Dopamine Receptor D1 Is Exempt from Transforming Growth Factor β-Mediated Antifibrotic G Protein-Coupled Receptor Landscape Tampering in Lung Fibroblasts[S]

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ABSTRACT

Pulmonary fibroblasts are the primary producers of extracellular matrix (ECM) in the lungs, and their pathogenic activation drives scarring and loss of lung function in idiopathic pulmonary fibrosis (IPF). This uncontrolled production of ECM is stimulated by mechanosignaling and transforming growth factor beta 1 (TGF-β1) signaling that together promote transcriptional programs including Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ). G protein-coupled receptors (GPCRs) that couple to G α s have emerged as pharmacological targets to inactivate YAP/TAZ signaling and promote lung fibrosis resolution. Previous studies have shown a loss of expression of "antifibrotic GPCRs"—receptors that couple to G α s, in IPF patient-derived fibroblasts compared with non-IPF samples. Of the 14 G α s GPCRs we found to be expressed in lung fibroblasts, the dopamine receptor D1 (*DRD1*) was one of only two not repressed by TGF-β1 signaling, with the β2-adrenergic receptor being the most repressed. We compared the potency and efficacy of multiple D1 and β2 receptor agonists $+/-$ TGF-β1 treatment in vitro for their ability to elevate cAMP, inhibit nuclear localization of YAP/TAZ, regulate expression of profibrotic and antifibrotic genes, and inhibit cellular proliferation and collagen deposition. Consistently, the activity of β2 receptor agonists was lost, whereas D1 receptor agonists was maintained, after stimulating cultured lung fibroblasts with TGF-β1. These data further support the therapeutic potential of the dopamine receptor D1 and highlight an orchestrated and pervasive loss of antifibrotic GPCRs mediated by TGF-β1 signaling.

SIGNIFICANCE STATEMENT

Idiopathic pulmonary fibrosis (IPF) is a deadly lung disease with limited therapies. GPCRs have emerged as a primary target for the development of novel antifibrotic drugs; however, a challenge to this approach is the dramatic changes in GPCR expression in response to profibrotic stimuli. Here, we investigate the impact of TGF-β1 on the expression of antifibrotic GPCRs and show the D1 dopamine receptor expression is uniquely maintained in response to TGF-β1, further implicating it as a compelling target to treat IPF.

Introduction

Despite continued therapeutic developments, idiopathic pulmonary fibrosis (IPF) remains a debilitating, rapidly progressive, and ultimately fatal lung disease (Nalysnyk et al., 2012) with a prevalence of $\sim$18 cases per 100,000 individuals in the United States and accounting for $\sim$40–80,000 deaths annually in the United States and Europe (Hutchinson et al., 2014; Raghu et al., 2016). Antifibrotic drugs have become available for IPF in the last decade; however, these drugs only slow the progression of the disease and have only a limited capacity to improve mortality (Richeldi et al., 2017; Valenzuela et al., 2020). Therefore, there remains a significant need to identify effective therapeutic strategies for IPF.

At the cellular level, activated fibroblasts are one of the core contributors to tissue fibrosis (Barkauskas and Noble, 2014), driving matrix deposition and tissue remodeling in response to profibrotic stimuli such as mechanotransduction and transforming growth factor beta 1 (TGF-β1) (Haak et al., 2018). Recent work has identified Yes-associated protein 1 (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) transcription cofactors as central regulators of fibroblast matrix deposition and proliferation (Liu et al., 2015; Mannaerts et al., 2015; Piersma et al., 2015; Martin et al., 2016; Szeto et al., 2016;

ABBREVIATIONS: *ADRB2*, β2-adrenergic receptor; BSA, bovine serum albumin; DAPI, 4′, 6-diamidino-2-phenylindole; *DRD1*, dopamine receptor D1; ECM, extracellular matrix; EMEM, Eagle's minimum essential medium; GPCR, G protein-coupled receptor; IPF, idiopathic pulmonary fibrosis; *LOXL2*, lysyl oxidase like-2; TAZ, transcriptional coactivator with PDZ-binding motif; TGF-β1, transforming growth factor beta 1; YAP, Yes-associated protein.

Noguchi et al., 2017; Stearns-Reider et al., 2017). Consistent with this, we have observed prominent nuclear staining for YAP and TAZ in interstitial cells of IPF, but not control lungs (Liu et al., 2015). In previous work, we performed a G protein-coupled receptor (GPCR)-focused transcriptome screen to reveal the D1 dopamine receptor as a target to inactivate YAP/TAZ in lung fibroblasts, and we showed that an agonist of the D1 receptor, dihydrexidine, blocks YAP/TAZ nuclear localization, stimulates collagen degradation, and promotes lung fibrosis resolution in a bleomycin lung injury model of fibrosis, consistent with the D1 receptor exclusively coupling to G α s (Haak et al., 2019; Diaz-Espinosa et al., 2020; Choi et al., 2021). Additional GPCRs which couple to G alpha s also block fibrotic signaling including the prostaglandin E2 receptor (Bozyk and Moore, 2011; Mukherjee et al., 2019), prostacyclin IP receptor (Roberts et al., 2021; Zmajkovicova et al., 2019), and the relaxin family peptide receptor 1 (Pini et al., 2010) through their elevation of cAMP and interaction with downstream effector proteins Exchange Factor directly Activated by cAMP1/2 (EPAC1/2) and protein kinase A (Insel et al., 2012). This trend is consistent with the known association of cAMP elevation with YAP/TAZ nuclear exclusion (Zmajkovicova et al., 2020). Intriguingly, ligands for both the prostaglandin E2 receptor and relaxin family peptide receptor 1 receptors lose efficacy in bleomycin injured lungs and IPF patient-derived cells due to the reduced expression of these receptors in pathologic tissue (Moore et al., 2005; Huang et al., 2010; Tan et al., 2016; Bahudhanapati et al., 2019).

Building on these previous findings, we sought to evaluate the expression of GPCRs under in vitro profibrotic conditions and found that the expression of antifibrotic G α s coupled receptors are almost universally repressed in cultured lung fibroblasts stimulated with the "master regulator of fibrosis" -transforming growth TGF-β1. To characterize the functional impact of changes in the receptor landscape, we focused on the D1 dopamine receptor, which was not repressed by TGF-β1, and the β2 adrenergic receptor, which displayed the most dramatic loss in expression, and measured changes in cAMP production, inhibition of YAP/TAZ nucleal localization, expression of fibrosis-associated genes, collagen deposition, and proliferation in response to TGF-β1 stimulation. Our results indicate that, in contrast to the β2 adrenergic receptor, the antifibrotic effects of D1 dopamine receptor activation persist even in the presence of TGF-β1. These findings suggest that receptor repression may compromise β2 adrenergic receptor antifibrotic signaling and further implicate the D1 dopamine receptor as a promising therapeutic target for IPF.

Materials and Methods

Chemical and Reagents. Y-27632, forskolin, SB431542, formoterol, fenoterol, salbutamol, salmeterol, and fenoldopam were purchased from Cayman Chemical (Ann Arbor, MI). Dihydrexidine, A77636, A68930, and fenoldopam were purchased from Tocris Bioscience (Minneapolis, MN). Recombinant human TGF-β1 was purchased from PeproTech (Rocky Hill, NJ). Bovine serum albumin (BSA), 10% formalin, and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO). PBS was purchased from Life Technologies (Carlsbad, CA).

Cell Culture. Primary human lung fibroblasts (two male donors, one female donor) were purchased from Lonza (Allendale, NJ) and ScienCell (Carlsbad, CA). All cells were cultured in Eagle's minimal essential medium (EMEM) containing 10% fetal bovine serum and antibiotic/antimycotic purchased from American Type Culture Collection (Manassas, VA) unless otherwise noted. Experiments were performed with cells between passages four and eight.

Quantitative PCR. Fibroblasts were plated into 6-well tissue culture plates purchased from Thermo Fisher Scientific, treated as indicated for each experiment. RNA isolation was performed using an RNeasy Plus Mini Kit purchased from Qiagen (Hilden, Germany) according to the manufacturer's instructions. Isolated RNA was used to synthesize cDNA using SuperScript Vilo purchased from Invitrogen. Quantitative PCR was performed using FastStart Essential DNA Green Master purchased from Roche and analyzed with a LightCycler 96. Data are expressed as a fold change by $\Delta\Delta$Ct relative to the level of the housekeeping gene (*GAPDH*) and normalized to an experimental control. Primer sequences were as follows: *GAPDH* (F: ACATCGCTCAGACACCATG, R: TGTAGTTGAGGTCAATGAAGGG), *DRD1* (F: CCCAGCCCTATCAGTCATATTG, R: AGGATTCATCTGCGAGTTCAG), *ADRB2* (F: TCTTCACGAACCAAGCCTATG, R: AGACCCTGGAGTAGACGAAG), *COL1A1* (F: AAGGGACACAGAGGTTTCAGTGG, R: CAGCACCAGTAGCACCATCATTTC), *COL1A2* (F: CTTGCAGTAACCTTATGCCTAGCA, R: CCCATCTAACCTCTCTACCCAGTCT), *LOXL2* (F: GTGCAGCGACAAAAGGATTC, R: GCGGTAGGTTGAGAGGATG), *CTSK* (F: CTCCTTCCAGTTTTACAGCAAAG, R: TTTCCCCAGTTTTCTCCCCC).

Microarray Analysis. These data are produced from our published microarray experiments (Link et al., 2022) and are available through the Gene Expression Omnibus (GEO) under the accession no. GSE175853. Briefly, transcript levels of human lung fibroblasts subjected to TGF-β1 (2 ng/ml) stimulation in conjunction with scrambled, YAP, TAZ, or both YAP and TAZ small interfering RNA ($n = 3$ replicates per condition) were evaluated using the Affymetrix GeneChip PrimeView Human Gene Expression Array.

Flow Cytometry. Fibroblasts were plated into 6-well tissue culture plates purchased from Thermo Fisher Scientific. After the cells attached, the media was exchanged for EMEM containing 0% FBS +/– 2 ng/ml TGF-β1 (PeProTech). After 72 hours, cells were collected and fixed in 1% paraformaldehyde for 10 minutes at room temperature. They were then washed and stained in autoMACS rinsing solution (130-091-222, Miltenyi Biotec) with 0.5% BSA and stained with anti-human DRD1:PE (366403, Biolegend) or isotype control (555574, BD Pharmingen), and 4′,6-diamidino-2-phenylindole (DAPI) for 30 minutes on ice in the dark. After a final wash, cells were acquired using BD LSRFortessa X-20 and analyzed using FlowJo software V10.8.1.

cAMP. Fibroblasts were plated into 96-well tissue culture plates purchased from Thermo Fisher Scientific. cAMP was measured using the cAMP-Glo Assay (Promega) according to manufacturer's suggestions. Thirty minutes prior to cell lysis, media was removed, and cells were treated with "induction buffer" containing nonselective phosphodiesterase inhibitors and the indicated concentration of compound(s) diluted in EMEM media without FBS. Luminescence was measured on a Promega GloMax Discover Microplate Reader.

Quantification of YAP/TAZ Localization. Experiments were performed as previously described (Haak et al., 2019; Aravamudhan et al., 2020; Choi et al., 2021; Zhang et al., 2022). Fibroblasts (1000 cells/well) were plated into 96-well plates and allowed to attach overnight. Cells were then treated with the indicated concentration of compounds for 2 hours and fixed in 37% formalin, permeabilized with 0.25% triton for 15 minutes at room temperature, blocked with 1% BSA for 60 minutes at room temperature, and immunostained with YAP/TAZ (D24E4) (Cell Signaling) overnight at 4°C. Finally, anti-rabbit secondary antibody was incubated for 1 hour at room temperature along with DAPI. Cells were imaged using a 4× objective, and YAP/TAZ nuclear localization was quantified as previously described using a Cytation5 (Biotek) high-content epifluorescent microscope. Briefly, cell nuclei "objects" were identified using DAPI, and mean YAP/TAZ nuclear intensity was determined for the control, vehicle-treated group. A threshold value of 85% of this intensity was then used to quantify the percentage of YAP/TAZ nuclear-positive cells after

treatments, and these data are plotted as %nuclear YAP/TAZ in Fig. 3. In initial experiments developing this image analysis protocol, we compared the automated software analysis to two different investigators asked to identify nuclear-positive YAP/TAZ cell populations, blinded to the conditions. The automated analysis was within 5% variance of the two investigators for each condition. We also previously confirmed robust correlation between this automated analysis and biochemical fractionation assays quantifying nuclear and cytoplasmic YAP/TAZ (Haak et al., 2019).

TEAD Luciferase Reporter. Fibroblasts were plated into 6-well tissue culture plates purchased from Thermo Fisher Scientific. After cells attached, 25 μl of 2.5 $\times$ 10^7 infectious units/ml lentivirus containing a TEAD luciferase reporter construct (Lipexogen, TEAD-TAL-BSD) were added to the cell culture media. Forty-eight hours later, cells were replated into 96-well plates (5000 cells/well). After cells attached, media was changed to EMEM containing 0% FBS and the indicated concentration of D1R agonist. Eighteen hours later, cells were lysed and treated with luciferin (Promega, Bright-Glo), and luminescence was measured on a Promega GloMax luminometer.

Collagen Deposition Assay. Fibroblasts (3000 cells/well) were plated into 96-well plates and allowed to attach overnight. As indicated for each specific experiment, media was then changed to either control (EMEM, 0% FBS) or EMEM containing 2 ng/ml TGF-β1, 50 μg/ml ascorbic acid, and 2% FBS (TAA2). TGF-β1 (2 ng/ml) was chosen based on dose–response studies we performed previously (Jones et al., 2019). This proliferation and matrix stimulating media was developed based on our own previous work in generating cell-derived matrices and other published work in this space. After 72 hours in culture, cells were either fixed or media was changed with fresh control or TAA2, with the indicated compounds. In "cellularized" experiments the cells were fixed, permeabilized, and blocked as described above for YAP/TAZ localization experiments. Primary antibody for type I collagen (NB600-408; Novus Biologicals Inc., Centennial, CO) was incubated overnight. Cells were then washed and anti-rabbit secondary antibody was incubated for 60 minutes at room temperature. Cells were imaged using a Cytation 5 inverted microscope. In "decellularized" experiments the procedure was identical, however, prior to fixation, the media was removed, and the cells were incubated for 10 minutes in PBS containing 20 mM ammonium hydroxide and 0.5% Triton X-100, consistent with our previous decellularization protocols (Diaz-Espinosa et al., 2020). For proliferation quantification, the number of DAPI objects/field of view was quantified using Gen5 software. For collagen intensity quantification, the pixel intensity across the entire field of view was determined, background (identical experiment, no primary antibody) was subtracted, and data are plotted as fold-increase relative to the indicated control.

Statistics. Data analysis and plotting were performed using Prism 9.0 (GraphPad Software, La Jolla, CA). Statistical comparison between two groups was performed by unpaired t test. A statistical analysis of three or more groups was performed by repeated measures one-way ANOVA with Dunnett's multiple comparison test, where the mean value of each group was compared against all groups. Results are expressed as the mean $\pm$ S.E.M. The sample number (n) indicates the number of independent samples in each experiment.

Results

Antifibrotic GPCRs Are Nearly Ubiquitously Repressed by TGF-β1. Based on previous findings that agonists of G α s coupled receptors lose their efficacy in fibrotic tissues (Moore et al., 2005; Huang et al., 2010; Tan et al., 2016), we analyzed a previously published RNA sequencing dataset comparing cultured IPF-patient derived fibroblasts +/− TGF-β1 treatment (GEO database series GSE136534) (Jones et al., 2019). Using previously published models and experimental data of GPCR/G protein coupling selectivity, we generated a

list of 42 GPCRs that exclusively or primarily couple to G α s (Inoue et al., 2019; Armstrong et al., 2020). Of these receptors, 14 were found to be expressed at a detectable level in all fibroblast samples, and all but dopamine receptor D1 (*DRD1*) and *GPR3* were repressed by TGF-β (Fig. 1A). *DRD1* and the β2-adrenergic receptor (*ADRB2*) are used as models for the remainder of this study because they highlight the extremes of receptor changes after TGF-β stimulation, and they both offer a rich pharmacological toolset for receptor agonism. The above RNA sequencing dataset was collected 12 hours after TGF-β treatment. To confirm this transcriptional program was not only a transient effect, we treated cultured lung fibroblasts for 24, 48, and 72 hours +/− TGF-β1 prior to collecting RNA and analyzing *DRD1* and *ADRB2* by quantitative PCR (Fig. 1, B and C). The effect of exogenous treatment was consistent with the changes observed at 12 hours and it was conserved over 72 hours. Interestingly, the effect of culturing fibroblasts in the absence of exogenous TGF-β1 appears to mimic these changes, just at a slower pace. We hypothesize this was driven by endogenous production of TGF-β leading to autocrine signaling in the culture well, because this effect has previously been described in fibroblasts cultured on tissue culture plastic (Hinz, 2015). In support of this, we cultured lung fibroblasts for 72 hours (without exogenous TGF-β1) in the presence of the TGF-β1 receptor/ALK5 inhibitor SB431542. Consistent with our findings, *COL1A1* and *DRD1* were repressed by inhibiting TGF-β signaling and *ADRB2* was enhanced (Fig. 1D). To confirm enhanced transcript levels of *DRD1* in response to TGF-β1 correlates with increased protein abundance for the dopamine receptor D1 (D1R), we treated cultured lung fibroblasts +/− TGF-β1 for 72 hours and quantified D1R expression by flow cytometry (Supplemental Fig. 1), which supported a positive correlation between transcript changes and membrane receptor density.

**TGF-β Treatment Prevents β2-Adrenergic Receptor-Mediated cAMP Elevation and YAP/TAZ Inhibition.** To investigate the functional impact of TGF-β stimulated loss of *ADRB2* expression in cultured lung fibroblasts, we compared the potency and efficacy of multiple β2-adrenergic receptor agonists (Fig. 2, A and B) and dopamine receptor D1 agonists (Fig. 2, C and D) for their ability to stimulate production of intracellular cAMP after 72 hours in culture with (Fig. 2, A and C) or without (Fig. 2, B and D) recombinant TGF-β1. In the absence of exogenous TGF-β1, all four β2 agonists potently stimulated cAMP production with salbutamol and salmeterol producing a partial effect, consistent with previously published reports of their intrinsic activity in other tissues (Grove et al., 1995; Lotvall, 2001). After exogenous treatment with TGF-β1, none of the β2 agonists produced an appreciable elevation of cAMP, consistent with a loss in receptor density (Fig. 1). Alternatively, D1 receptor agonists produced consistent results in elevating cAMP +/− TGF-β1 with the previously identified intrinsic activities for each agonist (Grenader and Healy, 1991; Martin, 2011; McCorvy et al., 2012), being observable in cultured lung fibroblasts (Fig. 2, C and D). Similarly, β2 agonists also lost their efficacy in reducing nuclear YAP/TAZ after 72 hours of exogenous TGF-β1 treatment (Fig. 3, A and B) whereas D1 receptor agonism remained effective with or without TGF-β1 treatment (Fig. 3, C and D). In comparing the control cell culture datasets for cAMP elevation and inhibition of nuclear YAP/TAZ, the dopamine D1 agonist efficacy was consistent,
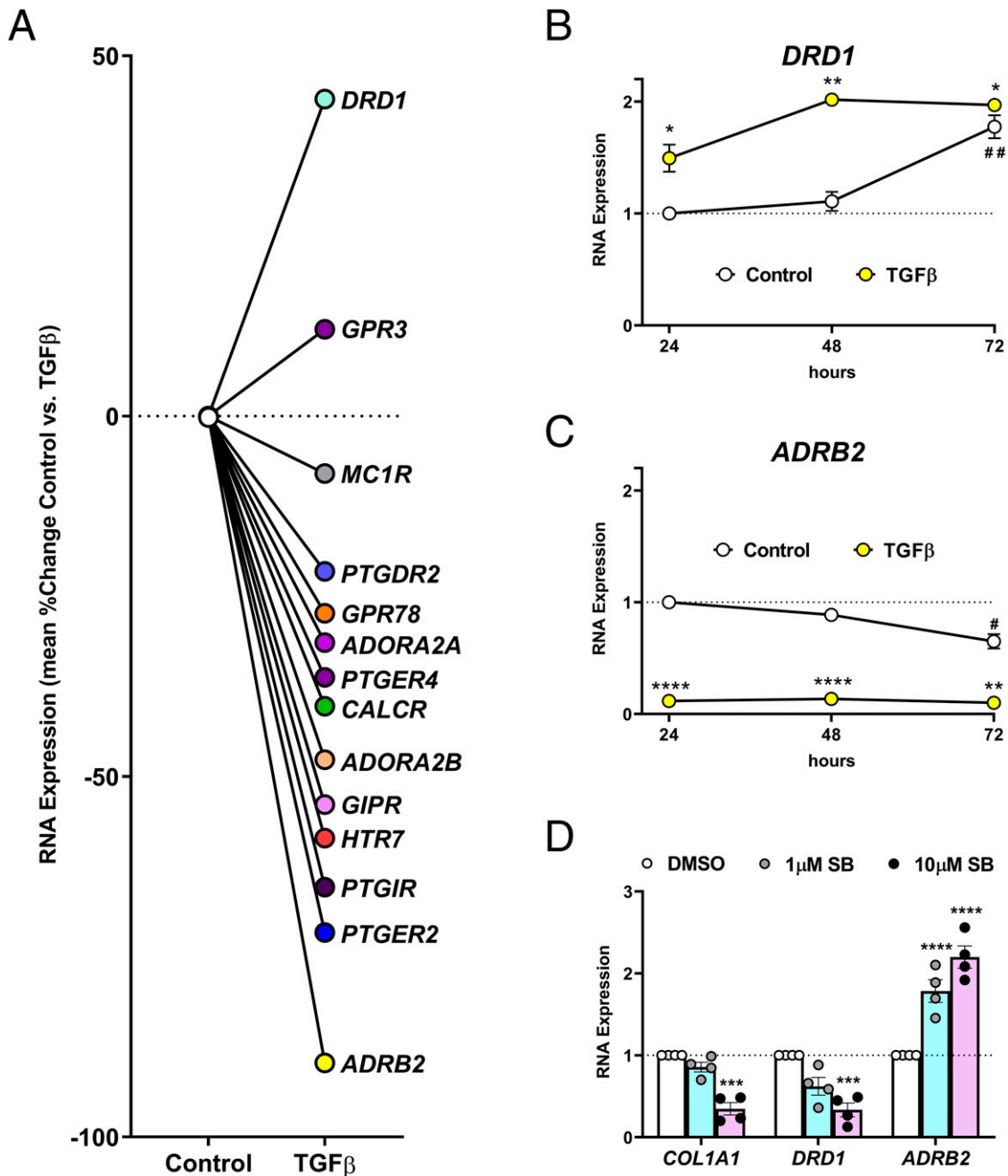
Fig. 1. TGF-β signaling regulates transcription of G α s coupled GPCRs in lung fibroblasts. (A) Analysis of a previously published RNA sequencing dataset. IPF patient-derived lung fibroblasts stimulated for 12 hours with 2 ng/ml TGF-β1 prior to RNA isolation and analysis. Shown are the mean % changes in expression of all GPCRs that couple exclusively to G α s and are expressed in these datasets. $N = 3$ unique patient samples. GEO database series GSE136534. (B and C) RNA expression of *DRD1* and *ADRB2* in cultured human lung fibroblasts $+/-$ 2 ng/ml TGF-β1 over a 72-hour time course. $N = 4$ independent experiments performed with unique donor samples (*$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$ vs. time matched control; #$P < 0.05$ and ##$P < 0.01$ vs. treatment matched 24-hour time point). (D) Human lung fibroblasts cultured for 72 hours with the indicated concentration of the TGF-β type I receptor/ALK5 inhibitor SB431542 prior to RNA isolation and assessment of known TGF-β/SMAD target—*COL1A1*, and *ADRB2*, and *DRD1*. $N = 4$ independent experiments performed with unique donor samples (***$P < 0.001$; ****$P < 0.0001$ vs. DMSO-treated cells).

with fenoldopam exhibiting the lowest efficacy followed by A68930, and DHX/A77636 displaying full efficacy (Fig. 2C; Fig. 3C) consistent with their intrinsic activity for the D1 receptor (Alexander et al., 2019). This direct relationship was not observed in the β2 agonist data and salbutamol was a partial agonist at elevating cAMP (Fig. 2A), however, it was able to fully inhibit YAP/TAZ nuclear localization (Fig. 3A). YAP and TAZ cofactors directly bind with TEAD family of

transcription factors to regulate expression of proliferative and profibrotic genes, and a TEAD-luciferase reporter construct can directly monitor the activity of YAP and TAZ (Piccolo et al., 2014; Dupont, 2019). To confirm the inhibition of nuclear localization was indeed inactivating YAP/TAZ, we transduced cultured lung fibroblasts with a lentiviral delivered TEAD-luciferase reporter and incubated cells with DHX and A77636 (Supplemental Fig. 2). These experiments
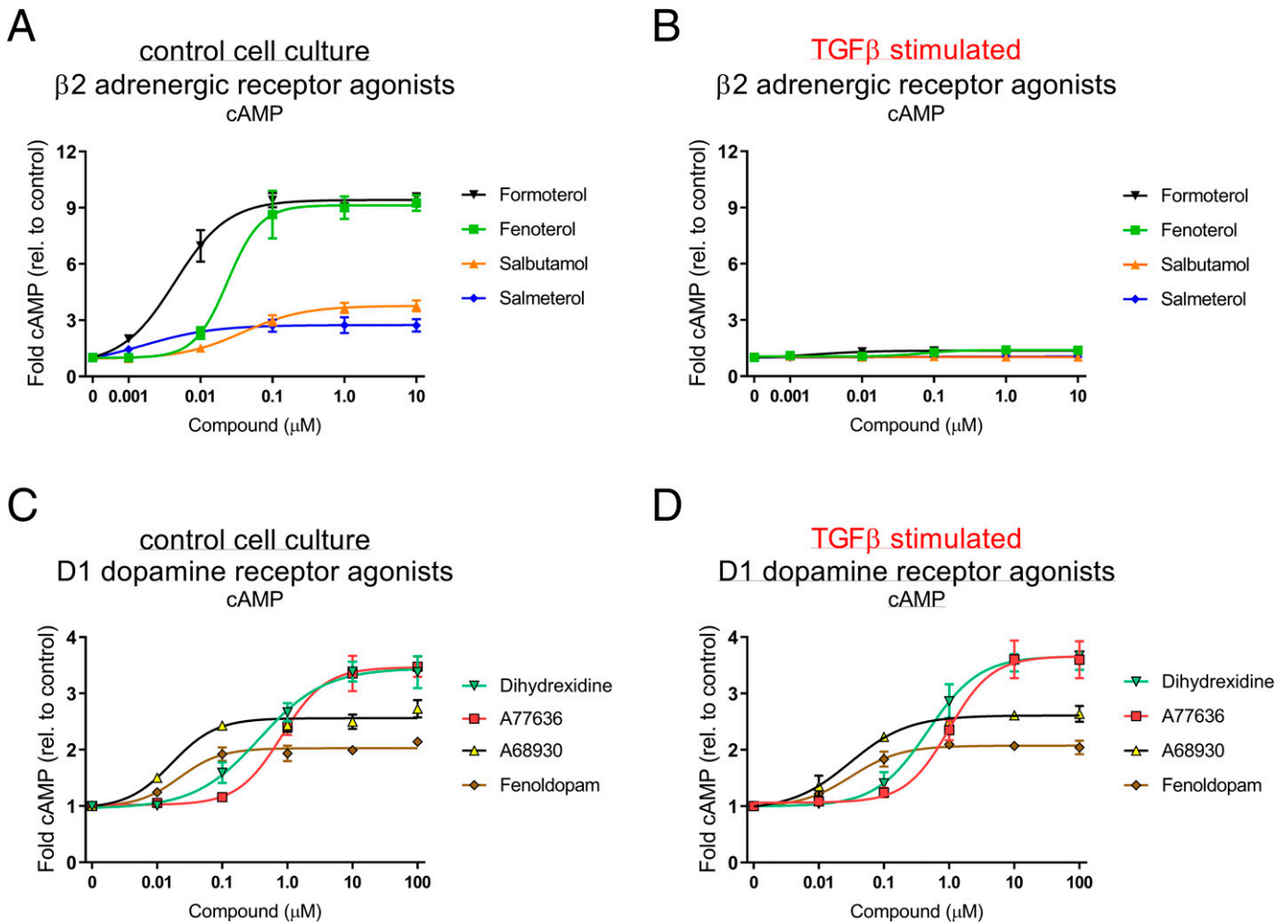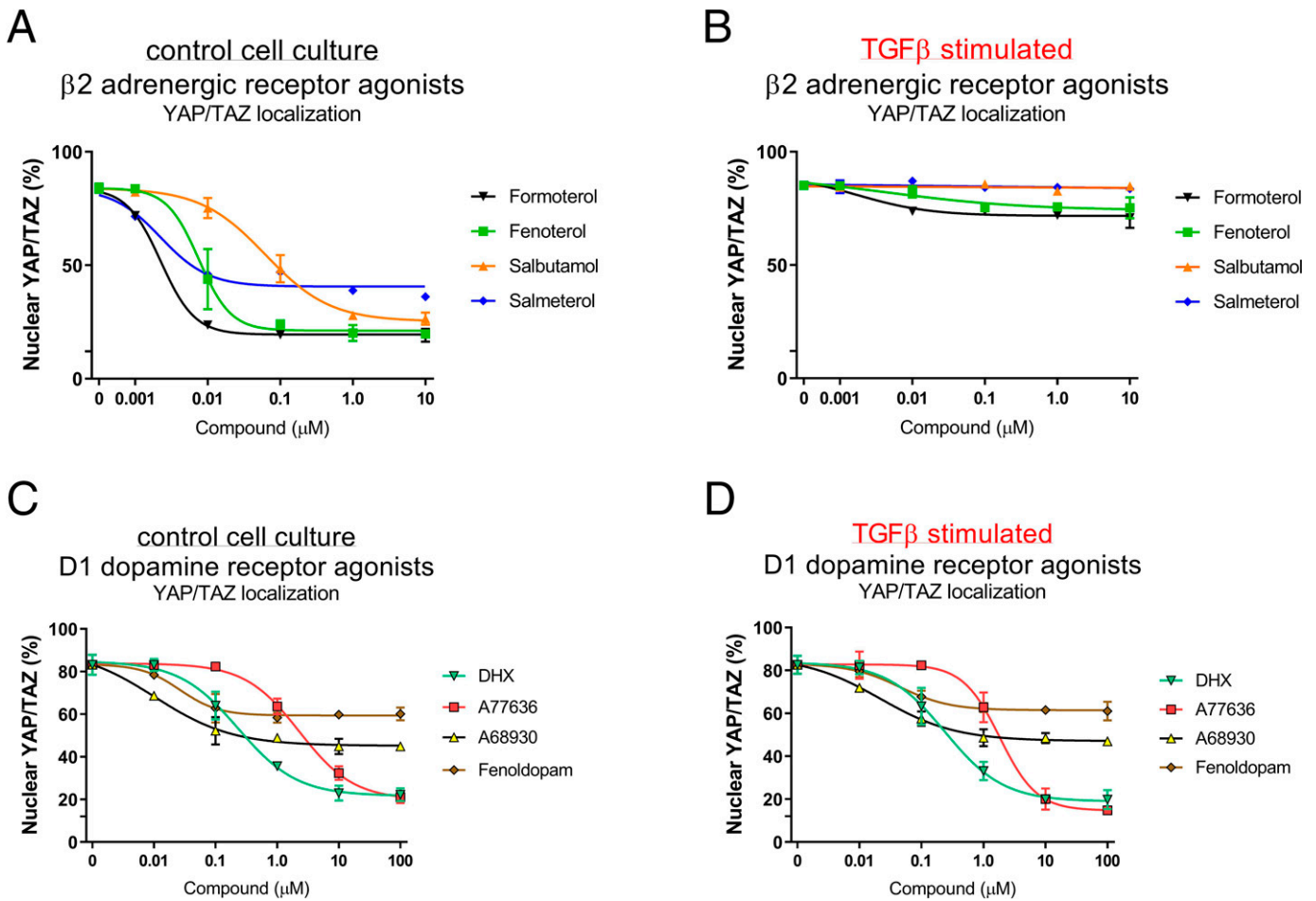
Fig. 2. TGF-β treatment induces loss of $\beta2$ adrenergic receptor-stimulated cAMP elevation. Human lung fibroblasts were treated for 72 hours with (B and D) or without (A and C) 2 ng/ml TGF-$\beta1$. Cells were then stimulated with the indicated concentration of $\beta2$ adrenergic receptor agonists (A and B) or D1 dopamine receptor agonists (C and D) for 30 minutes prior to measuring cAMP. Data are expressed as mean fold changes relative to untreated control. $N = 3$ independent experiments performed with unique donor samples.

phenocopied the potency and efficacy of our cAMP and nuclear localization experiments with the full D1 receptor agonists.

Receptor-Specific Effects on Fibrosis-Associated Gene Expression. Transcriptional changes downstream of G protein signaling and YAP/TAZ modulation regulate fibroblast biology in the setting of lung fibrosis (Haak et al., 2020; Zhu et al., 2020). We recently published microarray experiments of lung fibroblasts stimulated with TGF-β after knocking down YAP and TAZ expression by small interfering RNA and reported dramatic changes in genes that regulate cellular contractility (Link et al., 2022). Here, we reanalyzed these datasets and found YAP/TAZ also regulate expression of the two genes essential to type I collagen (*COL1A1* and *COL1A2*), the collagen crosslinking gene lysyl oxidase like-2 (LOXL2) (Puente et al., 2019), and inversely regulate expression of the collagen degrading enzyme- cathepsin K (*CTSK*) (Supplemental Fig. 3). CTSK plays an essential role in the resolution of lung fibrosis stimulated by dopamine D1 signaling (Diaz-Espinosa et al., 2020). We next determined if inhibiting YAP/TAZ by $\beta2$ or D1 receptor agonism would recapitulate our microarray findings. In control or TGF-β–treated lung fibroblasts, the D1

receptor agonists A77636 and dihydrexidine repressed expression of collagen genes *COL1A1*, *COL1A2*, and *LOXL2*. Consistent with their role in promoting fibrosis, all of these genes were enhanced in cells treated with TGF-$\beta1$, whereas CTSK, an antifibrotic gene was repressed by TGF-β but stimulated with D1 agonism (Fig. 4). Consistent with results shown in Figs. 2 and 3, the $\beta2$ agonists formoterol and fenoterol produced an antifibrotic response in control cultured lung fibroblasts but lost their efficacy in experiments where cells were treated with TGF-$\beta1$ (Fig. 4). The concentration used for these studies was based on an approximate minimum amount of compound required to generate a maximum response in the cAMP and YAP/TAZ experiments (Fig. 2; Fig. 3).

D1 Receptor Agonism Blocks Fibroblast Proliferation and Collagen Deposition. To monitor both proliferation and collagen deposition in vitro, we cultured lung fibroblasts in the presence or absence of 2 ng/ml TAA2 for 3 and 6 days and immunostained type I collagen deposition. Interestingly, both proliferation and collagen deposition were only modestly increased by TAA2 treatment within the first 3 days but were dramatically elevated from days 3 to 6 (Fig. 5A), with TGF-$\beta1$ and ascorbic acid being the primary

A
control cell culture
β2 adrenergic receptor agonists
YAP/TAZ localization

Legend: Formoterol, Fenoterol, Salbutamol, Salmeterol

B
TGFβ stimulated
β2 adrenergic receptor agonists
YAP/TAZ localization

Legend: Formoterol, Fenoterol, Salbutamol, Salmeterol

C
control cell culture
D1 dopamine receptor agonists
YAP/TAZ localization

Legend: DHX, A77636, A68930, Fenoldopam

D
TGFβ stimulated
D1 dopamine receptor agonists
YAP/TAZ localization

Legend: DHX, A77636, A68930, Fenoldopam

Fig. 3. TGF-β treatment induces loss of β2 adrenergic receptor-mediated inhibition of YAP/TAZ nuclear localization. Human lung fibroblasts were treated for 72 hours with (B and D) or without (A and C) 2 ng/ml TGF-β1. Cells were then stimulated with the indicated concentration of β2 adrenergic receptor agonists (A and B) or D1 dopamine receptor agonists (C and D) for 120 minutes prior to measuring fixing and staining using an antibody that detects YAP and TAZ transcription cofactors. Image analysis is performed using automated software. Data are expressed as mean % changes relative to untreated control. N = 3 independent experiments performed with unique donor samples.

drivers of collagen deposition and FBS supporting proliferation (Supplemental Fig. 4). To specifically assess if the collagen staining was truly deposited matrix or intracellular protein, we developed a parallel protocol where we decellularize matrices and then immunostained for type I collagen (Fig. 5B). In these experiments, we observed consistent collagen intensity and visual organization. This novel platform allowed us to robustly assess the potential for therapeutic ECM reductions; in this instance we waited until day 3 to allow TGF-β1 to prime the GPCR landscape of the fibroblasts prior to adding β2 or D1 agonists. Treatment with either D1 agonist reduced proliferation and collagen deposition in these experiments, in both cellularized and decellularized experiments (Fig. 6). In either scenario, β2 receptor agonists were unable to elicit an effect.

Discussion

In summary, our findings highlight the therapeutic potential of targeting G α s receptors, such as the D1 dopamine receptor. We show here that the antifibrotic effect of D1 dopamine receptor agonism in lung fibroblasts persists even in the presence of TGF-β1, suggesting that D1 dopamine receptor agonists may serve as effective therapeutic agents under the

physiologic context of IPF. TGF-β1 has been the primary growth factor of focus in IPF research since the mid-1980s, largely due to its sufficient and dramatic ability to activate fibroblasts in vitro and in vivo (Ye and Hu, 2021). We found TGF-β1 modestly upregulates the expression of the D1 dopamine receptor; however, we did not observe a functional impact in intracellular cAMP or YAP/TAZ localization inhibition by D1 agonists in cells treated +/− TGF-β. This could potentially be an example of a spare receptor phenomenon (Stephenson, 1956) where enough D1 receptor is present even in the absence of exogenous TGF-β to stimulate maximum cAMP and maximum YAP/TAZ localization inhibition, at least in vitro, in this context. TGF-β1 enhancing D1 expression may suggest an association between receptor upregulation and priming of cells to respond to antifibrotic signaling. These findings are consistent with previous work that has demonstrated a role for D1 dopamine receptor agonism in promoting lung fibrosis resolution (Haak et al., 2019; Diaz-Espinosa et al., 2020; Choi et al., 2021). Unlike multiple antifibrotic GPCRs, DRD1 was found to be unchanged in IPF patient-derived lung fibroblasts compared with non-IPF controls (Haak et al., 2019). D1 dopamine receptor activation has also been implicated in the resolution of other types of fibrosis, including cardiac and

COL1A1

COL1A2

LOXL2

CTSK
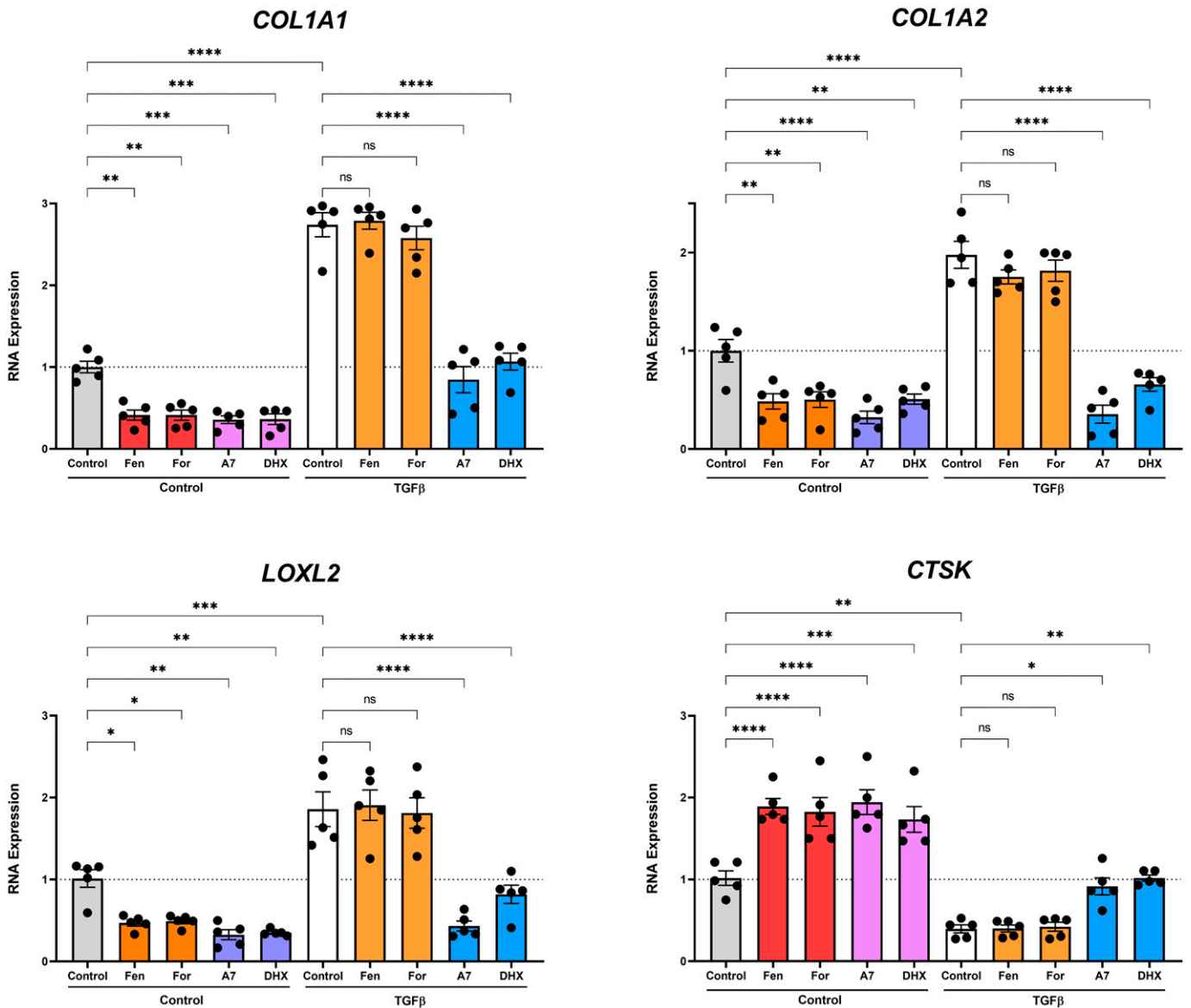
Fig. 4. TGF-β treatment induces loss of β2 adrenergic receptor-regulated antifibrotic transcriptional reprograming. Human lung fibroblasts were treated for 60 hours with or without 2 ng/ml TGF-β1. Cells were then stimulated with D1 dopamine receptor agonists dihydrexidine (DHX) or A77636 (A7) (both 10 μM), or β2 adrenergic receptor agonists formoterol (For) or fenoterol (Fen) (both 1 μM) for 12 hours prior to isolating RNA to measure transcription changes in collagen genes *COL1A1* and *COL1A2*, and collagen regulating genes *LOXL2* and *CTSK*. $N = 5$ independent experiments performed with three unique donor samples (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ vs. the indicated group).

liver fibrosis (Haak et al., 2019; Liu et al., 2021). It is important to consider that ligands such as dopamine cannot be definitively classified as antifibrotic. The physiologic response is dependent on which receptors will dominate the signaling cascade when the ligand is presented. For instance, a recent study found that signaling via the D3 dopamine receptor promoted cardiac fibroblast activation in vitro and in vivo (Kisling et al., 2021), and we also demonstrated high expression of the D2 dopamine receptor in retinal cells that proliferate and synthesize ECM in ocular fibrotic diseases (Gao et al., 2022). In these contexts, dopamine serves as a profibrotic ligand.

Our results demonstrate that the antifibrotic effect of β2 adrenergic receptor agonism in lung fibroblasts is lost in the presence of TGF-β1. TGF-β1 also inhibits the expression of

this receptor, suggesting an association between receptor repression and loss of antifibrotic signaling. Interestingly, previous work has shown efficacy of β2-adrenoceptor agonist olodaterol in an in vivo model of pulmonary fibrosis (Herrmann et al., 2017). Moreover, a trial examining the effect of combined treatment of beclomethasone, a corticosteroid, and formoterol, a long-acting β2-adrenoceptor agonist, in patients with IPF reported promising results, albeit in a small sample size (Wright et al., 2017). The efficacy of β2-adrenergic receptor agonism in these studies may be explained by the existence of a diversity of distinct fibroblast subtypes that contribute to pulmonary fibrosis (Habermann et al., 2020). Alternatively, the observed benefit in this trial could be driven by formoterol signaling in other pulmonary cells including alveolar epithelial cells
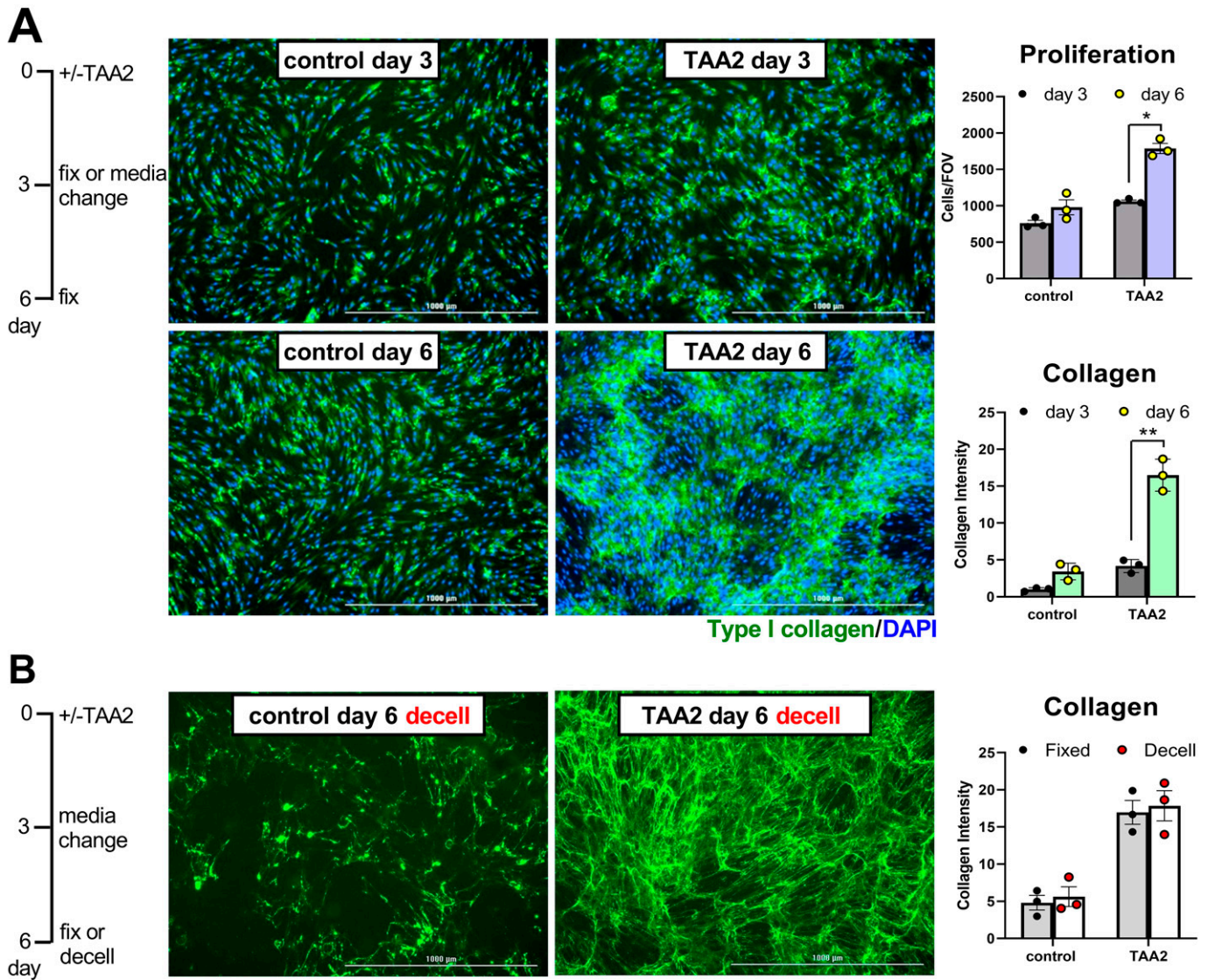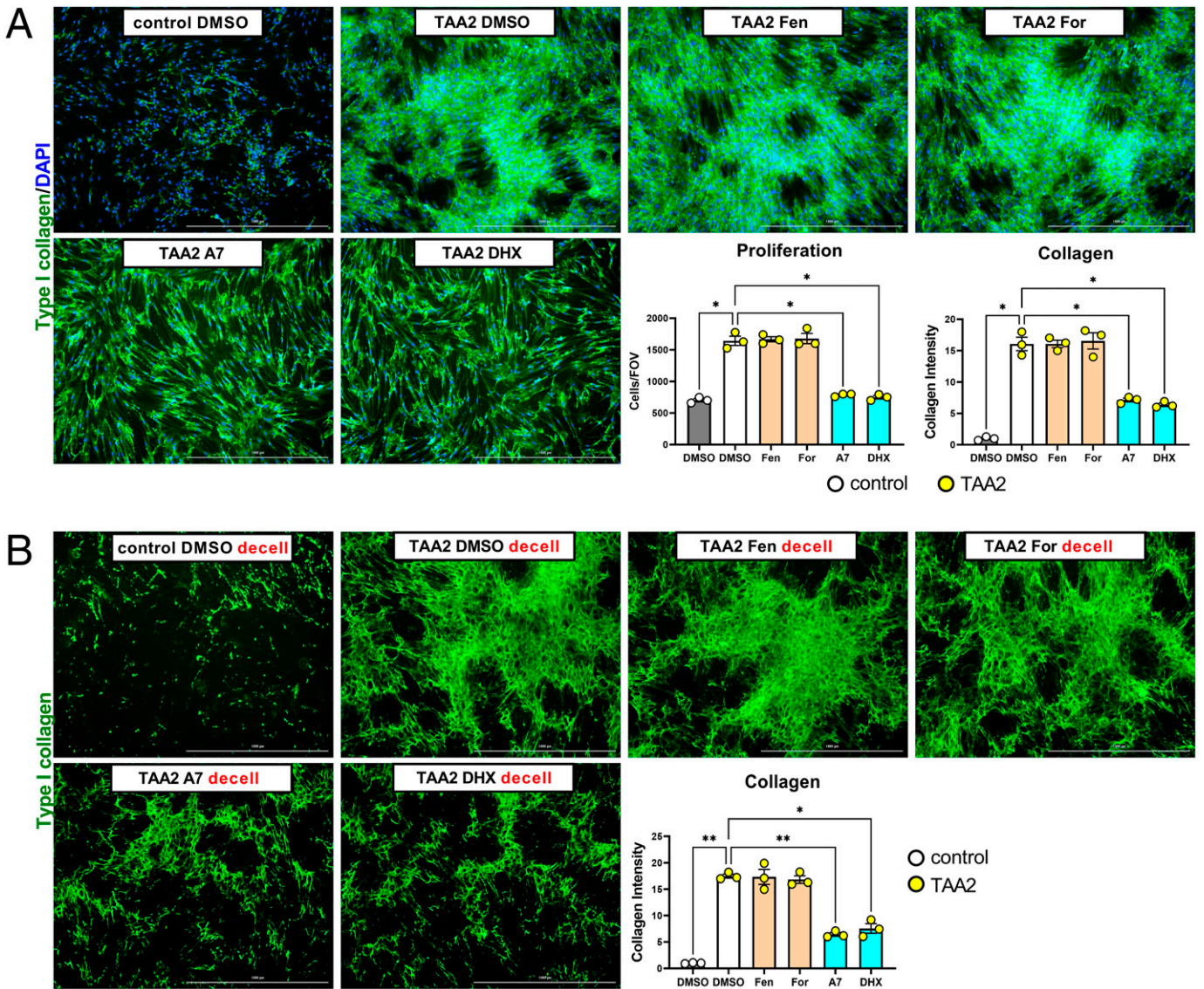
Fig. 5. Collagen deposition assay development. (A) Human lung fibroblasts were cultured +/− TAA2 for 3 days or media changed and cultured for another 3 days. Cells were fixed and immunostained for type I collagen and DAPI. Representative images from control (serum-starved media) compared with TAA2 stimulated cells on days 3 and 6. Proliferation and collagen intensity quantified by image analysis software. (B) Cells were plated and treated identical to above, prior to fixation the matrices were decellularized. Images were immunostained for type I collagen and DAPI. $N = 3$ independent experiments performed with three unique donor samples (*$P < 0.05$; **$P < 0.01$ vs. the indicated group).

where $\beta2$ receptors can enhance fluid clearance (Factor et al., 2002). Although $\beta2$ adrenergic receptor agonists did not sustain their antifibrotic effect under profibrotic conditions in the fibroblasts we examined, they may remain efficacious in other subtypes, driven by programs independent of TGF-$\beta1$.

Our study measured potency and efficacy (magnitude) of D1 and $\beta2$ agonists to elevate intracellular cAMP. Previous work found that cAMP response magnitude did not predict antifibrotic activity, including inhibition of proliferation and differentiation, in lung fibroblasts (Roberts et al., 2018). The authors of the study proposed the existence of a threshold of cAMP, above which further elevations in cAMP do not correlate with enhanced antifibrotic effects (Roberts et al., 2018). This proposition is strongly supported by our data comparing the impact of YAP/TAZ inhibition downstream of cAMP elevation. If we focus on our results from the D1 receptor agonists

in cells not stimulated with TGF-$\beta1$, there is a linear relationship between cAMP magnitude and YAP/TAZ inhibition best illustrated when comparing a full agonist such as dihydrexidine (maximum efficacy, $\sim$3-fold cAMP induction) to fenoldopam (maximum efficacy, $\sim$2-fold) that translates to full measured inhibition of YAP/TAZ localization with DHX but only partial inhibition with fenoldopam. However, this linear relationship is lost when examining the $\beta2$ agonists experiments, in which a full agonist such as formoterol elevates cAMP $\sim$9-fold and reduces nuclear YAP/TAZ localization equally to dihydrexidine that only elevates cAMP $\sim$3-fold. Suggesting a threshold around 3-fold cAMP could max out the antifibrotic potential of G α s coupled receptor agonists, and the threshold limitation is a product of a finite inhibition of YAP/TAZ nuclear localization and/or its finite impact on regulating profibrotic gene expression.

Fig. 6. D1 agonists inhibit fibroblast proliferation and collagen expression. Human lung fibroblasts were cultured +/− TAA2 for 3 days and media changed and cultured for another 3 days +/− TAA2 and the indicated compound: 1 μM fenoterol (Fen), 1 μM formoterol (For), 10 μM A 77636 (A7), and 10 μM dihydrexidine (DHX). (A) Cells were fixed and immunostained for type I collagen and DAPI, or (B) decellularized and immunostained for type I collagen. Representative images from each group are shown. Proliferation and collagen intensity quantified by image analysis software. $N = 3$ independent experiments performed with three unique donor samples (*$P < 0.05$ vs. the indicated group).

In the previous study identifying a loss of the antifibrotic, prostaglandin E2 receptor (*PTGER2*) in fibrotic fibroblasts, it was shown to be driven by promoter hypermethylation (Huang et al., 2010). Although this report did not investigate the role of TGF-β signaling, another later study found dramatic hypermethylation changes after exogenous TGF-β1 stimulation in cultured lung fibroblasts (Negreros et al., 2019). Alternatively, repression of the antifibrotic Relaxin 1 receptor (*RXFP1*) was shown to be driven by microRNA-144-3p. In our experiments, we were unable to detect expression of *RXFP1* by bulk RNA sequencing, however, microRNA-144-3p was shown to be regulated by TGF-β signaling so these findings could have overlapping mechanisms with our observations. Although not previously identified in the context of lung fibrosis, there have been multiple reports showing a loss of the β2-adrenergic receptor expression and function in response to TGF-β signaling in airway smooth

muscle cells (Nogami et al., 1994; Ojiaku et al., 2019; Mak et al., 2000). Intriguingly, one group also discovered that cotreatment with a corticosteroid, dexamethasone, rescued the loss of β2-adrenergic receptor mediated by TGF-β1 in airway smooth muscle cells. This offers another explanation to the observed benefits of combined treatment of beclomethasone, a corticosteroid, and formoterol, a long-acting β2-adrenoceptor agonist in IPF patients (Wright et al., 2017), although this would require further validation.

A remaining question remains as to why *DRD1* expression is regulated so uniquely? One potential hypothesis is that the ligand for dopamine signaling, rather than the receptor, is repressed during fibrosis and/or wound healing as a mechanism to allow for fibroblast expansion and ECM deposition. This is supported by our previous findings that show that the enzyme responsible for the biosynthesis of dopamine is repressed in

the lungs of patients with IPF (Haak et al., 2019). Overall, D1 dopamine receptor agonism results in a robust antifibrotic effect in lung fibroblasts and should be further explored as a treatment option for IPF.

Authorship Contributions

Participated in research design: Gao, Jones, Tschumperlin, Haak.

Conducted experiments: Gao, Diaz Espinosa, Nguyen, Link, Meridew, Gibbard, Haak.

Performed data analysis: Gao, Diaz Espinosa, Nguyen, Link, Meridew, Gibbard, Haak.

Wrote or contributed to the writing of the manuscript: Gao, Nguyen, Jones, Tschumperlin, Haak.

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