International Journal of
Molecular Sciences

MDPI

Article

Prostaglandin E2 (PGE2) and Roflumilast Involvement in IPF Progression

Noa Moshkovitz [1], Gali Epstein Shochet [1] and David Shitrit [1,2,*]

[1] Pulmonary Department, Meir Medical Center, Kfar Saba 44281, Israel; noamosh@gmail.com (N.M.); galieps@gmail.com (G.E.S.)
[2] Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv 69978, Israel
* Correspondence: davids3@clalit.org.il; Tel.: +972-9-747-2512

Abstract: The ECM propagates processes in idiopathic pulmonary fibrosis (IPF), leading to progressive lung scarring. We established an IPF-conditioned matrix (IPF-CM) system as a platform for testing drug candidates. Here, we tested the involvement of a PGE2 and PDE4 inhibitor, Roflumilast, in the IPF-CM system. Primary normal/IPF tissue-derived human lung fibroblasts (N/IPF-HLFs) were cultured on Matrigel and then removed to create the IPF-CM. N-HLFs were exposed to the IPF-CM/N-CM with/without PGE2 (1 nM) and Roflumilast (1 μM) for 24 h. The effect of the IPF-CM on cell phenotype and pro-fibrotic gene expression was tested. In addition, electronic records of 107 patients with up to 15-year follow-up were retrospectively reviewed. Patients were defined as slow/rapid progressors using forced vital capacity (FVC) annual decline. Medication exposure was examined. N-HLFs cultured on IPF-CM were arranged in large aggregates as a result of increased proliferation, migration and differentiation. A PGE2 and Roflumilast combination blocked the large aggregate formation induced by the IPF-CM ($p < 0.001$) as well as cell migration, proliferation, and pro-fibrotic gene expression. A review of patient records showed that significantly more slow-progressing patients were exposed to NSAIDs ($p = 0.003$). PGE2/PDE4 signaling may be involved in IPF progression. These findings should be further studied.

Keywords: IPF; PGE2; Roflumilast; extracellular matrix; fibroblast

Citation: Moshkovitz, N.; Epstein Shochet, G.; Shitrit, D. Prostaglandin E2 (PGE2) and Roflumilast Involvement in IPF Progression. *Int. J. Mol. Sci.* **2023**, *24*, 12393. https://doi.org/10.3390/ijms241512393

Academic Editor: Satish K. Madala

Received: 29 June 2023
Revised: 31 July 2023
Accepted: 3 August 2023
Published: 3 August 2023

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive interstitial pneumonia that is characterized by scar tissue formation and excessive extracellular matrix (ECM) deposition, resulting in the loss of lung function [1,2]. A major therapeutic challenge arises from the variability in the clinical course, as IPF patients may exhibit distinct, and unpredictable, patterns of disease progression [3–5]. Some patients show a slowly progressive disease ('slow' progressors (SP)), often with long duration of symptoms before diagnosis, while others display a rapidly progressive course ('rapid' progressors (RP)) [4]. Disease course may also be complicated by acute exacerbations, with dismal prognosis [3,6]. Although there are some distinguishing markers between the groups [3,7,8], most clinical trials do not take them into consideration [9–11]. At present, Nintedanib and Pirfenidone are the only FDA-approved IPF treatments [12,13]. Nonetheless, IPF still carries poor prognosis, as these treatments are only able to slow down disease progression. Thus, additional targets and treatment options are needed.

The ECM plays a significant role in fibrosis progression. As reviewed [14], IPF-ECM alone can induce normal lung fibroblasts to turn into activated myofibroblasts. Once formed, IPF-ECM sets up a pro-fibrotic feedback loop that is capable of sustaining progressive fibrosis. In support of this hypothesis, our group developed an in vitro IPF conditioned matrix (IPF-CM) system, which utilizes human primary lung fibroblasts (HLF) derived from patients with IPF. Normal tissue-derived HLFs (N-HLFs) added to IPF-CM were

shown to have increased migration and elevated pro-fibrotic markers. This platform allows us to investigate drug function with regard to fibroblast–ECM interaction [15–17].

COX-2 expression is regulated via several signals of injury and inflammation [18]. An abnormal expression of COX-2 has been linked to cancer and chronic inflammatory pathologies, including fibrosis [19]. Roflumilast, a long-acting selective inhibitor of the phosphodiesterase-4 (PDE4) enzyme, was shown to have anti-inflammatory effects in lung fibroblast models, and it is prescribed for other conditions, such as chronic obstructive pulmonary disease (COPD) [20,21]. PDE4 inhibition affects a broad spectrum of lung fibroblast functions, such as myofibroblast transition and ECM generation in vitro and mitigates bleomycin-induced lung fibrosis in vivo [22,23]. PDE4 inhibition was recently reviewed by Schick and Schlegel [24] as a possible therapeutic target.

In this study, we explored the effect of PGE2 and PDE4 inhibition on the IPF-CM system as further support to previously published evidence for this approach in IPF.

2. Results

2.1. PGE2 in Combination with Roflumilast Inhibits IPF-CMs' Pro-Fibrotic Effects on N-HLFs

The main outcome of the IPF-CM system is large aggregate formation [15]. As previously described, the IPF-CM elevates the aggregate size in comparison to N-CM after 24 h ($p < 0.001$) as well as the total number of cells. Recently, we combined the aggregate size and aggregate number values to create a new, more accurate parameter, the average aggregate size, as published in [17]. Here, we tested whether the PGE2 with/without the PDE4 inhibitor, Roflumilast, will have an effect in this system. The addition of PGE2 or Roflumilast alone showed a partial effect on relative aggregate size (IPF-CM vs. N-CM, Figure 1A) and cell counts (Figure 1B). However, the addition of PGE2 and Roflumilast resulted in a complete inhibition of large aggregate formation as well as a reduction in the relatively elevated cell counts ($p < 0.0001$ and $p < 0.05$, respectively, Figure 1A,B). To test whether this was a result of reduced cell migration or increased cell death, we performed a wound-healing assay and tested cell death by flow cytometry with PGE2 with/without Roflumilast. Results show that the addition of PGE2 and Roflumilast inhibits HLF migration ($p < 0.05$, Figure 1C) and total cell death ($p < 0.05$, Figure 1D). Thus, the combination of PGE2 and Roflumilast leads to a complete inhibition of IPF-CMs' effects on N-HLFs.

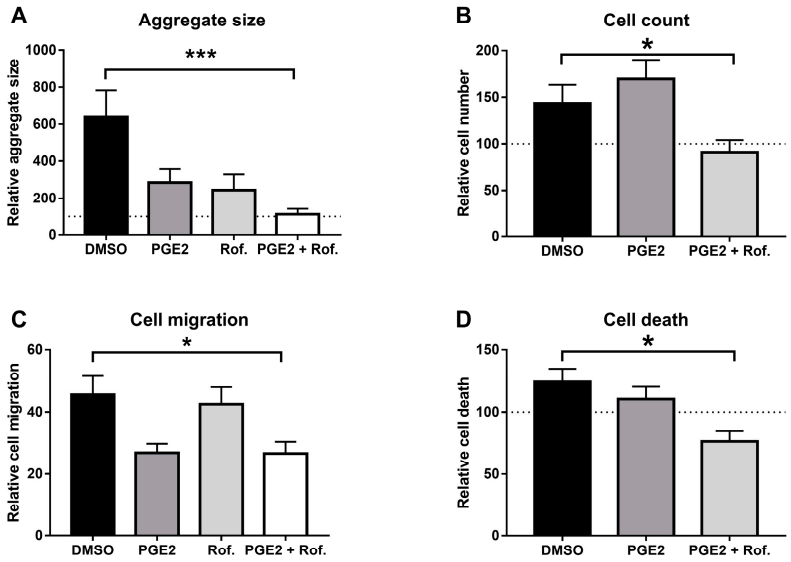

Figure 1. *Cont.*

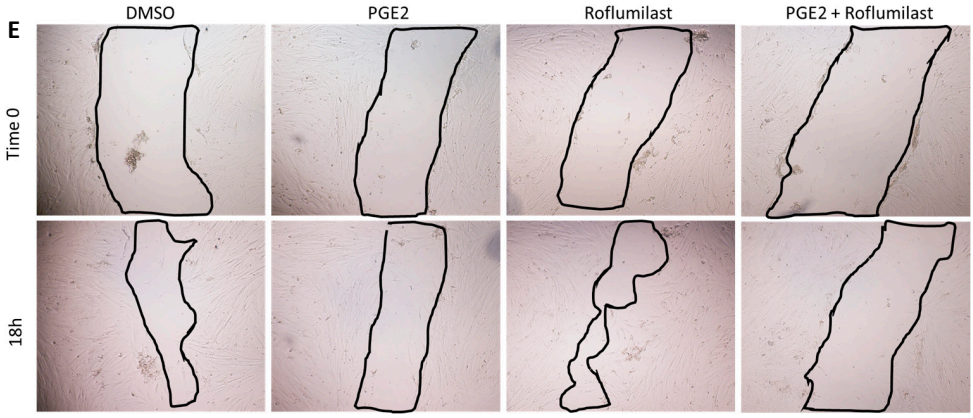

Figure 1. PGE2 and Roflumilast combination inhibits IPF-CMs large aggregate formation. Normal human lung fibroblasts (N-HLF) were cultured on N/IPF-CM for 24 h with/without PGE2 (1 nM) with/without Roflumilast (1 μM) or DMSO. Following culture, relative aggregate size was calculated (**A**), cells were harvested and counted (**B**) and then subjected to cell death analysis using flow-cytometry (**D**). To assess cell migration, N-HLFs were exposed to DMSO and PGE2 (1 nM) with/without Roflumilast (1 μM) by wound-healing assay (**C**,**E**). The results are presented as the relative aggregate size between IPF-CM and N-CM for each treatment with regard to the 100% dashed line, representing zero effect. PGE2—prostaglandin E2, IPF-CM—IPF-conditioned matrix, Rof.—Roflumilast. N ≥ 9, (* $p < 0.05$, *** $p < 0.0001$).

Pro-fibrotic gene expression was also tested. We found that COL1A, ACTA2, TGFB1 and MMP2, which were not affected by PGE2 alone, were completely inhibited by the addition of PGE2 + Roflumilast ($p < 0.05$, Figure 2A–D). IL-8 expression was not affected by the addition of PGE2 or Roflumilast (Figure 2E). Interestingly, HIF1A expression was completely inhibited by PGE2 alone, and it showed a similar result with the addition of Roflumilast ($p < 0.05$, Figure 2F). These findings may suggest that PGE2 pathway inhibition, using NSAIDs for instance, may inhibit disease progression.

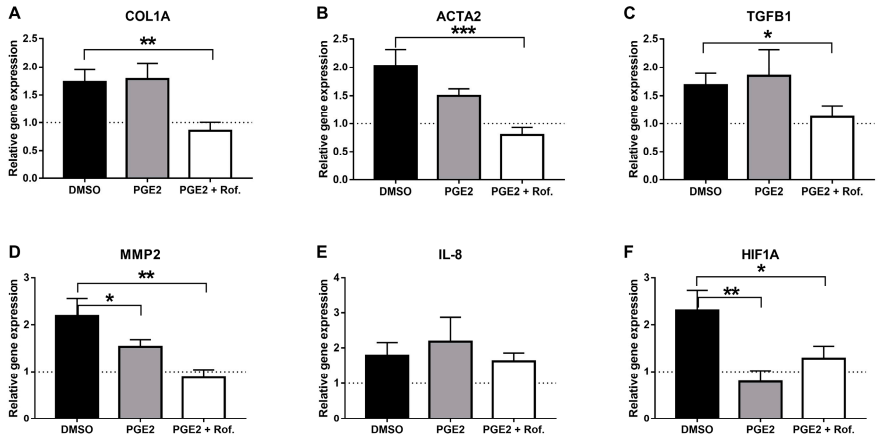

Figure 2. PGE2 and Roflumilast combination inhibits IPF-CMs' induction of pro-fibrotic gene expression. Normal human lung fibroblasts (N-HLF) were cultured on N/IPF-CM for 24 h with/without PGE2 (1 nM) with/without Roflumilast (1 μM) or DMSO. Following culture, cells were harvested for RNA extraction. The results are presented as the relative gene expression between IPF-CM and N-CM for each treatment, with regard to the 100% dashed line, representing zero effect. (**A**) Collagen type 1 alpha (COL1A), (**B**) alpha-SMA actin alpha 2 (ACTA2), (**C**) Transforming Growth Factor Beta 1 (TGFB1), (**D**) Matrix Metallopeptidase 2 (MMP2), (**E**) Interleukin 8 (IL-8), (**F**) hypoxia-inducible factor 1-alpha (HIF1A). PGE2—prostaglandin E2, IPF-CM—IPF-conditioned matrix. N ≥ 5 (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$).

In order to test the direct effect of PGE2 and Roflumilast on HLFs, cells were exposed to PGE2 and TGFβ with/without Roflumilast for 24 h. In accordance with the IPF-CMs' results, the addition of TGFβ increased, while PGE2 significantly decreased ACTA2 and COL1A mRNA expression. The addition of Roflumilast resulted in partial inhibition (Figure 3A,B). To assure this was not a result of reduced cell viability, cell death and numbers were also tested (Figure 3C,D). In fact, PGE2 addition to HLFs reduced cell death and slightly increased cell counts, suggesting that PGE2 addition promoted cell viability. Moreover, the addition of PGE2 together with TGFβ and Roflumilast did not change the rate of cell death or cell counts, indicating that the changes in pro-fibrotic gene expression were not directly associated with reduced cell viability in the IPF-CM system. Our previous work [17] showed that the HIF1A pathway is significantly activated in the IPF-CM using RNAseq and that the plasminogen activator inhibitor-1 (PAI1) protein (SERPINE1) is elevated. Thus, we tested PAI1 expression in the HLFs and found that exposure to PGE2 significantly reduced PAI1 protein expression. HIF1A protein levels were increased following TGFβ addition, yet they were not significantly changed by PGE2/Roflumilast directly (Figure 3E).

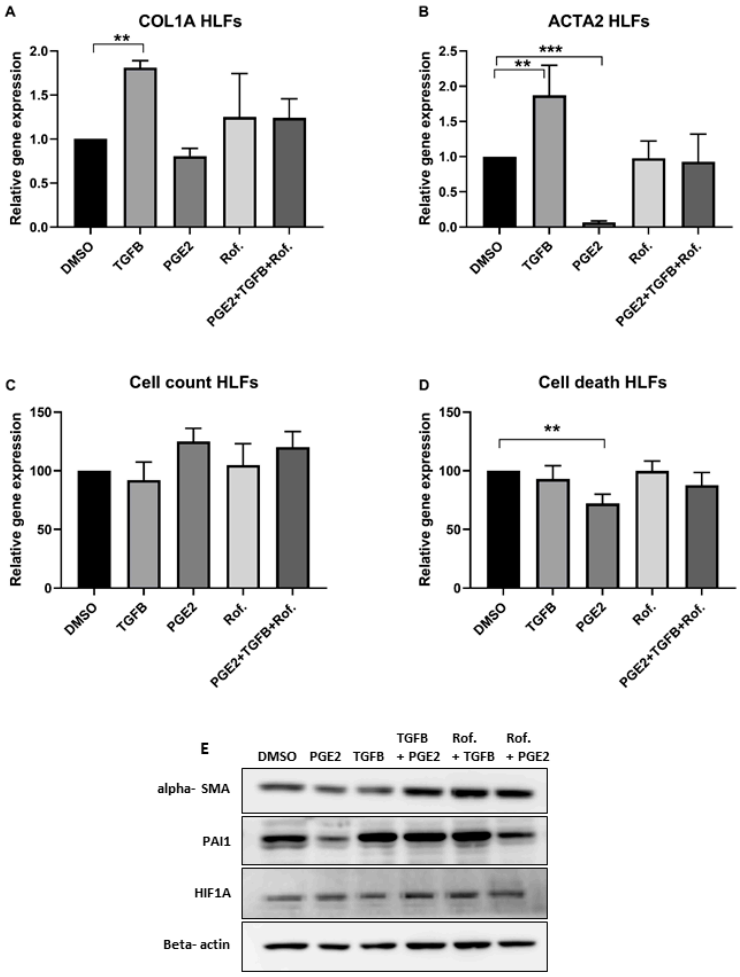

Figure 3. PGE2, TGFβ and Roflumilasts' direct effect of HLFs following 24 h. Normal human lung fibroblasts (N-HLF) were cultured for 24 h with/without PGE2 (1 nM), TGFβ with/without Roflumilast (1 μM) or DMSO. Following culture, cells were harvested for RNA/protein extraction (**A**,**B**,**E**) as well as counted (**C**) and analyzed for cell death using flow cytometry (**D**). The results are normalized to control DMSO. HIF1A—hypoxia-inducible factor 1-alpha. N ≥ 3. (** $p < 0.001$, *** $p < 0.0001$).

2.2. Slow-Progressing IPF Patients Were More Exposed to NSAIDs

To retrospectively test this hypothesis, we classified a cohort of IPF patients into slow and rapid progressors (SP/RP) and reviewed their medication intake. Study population included 107 patients diagnosed with IPF; of them, 62 were classified as SP and 45 as RP, as described in the Section 4. The average age at diagnosis was 66 ± 11 with 67% males. Patient demographics and comorbidities are presented in Table 1. Similar to previous reports [3], male predominance was significantly more pronounced in the RP group, which also included more patients with renal failure, yet it had no patients with malignancy (Table 1). Overall, survival, as well as transplant-free survival, was significantly higher in the SP group ($p < 0.001$, Figure 4).

Table 1. Patient characteristics according to the slow/rapid progressing IPF.

Parameter	Slow Progression N = 62	Rapid Progression N = 45	*p*-Value
Age, years	65.7 ± 12.3	66.3 ± 10.3	0.39
Gender (% male)	56%	67%	0.02
Smoking history	77%	78%	0.79
Cardiovascular disease	27%	24%	0.41
Renal failure	2%	9%	<0.001
Malignancy	25%	0	<0.001
Mortality	19%	54%	<0.001

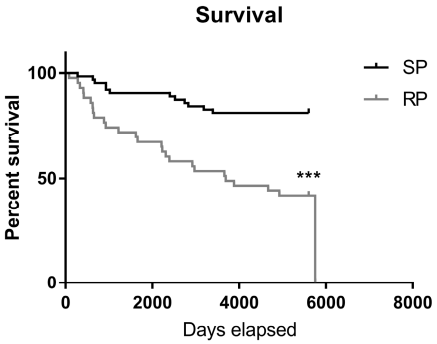

Figure 4. Survival curve of patients from SP and RP groups. Patient survival was analyzed using the Kaplan–Meier plot (*** indicates $p < 0.001$).

Moreover, during follow-up, pulmonary function tests were significantly better in patients from the SP group, including forced vital capacity (FVC), total lung capacity (TLC), DLCO and 6 min walking test (6MWT) parameters (Table 2).

Table 2. Pulmonary function tests of patients according to the slow/rapid progressing IPF.

Parameter	Slow Progression N = 62	Rapid Progression N = 45	*p*-Value
FVC, L	2.34 ± 0.82	1.85 ± 0.75	0.002
FVC, % predicted	77.51 ± 21	59.46 ± 17.9	0.001
FRC PL % predicted	79.24 ± 20.1	62.6 ± 15.4	<0.001
FRC, L	2.46 ± 0.66	1.98 ± 0.57	0.001
TLC, L	4.16 ± 1.11	3.22 ± 0.88	<0.001
TLC, % predicted	72.63 ± 16.6	57.23 ± 11.5	<0.001
DLCO, % predicted	54.51 ± 15	42.27 ± 13.2	<0.001
6 min walk test			
SAT rest, %	96.5 ± 2	95.13 ± 2.3	0.017
SAT exercise, %	90.90 ± 5.3	86.17 ± 4.8	0.001

Table 2. *Cont.*

Parameter	Slow Progression N = 62	Rapid Progression N = 45	*p*-Value
HR rest	78.31 ± 10.3	78.08 ± 12.6	0.940
HR exercise	110.2 ± 18	107.6 ± 20	0.619
Distance, m	384.4 ± 150.4	307 ± 153.9	0.053

Forced vital capacity (FVC), functional residual capacity (FRC), total lung capacity (TLC), single-breath diffusing capacity for carbon monoxide (DLCO), saturation (SAT), heart rate (HR).

Most patients with IPF, as with other patients over the age of 60, suffer from various comorbidities and are prescribed several concomitant medications. These include proton pump inhibitors, anticoagulants, statins, antihypertensive drugs, nonsteroidal anti-inflammatory drugs (NSAIDs), etc. [25]. Thus, we examined medication exposure to the five major groups of concomitant treatments between the SP and RP patients during the follow-up period. There were no significant differences between the groups regarding usage of statins (p = 0.833, Figure 5A), angiotensin-converting enzyme (ACE) inhibitors (p = 0.71, Figure 5B), beta blockers (p = 0. 409, Figure 5C) or serotonin-specific reuptake inhibitors (SSRI) (p = 0.271, Figure 5D). Interestingly, there was a significant difference in exposure to NSAIDs, with 15 SP patients being exposed to this class of drugs as opposed to only two patients in the RP group (p = 0.003, Figure 5E).

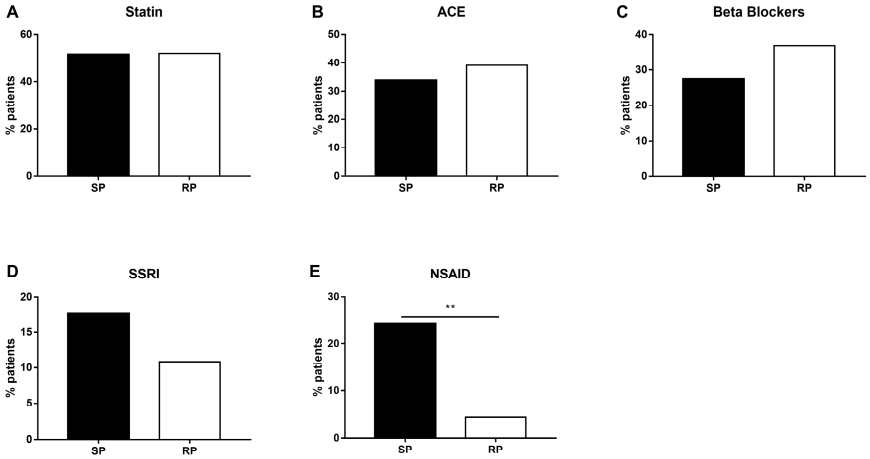

Figure 5. Medication exposure in IPF patients during the study period. Medication exposure to the following five classes of drugs: statins (**A**), angiotensin-converting enzyme (ACE) inhibitors/angiotensin type 1 receptor blockers (ARB1) (**B**), beta blockers (**C**), serotonin-specific reuptake inhibitors (SSRI) (**D**) and NSAIDs (including COX-2 inhibitors) (**E**) were examined, starting 6 months prior to diagnosis of IPF until end of follow-up. Comparison was made between the slow and rapid progressors (SP vs. RP). (** indicates $p < 0.01$).

3. Discussion

The ECM plays a major role in IPF progression [15]. In this work, we utilized the already established IPF-CM system to explore the involvement of PGE2 in the IPF–HLF–ECM interplay. We showed that PGE2 and Roflumilast combination completely inhibited IPF-CM pro-fibrotic effects on N-HLFs. These findings were then supported by a small cohort of patients, which were followed up for up to 15 years, showing that in fact, slow-progressing IPF patients were more exposed to NSAIDs than the rapidly progressing group.

PGE2 was shown to inhibit fibroblast proliferation, collagen synthesis and the modulation of TGFβ-induced fibroblast to myofibroblast transition [26–28]. Moreover, increased PGE2 levels were reported in bleomycin-induced lung fibrosis in murine models [29]. The crosstalk between TGFβ, which was previously found to be activated in the IPF-CM system [30], to PGE2 is complex [31–34]. On the one hand, PGE2 can inhibit fibroblast

proliferation, the synthesis of collagen and modulation of TGFβ-induced fibroblast to myofibroblast transition [19]. On the other hand, TGFβ is also known to increase PGE2 levels. This issue was reviewed by Bozyk et al. [19], where they suggested a way by which PGE2 can either promote or inhibit the fibrotic process, according to the four E Prostanoid (EP) receptors (EP1–EP4), while the cellular response to prostaglandins depends on the receptor they harbor and the PGE2 concentration [35,36]. Since these were not tested, it is hard to draw further conclusions. Moreover, matrix stiffness also affects prostaglandin expression [37]. Here, we showed that the addition of PGE2 alone was not sufficient, and the combination of several factors is required for complete inhibition.

Roflumilast, a long-acting selective inhibitor of PDE4, had a positive effect by reducing aggregate size and cell migration. The Roflumilast + PGE2 combination inhibited the increase in pro-fibrotic gene expression. This is supported by in vitro [22,23], as well as by in vivo [38] works that showed PDE4 inhibition to reduce lung fibrosis. Our results demonstrate these findings in a human-derived model of primary lung cells. This is important, as the UIP pattern is specific to humans [39], thus giving further validation to previously published results.

Interestingly, Roflumilast with PGE2 had a significantly stronger effect than Roflumilast alone. PDE4 inhibitors, such as Roflumilast, prevent the breakdown of cAMP, while TGFβ modulates cAMP by altering the metabolism of PGE2. The effect of PDE4 inhibitors may have been mediated through a cAMP-stimulated protein kinase, and it depended on fibroblast production of PGE2 and TGFβ-induced PGE2 production. From our results, which show a significant effect of PGE2 addition alone, we can carefully assume that PGE2 levels in the culture are not saturated. Thus, by adding PGE2, we somewhat increased the 'inflammatory' state of the culture, also affecting TGFβ signaling and mimicking an inflammation that often, but not always, is present in IPF. Cortijo et al. [23] suggested several mechanisms by which Roflumilast may attenuate lung fibrosis. Of them, it was shown that Roflumilast reduced collagen mRNA expression as well as TGFβ1 formation in BLM treated mice. Furthermore, an in vitro study using fetal lung fibroblasts stimulated with TNF-alpha highlighted that the effects of Roflumilast on intercellular adhesion molecule-1 (ICAM-1) and eotaxin release were more prominent in the presence of PGE2, as in our system. Moreover, Sabatini et al. [40] pointed out that Roflumilast N-oxide diminished the TGFB1-induced expression of alpha-SMA and transcripts of connective tissue growth factor (CTGF) and fibronectin in the presence of basic fibroblast growth factor (bFGF). This is interesting, as our previous reports showed increased levels of both bFGF and TNF-alpha in the supernatants of IPF HLF and IPF-CM cultures [41], and that nintedanib, which blocks the FGF receptor (FGFR), was shown to have a significant impact in the IPF-CM system [15,16]. Additional reports suggested that PDE4 inhibitors, together with TGFβ, resulted in augmented PGE2 production along with an increased expression of COX mRNA and protein [21].

As recently reviewed by Claire Lugnier, PDE4 inhibition has many possible downstream pathways, leading to reduced inflammation, reduced oxidative stress, TNF-α, and cytokine production [42], which are all relevant for IPF progression.

Our findings require further research, as there may be several mechanisms of action to achieve the reduction in aggregate formation, which was previously shown to be correlated with the pro-fibrotic phenotype of the HLFs. However, although we did not elucidate the exact mechanism, we prove further evidence of the potential benefit of this already approved treatment for another indication. Due to the inclusion criteria, patients with COPD were not included, and thus, no data regarding Roflumilast were available in this cohort.

HIF1A is a facilitator of fibrosis, and it has been considered a pro-inflammatory factor [43] as well as a regulator of pro-fibrotic mediator production [44,45]. An interesting finding was that the HIF1A was significantly inhibited by PGE2 itself. A recent study conducted in our lab showed that HLFs cultured on IPF-CM showed an over-expression of HIF1A. Moreover, the HIF1A signaling pathway was the most over-expressed pathway

in the analysis of RNA-sequencing of HLFs cultured on the IPF-CM with significant involvement of PAI1 (SERPINE1) [17]. Ivanova et al. showed that PGE2 suppresses HIF1A expression, thus supporting our results [46]. That study also deducted that PGE2 could be used to reduce fibrosis in IPF. Moreover, it was recently shown that PDE4 inhibition significantly decreased CTGF, PAI-1, collagen 1A1 and fibronectin mRNA in TGFβ-stimulated human mesangial cells [47]. These results are in accordance to our results, as the IPF-CM system was previously shown to activate TGF-β signaling [15].

Despite its inevitably progressive nature, IPF is characterized by a highly variable disease course, which makes the natural history of the disease largely unpredictable in individual patients [48]. Our study aimed to examine the influence of certain common medication groups, unrelated to IPF, on disease progression. The majority of patients with IPF, as other patients over the age of 60, suffer from various comorbidities and are prescribed several concomitant medications. These include proton pump inhibitors, anticoagulants, statins, antihypertensive drugs, nonsteroidal anti-inflammatory drugs (NSAIDs), etc. [25]. Some of these treatments were already implicated in having an effect on fibrotic processes. For instance, angiotensin type 1 receptor blockers (ARB1) and ACE inhibitors were previously shown to suppress the release of TGFβ and diminish connective tissue synthesis [49]. A recent study that retrospectively explored the effect of cardiovascular drugs on IPF progression in 323 patients suggested that statin therapy may be beneficial for IPF progression [50]. Similarly, SSRIs were also implicated to have an effect of ILD progression [51].

In this relatively preliminary analysis, we examined medication exposure to five major groups of concomitant treatments between the SP and RP patients during the follow-up period of up to 15 years. The medications were grouped into major categories, as the population size was limited. Here, we demonstrated that patients classified as RP were less exposed to NSAID treatment during the course of their illness. Other treatment groups did not reach significance, although some trends were observed. It is important to emphasize, however, that drug exposure in our study was not limited to selective COX-2 inhibitors. Naproxen, a classical NSAID, was found to be effective in reducing lung inflammation and preventing collagen accumulation in a murine model of bleomycin-induced pulmonary fibrosis [52]. Interestingly, metastatic and fibrotic processes were shown to share similar characteristics as well as signaling pathways (e.g., TGFβ and MAPK) (reviewed in [53]). One of the major pathways in cancer, c-Met, was shown to be inhibited by NSAIDs when administrated at an early stage [54,55]. In fact, studies suggested that the combination of COX-2 inhibitors with current treatments may improve survival from lung cancer [56]. Aspirin not only blocks the biosynthesis of prostaglandins but also stimulates the endogenous production of anti-inflammatory and pro-resolving mediators [57,58].

Our study has several limitations. First of all, it is a retrospective cohort based on electronic databases where medication exposure is based on those purchased by patients. We were also not able to assess the indications for which the medications were prescribed, which may also influence outcome.

Secondly, the follow-up period was very diverse among patients, (i.e., from 1 to 15 years). Hence, based on the inclusion criteria, a significant drug exposure could range from 6 months to several years, resulting in difficulty interpreting the actual influence of drug exposure among various patients. Thirdly, naturally due to the inconsistent nature of NSAID exposure, we had to define a certain cutoff level. However, as discussed above, the timing of exposure to this class of drugs during disease progression could prove important. Since this is an in vitro study, all drawn conclusions should be cautiously considered.

IPF fibroblasts modify the ECM differently than normal fibroblasts, thus creating a CM that further propagates the 'IPF-like' phenotype of normal fibroblasts. We found that PGE2 and Roflumilast inhibit the pro-fibrotic effects of IPF-CM. Further research is needed in order to establish this as a possible treatment for IPF.

4. Materials and Methods

4.1. IPF-CM Model

Primary HLFs were isolated from IPF tissues and control samples at the time of biopsy, as described [17,41]. Following extraction, HLFs were cultured in DMEM supplemented with 20% FCS, L-glutamine (2 mM) with antibiotics (IMBH, Beit Haemek, Israel) and maintained in 5% CO_2 at 37 °C. Experiments were performed as previously described [15]. Briefly, IPF/N-HLFs were cultured on Matrigel (Corning, NY, USA). Following 48 h, cells were removed by NH_4OH, and N-HLFs were added for further culture. PGE2 (1 nM, PeproTech, Cranbury, NJ, USA) and Roflumilast (1 μM, Boringher Ingelheim, Ingelheim am Rhein, Germany) were diluted in DMSO and added an hour prior to the addition of N-HLFs to the culture.

4.2. Aggregate Size Measurement

Cell cultures were inspected utilizing an Olympus IX71 microscope. The aggregate size and number were evaluated utilizing the ImageJ software: https://imagej.nih.gov/ij/download.html, accessed on 1 January 2023. Results were normalized to control (N-CM).

4.3. Real-Time Quantitative PCR

RNA was extracted by a RNeasy kit (Qiagen, Germany) and converted to cDNA using GeneAmp (Applied Biosystems, Weierstadt, Germany). Reactions were performed with SYBR Green (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's guidelines. Reactions were set to 40 cycles. Primers sequences (purchased from Hylabs, Rehovot, Israel) (5′-3′): GAPDH: F-CTCTGCTCCTCCTGTTCGAC, R-TTAAAAGCAGCCCTGGTGAC; MMP2: F-CAAGGACCGGTTTATTTGGC, R-ATTCCCTGCGAAGAACACAGC; IL8: F-CTCTTGGCAGCCTTCCTGATTT, R-TGGGGTGGAAAGGTTTGGAGTA; COL1A: F-CGAAGACATCCCACCAATCAC, R-CAGATCACGTCATCGCACAAC; ACTA2: F-TGAGAAGAGTTACGAGTTGCCTGAT, R-GCAGACTCCATCCCGATGAA; TGFB1: F-TTTTGATGTCACCGGAGTTG, R-AACCCGTTGATGTCCACTTG. GAPDH was used as control.

4.4. Cell Migration

HLFs (5×10^4) were placed in 96-well plates and allowed to adhere for 24 h. Wound closure was monitored immediately after scratching and at 24 h. Areas were measured using ImageJ (http://rsbweb.nih.gov/ij/, accessed on 1 January 2023).

4.5. Cell Death

Assessment of apoptosis/necrosis was conducted with AnnexinV-FITC supplemented with PI (MEBCYTO®, MBL international, Woburn, MA, USA) by flow cytometry according to the manufacturer's instructions. AnnexinV+/PI− cells were considered apoptotic, and AnnexinV+/PI+ cells were considered late apoptotic/necrotic. Total cell death was the sum of both. All results are expressed as the percent of the total cell number.

4.6. Western Blot

Western blot was performed as previously described [17,41]. Antibodies are listed in Supplementary Table S1. Bound antibodies were visualized using goat peroxidase-conjugated secondary antibodies (Supplementary Table S1, doi:10.6084/m9.figshare.23708280) followed by enhanced chemiluminescence detection (Millipore, Temecula, CA, USA). LAS3000 (Fujifilm, Tokyo, Japan) was used to quantify protein expressions.

4.7. Electronic Record Retrospective Study Population

The study group consisted of a cohort of individuals aged > 18 with IPF according to the guidelines [59].

4.8. Data Collection

Data were retrospectively retrieved from patients' electronical medical records from 5/2003 to 7/2018. Medical records were reviewed and data on serial lung function tests, HRCT of the chest, right heart catheterization and echocardiography were collected for all patients. None of the subjects had a history of relevant occupational or environmental exposure or clinical features of hypersensitivity pneumonitis or connective tissue disease. All patients had negative autoimmune serologic testing.

4.9. Statistical Analysis

Statistical analysis was done using GraphPad Prism version 8.00 for Windows (GraphPad Software, La Jolla, CA, USA) and by SPSS (IBM, Armonk, NY, USA). ANOVA was performed to compare differences between multiple cohorts. Paired Student's *t*-tests were employed to analyze differences between two groups. An effect was considered significant when the *p*-value was <0.05. All experiments were repeated at least three times.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms241512393/s1.

Author Contributions: Conceptualization, G.E.S. and D.S.; methodology, N.M. and G.E.S.; formal analysis, G.E.S.; investigation, N.M.; resources, D.S.; data curation, N.M.; writing—original draft preparation, N.M.; writing—review and editing, G.E.S. and D.S.; visualization, G.E.S. and N.M.; supervision D.S.; project administration, G.E.S.; funding acquisition, D.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: This study was conducted in accordance with the amended Declaration of Helsinki. The study is part of a large study named 'Microenvironments role in idiopathic pulmonary fibrosis (IPF) disease progression', which was approved by the Ethics Committee of the Meir Medical Center (MMC-016-16, approved on 15 March 2016).

Informed Consent Statement: Informed consent was obtained from all patients. The retrospective data collection was approved by the Institutional Review Board of Meir Medical Center (No. 177-17-MMC, approved on 15 October 2017). Individual patients' informed consent was not required.

Data Availability Statement: Data will be made available upon request.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Raghu, G.; Remy-Jardin, M.; Myers, J.L.; Richeldi, L.; Ryerson, C.J.; Lederer, D.J.; Behr, J.; Cottin, V.; Danoff, S.K.; Morell, F.; et al. Diagnosis of Idiopathic Pulmonary Fibrosis. An Official ATS/ERS/JRS/ALAT Clinical Practice Guideline. *Am. J. Respir. Crit. Care Med.* **2018**, *198*, e44–e68. [CrossRef] [PubMed]
2. American Thoracic Society. Idiopathic pulmonary fibrosis: Diagnosis and treatment. International consensus statement. American Thoracic Society (ATS), and the European Respiratory Society (ERS). *Am. J. Respir. Crit. Care Med.* **2000**, *161*, 646–664. [CrossRef] [PubMed]
3. Selman, M.; Carrillo, G.; Estrada, A.; Mejia, M.; Becerril, C.; Cisneros, J.; Gaxiola, M.; Perez-Padilla, R.; Navarro, C.; Richards, T.; et al. Accelerated variant of idiopathic pulmonary fibrosis: Clinical behavior and gene expression pattern. *PLoS ONE* **2007**, *2*, e482. [CrossRef] [PubMed]
4. McCormack, F.X.; King, T.E., Jr.; Bucher, B.L.; Nielsen, L.; Mason, R.J. Surfactant protein A predicts survival in idiopathic pulmonary fibrosis. *Am. J. Respir. Crit. Care Med.* **1995**, *152*, 751–759. [CrossRef]
5. Martinez, F.J.; Safrin, S.; Weycker, D.; Starko, K.M.; Bradford, W.Z.; King, T.E., Jr.; Flaherty, K.R.; Schwartz, D.A.; Noble, P.W.; Raghu, G.; et al. The clinical course of patients with idiopathic pulmonary fibrosis. *Ann. Intern. Med.* **2005**, *142*, 963–967. [CrossRef]
6. Ambrosini, V.; Cancellieri, A.; Chilosi, M.; Zompatori, M.; Trisolini, R.; Saragoni, L.; Poletti, V. Acute exacerbation of idiopathic pulmonary fibrosis: Report of a series. *Eur. Respir. J.* **2003**, *22*, 821–826. [CrossRef]
7. Trujillo, G.; Meneghin, A.; Flaherty, K.R.; Sholl, L.M.; Myers, J.L.; Kazerooni, E.A.; Gross, B.H.; Oak, S.R.; Coelho, A.L.; Evanoff, H.; et al. TLR9 differentiates rapidly from slowly progressing forms of idiopathic pulmonary fibrosis. *Sci. Transl. Med.* **2010**, *2*, 57–82. [CrossRef]

8. Balestro, E.; Calabrese, F.; Turato, G.; Lunardi, F.; Bazzan, E.; Marulli, G.; Biondini, D.; Rossi, E.; Sanduzzi, A.; Rea, F.; et al. Immune Inflammation and Disease Progression in Idiopathic Pulmonary Fibrosis. *PLoS ONE* **2016**, *11*, e0154516. [CrossRef]
9. Richeldi, L.; du Bois, R.M.; Raghu, G.; Azuma, A.; Brown, K.K.; Costabel, U.; Cottin, V.; Flaherty, K.R.; Hansell, D.M.; Inoue, Y.; et al. Efficacy and safety of nintedanib in idiopathic pulmonary fibrosis. *N. Engl. J. Med.* **2014**, *370*, 2071–2082. [CrossRef]
10. Azuma, A.; Nukiwa, T.; Tsuboi, E.; Suga, M.; Abe, S.; Nakata, K.; Taguchi, Y.; Nagai, S.; Itoh, H.; Ohi, M.; et al. Double-blind, placebo-controlled trial of pirfenidone in patients with idiopathic pulmonary fibrosis. *Am. J. Respir. Crit. Care Med.* **2005**, *171*, 1040–1047. [CrossRef]
11. Noble, P.W.; Albera, C.; Bradford, W.Z.; Costabel, U.; Glassberg, M.K.; Kardatzke, D.; King, T.E., Jr.; Lancaster, L.; Sahn, S.A.; Szwarcberg, J.; et al. Pirfenidone in patients with idiopathic pulmonary fibrosis (CAPACITY): Two randomised trials. *Lancet* **2011**, *377*, 1760–1769. [CrossRef]
12. Lederer, D.J.; Martinez, F.J. Comment on "Idiopathic Pulmonary Fibrosis". *N. Engl. J. Med.* **2018**, *379*, 797–798. [CrossRef]
13. Lederer, D.J.; Martinez, F.J. Idiopathic Pulmonary Fibrosis. *N. Engl. J. Med.* **2018**, *378*, 1811–1823. [CrossRef]
14. Herrera, J.; Henke, C.A.; Bitterman, P.B. Extracellular matrix as a driver of progressive fibrosis. *J. Clin. Investig.* **2018**, *128*, 45–53. [CrossRef]
15. Epstein Shochet, G.; Wollin, L.; Shitrit, D. Fibroblast-matrix interplay: Nintedanib and pirfenidone modulate the effect of IPF fibroblast-conditioned matrix on normal fibroblast phenotype. *Respirology* **2018**, *23*, 756–763. [CrossRef]
16. Epstein-Shochet, G.; Pham, S.; Beck, S.; Naiel, S.; Mekhael, O.; Revill, S.; Hayat, A.; Vierhout, M.; Bardestein-Wald, B.; Shitrit, D.; et al. Inhalation: A means to explore and optimize nintedanib's pharmacokinetic/pharmacodynamic relationship. *Pulm. Pharmacol. Ther.* **2020**, *63*, 101933. [CrossRef]
17. Epstein Shochet, G.; Bardenstein-Wald, B.; McElroy, M.; Kukuy, A.; Surber, M.; Edelstein, E.; Pertzov, B.; Kramer, M.R.; Shitrit, D. Hypoxia Inducible Factor 1A Supports a Pro-Fibrotic Phenotype Loop in Idiopathic Pulmonary Fibrosis. *Int. J. Mol. Sci.* **2021**, *22*, 3331. [CrossRef]
18. Kawano, T.; Anrather, J.; Zhou, P.; Park, L.; Wang, G.; Frys, K.A.; Kunz, A.; Cho, S.; Orio, M.; Iadecola, C. Prostaglandin E2 EP1 receptors: Downstream effectors of COX-2 neurotoxicity. *Nat. Med.* **2006**, *12*, 225–229. [CrossRef]
19. Bozyk, P.D.; Moore, B.B. Prostaglandin E2 and the pathogenesis of pulmonary fibrosis. *Am. J. Respir. Cell Mol. Biol.* **2011**, *45*, 445–452. [CrossRef]
20. Rennard, S.I.; Calverley, P.M.; Goehring, U.M.; Bredenbroker, D.; Martinez, F.J. Reduction of exacerbations by the PDE4 inhibitor roflumilast--the importance of defining different subsets of patients with COPD. *Respir. Res.* **2011**, *12*, 18. [CrossRef]
21. Togo, S.; Liu, X.; Wang, X.; Sugiura, H.; Kamio, K.; Kawasaki, S.; Kobayashi, T.; Ertl, R.F.; Ahn, Y.; Holz, O.; et al. PDE4 inhibitors roflumilast and rolipram augment PGE2 inhibition of TGF-{beta}1-stimulated fibroblasts. *Am. J. Physiol. Lung Cell Mol. Physiol.* **2009**, *296*, L959–L969. [CrossRef] [PubMed]
22. Hatzelmann, A.; Morcillo, E.J.; Lungarella, G.; Adnot, S.; Sanjar, S.; Beume, R.; Schudt, C.; Tenor, H. The preclinical pharmacology of roflumilast—A selective, oral phosphodiesterase 4 inhibitor in development for chronic obstructive pulmonary disease. *Pulm. Pharmacol. Ther.* **2010**, *23*, 235–256. [CrossRef] [PubMed]
23. Cortijo, J.; Iranzo, A.; Milara, X.; Mata, M.; Cerda-Nicolas, M.; Ruiz-Sauri, A.; Tenor, H.; Hatzelmann, A.; Morcillo, E.J. Roflumilast, a phosphodiesterase 4 inhibitor, alleviates bleomycin-induced lung injury. *Br. J. Pharmacol.* **2009**, *156*, 534–544. [CrossRef] [PubMed]
24. Schick, M.A.; Schlegel, N. Clinical Implication of Phosphodiesterase-4-Inhibition. *Int. J. Mol. Sci.* **2022**, *23*, 1209. [CrossRef] [PubMed]
25. Brunnemer, E.; Walscher, J.; Tenenbaum, S.; Hausmanns, J.; Schulze, K.; Seiter, M.; Heussel, C.P.; Warth, A.; Herth, F.J.F.; Kreuter, M. Real-World Experience with Nintedanib in Patients with Idiopathic Pulmonary Fibrosis. *Respir. Int. Rev. Thorac. Dis.* **2018**, *95*, 301–309. [CrossRef]
26. Kolodsick, J.E.; Peters-Golden, M.; Larios, J.; Toews, G.B.; Thannickal, V.J.; Moore, B.B. Prostaglandin E2 inhibits fibroblast to myofibroblast transition via E. prostanoid receptor 2 signaling and cyclic adenosine monophosphate elevation. *Am. J. Respir. Cell Mol. Biol.* **2003**, *29*, 537–544. [CrossRef]
27. McAnulty, R.J.; Hernandez-Rodriguez, N.A.; Mutsaers, S.E.; Coker, R.K.; Laurent, G.J. Indomethacin suppresses the anti-proliferative effects of transforming growth factor-beta isoforms on fibroblast cell cultures. *Biochem. J.* **1997**, *321 Pt 3*, 639–643. [CrossRef]
28. Thomas, P.E.; Peters-Golden, M.; White, E.S.; Thannickal, V.J.; Moore, B.B. PGE(2) inhibition of TGF-beta1-induced myofibroblast differentiation is Smad-independent but involves cell shape and adhesion-dependent signaling. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **2007**, *293*, L417–L428. [CrossRef]
29. Moore, B.B.; Ballinger, M.N.; White, E.S.; Green, M.E.; Herrygers, A.B.; Wilke, C.A.; Toews, G.B.; Peters-Golden, M. Bleomycin-induced E prostanoid receptor changes alter fibroblast responses to prostaglandin E2. *J. Immunol.* **2005**, *174*, 5644–5649. [CrossRef]
30. Epstein Shochet, G.; Brook, E.; Bardenstein-Wald, B.; Shitrit, D. TGF-beta pathway activation by idiopathic pulmonary fibrosis (IPF) fibroblast derived soluble factors is mediated by IL-6 trans-signaling. *Respir. Res.* **2020**, *21*, 56. [CrossRef]
31. Ramirez-Yanez, G.O.; Hamlet, S.; Jonarta, A.; Seymour, G.J.; Symons, A.L. Prostaglandin E2 enhances transforming growth factor-beta 1 and TGF-beta receptors synthesis: An in vivo and in vitro study. *Prostaglandins Leukot. Essent. Fat. Acids* **2006**, *74*, 183–192. [CrossRef]

32. Hui, A.Y.; Dannenberg, A.J.; Sung, J.J.; Subbaramaiah, K.; Du, B.; Olinga, P.; Friedman, S.L. Prostaglandin E2 inhibits transforming growth factor beta 1-mediated induction of collagen alpha 1(I) in hepatic stellate cells. *J. Hepatol.* **2004**, *41*, 251–258. [CrossRef]
33. Boak, A.M.; Roy, R.; Berk, J.; Taylor, L.; Polgar, P.; Goldstein, R.H.; Kagan, H.M. Regulation of lysyl oxidase expression in lung fibroblasts by transforming growth factor-beta 1 and prostaglandin E2. *Am. J. Respir. Cell Mol. Biol.* **1994**, *11*, 751–755. [CrossRef]
34. Mukherjee, S.; Sheng, W.; Michkov, A.; Sriarm, K.; Sun, R.; Dvorkin-Gheva, A.; Insel, P.A.; Janssen, L.J. Prostaglandin E2 inhibits profibrotic function of human pulmonary fibroblasts by disrupting Ca^{2+} signaling. *Am. J. Physiol. Lung Cell Mol. Physiol.* **2019**, *316*, L810–L821. [CrossRef] [PubMed]
35. Huang, S.K.; Wettlaufer, S.H.; Hogaboam, C.M.; Flaherty, K.R.; Martinez, F.J.; Myers, J.L.; Colby, T.V.; Travis, W.D.; Toews, G.B.; Peters-Golden, M. Variable prostaglandin E2 resistance in fibroblasts from patients with usual interstitial pneumonia. *Am. J. Respir. Crit. Care Med.* **2008**, *177*, 66–74. [CrossRef] [PubMed]
36. White, K.E.; Ding, Q.; Moore, B.B.; Peters-Golden, M.; Ware, L.B.; Matthay, M.A.; Olman, M.A. Prostaglandin E2 mediates IL-1beta-related fibroblast mitogenic effects in acute lung injury through differential utilization of prostanoid receptors. *J. Immunol.* **2008**, *180*, 637–646. [CrossRef] [PubMed]
37. Berhan, A.; Harris, T.; Jaffar, J.; Jativa, F.; Langenbach, S.; Lonnstedt, I.; Alhamdoosh, M.; Ng, M.; Lee, P.; Westall, G.; et al. Cellular Microenvironment Stiffness Regulates Eicosanoid Production and Signaling Pathways. *Am. J. Respir. Cell Mol. Biol.* **2020**, *63*, 819–830. [CrossRef]
38. Sisson, T.H.; Christensen, P.J.; Muraki, Y.; Dils, A.J.; Chibucos, L.; Subbotina, N.; Tohyama, K.; Horowitz, J.C.; Matsuo, T.; Bailie, M.; et al. Phosphodiesterase 4 inhibition reduces lung fibrosis following targeted type II alveolar epithelial cell injury. *Physiol. Rep.* **2018**, *6*, e13753. [CrossRef]
39. McDonough, J.E.; Ahangari, F.; Li, Q.; Jain, S.; Verleden, S.E.; Herazo-Maya, J.; Vukmirovic, M.; DeIuliis, G.; Tzouvelekis, A.; Tanabe, N.; et al. Transcriptional regulatory model of fibrosis progression in the human lung. *JCI Insight* **2019**, *4*, e131597. [CrossRef]
40. Sabatini, F.; Petecchia, L.; Boero, S.; Silvestri, M.; Klar, J.; Tenor, H.; Beume, R.; Hatzelmann, A.; Rossi, G.A. A phosphodiesterase 4 inhibitor, roflumilast N-oxide, inhibits human lung fibroblast functions in vitro. *Pulm. Pharmacol. Ther.* **2010**, *23*, 283–291. [CrossRef]
41. Epstein Shochet, G.; Brook, E.; Israeli-Shani, L.; Edelstein, E.; Shitrit, D. Fibroblast paracrine TNF-alpha signaling elevates integrin A5 expression in idiopathic pulmonary fibrosis (IPF). *Respir. Res.* **2017**, *18*, 122. [CrossRef]
42. Lugnier, C. The Complexity and Multiplicity of the Specific cAMP Phosphodiesterase Family: PDE4, Open New Adapted Therapeutic Approaches. *Int. J. Mol. Sci.* **2022**, *23*, 10616. [CrossRef]
43. Palazon, A.; Goldrath, A.W.; Nizet, V.; Johnson, R.S. HIF transcription factors, inflammation, and immunity. *Immunity* **2014**, *41*, 518–528. [CrossRef]
44. Ueno, M.; Maeno, T.; Nomura, M.; Aoyagi-Ikeda, K.; Matsui, H.; Hara, K.; Tanaka, T.; Iso, T.; Suga, T.; Kurabayashi, M. Hypoxia-inducible factor-1alpha mediates TGF-beta-induced PAI-1 production in alveolar macrophages in pulmonary fibrosis. *Am. J. Physiol. Lung Cell Mol. Physiol.* **2011**, *300*, L740–L752. [CrossRef]
45. Moon, J.O.; Welch, T.P.; Gonzalez, F.J.; Copple, B.L. Reduced liver fibrosis in hypoxia-inducible factor-1alpha-deficient mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2009**, *296*, G582–G592. [CrossRef]
46. Ivanova, V.; Garbuzenko, O.B.; Reuhl, K.R.; Reimer, D.C.; Pozharov, V.P.; Minko, T. Inhalation treatment of pulmonary fibrosis by liposomal prostaglandin E2. *Eur. J. Pharm. Biopharm.* **2013**, *84*, 335–344. [CrossRef]
47. Nio, Y.; Ookawara, M.; Yamasaki, M.; Hanauer, G.; Tohyama, K.; Shibata, S.; Sano, T.; Shimizu, F.; Anayama, H.; Hazama, M.; et al. Ameliorative effect of phosphodiesterase 4 and 5 inhibitors in deoxycorticosterone acetate-salt hypertensive uni-nephrectomized KKA(y) mice. *FASEB J.* **2020**, *34*, 14997–15014. [CrossRef]
48. Ley, B.; Collard, H.R.; King, T.E., Jr. Clinical course and prediction of survival in idiopathic pulmonary fibrosis. *Am. J. Respir. Crit. Care Med.* **2011**, *183*, 431–440. [CrossRef]
49. Molteni, A.; Wolfe, L.F.; Ward, W.F.; Ts'ao, C.H.; Molteni, L.B.; Veno, P.; Fish, B.L.; Taylor, J.M.; Quintanilla, N.; Herndon, B.; et al. Effect of an angiotensin II receptor blocker and two angiotensin converting enzyme inhibitors on transforming growth factor-beta (TGF-beta) and alpha-actomyosin (alpha SMA), important mediators of radiation-induced pneumopathy and lung fibrosis. *Curr. Pharm. Des.* **2007**, *13*, 1307–1316. [CrossRef]
50. Lambert, E.M.; Wuyts, W.A.; Yserbyt, J.; De Sadeleer, L.J. Statins: Cause of fibrosis or the opposite? Effect of cardiovascular drugs in idiopathic pulmonary fibrosis. *Respir. Med.* **2021**, *176*, 106259. [CrossRef]
51. Rosenberg, T.; Lattimer, R.; Montgomery, P.; Wiens, C.; Levy, L. The relationship of SSRI and SNRI usage with interstitial lung disease and bronchiectasis in an elderly population: A case-control study. *Clin. Interv. Aging* **2017**, *12*, 1977–1984. [CrossRef] [PubMed]
52. Rosa, A.C.; Pini, A.; Lucarini, L.; Lanzi, C.; Veglia, E.; Thurmond, R.L.; Stark, H.; Masini, E. Prevention of bleomycin-induced lung inflammation and fibrosis in mice by naproxen and JNJ7777120 treatment. *J. Pharmacol. Exp. Ther.* **2014**, *351*, 308–316. [CrossRef] [PubMed]
53. Lopez-Novoa, J.M.; Nieto, M.A. Inflammation and EMT: An alliance towards organ fibrosis and cancer progression. *EMBO Mol. Med.* **2009**, *1*, 303–314. [CrossRef] [PubMed]
54. Kankuri, E.; Cholujova, D.; Comajova, M.; Vaheri, A.; Bizik, J. Induction of hepatocyte growth factor/scatter factor by fibroblast clustering directly promotes tumor cell invasiveness. *Cancer Res.* **2005**, *65*, 9914–9922. [CrossRef]

55. Bizik, J.; Kankuri, E.; Ristimaki, A.; Taieb, A.; Vapaatalo, H.; Lubitz, W.; Vaheri, A. Cell-cell contacts trigger programmed necrosis and induce cyclooxygenase-2 expression. *Cell Death Differ.* **2004**, *11*, 183–195. [CrossRef]
56. Lippman, S.M.; Gibson, N.; Subbaramaiah, K.; Dannenberg, A.J. Combined targeting of the epidermal growth factor receptor and cyclooxygenase-2 pathways. *Clin. Cancer Res.* **2005**, *11*, 6097–6099. [CrossRef]
57. Gilligan, M.M.; Gartung, A.; Sulciner, M.L.; Norris, P.C.; Sukhatme, V.P.; Bielenberg, D.R.; Huang, S.; Kieran, M.W.; Serhan, C.N.; Panigrahy, D. Aspirin-triggered proresolving mediators stimulate resolution in cancer. *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 6292–6297. [CrossRef]
58. Lucotti, S.; Cerutti, C.; Soyer, M.; Gil-Bernabe, A.M.; Gomes, A.L.; Allen, P.D.; Smart, S.; Markelc, B.; Watson, K.; Armstrong, P.C.; et al. Aspirin blocks formation of metastatic intravascular niches by inhibiting platelet-derived COX-1/thromboxane A2. *J. Clin. Investig.* **2019**, *129*, 1845–1862. [CrossRef]
59. Raghu, G.; Collard, H.R.; Egan, J.J.; Martinez, F.J.; Behr, J.; Brown, K.K.; Colby, T.V.; Cordier, J.F.; Flaherty, K.R.; Lasky, J.A.; et al. An official ATS/ERS/JRS/ALAT statement: Idiopathic pulmonary fibrosis: Evidence-based guidelines for diagnosis and management. *Am. J. Respir. Crit. Care Med.* **2011**, *183*, 788–824. [CrossRef]