into the activated phenotype, and resistance to apoptosis. In addition to direct deposition of ECM proteins, especially type I collagen, activated fibroblasts secrete a number of profibrotic signaling ligands that lead to paracrine activation of neighboring cells (6).

Although primarily regarded as the scaffolding protein of fibrotic matrix, type I collagen can initiate cell signaling through activation of a number of cell surface receptors with significant influence on cell behavior (7–9). Type I collagen signaling has been shown to be upregulated in cancer progression, and inhibition of collagen I synthesis can lead to decreased tumor invasion. In the lung, type I collagen has

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been shown to be upregulated quickly after acute injury, both in human patients and in animal models, implicating a role for collagen I signaling as a regulator of progressive fibrosis rather than serving solely as the end product of fibrosis. We have previously shown that selective deletion of collagen I from alveolar epithelial cells surprisingly led to robust protection from fibrosis (10). We found that early production of collagen activated collagen receptors *in vivo*, which promoted further fibroblast activation, potentially establishing an important positive feedback loop in progressive fibrosis (10, 11). Thus, type I collagen is both an important physical component of the fibrotic matrix and a potential profibrotic signaling molecule.

Collagens can signal through several different families of cell surface receptors, including several integrins, especially $\alpha_2\beta_1$ integrin, and the discoidin domain receptors (DDRs) (7, 8, 12). The DDRs are receptor tyrosine kinases that are activated upon binding to collagens. DDR1 primarily binds type IV collagen and has already been implicated in mediating the fibrotic response (13). Fibrosis is characterized by increased production of fibrillar collagens, especially type I collagen, but a role for type I collagen signaling during progressive fibrosis has not been well characterized. DDR2 is the primary receptor for fibrillar collagens, including type I and type III collagen (7, 8, 12). There is increased recognition of an important role of DDR2 in a variety of malignancies, leading to interest in generating DDR2-specific inhibitors (14). The role of DDR2 in fibrosis is mixed. Mice deficient in DDR2 have an increased propensity for liver fibrosis (15), but knockdown of DDR2 reduced angiotensin-induced cardiac fibrosis (16). Authors of a recent report found attenuated lung fibrosis in mice deficient in DDR2; however, the molecular mechanisms involved in DDR2 regulation of fibrosis remain poorly explored (17). Most of the research has focused on DDR2-mediated fibroblast activation. Notably, nintedanib, one of the approved therapies for IPF, is a nonspecific tyrosine kinase inhibitor with known activity against DDR2 (18). Several other tyrosine kinase inhibitors that are known to block DDR2 have also been shown to attenuate experimental fibrosis (19).

In the present study, we explored the mechanisms by which collagen signaling through DDR2 regulates fibroblast behavior during lung fibrosis. We found that DDR2 is a major regulator of fibroblast apoptosis through activation of a 3-phosphoinositide dependent protein kinase-1 (PDK1)/Akt pathway. In addition, we found that DDR2 expression is skewed to a mesenchymal population and that IPF fibroblasts demonstrate upregulation of this pathway, offering a potentially attractive target for fibrosis therapy (20, 21).

Methods

Reagents

Phosphorylated DDR2 (phospho-DDR2; Y740) antibody and $\alpha_2\beta_1$ integrin inhibitor TC-I15 were purchased from R&D Systems. Phospho-tyrosine 9-PDK1, DDR2, and integrin-α_2 antibodies were obtained from Abcam. Collagen, type I, alpha 1 (Col1a1) antibody was purchased from Thermo Fisher Scientific. Hydroxyproline reagent and cycloheximide (CHX), as well as α-smooth muscle actin (ACTA2), SMA-FITC, β-actin, and FLAG M2 antibodies, were obtained from Sigma-Aldrich. AKT, phospho-AKT (Ser473), phospho-AKT (Thr308), extracellular signal-regulated kinase 1/2 (ERK1/2), phospho-ERK1/2 (T202/Y204), Src, phospho-Src, XIAP (X-linked inhibitor of apoptosis protein), GAPDH, phospho-SMAD3, phospho-Tyr, and phospho-Tyr-horseradish peroxidase antibodies were purchased from Cell Signaling Technology. SMAD2/3 primary antibody and horseradish peroxidase–conjugated secondary antibodies were obtained from Santa Cruz Biotechnology. Phospho-tyrosine 376-PDK1 antibody was purchased from Signalway Antibody. Phospho-SMAD2 antibody, phosphatase inhibitor cocktail, and bleomycin were purchased from MilliporeSigma. [^{3}H]thymidine was purchased from GE Healthcare PerkinElmer. Collagen I and antimouse CD95 were obtained from BD Biosciences. Protein A– and protein G–conjugated agarose beads and the *In Situ* Cell Death Detection Kit (TMR red) were obtained from Roche. Caspase-Glo 3/7 reagent was purchased from Promega. IncuCyte Caspase-3/7 Apoptosis Assay Reagent was obtained from Essen BioScience. PDK1 inhibitors BX-795 and BX-912 were purchased from Selleckchem. Phosphatase inhibitor cocktail and bleomycin were purchased from MilliporeSigma. FLAG-tagged PDK1 cloned into a retroviral expression vector was purchased from Addgene.

Mice

DDR2-null mice (slie/slie) were previously described (22) and were purchased from The Jackson Laboratory. Floxed Col1a1 mice were previously described (10). Six- to eight-week-old wild-type (WT) and DDR2-null mice were injured with 40 μl of 2 U/kg of bleomycin dissolved in saline versus saline alone by oropharyngeal aspiration as previously described (23). Mice were killed at the specified time points after treatment in each respective experiment and analyzed as described. All mice were maintained in a specific pathogen–free environment until the time of mice were killed. All animal experiments were approved by the animal care and use committee at the University of Michigan.

BAL and Lung Myeloid Cell Isolation

For BAL, mice were killed and lavaged with 1 ml of PBS. Total cells were quantified by counting with a hemocytometer. For cell differential calculation, cells were fixed, cytofuged onto glass slides, stained with hematoxylin and eosin, and visualized by light microscopy. Lung myeloid cells were isolated as previously described (24, 25). Briefly, lungs were removed from killed mice, minced, and digested with collagenase and dispase. Erythrocytes were lysed, and the myeloid cells were isolated by filtration and centrifugation in Percoll.

Hydroxyproline Assay

Whole-lung hydroxyproline was measured as previously described (26, 27). Mice were killed at the specified time points in each experiment. Whole lung was harvested, homogenized in water, and baked in 12N hydrochloric acid at 120°C overnight. Samples were then neutralized with citrate buffer and incubated in chloramine-T solution at room temperature. Ehrlich's solution was added before incubation at 65°C. The absorbance at 540 nm was measured, and the hydroxyproline concentration was quantified against a standard.

Histology

Lung section trichrome staining was previously described (10). Briefly, whole lungs harvested from killed mice were fixed by inflation with formaldehyde to a

pressure of 25 cm H_2O. Lungs were then embedded in paraffin. Sections were stained with Masson's trichrome by the McClinchey Histology Lab.

Immunofluorescence Staining

Lungs were perfused with PBS, inflated and embedded in optimal cutting temperature compound, and frozen in a dry ice and alcohol bath. Ten-micrometer lung sections were then stained as previously described (10). Briefly, lung sections were fixed and permeabilized with methanol and blocked with 5% goat serum and 1% BSA in PBS. The slides were stained with primary antibody overnight at 4°C. After washing, slides were stained with appropriate secondary antibody and for TUNEL by using the In Situ Cell Death Detection Kit (TMR red). Slides were mounted in Molecular Probes ProLong Gold mountant containing DAPI (Thermo Fisher Scientific). Lung sections were visualized on an Olympus BX-51 fluorescence microscope, and images were captured with an Olympus DP-70 camera and analyzed with DP controller software version 3.1.1.267 (Olympus). Quantification of costained cells was completed in a blinded fashion.

Mouse and Human Lung Fibroblast Isolation and Culture

Primary murine lung fibroblasts were isolated and cultured as previously described (10). Ten- to 24-week-old mice were killed, and whole lungs were removed after perfusion and lavage with PBS. Whole-lung samples were finely minced and incubated in Dulbecco's modified Eagle medium with 10% FBS, penicillin, and streptomycin until fibroblasts adhered to the plate. Human lung fibroblasts were isolated from IPF and normal patients as previously described (28) and cultured in Dulbecco's modified Eagle medium with 10% FBS, penicillin, streptomycin, and amphotericin B. All cells were cultured in a 37°C incubator supplemented with 5% CO_2.

For cell expression experiments, equal numbers of cells were seeded on tissue culture plates in serum-containing media and allowed to adhere overnight. Cells were rinsed and serum starved for 24 hours. Cells were then treated with transforming growth factor (TGF)-β (4 ng/ml) or vehicle control for the time points indicated. Some cells were treated with TC-I15 (2.5 μM) versus vehicle control for 1 hour, followed by treatment with TGF-β.

Retroviral and Lentiviral Infection of Cells

Murine DDR2 was cloned into a retroviral expression vector (pWZL-blast) by PCR and standard cloning techniques. Retroviral supernatants were generated using Phoenix-E cells as previously described (29). Briefly, 1 day after seeding, primary WT or DDR2-null murine lung fibroblasts were treated with retroviral supernatants to overexpress DDR2 or FLAG-PDK1 (vs. vehicle control) as indicated. After 2 days, the media were replaced, and cells were treated and analyzed as indicated. For DDR2 shRNA, lentivirus was generated by the University of Michigan Vector Core using RNA interference vectors purchased from Open BioSystems. One day after seeding, primary WT murine fibroblasts were treated with DDR2 shRNA lentivirus (5 plaque-forming units per cell) or scrambled control lentivirus. After 2 days, the media were replaced, and cells were treated and analyzed as described previously (30).

IB and Immunoprecipitation

IB and immunoprecipitation of samples were performed as previously described (10). Immunoblots are representative of a minimum of three separate experiments.

Cell Proliferation Assays

Cellular proliferation was measured by the [^{3}H]thymidine incorporation method as previously described (30). Briefly, equal numbers of stimulated cells were incubated with the addition of 5 μl of 0.1 mCi/ml [^{3}H]thymidine per well in a 96-well plate overnight. Sixteen hours after addition of [^{3}H]thymidine, cells were harvested for incorporated [^{3}H]thymidine using scintillation fluid in a β-scintillation counter.

Cell Death Assays

Equal numbers of WT and DDR2-null cells were seeded on 24-well plates in serum-containing media. The next day, the cells were washed and covered with serum-free media. After an additional 24 hours, cells were treated with vehicle control or Fas-activating antibody (0.5 μg/ml) and CHX (5 μg/ml) to induce apoptosis. Some cells were pretreated for 1 hour with PDK1 inhibitor BX-795 (1 nM) or BX-912 (2 nM) before addition of Fas-activating antibody or CHX. After the time points indicated, cell death was assessed by caspase 3/7 activity using the Caspase-Glo 3/7 Assay (Promega) per the product protocol and as previously described (23). Briefly, treated cells were washed with PBS and lysed in radioimmunoprecipitation assay buffer. Active caspase 3/7 activity was captured using a Veritas Microplate Luminometer (Promega), normalized to protein concentration per sample, and expressed as fold differences in relative luminescence. Cell death by caspase 3/7 activity was also captured using the IncuCyte Live Cell Analysis System by incubating treated cells with IncuCyte Caspase-3/7 Apoptosis Assay Reagent. At least four images were captured per well in a 96-well plate. Discrete fluorescent apoptotic cells were counted using the IncuCyte basic analyzer and expressed as fold differences in relative numbers over time.

Gene Expression Analysis

qRT-PCR was performed as previously described (10). Briefly, RNA was isolated from lung tissue and cells with TRIzol reagent (Life Technologies) per the manufacturer's protocol. Reverse transcription was performed with the SuperScript III First-Strand Synthesis Kit (Life Technologies), and RT-PCR was performed using the *Power* SYBR Green PCR Master Mix Kit (Life Technologies) and the ABI PRISM 7000 Sequence Detection System (Life Technologies). Relative expression of genes was normalized to β-actin and GAPDH as previously described (30) using the following primers: α-actin forward: 5′-CCGTGAAAAGATGACCCAGATC-3′, β-actin reverse: 5′-CACAGCCTGGATGG CTACGT-3′; GAPDH forward: 5′-AACTT TGGCATTGTGGAAGG-3′, GAPDH reverse: 5′-ACACATTGGGGGTAGGAAC A-3′; COL1A1 forward: 5′-GCCAAGAAG ACAAACTTT-3′, COL1A1 reverse: 5′-GGCCTTGGAAACCTTGTGGAC-3′; COL1A2 forward: 5′-GGAGGGAACGGT CCACGAT-3′, COL1A2 reverse: 5′-GAGT CCGCGTATCCACAA-3′; COL3A1 forward: 5′-CAAGGTCTTCCTGGTCAGC CT-3′, COL3A1 reverse: 5′-TGCCACGAG ATCCATCTC-3′; ACTA2 forward: 5′-AGAGACTCTCTTCCAGCCATC-3′, ACTA2 reverse: 5′-GACGTTGTTAGCA TAGAGATC-3′; TGFβ1 forward:

5′-ATCCTGTCCAAACTAAGGCTCG-3′, TGFβ1 reverse: 5′-ACCTCTT TAGCATAGTAGTCCGC-3′; IL1β forward: 5′-TGTTCTTTGAAGTTG ACGGAC-3′, IL1β reverse: 5′-GATACTG CCTGCCTGAAGCTC-3′; CCL2 forward: 5-TTCTGGGCCTGCTGTTCACAG-3′, CCL2 reverse: 5′-CTACTCATTGGGATC ATCTTGC-3′; CCL7 forward: 5′-TGCTTTCAGCATCCAAGTGTG-3′, CCL7 reverse: 5′-GGACACCGACTACTG GTGATC-3′; CCL12 forward: 5′-TAGCTA CCACCATCAGTCCTC-3′, CCL12 reverse; 5′-GGGACACTGGCTGCTTGTG AT3′; TNFα forward: 5′-CCAAAGGGAT GAG AAGTTCCC-3′, TNFα reverse: 5′-GCTCCTCCACTTGGTGGTTTG-3′.

Statistical Analysis

Data are expressed as means, and error bars indicate the SEM. For evaluation of group differences, the two-tailed Student's *t* test was used. A *P* value less than 0.05 was accepted as significant.

Results

Mice Deficient in DDR2 Are Protected from Fibrosis

We have previously shown that mice deficient in type I collagen expression have decreased fibrosis and decreased numbers of active fibroblasts, suggesting an important role for collagen signaling in propagating progressive fibrosis (10). We first examined the relative expression of two of the best-described collagen I receptors, $\alpha_2\beta_1$ and DDR2, in alveolar epithelial cells and fibroblasts (Figure 1A). DDR2 was expressed almost exclusively on fibroblasts as compared with alveolar epithelial cells, consistent with prior reports (7, 8, 18). Integrin $\alpha_2\beta_1$ was expressed in both cell types, but in higher amounts in fibroblasts. We have previously shown increased activation and total concentrations of DDR2 in mouse lungs after bleomycin-induced injury (10). We therefore focused on DDR2 as a potential mediator of collagen-induced fibroblast activation. To determine if DDR2 regulated the fibrotic response, DDR2-null and WT mice were injured with bleomycin. After 21 days, DDR2 mice had no increase in fibrosis as determined in trichrome-stained lung sections or by hydroxyproline assay, indicating that collagen signaling through the DDR2 receptor regulates subsequent collagen deposition and fibrosis (Figure 1; *see also* Figure E1 in the data supplement). Notably, DDR2 is not highly expressed on myeloid cells, and the absence of DDR2 did not affect myeloid cell recruitment or expression of several inflammatory and profibrotic cytokines and chemokines (Figure E2).

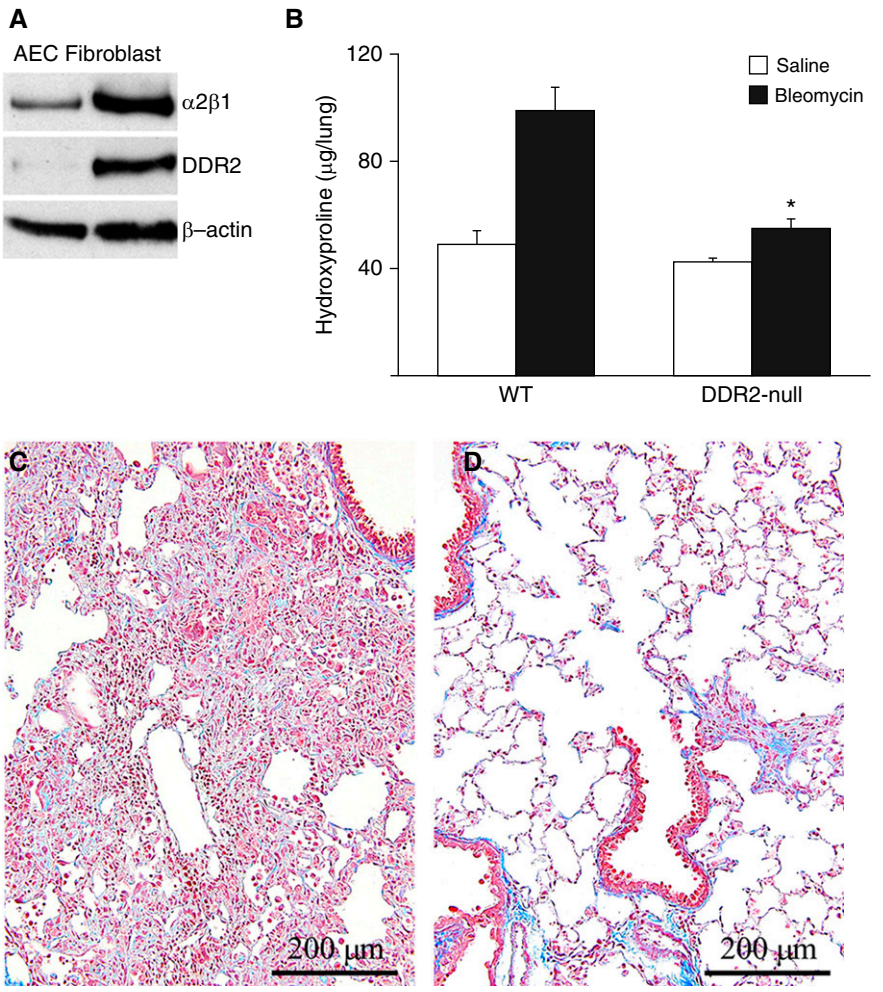

Figure 1. Discoidin domain receptor 2 (DDR2) regulates lung fibrosis. (*A*) Amounts of collagen type I receptors DDR2 and α2 integrin in primary murine alveolar epithelial cells (AECs) and primary murine lung fibroblasts were determined by IB. (*B*) Hydroxyproline assay of total lungs 21 days after intratracheal saline or bleomycin in DDR2-null or littermate control mice. DDR2-null mice have less fibrosis after bleomycin than control mice treated with bleomycin ($n = 6$–10 per group). *$P < 0.05$. (*C* and *D*) Trichrome stain of wild-type (WT) (*C*) and DDR2-null (*D*) lung sections (20 ×) 21 days after injury with intratracheal bleomycin. DDR2-null mice are protected from bleomycin-induced fibrosis compared with control. Scale bars: 200 μm.

DDR2 Does Not Regulates Fibroblast Activation or Proliferation

We have previously shown that fibroblasts with deletion of Col1a1 have reduced expression of fibroblast activation markers, including Col1a2, Col3a1, and ACTA2 (10). DDR2 is highly expressed on fibroblasts that accumulate during fibrosis and are believed to be the primary fibrotic effector cells. To determine if DDR2 regulates fibroblast activation, WT and DDR2-null primary lung fibroblasts were isolated and treated with TGF-β. After 24 hours, cells were analyzed by qPCR and IB for several well-described markers of fibroblast activation, including type I collagen and ACTA2. Contrary to our expectations, DDR2-null fibroblasts did not have decreased concentrations of fibroblast activation markers and indeed showed a trend toward increased concentrations of ACTA2 and collagen I (Figures 2A and 2B). We also found no differences in TGF-β–induced activation of Smad2 and Smad3 (Figure 2C).

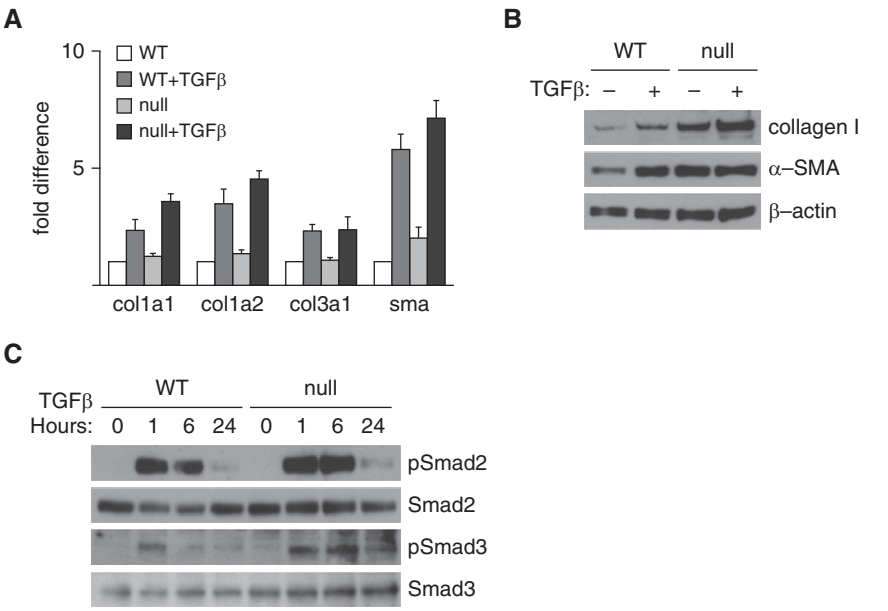

Figure 2. DDR2 does not regulate lung fibroblast activation. (*A*) Real-time qPCR of primary lung fibroblasts from control (WT) and DDR2-null (null) mice with or without treatment with transforming growth factor (TGF)-β. Loss of DDR2 does not significantly alter expression of collagen, type I, alpha 1 (Col1a1), Col1a2, Col3a1, and ACTA2 (sma) ($n = 4$). (*B*) IB for collagen I and ACTA2 (α-SMA) of primary lung fibroblasts from WT and null mice. Presence of DDR2 did not influence fibroblast production of collagen I and α-SMA. (*C*) IB of primary lung fibroblasts from WT and null mice after treatments with TGF-β at 0, 1, 6, and 24 hours. Phospho-Smad2 (pSmad2), Smad2, phospho-Smad3 (pSmad3), and Smad3 are shown. The presence of DDR2 does not influence Smad phosphorylation.

Fibroblast proliferation has been implicated in the progression of fibrosis. We cultured WT and DDR2-null fibroblasts in serum-free or 10% serum-containing conditions and measured proliferation with the incorporation of [^{3}H]thymidine. Again, contrary to our expectations, the presence of DDR2 did not influence fibroblast proliferation (Figure 4A).

DDR2 Regulates Fibroblast Apoptosis

Fibroblast resistance to apoptosis has emerged as a critical regulator of fibrosis. WT and DDR2-null fibroblasts were treated with CHX and a Fas-activating antibody (or vehicle control) to induce robust apoptosis. To track the induction of apoptosis over time, WT and DDR2-null fibroblasts were cultured with a reagent that fluoresces when cleaved by caspase 3/7. Cells were imaged over time using the IncuCyte system, and numbers of fluorescent cells were quantified over time. Greater induction of apoptosis in the DDR2-null fibroblasts was apparent within 20 hours after addition of Fas/CHX (Figure 4B). To confirm increased activation of caspase 3/7 in DDR2-null fibroblasts, WT and DDR2-null cells were treated with Fas/CHX as before, then lysed after 24 hours. Caspase 3/7 activity was

To determine if collagen I/DDR2 signaling regulates expression of the other major fibroblast fibrillar collagen receptor, fibroblasts from floxed type I collagen mice treated with adenovirus expressing Cre recombinase or control adenovirus were analyzed for expression of α2 integrin. Indeed, fibroblasts deficient in Col1a1 had markedly increased expression of α2 integrin (Figure 3A). Similarly, DDR2-null fibroblasts had increased expression of α2 integrin, suggesting that collagen I/DDR2 signaling negatively regulates expression of α2 integrin and that higher expression of α2 integrin might regulate the increased expression of profibrotic markers (Figure 3B). WT fibroblasts were treated with TGF-β1 with or without pretreatment with an α2 integrin–specific inhibitor, TC-I15 (2.5 μM). Indeed, fibroblasts treated with TC-I15 had reduced expression of type I collagen and ACTA2 (Figure 3C). These results demonstrate one aspect by which type I collagen signaling regulates accumulation of activated fibroblasts, but it does not clarify the mechanism by which DDR2-null mice are protected from fibrosis.

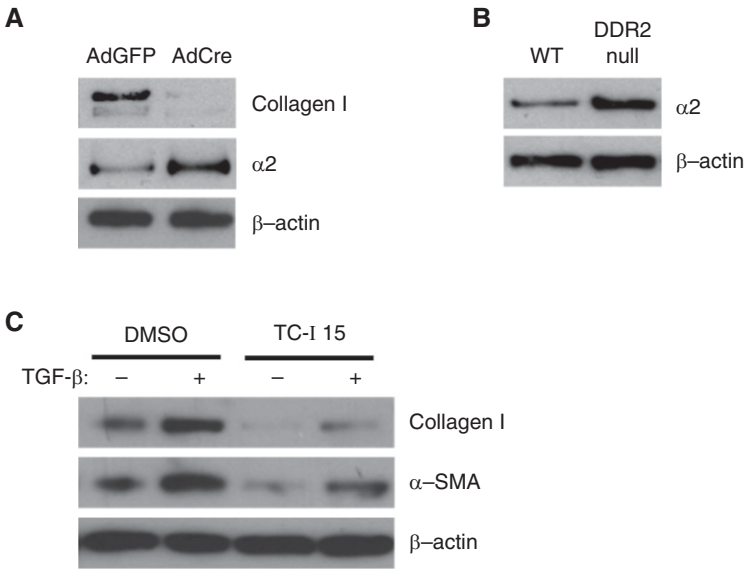

Figure 3. Collagen I/DDR2 signaling regulates expression of α2 integrin. (*A*) Lung fibroblasts from floxed Col1a1 were treated with an adenovirus expressing Cre recombinase (AdCre) or a control adenovirus expressing GFP (AdGFP). After 1 week, cells were analyzed from expression of collagen I and α2 integrin by IB. (*B*) Primary lung fibroblasts from WT and DDR2-null mice were analyzed from expression of α2 integrin by IB. (*C*) WT primary lung fibroblasts were treated with or without TGF-β and an α2 integrin inhibitor, TC-I15, and analyzed for expression of collagen I and ACTA2 (α-SMA) by IB.

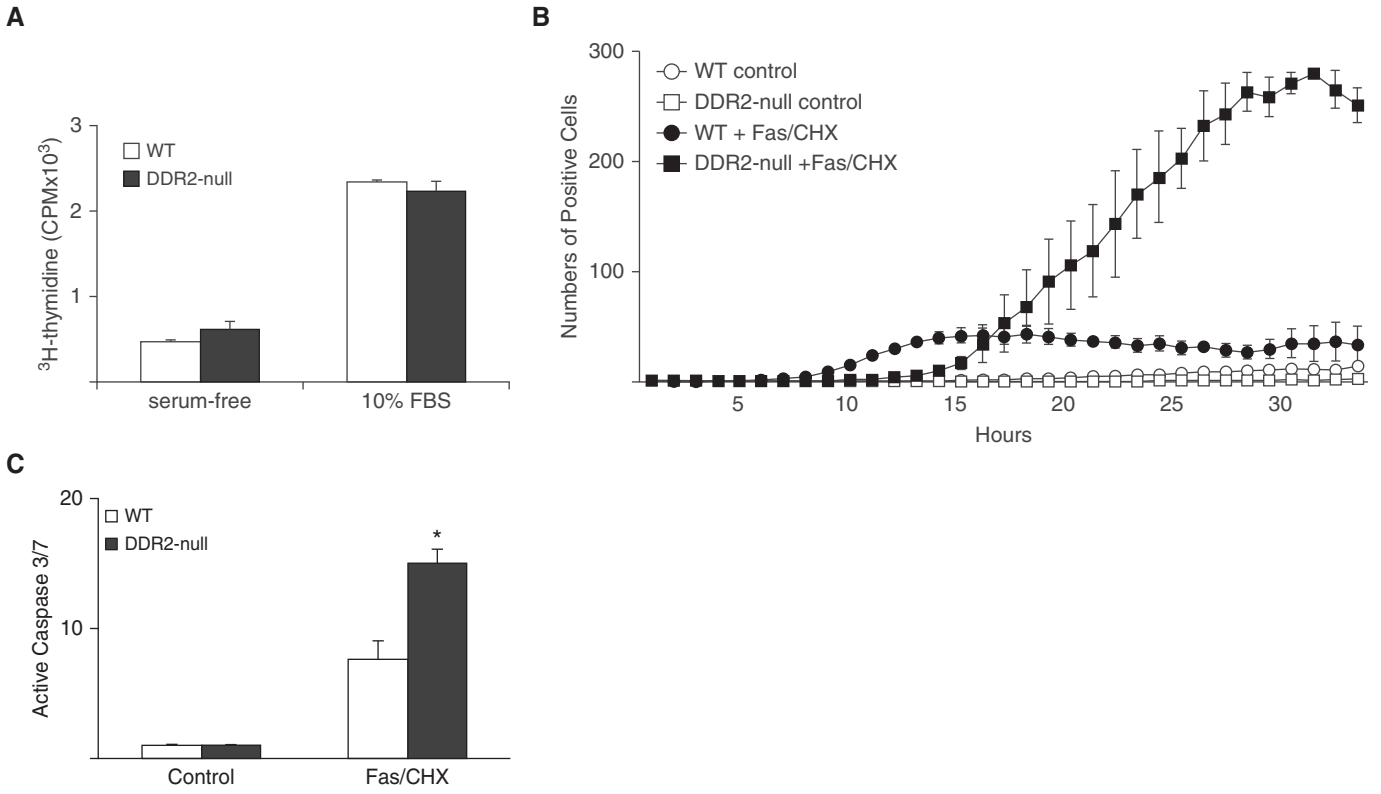

Figure 4. DDR2 regulates fibroblast apoptosis but not proliferation. (*A*) Cellular proliferation assessed by [3H]thymidine incorporation for 16 hours in WT and DDR2-null primary lung fibroblasts under serum-free or 10% serum-containing media measured by counts per minute (CPM) in a scintillation counter. DDR2 does not regulate lung fibroblast proliferation ($n = 4$). (*B*) Activation of caspase 3/7 using a fluorescent reagent and the IncuCyte System was trended over time in WT or DDR2-null primary lung fibroblasts cultured with and without a Fas-activating antibody and cycloheximide (Fas/CHX). Values are expressed as numbers of fluorescent cells per image field ($n = 4$). (*C*) Activation of caspase 3/7 in WT or DDR2-null primary lung fibroblasts upon induction of apoptosis with Fas/CHX was measured by using the Caspase-Glo luciferase-based assay 24 hours after treatment with Fas/CHX. Values are expressed as fold differences in relative luminescence units ($n = 5$). *$P < 0.01$ compared with WT fibroblasts.

measured in cell lysates using a luciferase-based Caspase-Glo system. As expected, DDR2-null fibroblasts demonstrated a more robust activation of caspase 3/7 than WT fibroblasts.

To determine if DDR2 contributes to fibroblast survival *in vivo*, WT and DDR2-null mice were treated with bleomycin. After 14 days, lung sections were stained for TUNEL and counterstained with ACTA2 to mark the active myofibroblasts. Consistent with the *in vitro* data, the numbers of TUNEL-positive myofibroblasts in bleomycin-treated DDR2-null lungs was significantly greater than bleomycin-injured WT lungs (Figure 5).

DDR2 Regulates Fibroblast Apoptosis through a PDK1/Akt Pathway

We next interrogated several well-described pathways involved in fibroblast survival that might predispose DDR2-null cells to greater apoptosis. Some of these pathways, most notably Src signaling, have been linked to DDR2 activation as well as fibroblast survival (19). However, we found no differences between WT and DDR2-null fibroblasts in concentrations of total or phospho-Src. We recently reported that XIAP is a major regulator of fibroblast survival in the context of lung fibrosis (31, 32), but again we found no differences between WT and DDR2-null fibroblasts (Figure 6A). Erk and Akt signaling are among the best-studied survival pathways. WT and DDR2 fibroblasts had no differences in Erk phosphorylation. Akt phosphorylation at serine 473 (Ser473) and threonine 308 (Thr308) lead to Akt activation and increased survival in several cell types. We found no difference in pSer473-Akt; however, DDR2-null fibroblasts had marked reduction in pThr308-Akt, suggesting that decreased Akt activation may be linked to the observed increased apoptosis (Figure 6B).

PDK1 is the best-described kinase in phosphorylating Akt at Thr308, and PDK1 activity is known to be regulated by tyrosine phosphorylation (33). DDR2 has not previously been described to regulate PDK activity. To determine if the presence of DDR2 regulates tyrosine phosphorylation of PDK1, we used a FLAG-tagged PDK1 vector. WT and DDR2-null fibroblasts were treated with retrovirus-encoding control vector or FLAG-PDK1. FLAG-PDK1 was immunoprecipitated, and the amounts of tyrosine phosphorylation were determined by IB for phospho-tyrosine. DDR2-null fibroblasts exhibited markedly decreased concentrations of phospho-tyrosine PDK1 (Figure 7A). We next obtained antibodies specific for PDK1 phosphorylation at PDK1's two major regulatory tyrosine residues, tyrosine 9 (Tyr9) and tyrosine 376 (Tyr376). Consistent with the FLAG-PDK1 overexpression experiment, IB of WT and DDR2-null fibroblasts demonstrated reduced numbers of pTyr9-PDK1 in DDR2 fibroblasts compared with WT fibroblasts (Figure 7B).

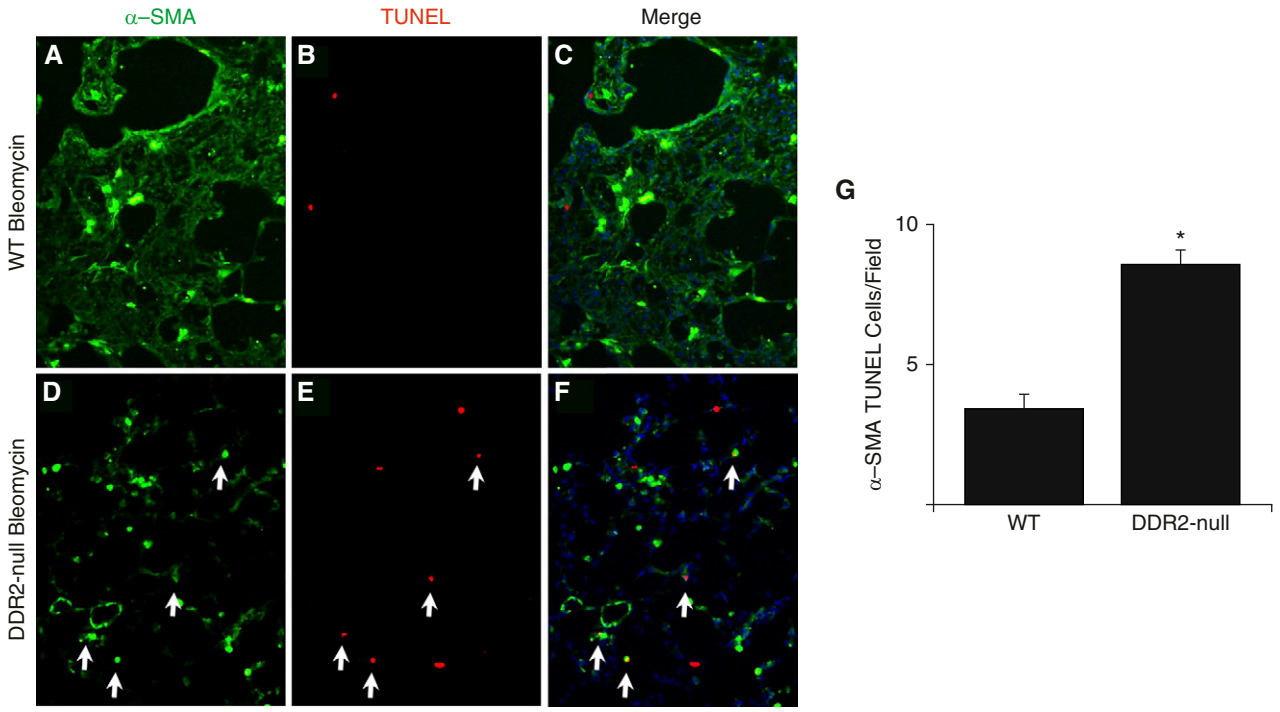

Figure 5. DDR2 regulates fibroblast apoptosis *in vivo*. Fourteen days after bleomycin injury, lung sections from WT (*A–C*) and DDR2-null (*D–F*) mice were stained for ACTA2 (α-SMA; green, *A* and *D*) and TUNEL (red; *B* and *E*), and images were merged (*C* and *F*). Compared with WT lungs, the DDR2-null lung sections had more TUNEL-positive myofibroblasts (*G*) ($n = 4$). *$P < 0.05$ compared with WT. Several cells co-positive for ACTA2 and TUNEL are indicated (arrows).

To our knowledge, PDK1 has not previously been reported to be involved in fibroblast survival. To confirm that PDK1-mediated Akt phosphorylation at Thr308 regulates fibroblast apoptosis, WT lung fibroblasts were treated with two different PDK1 inhibitors. PDK1 inhibition demonstrated rapid and sustained reduction in pThr308-Akt compared with fibroblasts treated with vehicle-alone control (Figure 7C). Consistent with DDR2 regulation of Akt phosphorylation, compared with DDR2-null fibroblasts treated with control retrovirus, WT lung fibroblasts in which expression of DDR2 was knocked down by shRNA had reduced amounts of pThr308-Akt (Figure 7D), and DDR2-null lung fibroblasts with retrovirus-mediated overexpression of DDR2 had increased amounts of pThr308-Akt (Figure 7E). Next, WT lung fibroblasts were treated with PDK1 inhibitors or vehicle control and stimulated with Fas/CHX to induce apoptosis. As before, Fas/CHX induced robust activation of caspase 3/7; however, the amount of caspase activation was much greater in the cells treated with the PDK1 inhibitors (Figure 7F).

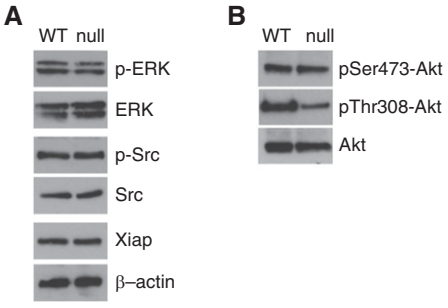

Figure 6. DDR2 regulates fibroblast Akt pathway. (*A*) IB for phospho-extracellular signal-regulated kinase (p-ERK), ERK, phospho-Src (p-Src), Src, and XIAP (X-linked inhibitor of apoptosis protein) in primary lung fibroblasts isolated from DDR2-null (null) and littermate control (WT) mice. (*B*) IB for phospho-Ser473-Akt (pSer473-Akt), phospho-Thr308-Akt (pThr308-Akt), and total Akt in primary lung fibroblasts isolated from null and WT mice.

Human Fibroblasts Have Increased Activation of DDR2 and PDK

IPF lungs are characterized in part by the accumulation of fibroblasts and type I collagen. Because DDR2 is known to be highly expressed by fibroblasts and phosphorylated in response to type I collagen, we confirmed that IPF lungs demonstrate increased concentrations of DDR2 and pDDR2 (Figure 8A). Compared with normal human lung fibroblasts, IPF fibroblasts also have greater collagen production and are resistant to apoptosis. To assess whether IPF fibroblasts exhibit greater activation of DDR2 and PDK1, we used primary human lung fibroblasts isolated from IPF lungs or normal human lungs. Consistent with the murine data, IPF fibroblasts exhibited increased concentrations of p-DDR2 and pTyr9-PDK1 (Figure 8B). Finally, frozen optimal cutting temperature compound–embedded lung tissue from IPF and normal human lungs were stained for pTyr9-PDK1 and ACTA2. As expected, IPF lungs demonstrated increased staining for pTyr9-PDK1, primarily within ACTA2-positive myofibroblasts (Figure 8). Collectively, these results suggest that collagen I signaling mediated through pDDR2/pTyr9-PDK1/pThr308-Akt contributes to increased fibroblast survival in progressive lung fibrosis.

Discussion

Our results support a model in which fibroblast resistance to apoptosis is a major driver of sustained and progressive fibrosis

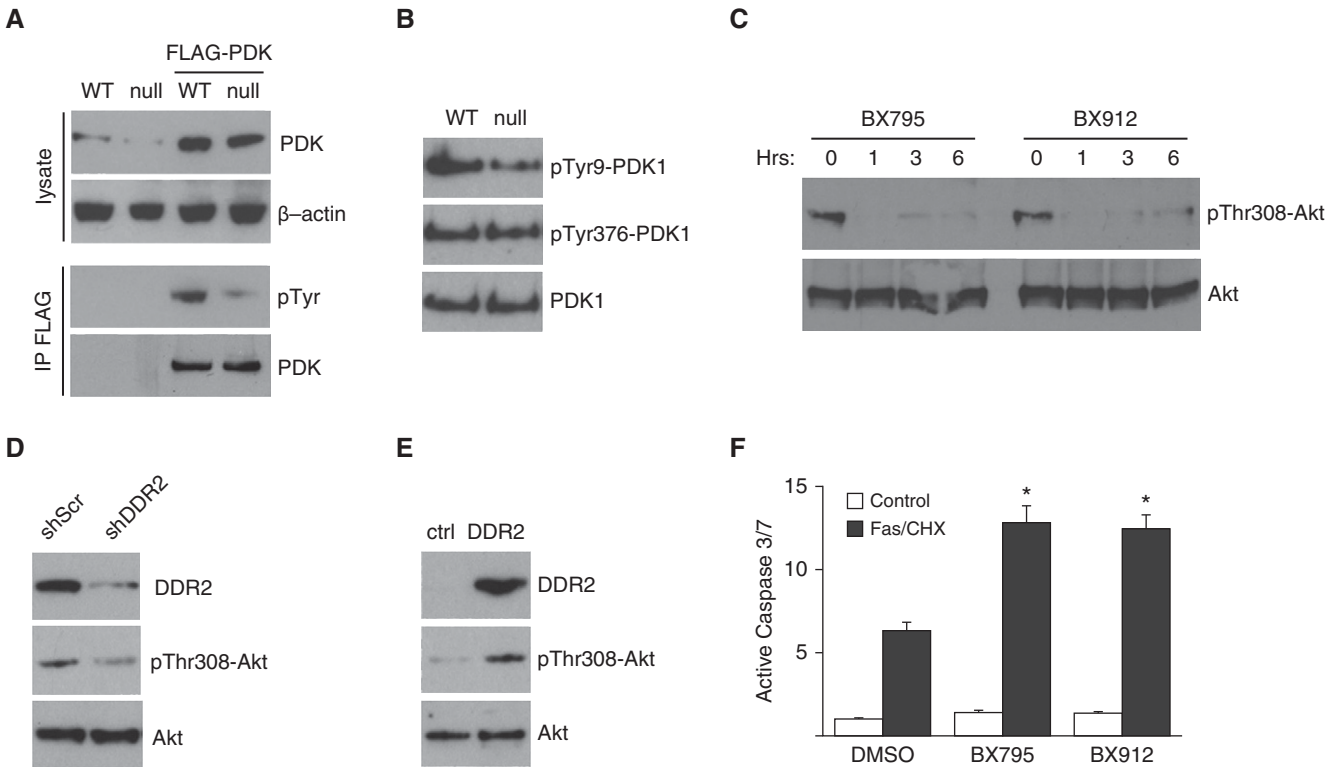

Figure 7. DDR2 regulates fibroblast survival through 3-phosphoinositide dependent protein kinase-1 (PDK1)/Akt. (*A*) Lysate from WT and DDR2-null (null) lung fibroblasts overexpressing FLAG-PDK1 were immunoprecipitated using a FLAG antibody and immunoblotted for phospho-tyrosine. Null cells have less tyrosine phosphorylation of FLAG-PDK1. (*B*) Null cells have less phospho-Tyr9-PDK1 (pTyr9-PDK1) and equal concentrations of phospho-Tyr376-PDK1 (pTyr376-PDK1) and total PDK1 compared with WT primary lung fibroblasts assessed by IB. (*C*) IB for pThr308-Akt and total Akt in WT primary lung fibroblasts treated with two small-molecule inhibitors of PDK1 (BX795 and BX912) for 0, 1, 3, and 6 hours. (*D*) IB of WT lung fibroblasts treated with lentivirus-mediated shRNA to DDR2 (shDDR2) or scrambled control (shScr) for DDR2 and pThr308-Akt. (*E*) DDR2-null lung fibroblasts treated with retrovirus-mediated overexpression of DDR2 (DDR2) or empty control retrovirus (ctrl) and immunoblotted for DDR2 and pThr308-Akt. (*F*) Activation of caspase 3/7 in WT primary lung fibroblasts upon induction of apoptosis by treatment with a Fas-activating antibody and cycloheximide (Fas/CHX) with or without BX795 and BX912. Caspase 3/7 activity was significantly higher in lung fibroblasts with additional inhibition of PDK1 than with Fas/CHX induction of apoptosis alone. Values are expressed as fold differences in relative luminescence units ($n = 6$). $^*P < 0.01$ compared with treatment with Fas/CHX and vehicle control.

(31, 32). The current paradigm suggests that in normal wound healing after injury, there is measured activation of fibroblasts with appropriate scar formation and disappearance of activated myofibroblasts through apoptosis or de-differentiation. In contrast, in fibrosis, there is exuberant activation of fibroblasts and resistance to apoptosis, leading to exaggerated and progressive fibrosis. The fibrosis itself can propagate this sustained fibrotic response (34, 35). Prior research has focused primarily on the mechanical properties of the fibrotic matrix leading to activation of intracellular pathways such as focal adhesion kinase (FAK) and Rho, as well as to greater fibroblast activation (36–38). We have recently shown that resistance to fibroblast apoptosis may actually be the more significant component of this progressive fibrotic response (31, 32). In support of this model, we find that DDR2-null fibroblasts actually have a tendency toward greater activation, even though DDR2-null mice have a significantly attenuated fibrosis.

The role of signaling through the ECM has been well studied as a regulator of fibrosis (4, 38). Prior studies have focused primarily on provisional matrix proteins such as fibronectin (39, 40). The role of collagen signaling during progressive fibrosis is not well studied. There is clearly upregulation of fibrillar collagens, including type I, type III, and others. The role of type IV collagen is less clear, although one of its main receptors, DDR1, has been shown to regulate lung fibrosis (13). Type I collagen is the most abundant protein in the fibrotic matrix, and we and others have found that collagen I expression is induced early after injury, suggesting that collagen signaling might have a role in regulating continued collagen expression and the development of fibrosis (10). Like many matrix proteins, collagen can activate a number of integrins, especially $\alpha_2\beta_1$ (41, 42). Collagen is fairly unique among matrix proteins in its ability to activate a receptor tyrosine kinase (8, 43). Our results suggest that these receptors have differing roles in regulating the cell response to type I collagen, with DDR2 regulating cell survival and $\alpha_2\beta_1$ regulating myofibroblast activation. It is noteworthy that deletion of DDR2 or type I collagen led to increased amounts of α2 integrin, which may complicate attempts to target this pathway for therapy. Consistent with collagen I–mediated inhibition of α2 integrin expression, authors of a recent report found reduced amounts of α2 integrin among IPF fibroblasts (44). Notably, there is growing

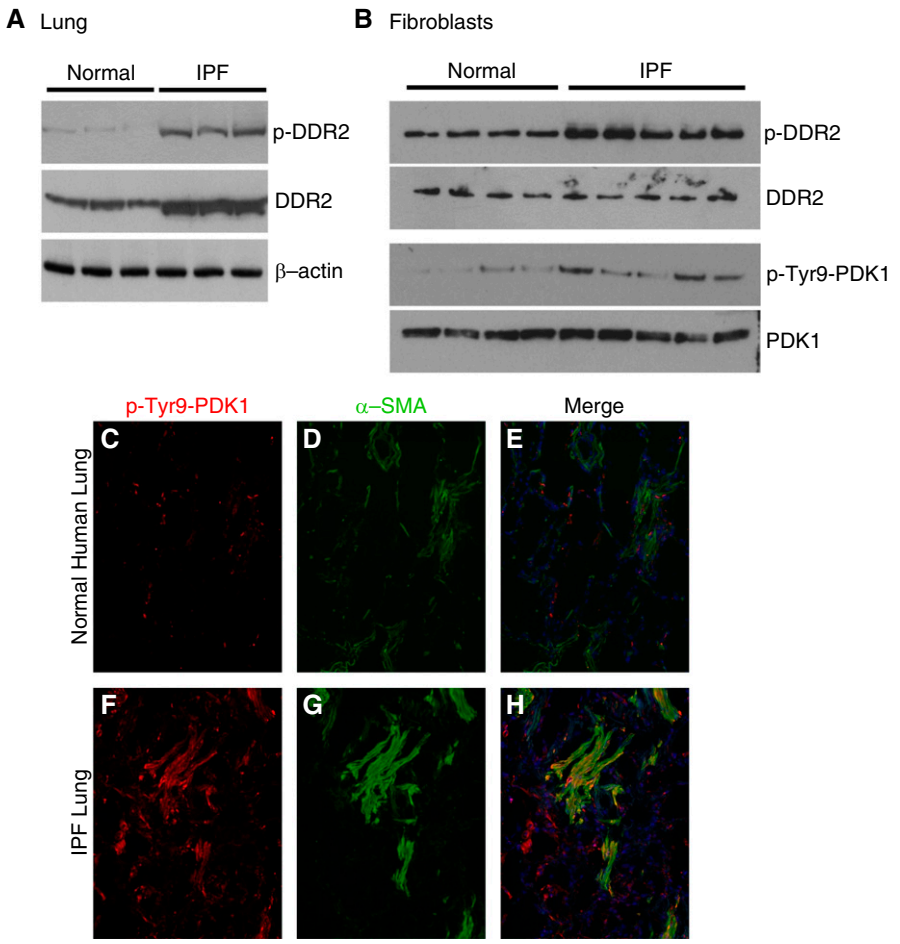

Figure 8. Idiopathic pulmonary fibrosis (IPF) fibroblasts have greater concentrations of phosphorylated DDR2 (pDDR2) and phosphorylated PDK1 (pPDK1). (*A*) IPF and normal human lung tissues were analyzed by IB for pDDR2 and DDR2. (*B*) IPF and normal human primary lung fibroblasts were analyzed by IB for pDDR2, DDR2, pTyr9-PKD1, and PDK1. (*C–H*) Normal human (*C–E*) and IPF (*F–H*) lungs were stained for pTyr9-PDK1 (red; *C* and *F*) and ACTA2 (α-SMA) (green; *D* and *G*), and the images were merged (*E* and *H*).

evidence for matrix/integrin signaling having a key role in translating matrix mechanotransduction signaling to cell behavior, including fibroblast activation and survival through well-known integrin intracellular signaling pathways involving FAK, Rho, and myocardin-related transcription factor (45). DDR2 signaling may involve an independent survival mechanism in response to early collagen production that might precede formation of a mature dense collagen matrix. Although our interest in DDR2 signaling arose from our prior studies of type I collagen deletion (10), DDR2 can be activated by a number of collagens, including type III collagen, which is also upregulated during fibrosis (46). The extent to which type I and other collagens promote fibroblast DDR2 activation during fibrosis is not well characterized.

A unique attractive feature of DDR2 is its extremely skewed high expression on mesenchymal cells and limited expression on other cell types, including epithelial cells and myeloid cells. Attempts to target survival and activation pathways have been challenging, owing to the commonality of these pathways among multiple cell types. For example, imatinib was shown to be a potent inhibitor of fibroblast activation and survival through inhibition of platelet-derived growth factor receptor and Abl, but it was later shown to influence epithelial cell survival as well (47–49). Similarly, we recently reported that inhibition of FAK signaling, which has been proposed as another potential therapeutic target in fibrosis, can also influence epithelial cell survival, potentially leading to sustained injury and fibrosis (26, 50). DDR2 expression is not limited to fibroblasts and mesenchymal cells. DDR2 expression on myeloid cells has been reported (51, 52), although we found very low expression on myeloid cells isolated from WT murine lungs (Figure E2). Recently, mutations in DDR2 have been linked to a subset of squamous cell lung cancers, and DDR2 signaling has been linked to epithelial–mesenchymal transition in other malignancies (53, 54). It is possible that DDR2 expression can be induced in alveolar epithelial cells; however, this population is likely to be profibrotic as well.

On the basis of lung cancer findings, there has been a concerted effort to develop DDR2-specific inhibitors (21). Indeed, many tyrosine kinase inhibitors are fairly nonspecific. As noted above, imatinib has activity against Abl, platelet-derived growth factor receptor, and many other tyrosine kinases. It is worth noting that dasatinib, which has been shown to block fibrosis *in vivo*, has significant activity against DDR2 (55, 56). Furthermore, nintedanib, which is approved therapy for IPF, is a fairly nonspecific tyrosine kinase inhibitor with activity against DDR2 (18, 57).

Although DDR1 and DDR2 have previously been studied in different fibrosis models, the results have been mixed, and the direct downstream targets are not well described (13, 15, 17, 58). These mixed results may be due to the complexity of the signaling networks that intersect with the DDRs. As noted above, deletion of DDR2 or collagen led to increased amounts of α2 integrin, which may lead to greater myofibroblast activation. Indeed, other studies have found a complex regulation of collagen receptor signaling influencing the expression and function of other collagen receptors and other intracellular signaling pathways (7, 8, 59, 60). Our findings also link collagen/DDR2 signaling to a signaling network that intersects with other pathways implicated in fibrosis. We found that the presence of DDR2 signaling promoted PDK phosphorylation at tyrosine 9. Because DDR2 is a well-described tyrosine kinase, it is possible that PDK is a direct downstream target of DDR2. Alternatively, DDR2 is well known to interact with a number of other kinases, and PDK phosphorylation may be indirectly regulated by DDR2 (43, 61). More detailed *in vitro* studies are

underway to help distinguish between these possibilities. The role of different tyrosine sites in regulating PDK activity at remains controversial, with some reports suggesting a more prominent role for tyrosine 376 and others suggesting an important role for tyrosine 9 (62–64). It may be that regulation of PDK activity is dependent on different cell types or other cell culture conditions. Inhibition of Akt has also been shown to promote myofibroblast apoptosis and block fibrosis (35, 65, 66). Akt can be activated at two main sites: Ser473, which is primarily regulated by mTOR, and Thr308, which is regulated primarily by PDK1 (33). Notably, mTOR inhibition is actively being pursued as a potential therapy for fibrosis (67), and it is possible that dual inhibition of Akt with PDK1 inhibition will augment the effect.

In summary, we found that collagen/DDR2 signaling can activate the Akt prosurvival pathway through activation of PDK1, leading to fibroblast survival and progressive fibrosis. DDR2 expression is skewed to the mesenchymal cell population, making it an attractive target for therapy. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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