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Antifibrotic effects of vitamin D3 on human lung fibroblasts derived from patients with idiopathic pulmonary fibrosis

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List of abbreviations: IPF, Idiopathic pulmonary fibrosis; VD, 1,25-dihydroxyvitamin D3; VDR, vitamin D receptor; HLFs, human lung fibroblasts; PDGF, platelet-derived growth factor; TGF- β , transforming growth factor- β ; DMEM, Dulbecco's Modified Eagle Medium; EtOH, ethanol; α -SMA, α -smooth muscle actin; OCR, oxygen consumption rate; ECAR, the extracellular acidification rate; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; LDH, lactate deshydrogenase.

Abstract

Idiopathic pulmonary fibrosis (IPF) is a progressive and fatal interstitial lung disease. Up to now, no treatment can stop the progression of IPF. Vitamin D3 (VD) reduces experimental lung fibrosis in murine models and depletion of vitamin D3 might be associated with the reduced survival of patients with IPF. In this context, we determined if VD can prevent the pro-fibrotic functions of human lung fibroblasts (HLFs) isolated from patients with IPF. IPF and control HLFs were derived from surgical lung biopsies collected from patients with IPF or with primary lung cancer, respectively. VD (3-100 nM) markedly reduced the basal and PDGF-induced proliferation of HLFs. VD also altered cell cycle by increasing the percentage of IPF HLFs arrested in the G0/G1 phase, and by downregulating the expression of various cell cycle regulatory proteins. In addition, VD barely prevented the TGF- β 1-induced differentiation in HLFs. At 100 nM, VD slightly reduced the expression of the pro-fibrotic marker α -smooth muscle actin, and had no effect on fibronectin and collagen-1 expression. In contrast, 100 nM VD strongly inhibited the aerobic glycolytic metabolism induced by TGF- β 1. Finally, VD reduced both the secretion of lactate, the levels of lactate dehydrogenase mRNA and the activity of intracellular LDH in IPF HLFs. In conclusion, our study shows that VD reduced pro-fibrotic functions of HLFs. These findings suggest that it might be interesting to assess the potential clinical benefits of vitamin D supplementation in patients with IPF, especially on lung function decline.

Keywords: Idiopathic pulmonary fibrosis, vitamin D, pulmonary fibroblasts, proliferation, differentiation, metabolism

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic and irreversible interstitial lung disease with an average life expectancy of 3-5 years after diagnosis [1]. An aberrant process of chronic healing, that stiffens the lungs and definitively reduces the airflow exchanges at pulmonary alveoli, defines IPF. The migration and proliferation of lung fibroblasts and then their differentiation into myofibroblasts constitute the central pathophysiological mechanisms that control the development of this disease [2]. Profibrotic factors like platelet-derived growth factor (PDGF) and transforming growth factor (TGF)- β , that are produced by epithelial and immune cells, orchestrate this process [3,4]. PDGF induces the migration and proliferation of fibroblasts [5] while TGF triggers the differentiation and the metabolic shift to aerobic glycolysis in myofibroblasts [6]. This glycolytic reprogramming contributes to the development of pulmonary fibrosis notably by increasing the secretion of lactic acid, a potent activator of latent TGF- β [6,7].

Nowadays, nintedanib and pirfenidone are the only drugs approved for treating IPF [8]. These antifibrotic drugs slow down but do not stop the progression of the disease [9,10]. Vitamin D3 is a fat-soluble pro-hormone that is metabolized by the kidneys and liver to produce 1,25-dihydroxyvitamin D3 (VD), its active metabolite [11]. VD is well-known to regulate mineralization and calcium homeostasis in bones and muscles by activating the vitamin D receptor (VDR), a ligand-dependent transcription factor [11]. In addition, VD could interfere with the fibrotic process in various organs. It decreases *in vitro* the differentiation of human dermal, corneal, cardiac and nasal polyp-derived fibroblasts induced by TGF- β [12–15]. It also limits the proliferation of primary murine lung fibroblasts [16]. In murine and rat models, VD attenuates renal fibrosis and bleomycin-induced lung fibrosis notably by decreasing the extracellular matrix production and deposition [17–19]. Conversely, vitamin D deficiency promotes lung fibrosis and especially exacerbates bleomycin-induced pulmonary

fibrosis [14,20]. Finally, recent clinical studies have shown that many patients with IPF have low serum vitamin D concentrations and that vitamin D deficiency may constitute a negative prognostic factor in IPF [20–22].

Altogether, these results suggest that vitamin D can interfere with the development of pulmonary fibrosis in humans. However, the ability of VD to inhibit the profibrotic functions of human lung fibroblasts (HLFs) still remains unknown. In this context, the present study was thus designed to determine whether VD could prevent the proliferation, migration and differentiation of HLFs isolated from patients with IPF.

2. Materials and Methods

2.1. Isolation of HLFs

HLFs derived from surgical lung biopsies isolated from patients with IPF (median age 68 years; range 51-77 years) [23]. IPF was diagnosed according to the 2022 ATS/ERS/JRS/ALAT criteria, including histopathological features of usual interstitial pneumonia [24]. Control HLFs derived from lung samples isolated from patients with cancer undergoing lobectomy or pneumonectomy, away from the tumor (median age 64.5 years; range 59-74 years). The absence of tumor tissue in control samples was verified histologically. Cell morphology was checked by phase contrast microscopy. This study was approved by the local ethics committee (Ethics Committee CHU Rennes, n° 16123). Written informed consent was obtained from all subjects. All experiments were performed following relevant guidelines and regulations.

2.2. Cell culture and treatments

IPF and control HLFs were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco™, Life Technologies, Courtaboeuf, France) supplemented with 10% fetal calf serum (FCS) (Eurobio scientific, Evry, France), L-glutamine, antibiotic-antimycotic solution (Gibco™, Life Technologies), and used at passages 5 and 6. Cells were cultured in the absence or presence of VD (Sigma-Aldrich, St. Quentin Fallavier, France). VD was first dissolved in ethanol (EtOH) to constitute a stock solution. EtOH was used as vehicle in each experiment. To study mRNA and protein expression, HLFs were seeded in 6-well plates for 24 h and then starved in FCS-free DMEM for 16 h. To induce the differentiation of myofibroblasts, serum-starved HLFs were first cultured in the absence or presence of 3-100 or 1000 nM VD for 1 h and then stimulated with TGF- β 1 (1 ng/ml, Preprotech, Neuilly-sur-Seine, France) for 24 h. The HLFs used for cell growth and cell cycle studies were serum-starved for 16 h, cultured in DMEM containing 1 % FCS with 3-100 nM VD for 1 h, and

stimulated with PDGF-BB (50 ng/ml) (R&D Systems, Bio-Techne, Lille, France) for a further 96 h.

2.3. Proliferation

Incucyte® live-cell analysis system (Essen BioScience, Sartorius, USA) was used to determine cell proliferation in a stable culture environment (37°C and 5% CO₂). The fluorescent dye NRR® (Nuclight Rapid Red Dye® diluted at 1/1000) specifically marked the nucleus of living HLFs. The number of HLFs was determined using the Incucyte® analysis software by creating a specific counting mask that identified and delimited the nuclei at each acquisition. The acquisition was performed every 8 hours for 96 hours using Incucyte® microscope, objective X4. Each point was performed in triplicate. The proliferation index was calculated relatively to the number of cells measured at 8 h and arbitrarily set at 1.

2.4. Cell cycle analysis

To analyse the cell cycle distribution, HLFs were fixed in ice-cold 70 % (v/v) ethanol for 30 min at 4°C and then collected by centrifugation (900g/5 min). The cell pellets were washed, suspended in PBS (pH 7.4) containing propidium iodide (50 µg/mL) and DNase-free RNase A (100 µg/mL) (Sigma-Aldrich, St. Quentin Fallavier, France), and incubated at 4°C for 1 h. DNA content was quantified by flow cytometry using a LRSFortessa™X20 flow cytometer (BD Biosciences, Paris, France). Data were analysed with the flow cytometry data analysis software FlowLogic (Miltenyi Biotech, Paris, France) using the Watson improved method.

2.5. Genetic invalidation by siRNA transfection

HLFs were transfected in optiMEM medium (Gibco, Waltham, USA) for 6 h with 25 nM VDR siRNA (J-003448-07-0005, ONTARGETplus siRNA Human VDR (7421) 5 nmol; Dharmacon, Horizon discovery) or 25 nM non-targeting control siRNA (ONTARGETplus Non-targeting Pool, 5 nmol; Dharmacon, Horizon discovery). Then, cells were washed and

cultured in antibiotic-free DMEM 1X Glutamax for 18 h before the study of cell proliferation and metabolism. siRNA efficacy was verified by western-blotting.

2.6. Western blot analysis

To analyse protein expression, cOmplete™ Protease Inhibitor Cocktail (Roche, Sigma-Aldrich) and phosphatase inhibitor cocktail (Sigma-Aldrich) in ice-cold RIPA buffer were used to lyse HLFs. Proteins separation was performed by electrophoresis on polyacrylamide gels containing 10 % Tris-glycine SDS and transferred to nitrocellulose membranes, as previously described [25]. Free binding sites were blocked by incubation in 5 % milk or bovine serum albumin (BSA) (Sigma-Aldrich, St. Quentin Fallavier, France) for 2 h at room temperature. The appropriate primary human antibody (Ab) was then added to the membranes overnight at 4°C. The primary Abs were anti- α SMA (clone 1A4, Sigma-Aldrich), anti-collagen I (Southern Biotechnology Associates, Birmingham, AL), anti-fibronectin (Abcam), anti-GAPDH (mab90009-P, Covalab, Bron, France) Abs. Anti-phospho SMAD2 (3104), anti-SMAD2 (12584), anti-phospho SMAD3 (9520), anti-SMAD3 (9523), anti-phospho PDGF receptor (PDGFR) (3161), anti-PDGFR (3169), anti-CDK2 (2546), anti-CDK4 (12790), anti-CDK1 (9112S) and anti-Cyclin D1 (55506) Abs were from Cell Signaling Technology (Massachusetts, USA). Anti-Cyclin E (sc-56311), anti-Cyclin A (sc-751) and anti-Cyclin B1 (sc-245) Abs were from Santa Cruz Biotechnology, USA. A gel imaging system (Chemi doc, BIO-RAD Laboratories, Marnes-La-Coquette, France) equipped with a CCD camera was used to acquire digital images. Western blots were quantified using the Image Lab™ software (BIO-RAD) by densitometry and normalized to GAPDH. The relative protein expression in stimulated cells was determined by arbitrarily setting at 1 the protein expression measured in untreated cells cultured in the presence of vehicle (EtOH).

2.7. RNA isolation and RT-PCR assays

The extraction kit from Macherey Nagel (Hoerdt, France) was used to extract total RNAs according to the manufacturer's instructions. NanoDrop 1000 (Thermo Fisher Scientific, Saint-Herblain, France) was used to quantify RNA concentrations by spectrofluorimetry. mRNAs were reverse transcribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Thermo Fisher Scientific). Quantitative PCR (polymerase chain reaction) was performed using the SYBR Green methodology on a CFX384 Real-Time PCR System (Bio-Rad Laboratories) in duplicates. All the primers were provided by Sigma-Aldrich (KiCqStart™ SYBR® Green Primers, St. Quentin Fallavier, France). The specificity of gene amplification was checked at the end of PCR using the comparative cycle threshold method (CFX Manager Software). The mean Cq values were used to normalize the target mRNA concentrations to those of the 18S ribosomal protein by the $2(-\Delta\Delta Cq)$ method. The mRNA levels were defined relatively to those measured in untreated cells and arbitrarily set at 1.

2.8. Mitochondrial respiration and glycolytic function

A Seahorse XFe24® Analyzer (Agilent Technologies) was used to determine the basal mitochondrial respiration and the basal glycolysis by directly measuring the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) in IPF and control HLFs, respectively. Parameters of the mitochondrial respiration were determined using the Seahorse XF Cell Mito Stress kit, in both basal condition and after the injection of mitochondrial respiration inhibitors in the system. These inhibitors were oligomycin (inhibitor of ATP synthase), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP, uncoupling agent that collapses the proton gradient and disrupts the mitochondrial membrane potential), antimycin A (complex III inhibitor) and rotenone (complex I inhibitor). The glycolytic function was determined using a Seahorse® XF Glycolysis Stress Test kit that assesses the basal extracellular acidification rate (ECAR) in the absence of glucose in culture media. The

main parameters of the glycolytic flux, i.e glycolysis, glycolytic capacity and glycolytic reserve, were then determined after the injection of glucose, oligomycin (shifted the energy production to glycolysis) and 2-deoxy-glucose (an inhibitor of glycolysis through competitive binding to glucose hexokinase), respectively.

2.9. Lactate secretion

The secretion of lactate in culture supernatant was quantified on the Roche Cobas 8000 c502 analyser (Hoffmann-La Roche, Basel, Switzerland). Lactate concentrations were determined using a spectrophotometric enzymatic-based method catalysed by a lactate oxidase-peroxydase reaction that produced a chromogen quinone-imine product at 552 nm. The intensity of the color formed was proportional to the lactate concentration present in the sample.

2.10. Lactate deshydrogenase activity

The activity of lactate deshydrogenase (LDH) present in cell lysate was measured using the LDH IFCC assay from Sigma-Aldrich (MAK066) in duplicates. LDH catalysed the conversion of lactate to pyruvate in the presence of NAD that is reduced to NADH during this process. The rate of NADH formation, directly proportional to the catalytic LDH activity, was determined by measuring the increase in absorbance at 340 nm.

2.11. Statistical analysis

All results are presented as means \pm standard errors of mean (SEM) of the indicated numbers of independent biological experiments. Each biological experiment was performed with different cell cultures deriving from different donors. Statistical analyses were performed with Prism 5 (GraphPad Software, La Jolla, CA). Significant differences were determined by the Student's *t*-test when two groups were compared, or by a repeated ANOVA followed by the

Dunnett's Multiple comparison *t*-test to study the effects of increasing VD concentrations. #, * p<0.05; ##, ** p<0.01; ###, *** p<0.001. #: versus PDGF-BB or TGF- β 1, *: versus VD.

3. Results

3.1. VDR was expressed and active in HLFs.

We first determined if VDR expression could be detected in control and IPF HLFs. The Figure 1A shows that the VDR protein expression was present in all HLF culture tested. No differences were observed between control and IPF HLFs (Fig. 1A). To verify the activity of VDR, we quantified mRNA levels of the *CYP24A1* gene that is highly activated in response to the binding of VD to VDR. As expected, *CYP24A1* mRNA levels were strongly induced in HLFs exposed to VD (Fig. 1C). This expression was almost totally inhibited when VDR protein expression was deleted by genetic invalidation (Fig. 1B-C). This result thus confirms that VDR was active in HLFs.

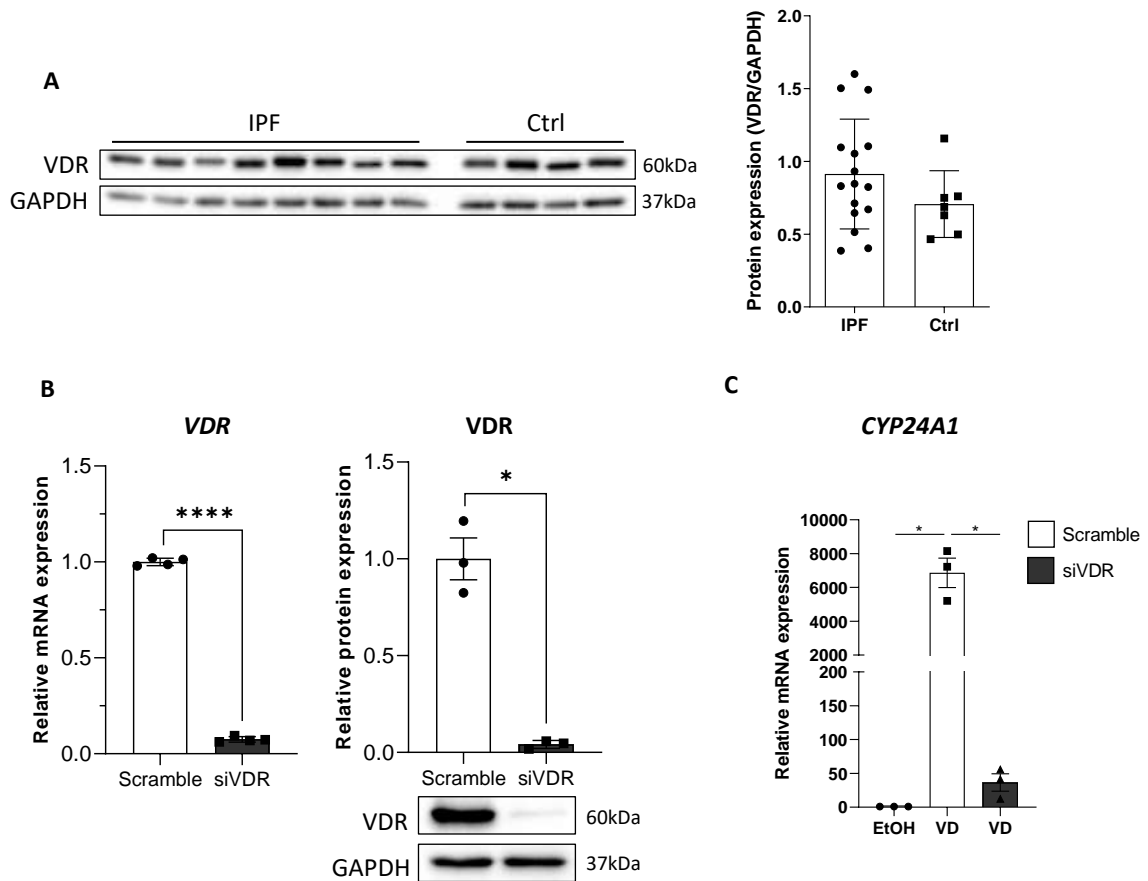


Figure 1. Vitamin D Receptor (VDR) was expressed and active in human HLFs. (A) Representative Western blot analysis of VDR expression in IPF and control (Ctrl) HLFs. Protein expression was quantified by densitometry and normalized to GAPDH protein expression. (B, C) IPF HLFs were transfected with control siRNA (scramble) or VDR-siRNA (siVDR) for 24 h (B), and then treated with vehicle (EtOH) or 100 nM VD for 24 h (C). Relative *VDR* mRNA (B) and *CYP24A1* mRNA (C) levels were measured by quantitative RT-PCR and normalized to ribosomal 18S RNA. The mRNA levels measured in HLFs transfected with scramble (B) and treated with EtOH (C) were arbitrarily set at 1. VDR protein expressions in HLFs transfected with scramble or SiVDR were analysed by Western blot (B). Results are expressed as means \pm SEM of 3-16 independent experiments. Significant differences were determined by unpaired (A) or paired (B, C) t-tests.

3.2.VD inhibited basal and PDGF-BB-induced proliferation of HLFs.

Fibroblast proliferation is a major event contributing to the accumulation of fibroblastic foci in the pulmonary tissues of patients with IPF [26]. We thus determined if VD could prevent cell proliferation. IPF and control HLFs were cultured with VD, in the absence or presence of PDGF-BB for 96 h to determine its effects on basal and stimulated cell growth, respectively. VD significantly inhibited, in a concentration-dependent manner, the basal proliferation of both IPF and control HLFs (Fig. 2A). The inhibition was higher when cells were exposed to 100 nM VD. As expected, PDGF-BB significantly increased the proliferation of control and IPF HLFs (Fig. 2B). VD inhibited the PDGF-BB-induced proliferation with a dose-response similar to that reducing the basal proliferation. Finally, we assessed if VDR mediated the VD effects on cell proliferation. Figure 2C indicated that genetic invalidation of VDR expression blocked the ability of VD to reduce basal and PDGF-BB-induced proliferation of HLFs. Thus, VD likely prevented cell growth through a molecular VDR-dependent mechanism. PDGF-BB stimulates cell proliferation by its binding to the PDGFR and the subsequent activation of various signalling pathways involving the kinases ERK and AKT. Figure S1 shows that PDGF-BB strongly increased the phosphorylation of the PDGFR, ERK and AKT. However, VD could not prevent the activation of such a signalling.

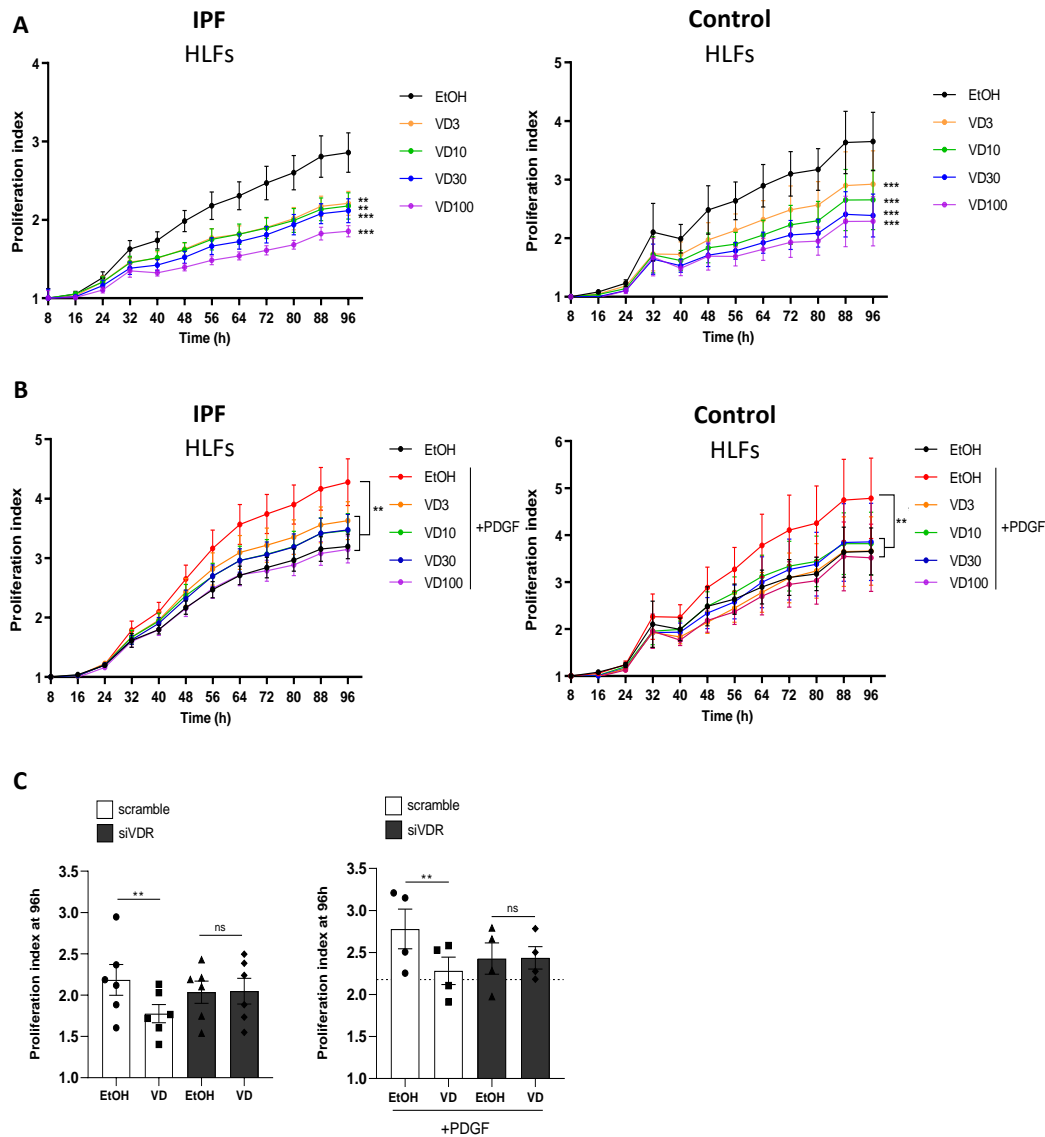


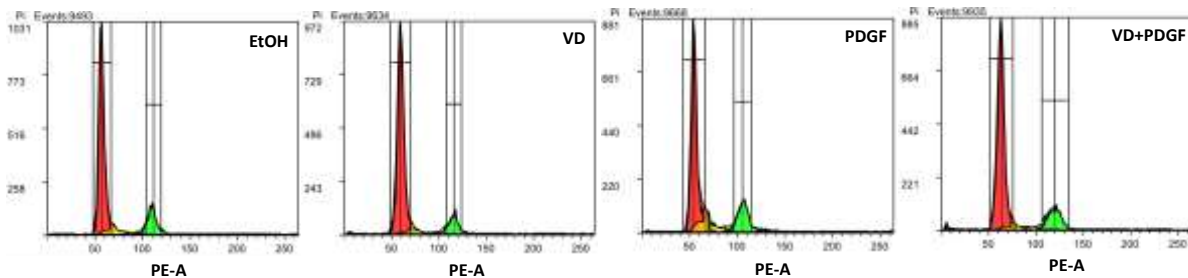
Figure 2. VD reduced the proliferation of HLFs in a VDR-dependent manner. (A, B) IPF and control HLFs were treated with vehicle (EtOH) or 3-100 nM VD in absence (A) or presence of 50 ng/mL PDGF-BB (B) for 96 h. (C) IPF HLFs were transfected with control siRNA (scramble) or VDR-siRNA (siVDR) for 24 h, and then treated with EtOH or 100 nM VD in the absence or presence of 50 ng/mL PDGF-BB for 96 h. The proliferation was determined by Incucyte® quantification of fluorescent stained cells with acquisition every 8 h. The proliferation index was calculated by arbitrarily setting the first cell number acquisition at 1. In the right panel (C), the dashed line represents the basal proliferation index for cells

treated with EtOH in the absence of PDGF-BB. Results are expressed as means \pm SEM of 8 (A) and 6 (B,C) independent experiments. Significant differences were determined by a repeated ANOVA followed by Dunnett's multiple comparisons t-test (A, B) or by a paired t-test (C).

3.3.VD altered the cell cycle of IPF HLFs and inhibited the expression cyclin/cdk partners.

We then investigated the effects of VD on the cell cycle of basal and PDGF-BB-stimulated IPF HLFs. As shown in Figure 3A and 3B, a 48 h-treatment with 100 nM VD significantly increased the percentage of non-stimulated IPF HLFs arrested in G0/G1, while it numerically decreased the percentage of cells in the S and G2/M phases. Cell stimulation with PDGF-BB significantly decreased the percentage of cells in the G0/G1 phase and increased those of cells detected in the S and G2/M phases. As observed for basal IPF HLFs, VD specifically enhanced the percentage of PDGF-BB-activated cells arrested in the G0/G1 phase.

A



B

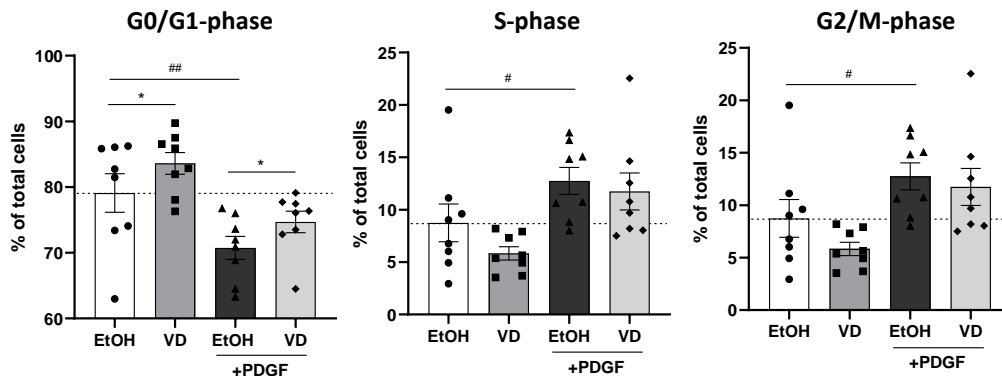
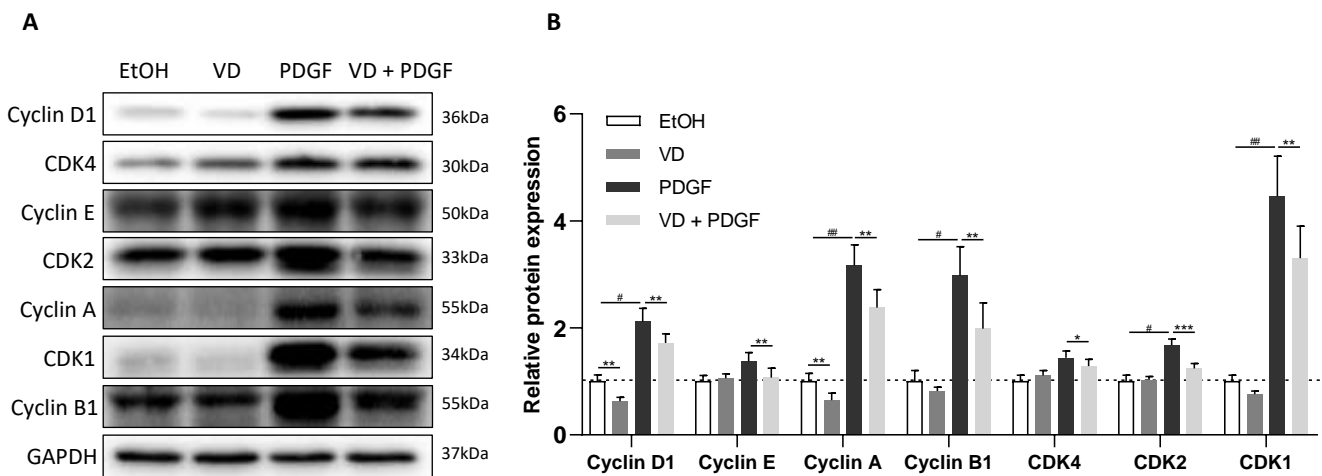


Figure 3. VD altered the cell cycle distribution in IPF HLFs. Cells were treated with vehicle (EtOH) or 100 nM VD for 1 h, and then left untreated or stimulated with 50 ng/mL PDGF-BB for 48 h. Cells were washed, fixed and stained with propidium iodide to analyse the cell cycle by flow cytometry. (A) Representative graphs saved during flow cytometry analysis. (B) Data for each cell cycle phase were expressed as percentages of total IPF HLFs. Results are expressed as means \pm SEM of 8 independent experiments. Significant differences were determined by paired t-tests.

To further specify the mechanism controlling the inhibition of cell proliferation, we next determined if VD could alter the expression of cell cycle regulatory proteins. Figure 4 shows that 100 nM VD significantly decreased the basal expression of cyclin D1 and cyclin A. In addition, it reduced the expression of cyclins A, B1, D1 and E induced by PDGF-BB in IPF HLFs. It also limited expression of the cyclin partners Cdk1, Cdk2 and Cdk4 in these



activated cells (Figure 4B).

Figure 4. VD altered the protein expression of cyclins and cyclin-dependent kinases in IPF HLFs. Cells were treated with vehicle (EtOH) or 100 nM VD in the absence or presence of 50 ng/mL PDGF-BB for 48 h. (A) Representative Western blot showing the expression of cell cycle-related proteins in IPF HLFs. (B) Protein expression was quantified by densitometry and normalized to GAPDH. Basal protein expressions measured in cells treated with EtOH (dashed lines) were arbitrarily set at 1. Results are expressed as means \pm SEM of 8 independent experiments. Significant differences were determined by paired t-tests.

3.4. VD did not prevent the migration of HLFs induced by PDGF-BB.

Besides proliferation, the migration of HLFs also promotes the formation of pulmonary fibroblastic loci. As expected, PDGF-BB increased the migration of IPF HLFs stimulated for 24 h (Figure S2). However, co-treatment with 100 nM VD did not prevent this cell migration.

3.5. VD slightly reduced the TGF- β 1-induced differentiation of HLFs.

TGF- β plays a key role in the development of pulmonary fibrosis by inducing the differentiation of fibroblasts into myofibroblasts. These cells express major pro-fibrotic genes controlling the synthesis of contractile actin microfilaments containing α -SMA and the production of ECM such as fibronectin and collagen 1 [27]. In this next set of our experiments, we thus determined if VD could prevent the differentiation of pulmonary myofibroblasts by altering the mRNA and protein expression of various pro-fibrotic genes. Figures 5A-B show that VD did not modify the basal mRNA expression in IPF HLFs whatever the gene tested. In contrast, 100 nM VD slightly but significantly reduced the *ACTA2* mRNA level and the α -SMA protein expression in cells stimulated with TGF- β 1 for 24 h (Figures 5C-D). Several previous studies demonstrated that, *in vitro*, micromolar concentrations of VD can markedly inhibit TGF- β 1-induced differentiation in different types of fibroblasts [12,16,20]. Thus, we next determined if 1 μ M VD could more strongly prevent the differentiation of IPF HLFs. To this goal, we first compared VDR activity in IPF HLFs treated with 100 nM or 1 μ M VD. The Figure S3A shows that *CYP24A* mRNA levels were similarly increased in cells exposed to 100 nM and 1 μ M VD. In addition, Figure S3C-D demonstrates that, as observed with a 100 nM VD treatment, 1 μ M VD only weakly prevented the induction of *ACTA2* mRNA and α -SMA protein levels, and it did not inhibit the expression of collagen and fibronectin in IPF HLFs. As described in IPF HLFs, 100 nM VD slightly reduced the expression of the *ACTA2* gene at mRNA and protein levels in control

HLFs (Figure S4). We next determined if VD could limit the *ACTA2* gene expression by interfering with the phosphorylation of SMAD2 and SMAD3, which mediate most TGF- β 1 effects. Figure 5E and S4C indicates that 100 nM VD did not significantly reduce the phosphorylation of SMAD2 and SMAD3 in IPF and control HLFs, respectively.

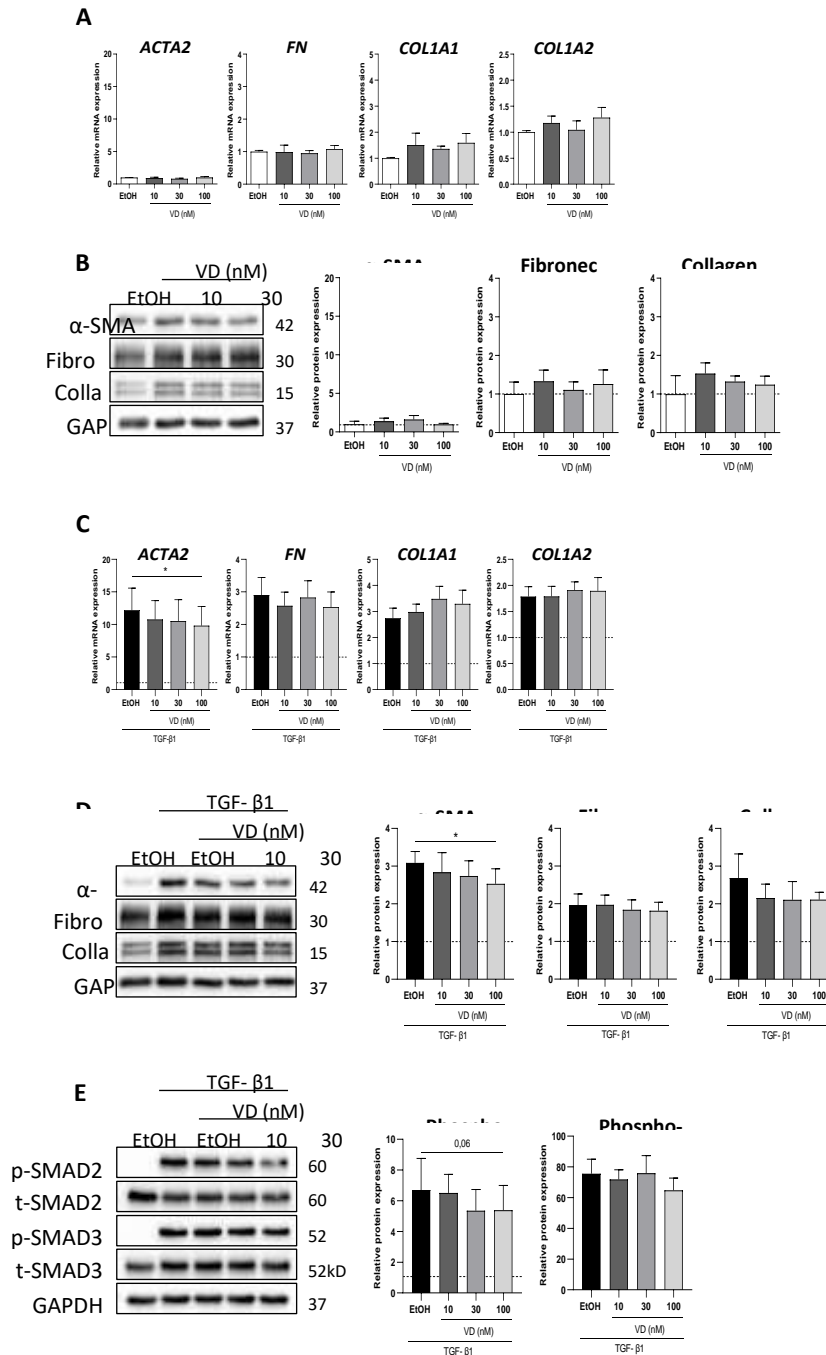


Figure 5. VD reduced ACTA2 mRNA levels and α -SMA protein expression in IPF HLFs.

Cells were treated with vehicle (EtOH) or 100 nM VD in the absence (A, B) or presence (C-E) of 1 ng/mL TGF- β 1 for 24 h. (A, C) Relative *ACTA2*, *COL1A1*, *COL1A2* and *FNI* mRNA levels were measured by quantitative RT-PCR and normalized to ribosomal 18S RNA. The mRNA levels measured in cells treated with EtOH were arbitrarily set at 1. (B, D, E) Representative Western blot showing the expression of α -SMA, Fibronectin, collagen I and phospho-SMAD2/3 in IPF HLFs (left panels). Protein expressions were quantified by densitometry and normalized to GAPDH (bar plots). Basal protein expressions measured in cells treated with EtOH (dashed lines) were arbitrarily set at 1. Results are expressed as means \pm SEM of 4-6 independent experiments. Significant differences were determined by a repeated ANOVA followed by Dunnett's multiple comparison t-tests.

3.6. VD inhibited the TGF- β 1-induced glycolytic metabolism in IPF HLFs.

Recently, glycolytic reprogramming has been associated with the differentiation of myofibroblast and has emerged as a targetable pathway in IPF [28]. To examine the ability of VD to prevent this stimulation of the glycolytic metabolism in HLFs, we evaluated its effects on mitochondrial and glycolysis-related respiration using a Seahorse extracellular flux analyser. TGF- β 1 increased mitochondrial respiration assessed by OCR consumption (Figure 6A). Particularly, the Figure 6B shows that TGF- β 1 significantly enhanced the basal mitochondrial respiration, thereby emphasizing an increased oxidative phosphorylation capacity, and OCR-related ATP production. VD reduced, although not significantly, these effects. In addition, TGF- β 1 increased the basal acidification rate (ECAR) and VD blocked this induction (Figure 6C). The metabolic profile (Figure 6D) indicates that TGF- β 1 promoted an energetic phenotype in HLFs that was in part prevented by VD.

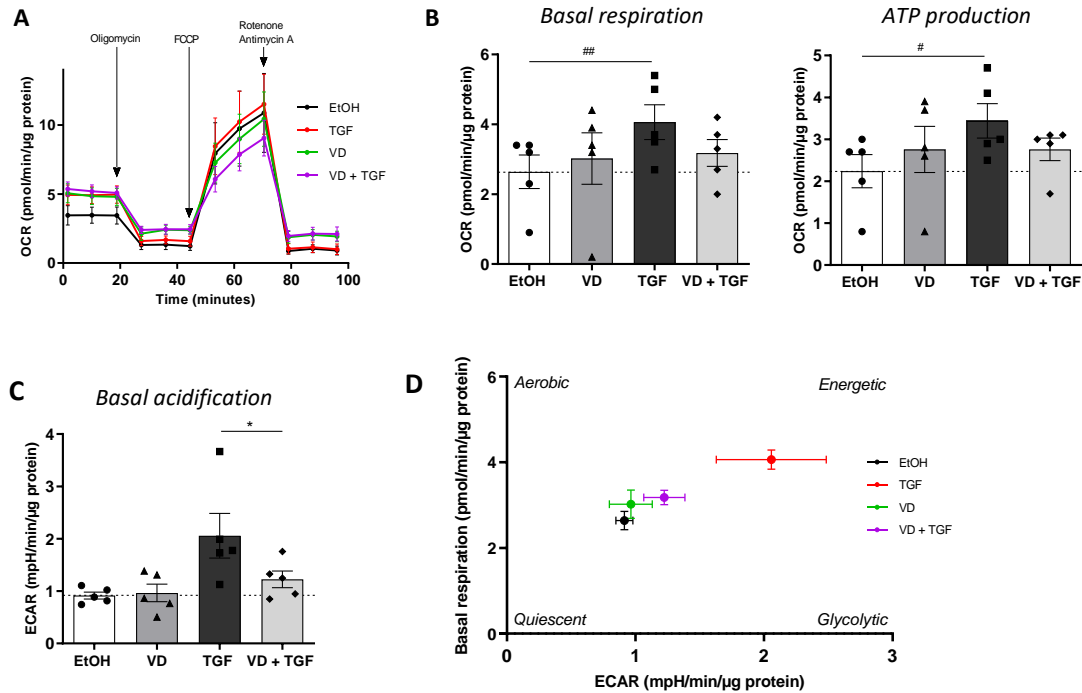


Figure 6. VD prevented the basal extracellular acidification induced by TGF- β 1 in IPF HLFs. Cells were treated with vehicle (EtOH) or 100 nVD, in the absence or presence of TGF- β 1 (TGF), for 24 h before analyzing of the cell metabolism by Seahorse technology. (A) Representative graph of oxygen consumption rate (OCR) by IPF HLFs using a Seahorse® XF Cell Mito Stress kit. (B) mitochondrial basal respiration and OCR-related ATP production were quantified after injection of oligomycin, FCCP, rotenone and antimycin A in the system. (C) The basal extracellular acidification was determined by measuring the extracellular acidification rate (ECAR). (D) The cell energy phenotype profile determined by the simultaneous measurement of OCR and ECAR values is shown. Results are expressed as means \pm SEM of 5 independent experiments. Significant differences were determined by paired t-tests.

We next specified the impact of VD on the glycolytic metabolism using the Seahorse XF Glycolysis Stress. Figure 7A shows that TGF- β 1 increased ECAR in the absence and presence of glucose. TGF- β 1 significantly stimulated glycolysis, glycolytic capacity and glycolytic reserve (Figure 7B). 100 nM VD did not affect the basal aerobic glycolysis of HLFs but totally inhibited the glycolytic shift induced by TGF- β 1 (Figure 7B). Genetic

invalidation of VDR expression blocked the inhibitory effects of VD on the glycolytic function in HLFs stimulated with TGF- β 1 (Figure 7C). TGF- β 1 also enhanced, although not significantly, the glycolytic functions in control HLFs (Figure S5). As observed with IPF HLFs, VD seems to reduce the basal glycolysis, glycolytic capacity and glycolytic reserve in control HLFs.

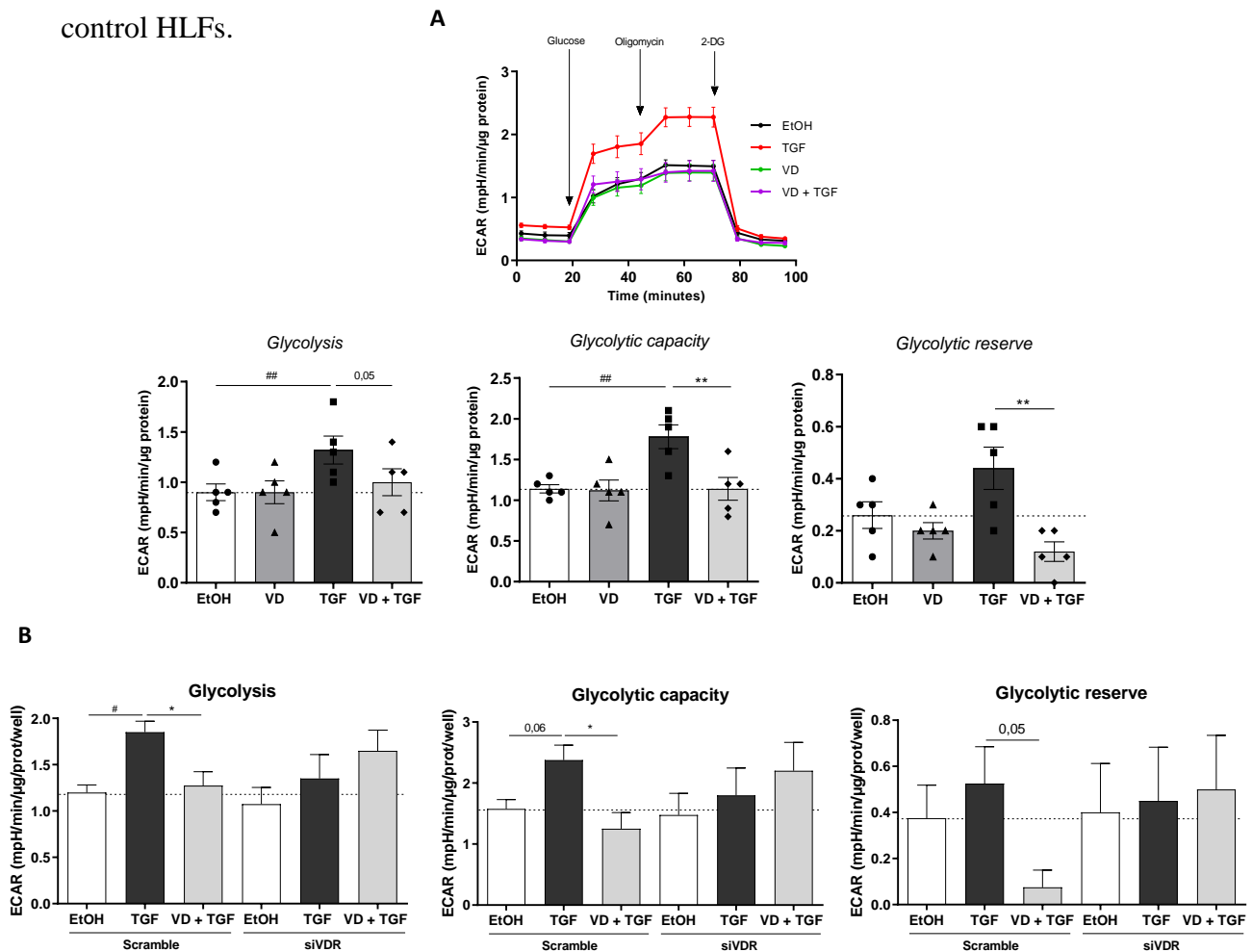


Figure 7. VD inhibited the glycolysis induced by TGF- β 1 in IPF HLFs. Cells were treated with vehicle (EtOH) or 100 nM VD, in the absence or presence of 5 ng/mL TGF- β 1 (TGF) for 24 h, before analysis of glycolysis by Seahorse technology. (A) Representative graph of extracellular acidification rate (ECAR) from IPF HLF culture media using a Seahorse® XF Glycolysis Stress Test kit. (B) The glycolytic metabolism was determined after injection of glucose, oligomycin and 2-DG in the system. (C) Cells were transfected with control siRNA (scramble) or VDR-siRNA (siVDR) for 24 h, and then treated with EtOH or 100 nM VD, in the absence or presence of TGF- β 1 for 24 h, before the analysis of the glycolytic metabolism.

Results are expressed as means \pm SEM of 5 independent experiments. Significant differences were determined by paired t-tests.

The secretion of lactate, produced by the reduction of pyruvate, contributes to the increase of ECAR when the glycolytic function is stimulated. Figure 8A indicates that TGF- β 1 significantly increased the concentration of lactate in cell culture media. In addition, it induced the expression of the *LDHA* gene (Figure 8B) and stimulated the LDH activity in IPF HLFs (Figure 8C). 100 nM VD significantly reduced the secretion of lactate from IPF HFLs. It also limited the induction of both *LDHA* mRNA level and LDH activity in HLFs stimulated with TGF- β 1.

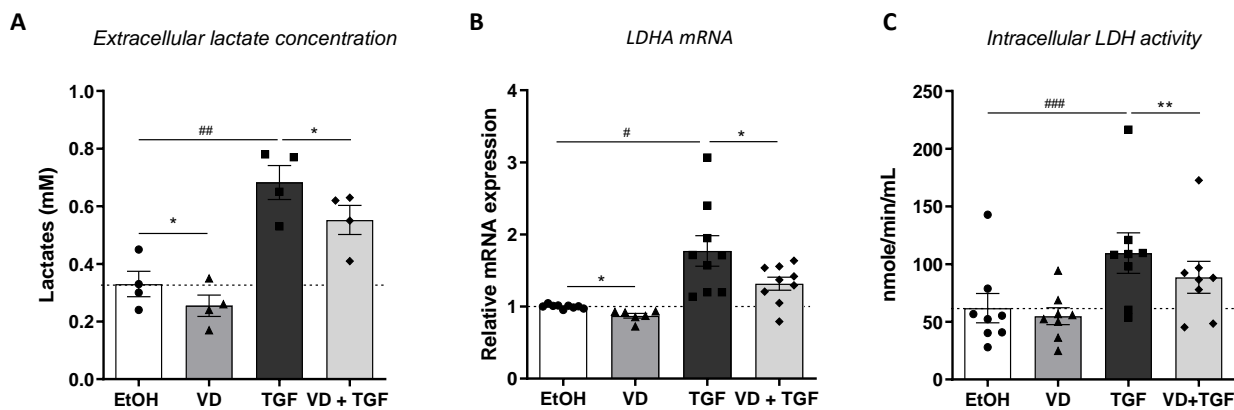


Figure 8. VD reduced lactate secretion, *LDHA* mRNA levels and intracellular LDH activity in IPF HLFs. Cells were treated with vehicle (EtOH) or 100 nM VD in the absence or presence of 5 ng/mL TGF- β 1 (TGF) for 24 h. Extracellular lactate concentrations (A) and LDH activity (C) were measured as described in the materials and methods section. (B) *LDHA* mRNA levels were measured by quantitative RT-PCR and normalized to ribosomal 18S RNA. The mRNA levels measured in cells treated with EtOH (dashed line) were arbitrarily set at 1. Results are expressed as means \pm SEM of 4 (A) or 8 (B, C) independent experiments. Significant differences were determined by paired t-tests.

4. Discussion

Our study demonstrates for the first time that VD inhibited major profibrotic processes in primary HLFs isolated from IPF and control lung parenchyma, likely through a VDR-dependent signalling.

We first reported that VDR was functional in HLFs as its genetic deletion totally blocked the induction of CYP24A1 mRNA levels in cells exposed to VD. CYP24A1 is one of the main target genes of VD that controls its oxidation and subsequent elimination [29]. In addition, we found that IPF and control HLFs similarly expressed VDR, which thus suggests that the VDR signalling may not be altered in pulmonary fibroblasts isolated from patients with IPF. This result contrasts with a recent report showing that VDR expression was negatively correlated with the development of liver fibrosis in patients suffering from chronic hepatitis B virus infection [30].

We next showed that increasing doses of VD significantly inhibited the basal and PDGF-BB-stimulated proliferation. PDGF-BB, that is frequently overexpressed in lung parenchyma of IPF patients, enhances the proliferation of lung fibroblasts and promotes their accumulation in pathologic foci [26]. Pharmacological inhibition of PDGFR reduced the induction of lung fibrosis in different mice models thereby strengthening a role for PDGF in the development of IPF [31,32]. These negative effects of VD on HLF proliferation were associated with a significant alteration of the cell cycle. Indeed, VD increased the percentage of HLFs arrested in G0/G1 and limited the expression of various cyclins and CDK controlling the cell cycle progression. Particularly, VD reduced the expression of cyclin D1, known to promote the G0/G1 transition. These mechanisms likely contribute to the antiproliferative of VD since previous reports showed that the hormone also inhibited the proliferation of various cell lines by arresting the cell cycle in the G0/G1 phase and by reducing cyclin and cdk expressions [33–35]. Our results further indicate that VD could not prevent the PDGF-induced

proliferation of HLFs by inhibiting the canonical PDGFR signalling since it did not prevent the activation of ERK, which markedly contributes to the growth of lung fibroblasts [36]. In contrast, we can hypothesize that VD inhibited cell growth by directly reducing cell cycle gene expression through a VDR-dependent mechanism, since the genetic deletion of its receptor blocked its effects. Additional investigations are however required to determine whether VD could stimulate the binding of VDR to specific responsive elements in genes controlling the expression of cyclin/CDK proteins.

In addition, our results demonstrated that VD slightly reduced the differentiation of HLFs into myofibroblasts. These cells play a major role in the development of lung fibrosis by the production of α -SMA-containing contractile filaments and extracellular matrix, which contributes to lung stiffness [26]. VD significantly, although slightly, prevented the increase in *ACTA2* mRNA and α -SMA protein levels in both control and IPF HLFs. In addition, it had no effect on the expression of collagen 1 and fibronectin induced by TGF- β 1. These latter results were unexpected since VD blocked the expression of major pro-fibrosis genes like *ACTA2*, *COL1A1*, *COL1A2*, and *FN1* induced by TGF- β 1 in various murine models and diverse cell lines [12–15,19,20]. These discordant results are likely due to the inability of VD to inhibit the TGF- β 1/SMAD signalling in HLFs. To stimulate fibroblast differentiation, TGF- β 1 activates the transcription factors SMAD2/3, their translocation into nuclei and their subsequent binding to the specific DNA-binding elements present on several profibrotic genes such as *ACTA2* and *COL1A1* [37]. VD-bound VDR can physically interact with phosphorylated SMAD2/3 and block their binding on various gene promoters in TGF- β 1-stimulated fibroblasts [13,38]. VD can also inhibit the phosphorylation of SMAD2/3 induced by TGF- β 1 [39]. We did not investigate if VD could block the binding of activated SMAD2/3 on their cognate responsive element in HLFs. However, we show that the hormone had very limited effects on the phosphorylation of the two SMAD proteins in HLFs. VD was thus

unlikely to inhibit the binding of VDR on SMAD proteins. This result might explain why VD could not actively prevent the differentiation of myofibroblasts induced by TGF- β 1. It may also be hypothesized that concentrations of VD higher than 100 nM may be required to fully block the TGF- β 1 signalling in these cells. However, we demonstrated that 1 μ M VD neither further increased the activity of VDR nor more strongly inhibited the differentiation of IPF HLFs when compared to cells treated with 100 nM. In addition, it should be noted that μ M concentrations of vitamin D, and this metabolite, are hardly reached in humans supplemented with the hormone [40]. It thus appears that, *in vitro*, VD only weakly prevented the TGF- β -dependent expression of profibrotic markers in IPF HLFs.

Finally, our results demonstrate that VD prevented the induction of aerobic glycolysis in myofibroblasts likely *via* VDR-dependent signalling. The stimulation of aerobic glycolysis has been identified in several pulmonary cells of patients with IPF, including alveolar epithelial cells, macrophages and fibroblasts [41]. This metabolic reprogramming is increasingly described as an essential mechanism promoting the development of IPF [41]. Indeed, *in vitro*, inhibitors of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, that prevent the metabolic reprogramming induced by TGF- β 1 in HLFs, reduce the profibrotic effects of TGF- β 1 [6,42]. *In vivo* inhibition of glycolysis also attenuates bleomycin-induced pulmonary fibrosis [6,42]. Aerobic glycolysis appears to promote the differentiation of myofibroblasts first by stimulating α -SMA expression [6]. Our results are concordant with this mechanism although we found that VD almost totally blocked the increase in aerobic glycolysis while it only slightly decreased the expression of α -SMA in TGF- β 1-stimulated HLFs. In addition, glycolysis leads to the ultimate production of lactic acid by the LDH-dependent reduction of pyruvic acid. Different studies have reported that lactic acid concentrations could be elevated in lung parenchyma and bronchoalveolar lavages in patients with IPF [7,43]. The secretion of lactic acid, notably through specific transporters, favours a

decrease in the extracellular pH, with secondary increases in the activation of latent TGF- β [7]. Interestingly, our findings demonstrate that VD significantly reduced LDH expression, LDH activity and lactic acid production. It may thus be hypothesized that VD could limit the formation of active TGF- β by preventing extracellular acidification due to the secretion of lactic acid. Additional experiments are however required to determine if VD may reduce the acidification in the lung microenvironment and block the loop of profibrotic response linked to glycolysis in patients with IPF.

In conclusion, the present study indicates that, *in vitro*, VD could prevent major profibrotic parameters in HLFs, including the metabolic reprogramming. Taking into account these results, and the ability of VD to prevent lung fibrosis *in vivo* in experimental models, it may be interesting in future to determine if a supplementation with vitamin D could contribute to limit the decline in lung function in patients with IPF.

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Authors' contributions

AJ, TV, SJ, LV conceived and designed the study. AJ, CM, TV performed the experiments. TV, AJ, SJ, LV interpreted the data and wrote the manuscript. CLN, BDLT, SR provided lung biopsy. DLG revised and edited the manuscript. Boehringer Ingelheim Pharma GmbH had no role in the design, analysis or interpretation of the results in this study but it was given the opportunity to review the manuscript for medical and scientific accuracy. All authors read and approved the final manuscript.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files]

Ethics approval and consent to participate

This study was approved by the local ethics committee (Ethics Committee CHU Rennes, n° 16123). Written informed consent was obtained from all subjects. All experiments were performed following relevant guidelines and regulations.

Consent for publication

Not applicable.

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Authors' Contributions

AJ, TV, SJ, LV conceived and designed the study. AJ, CM, TV performed the experiments. TV, AJ, SJ, LV interpreted the data and wrote the manuscript. CLN, BDLT, SR provided lung biopsy. DLG revised and edited the manuscript. Boehringer Ingelheim Pharma GmbH had no role in the design, analysis or interpretation of the results in this study but it was given the opportunity to review the manuscript for medical and scientific accuracy. All authors read and approved the final manuscript.

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