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15-Deoxy-Δ-12, 14-prostaglandin J2 acts cooperatively with prednisolone to reduce TGF-β-induced pro-fibrotic pathways in human osteoarthritis fibroblasts

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Abstract
Background/Aims. Synovial fibrosis is a pathological process that is observed in several musculoskeletal disorders and characterized by the excessive deposition of extracellular matrix, as well as cell migration and proliferation. Despite the fact that glucocorticoids are widely employed in the treatment of rheumatic pathologies such as osteoarthritis (OA) and rheumatoid arthritis, the mechanisms by which glucocorticoids act in the joint and their impacts on pro-fibrotic pathways are still unclear.
Materials. Human OA synovial fibroblasts were obtained from knee and hip joints. Cells were treated with prednisolone (1 mM) or transforming growth factor-beta 1 (TGF-β1) (10 ng/ml) for 1 and 7 days for quantification of RNA and protein expression (by real-time quantitative reverse transcription-PCR and western blot, respectively), 72 h for immunocytochemistry analysis, and 48 h for proliferation (by BrdU assay) and migration (by wound assay) studies. In addition, cells were preincubated with prednisolone and/or the peroxisome proliferator-activated receptor gamma (PPAR-γ) agonist 15-deoxy-Δ-12,14-prostaglandin J2 (15d-PGJ2) for 6 h before adding TGF-β1. pSmad1/5, pSmad2 and β-catenin levels were analyzed by Western blot. The activin receptor-like kinase-5 (ALK-5) inhibitor (SB-431542) was employed for the mechanistic assays.
Results. Prednisolone showed a predominant anti-fibrotic impact on fibroblast-like synoviocytes as it attenuated the spontaneous and TGF-β-induced gene expression of pro-fibrotic markers. Prednisolone also reduced α-sma protein and type III collagen levels, as well as cell proliferation and migration after TGF-β stimulation. However, prednisolone did not downregulate the gene expression of all the pro-fibrotic markers tested and did not restore the reduced PPAR-γ levels after TGF-β stimulation. Interestingly, anti-fibrotic actions of the glucocorticoid were reinforced in the presence of the PPAR-γ agonist 15d-PGJ2. Combined pretreatment modulated Smad2/3 levels and, similar to the ALK-5 inhibitor, blocked β-catenin accumulation elicited by TGF-β.
Conclusions. Prednisolone, along with 15d-PGJ2, modulates pro-fibrotic pathways activated by TGF-β in synovial fibroblasts at least partially through the inhibition of ALK5/Smad2 signaling and subsequent β-catenin accumulation. These findings shed light on the potential therapeutic effects of glucocorticoids treatment combined with a PPAR-γ agonist against synovial fibrosis, although future studies are warranted to further evaluate this concern.

Keywords: Fibroblast-like-synoviocytes; Fibrosis; Glucocorticoids; Osteoarthritis; Peroxisome proliferator-activated receptor-γ agonist; Transforming growth factor-β1.

1. Introduction

Fibrosis is abnormal wound healing defined by overgrowth, hardening and/or scarring of one or more tissues. This event is observed in synovial tissue in different rheumatic pathologies such as rheumatoid arthritis and osteoarthritis (OA) [1], [2], [3]. Synovial fibrosis contributes to articular pain and stiffness and is a hallmark of these diseases [1], [4], [5], and features a process likely associated with joint trauma or ligament knee surgery known as arthrofibrosis [2]. Fibrosis typically results from chronic inflammation or tissue injury, although the precise mechanisms triggering this pathological process in the joint are still evasive.

Synovial fibrosis is characterized by fibroblast activation and transformation into myofibroblasts, which are cells with contractile capacity that express alpha-smooth muscle actin 2 (α-sma). Myofibroblasts are responsible for the excessive secretion of extracellular matrix components including collagen (Col) type I and III, fibronectin or thrombospondin [1]. Transforming growth factor beta (TGF-β) signaling is the main inducer of this process, as well as cell migration and proliferation [6], [7]. In this sense, Smad signaling is recognized as a major pathway of TGF-β signaling for fibrosis [7]. The binding of TGF-β to its type II receptor recruits two different type I receptors, activin receptor-like kinase 5 (ALK5) and ALK1, which subsequently phosphorylate cytoplasmatic receptor Smad2/3 and Smad1/5/8, respectively [3]. Although growing numbers of studies suggest the involvement of ALK1-mediated pathways in fibrotic processes [8], [9], most of the literature indicates that the pro-fibrotic actions of TGF-β are mainly regulated by ALK5-Smad2/3, whereas ALK1-Smad1/5/8 signaling plays a role in its anti-fibrotic activities [3], [10]. Additionally, the Wnt/β-catenin pathway is also involved in TGF-β signaling [11], and cross-talk between both pathways has been described [12]. Wnt/β-catenin is pivotal in normal wound healing, although its sustained activation is commonly associated with fibrogenesis [11]. Thus, TGF-β signaling is very complex, as it participates in many other pivotal cellular processes. As a consequence, TGF-β blockade is an inadvisable therapy against fibrosis.

Glucocorticoids (GCs) are endogenously produced steroid hormones that act through glucocorticoid receptors to induce the expression of GC-sensitive genes. Synthetic GCs such as dexamethasone or prednisolone are widely employed in the treatment of rheumatic pathologies due to their powerful anti-inflammatory properties [13], [14], [15]. However, these hormones also present adverse events such as diabetes and osteoporosis [15], [16]. Nonetheless, the mechanisms by which GCs act in joints and their impacts on pro-fibrotic signaling pathways have not been completely delineated, despite the fact that it could inform the quest for more suitable treatments. Likewise, GCs present inconsistent effects on cell phenotype shift and organ fibrosis, likely due to their differential impacts on TGF-β pro-fibrotic signaling in different cells and organs [16], [17]. In this sense, we have previously described that prednisolone favored TGFβ signaling through Smad1/5 phosphorylation to the detriment of Smad2 phosphorylation pathway in joint cells [15], [18], [19].

Peroxisome proliferator-activated receptor gamma (PPARγ) is a ligand-dependent nuclear receptor involved in adipocyte differentiation and lipid metabolism [20], [21]. The inverse relationship between fibrosis and PPARγ expression/function was reported in multiple human fibrosing disorders as well as in animal models of fibrosis [20]. In the joint, PPARγ agonists show potential therapeutic effects against catabolic and inflammatory mediators involved in OA pathogenesis, as well as anti-fibrotic properties [21]. Moreover, PPARγ knockout mice present an accelerated spontaneous OA phenotype characterized by synovial inflammation and fibrosis [22]. However, the specific pro-fibrotic pathways and processes that PPAR-γ modulates in synovial fibrosis remain to be defined. Besides, it has been shown that PPAR-γ attenuates fibrotic processes in skin fibroblasts by blocking the activation of the TGF-β /Smad signaling pathway [23], [24], whereas in turn, TGF-β downregulates PPARγ expression through the activation of the same Smad2/3 signaling pathway [25].

In this study, we investigated for the first time to our knowledge the role of GCs on synovial fibrosis by evaluating their capacity to activate pro-fibrotic pathways per se and modulate fibrosis induced by TGF-β in OA synovial fibroblasts. We also tested the anti-fibrotic properties and mechanisms of the PPARγ agonist 15-deoxy-Δ-12,14-prostaglandin J2 (15d-PGJ2) alone or in combination with GCs.

2. Materials and methods

2.1. Cell culture

Synovial tissue was obtained from 20 osteoarthritic patients during joint replacement surgery (12 females and 8 males). The median age was 65 [40–77] years and median BMI was 30.1 [19.72–38.02] kg/m2. The institutional review boards (Research Ethics Committee) of CHU de Liège, Belgium approved the study protocol and the use of verbal informed consent to allow research procedures on the tissues collected. Synovial fibroblasts were isolated as previously described [26]. Cells were cultured in DMEM (Cambrex Bio Science, Baltimore, MD, USA) containing 10% fetal bovine serum (FBS; Lonza, Basel, Switzerland), L-glutamine (2 mM; Lonza, Basel, Switzerland), streptomycin (100 mg/ml; Lonza) and penicillin (100 U/ml; Lonza). Fibroblast subcultures were created with trypsin-EDTA (Lonza) and cells between the second and sixth passage were used for the experiments. Cells were seeded into 24-well plates (BD Biosciences, San Jose, CA, USA) for RNA and protein extraction or 96-well plates (BD Biosciences) for proliferation, migration and immunocytochemistry studies.

2.2. Reagents and treatments

Prednisolone (Pred, 1 μM) (Sigma-Aldrich, St Louis, MI, USA) was used as a glucocorticoid treatment [15], [18], [19], [26], [27]. TGF-β1 (10 ng/ml) (GIBCO-BRL, San Francisco, CA, USA) was employed to induce a fibrotic response based in our previous experience and the literature [18], [19], [28], [29], [30]. To activate the PPAR-γ pathway, the agonist 15d-PGJ2 (10 μM) (BioMol, Plymouth Meeting, PA, USA) was used as we and others have previously described [27], [31], [32], [33]. When indicated, synoviocytes were preincubated with prednisolone and/or PPAR-γ agonist for 6 h before adding TGF-β1 for 1 or 7 days for RNA and protein extraction, 72 h for immunocytochemistry studies, and 48 h for proliferation and migration assays. Additionally, an ALK-5 inhibitor, SB-431542 10 μM (Sigma-Aldrich) was also employed [26].

2.3. Reverse transcription qPCR experiments

Total RNAs were extracted and purified from cultured synoviocytes using RNeasy mini kit columns (Qiagen, Leiden, The Netherlands) and digested with DNAse I (#AM190, Ambion, Life Technologies, Gent, Belgium). cDNA was synthesized by the reverse transcription of 500 ng of RNA (in each reaction) with the RevertAid H Minus First Strand cDNA Synthesis Kit (#K1632, Thermo Scientific, Erembodegem – Aalst, Belgium) according to the manufacturer's instructions. cDNA products were then amplified by PCR using the KAPA SYBR FAST detection system (#KK4611, Sopachem, Eke, Belgium). qPCR experiments were run on a LightCycler 480 instrument (Roche Applied Science, Vilvoorde, Belgium) and the data were analyzed using the LC480 software release 1.5.0 SP4. The 2−ΔCT method was used to calculate the relative gene expression between the differently stimulated fibroblasts. Input amounts were normalized with the endogenous hypoxanthine phosphoribosyltransferase reference gene. All primers were purchased from Eurogentec (Seraing, Belgium). The primer sequences used are listed in Table 1.

Table 1. Primer sequences used for real-time PCR.

Gene	Forward	Reverse
Collagen I (COL1A1)	AGTTCGAGTATGGCGG	CAGTGACGCTGTAGGT
Collagen III (COL3A1)	GCGGTTTTGCCCCGTATTAT	TGCAGTTTCTAGCGGGGTTT
Thrombospondin 1 (THBS1)	CAGACCGCATTGGAGATAC	CCATCGTTGTCATCATCGTG
Fibronectin 1 (FN1)	CTGGCCGAAAATACATTGTAAA	CCACAGTCGGGTCAGGAG
Connective tissue growth factor (CTGF)	TTGGCAGGCTGATTTCTAGG	GGTGCAAACATGTAACTTTTGG
Metalloproteinase 13 (MMP13)	CAACGGACCCATACAG	ACAGACCATGTGTCCC
Tissue inhibitor of metalloproteinases 1 (TIMP1)	TTCCGACCTCGTCATCAG	TGAGAAACTCCTCGCT
Alpha smooth muscle actin 2 (ACTA2)	CGTGTTGCCCCTGAAGAGCAT	ACCGCCTGGATAGCCACATACA
Serum or glucocorticoid inducible kinase 1 (SGK1)	GACAGGACTGTGGACTGGTG	TTTCAGCTGTGTTTCGGCTA
E-Cadherin (CDH1)	TGGAGGAATTCTTGCTTTGC	CGCTCTCCTCCGAAGAAAC
Cadherin 11 (CDH11)	GATCGTCACACTGACCTCGACA	CTTTGGCTTCCTGATGCCGATTG
Twist-1 (TWIST1)	AGCTACGCCTTCTCGGTCT	CCTTCTCTGGAAACAATGACATC
Hypoxanthine-guanine phosphoribosyltransferase (HPRT)	TGTAATGACCAGTCAACAGGG	TGCCTGACCAAGGAAAGC

2.4. Western blot

Cells were lysed with RIPA buffer (150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS and 50 mM Hepes pH 7.5) containing phosphatase inhibitors (25 mM β-glycerophosphate, 1 mM Na3VO4, and 1 mM NaF) and complete protease inhibitor mixture (Roche Applied Science) and the total proteins were separated by SDS-PAGE as previously described [26]. Membranes were incubated with anti-α-sma (DAKO A/S, Glostrup, Denmark), anti-p-Smad2 (S465/467), anti-Smad2, anti-p-Smad1/5 (S463/465), anti-Smad1 (Cell Signaling Technology, Leiden, Netherlands), anti-β-catenin, anti-PPAR-γ or anti-HSP-90 (Santa Cruz Technologies, Heidelberg, Germany) antibodies overnight. Western blots were visualized with 1:2000-diluted anti-mouse or anti-rabbit (DAKO A/S) secondary antibodies and ECL chemiluminescent reagents (GE Healthcare, Buckinghamshire, UK). The ImageJ image processing software (http://imagej.nih.gov/) was used to quantify the protein bands by densitometry. The band intensity of a targeted protein was divided by its related HSP90 band intensity for the normalization process. The ratios «protein/HSP90» (arbitrary units) from several experiments ($n \geq 5$) are presented on a graph.

2.5. Immunocytochemistry

Synovial fibroblasts were fixed in ice-cold methanol for 15 min at −20 °C. The cells were washed three times in PBS, blocked in PBS-0.1% Tween 20 (PBST) + 2% BSA for 30 min, and incubated with mouse anti-human α-sma (DAKO A/S) or rabbit anti-human collagen III (Abcam, Cambridge, UK) for 1 h. The wells were then washed with PBST and peroxidase-labeled goat anti-mouse or anti-rabbit secondary antibody (DAKO A/S) was added and incubated for 30 min. The cells were then counterstained with hematoxylin (Merck, Overijse, Belgium) and examined using an inverted microscope CKX41 (Olympus, Antwerpen, Belgium). ImageJ was used to measure the percentage of positive area among the synovial cells.

2.6. Cell proliferation assay

Synovial fibroblasts were grown in 96-well plates in DMEM supplemented with 10% FBS for 24 h. The cells were then made quiescent by overnight incubation in medium containing 0.5% FBS. Subsequently, the cells were treated with different stimuli in DMEM with 2% FBS for 48 h. Cell proliferation was evaluated by the measurement of 5-bromo-2′deoxyuridine (BrdU) incorporation using a BrdU Cell Proliferation Assay Kit (Cell Signaling Technology) according to the manufacturer's recommendations.

2.7. Cell wound assay

Cells were cultured in 96-well plates in DMEM supplemented with 10% FBS. When the cells reached confluence, they were made quiescent by overnight incubation in medium containing 0.5% FBS. Then, cell monolayers were scratched with a 10 μl pipette tip to generate a linear wound. Subsequently, the wells were washed with medium to remove the detached cells and differently stimulated in DMEM with 0.5% FBS. Digital captures (4X) were taken using a CKX41 microscope (Olympus) after scratching and after 48 h and wound healing was assayed using the ImageJ software (http://imagej.nih.gov/). Cell migration was calculated as the percentage of the recovered distance of wound separation after 48 h stimulation compared with the initial wound separation.

2.8. Intracellular collagen quantification

Intracellular collagen was assayed by picrosirius red (PSR) (Sigma-Aldrich) staining, a well-established histological technique for visualizing collagen [34]. Briefly, cells were fixed in methanol for 15 min at −20 °C, washed with PBS, and incubated in 0.1% picrosirius red staining solution for 3 h. Then, the solution was removed and the cells were washed with 0.1% acetic acid. Intracellular retained PSR was solubilized in 0.1 M sodium hydroxide and the absorbance was read at 550 nm on an EnSpire® 2300 Multilabel Reader (PerkinElmer, Zaventem, Belgium). Additionally, photographs of cell staining were captured before solubilization using a digital camera under a CKX41 inverted microscope (Olympus) at 4× magnification.

2.9. Statistical analysis

The results in the graphs in the figures represent the means from «n» independent experiments (n = number of patients), with boxes with whisker lines indicating the maximum and minimum values detected. Comparisons between two conditions were analyzed using the nonparametric Wilcoxon-test and were considered as significantly different when $p < 0.05$.

3. Results

3.1. Prednisolone modulates pro-fibrotic markers in synovial cells

To elucidate whether GCs per se modulate fibrosis in synovial tissue, we initially evaluated the in vitro effects of the widely used glucocorticoid prednisolone on the expression of a battery of genes commonly associated with cellular phenotype (alpha smooth muscle actin 2 [ACTA2], E-cadherin [CDH1], and cadherin 11 [CDH11]), matrix synthesis and turnover (collagen I [COL1A], collagen III [COL3A1], thrombospondin 1 [THBS1], fibronectin 1 [FN1], metalloproteinase 13 [MMP13], and tissue inhibitor of metalloproteinases 1 [TIMP1]) and pro-fibrotic signaling (connective tissue growth factor [CTGF], serum or glucocorticoid inducible kinase 1 [SGK1], and Twist-1 [TWIST1]). They are illustrated in Fig. 1. To pursue this goal, we stimulated OA synovial fibroblasts with prednisolone for 1 or 7 days. We also assessed the effects of TGF-β.

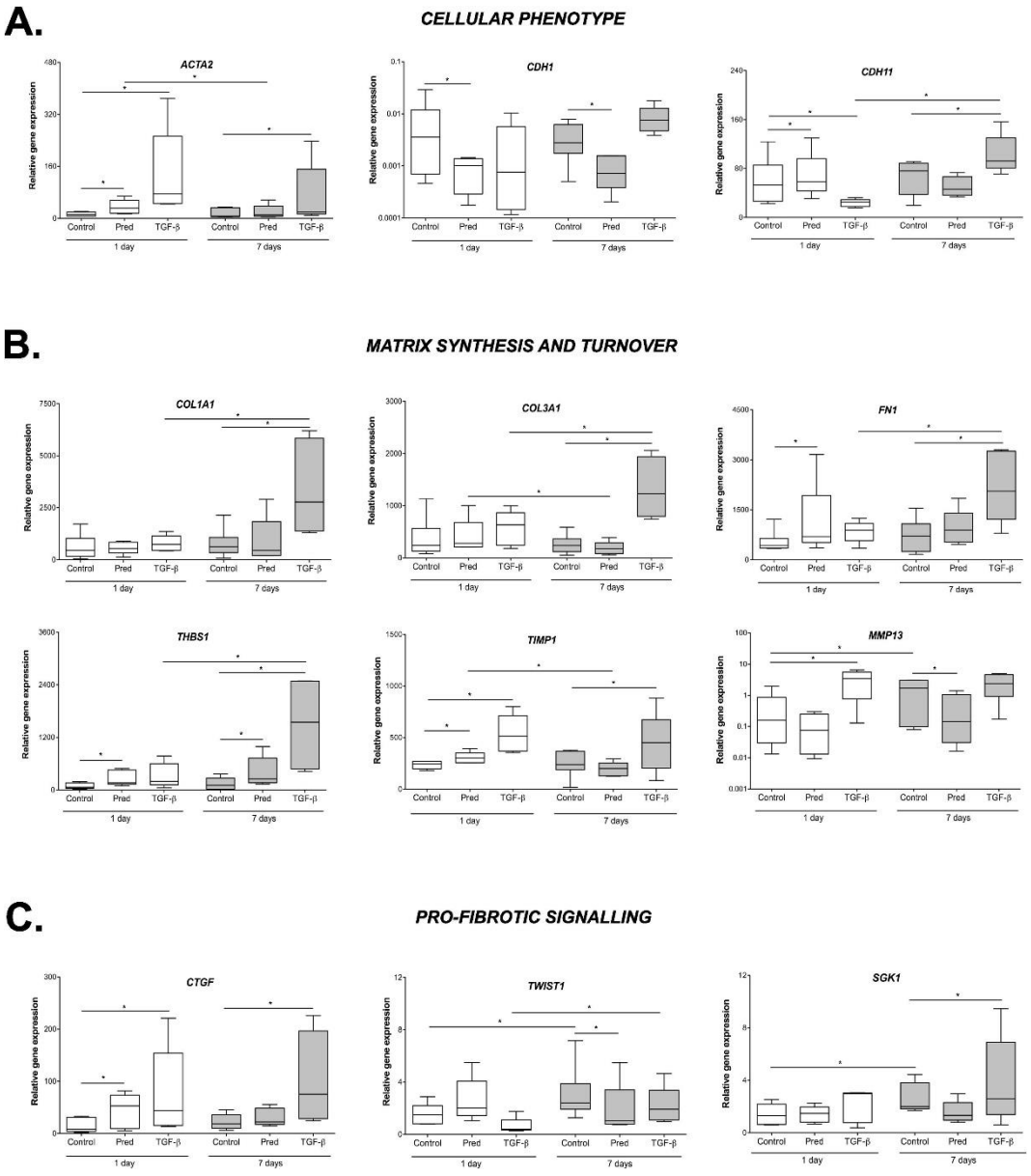

Fig. 1. Prednisolone modulated the expression of genes associated with pro-fibrotic pathways in OA fibroblast-like synoviocytes. Synovial fibroblasts were incubated in the presence or absence of prednisolone (Pred) (1 μM) or TGF-β (10 ng/ml) for 1 or 7 days. (A) Expression of genes commonly associated with cellular phenotype (alpha smooth muscle actin 2 [ACTA2], E-cadherin [CDH1], and cadherin 11 [CDH11])), (B) matrix synthesis and turnover (collagen I [COL1A], collagen III [COL3A1], fibronectin 1 [FN1], thrombospondin 1 [THBS1], tissue inhibitor of metalloproteinases 1 [TIMP1], and metalloproteinase 13 [MMP13]), and (C) pro-fibrotic signaling (connective tissue growth factor [CTGF], Twist-1 [TWIST1], and serum or glucocorticoid inducible kinase 1 [SGK1]) were analyzed by quantitative RT-PCR. The values were normalized to hypoxanthine guanine phosphoribosyltransferase (HPRT) expression ($n = 6$) and the control 1-day conditions were used as a reference. Whisker lines indicate the maximum and minimum values detected. Significance was set at $*=P \leq 0.05$.

We first observed a significant increase in the expression of genes such as MMP13 and TWIST1 as well as SGK1 after 7 days of culture without stimulation (*=$P < 0.05$). Interestingly, all the spontaneous increases in expression were downregulated under prednisolone stimulation. Prednisolone also significantly decreased its initial induction of ACTA2, COL3A, and TIMP1 expression over time (*=$P < 0.05$). In contrast, the glucocorticoid treatment upregulated THBS1 levels and reduced CDH1 expression at both stimulation times (*=$P < 0.05$).

We next observed the effects of TGF-β. As expected, compared to control conditions, we detected that TGF-β is a good inducer of genes involved in fibrosis. Indeed, after 7 days of treatment, ACTA2, TIMP1, COL3A1, THSB1, and CTGF as well as COL1A1, FN1, and CDH11 were significantly increased (*=$P < 0.05$). In contrast, after 1 day of TGF-β stimulation, only ACTA2, MMP13, CTGF, and TIMP1 were significantly upregulated (*=$P < 0.05$). Accordingly, when comparing TGF-β stimulation over time, we observed that the expression of COL3A1, THSB, and TWIST1 as well as COL1A1, CDH11, and FN1 were significantly enhanced (*=$P < 0.05$).

Briefly, we observed that prednisolone treatment showed some anti-fibrotic properties after 7 days of incubation, as it attenuated the spontaneous secretion of markers (e.g., MMP13, SGK1 or TWIST1), as well as induced a significant reduction of others initially upregulated at day 1 of treatment, such as ACTA2 and TIMP1.

3.2. Prednisolone regulates TGF-ß-induced pro-fibrotic pathways

To confirm our previous results at the protein level and evaluate the effects of prednisolone on pro-fibrotic signaling induced by TGF-β, we pretreated synoviocytes with prednisolone in the presence or absence of TGF-β and then examined pro-fibrotic hallmarks. First, the protein expression of the myofibroblastic marker, α-sma, was assessed by Western blot. TGF-β but not prednisolone increased the expression of α-sma after 7 days (Fig. 2A). Besides, prednisolone pretreatment attenuated the effects of TGF-β (Fig. 2A). In agreement, similar results were detected by immunocytochemistry (Fig. 2B). We also analyzed intracellular collagen levels and specifically type III collagen expression. By staining with PSR, we observed that glucocorticoid treatment slightly increased intracellular collagen but nonsignificantly modulated collagen-III production assessed by immunocytochemistry (Fig. 2C,D). In contrast, intracellular and type III collagen levels were both increased after TGF-β stimulation. Additionally, prednisolone preincubation did not modulate intracellular collagen accumulation but reduced type III collagen levels induced by TGF-β in FLS.

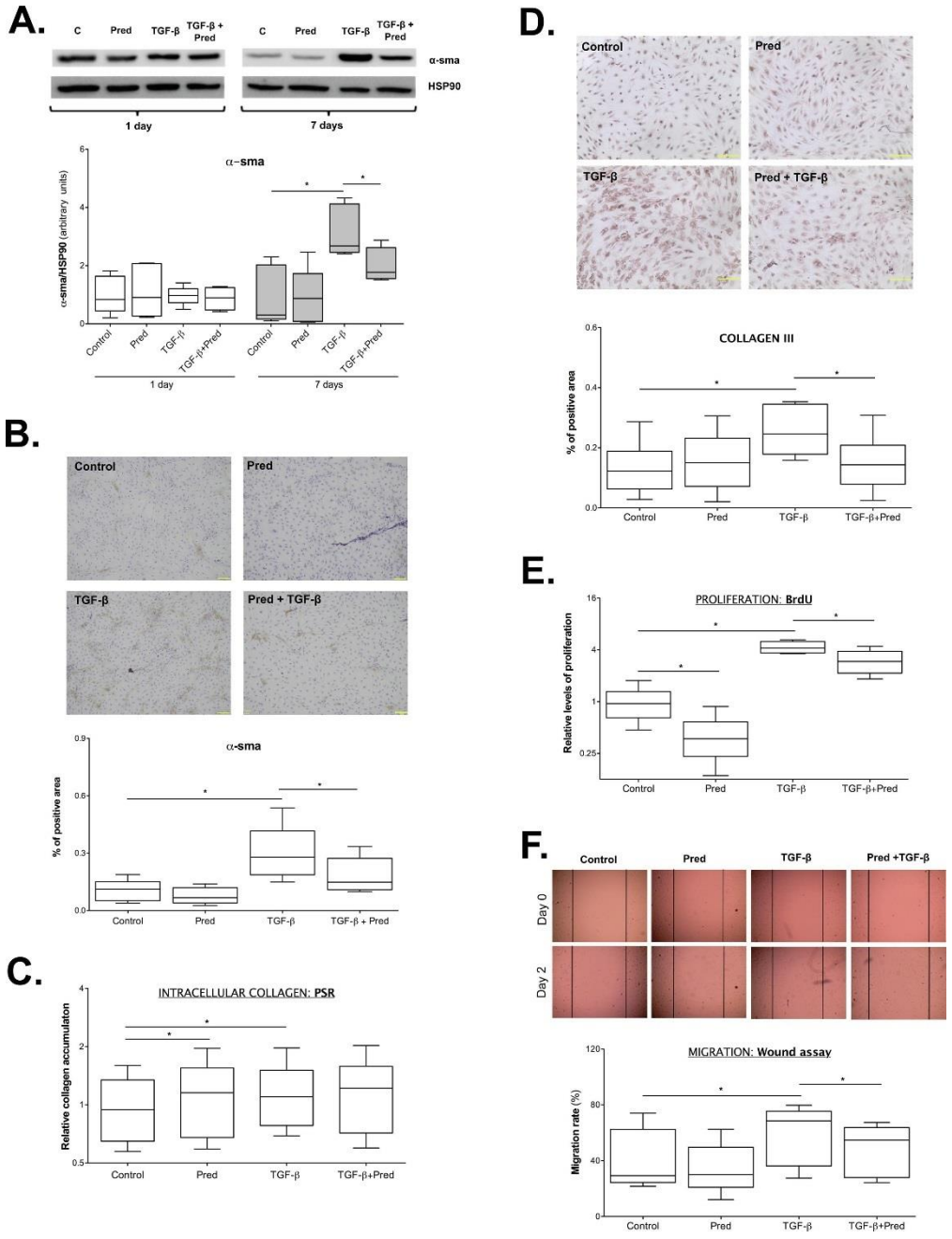

Fig. 2. Prednisolone regulated TGF-ß-induced pro-fibrotic pathways in OA fibroblast-like synoviocytes. (A) Synovial fibroblasts were preincubated with prednisolone for 6 h before TGF-β stimulation for 1 or 7 days. The protein expression of α-smooth muscle actin 2 (α-sma) was analyzed by Western blot. The values were normalized to heat shock protein 90 (HSP90) levels and the control 1-day conditions were used as a reference. The upper panel shows images obtained from a representative experiment and below are the quantification results for 6 performed Western blots. (B) α-sma levels were also assayed by immunocytochemistry. Representative images are illustrated. The lower panel shows the analysis of the percentage of positive area quantification (n = 5). (C) Intracellular collagen and (D) collagen type III were quantified by picrosirius red (PSR) and immunocytochemistry, respectively. Representative images are in the superior panel. Quantitative graphs are shown in the lower panel (n = 6). Proliferation analysis was performed with (E) BrdU assays (n = 6). (F) Measurement of the cell migratory capacity was assayed by wound assay. The represented data are the percentage of migration compared to the respective condition at day 0 (n = 5). The upper panel shows images obtained from a representative experiment. Whisker lines indicate the maximum and minimum values detected. Significance was set at *=P ≤ 0.05. Bar = 150 μm.

To evaluate cell proliferation, we performed BrdU proliferation assays. TGF-β significantly increased cell proliferation, while prednisolone decreased that observed in non- and TGF-β-stimulated fibroblasts (Fig. 2E). Finally, a wound assay performed with FLS showed that prednisolone per se failed to modulate cell migration, whereas its pretreatment reduced cell mobility elicited by TGF-β stimulation (Fig. 2F).

In conclusion, we observed that TGF-β behaves such as a fibrosis inducer in OA fibroblasts. Interestingly, although prednisolone slightly induced intracellular collagen accumulation, it significantly attenuated pro-fibrotic processes after TGF-β stimulation, suggesting that prednisolone might have some anti-fibrotic actions.

3.3. PPAR-γ agonists collaborate with prednisolone to modulate fibrotic markers

PPARγ is described as a ligand-dependent nuclear receptor with protective effects against fibrosis and TGF-β pro-fibrotic signaling in different cell types, such as fibroblasts and epithelial cells from skin, lung or kidney. Here, we observed that TGF-β slightly but significantly reduced PPAR-γ expression at 1 day of stimulation. Interestingly, prednisolone failed to rescue PPAR-γ levels, suggesting that its anti-fibrotic effects are independent from its modulation of PPAR-γ levels (Fig. 3A). To explore the possibility that PPAR-γ pathway activation can modulate the pro-fibrotic markers induced by TGF-β, prednisolone, or by a combination of both, we first pretreated FLS with the PPAR-γ agonist 15d-PGJ2 in the presence or absence of TGF-β and prednisolone. Subsequently, we analyzed the expression of those genes that we previously detected as significantly modulated by TGF-β and prednisolone (Fig. 3B). 15d-PGJ2 significantly decreased TGF-β-induced COL1A, COL3A1, THSB1 and MMP13 expression (Fig. 3B), but it failed to significantly modulate TGF-β-induced ACTA2 and CTGF levels. Interestingly, the PPAR-γ agonist also decreased prednisolone-induced ACTA2 and CTGF (Fig. 3B) as well as FN1. The combined treatment further improved the effects of prednisolone on TGF-β-induced COL1A1, COL3A1, and MMP13 levels (Fig. 3B).

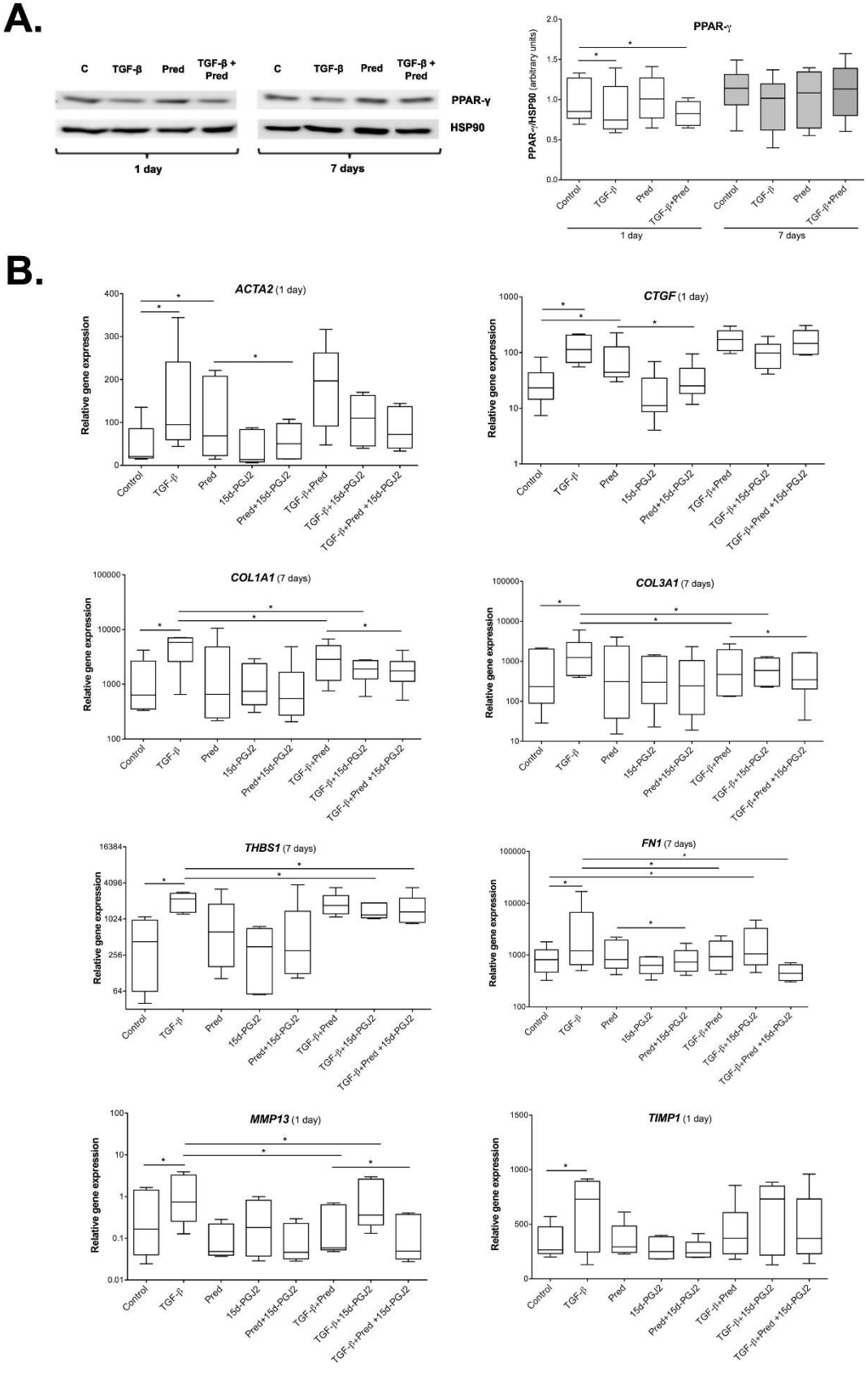
A.
C
TGF-β
Pred
TGF-β + Pred
PPAR-γ
HSP90
1 day
7 days
PPAR-γ/HSP90 (arbitrary units)
Control
TGF-β
Pred
TGF-β+Pred
B.
ACTA2 (1 day)
CTGF (1 day)
COL1A1 (7 days)
COL3A1 (7 days)
THBS1 (7 days)
FN1 (7 days)
MMP13 (1 day)
TIMP1 (1 day)
Relative gene expression
15d-PGJ2
Pred+15d-PGJ2
TGF-β+15d-PGJ2
TGF-β+Pred +15d-PGJ2

Fig. 3. PPAR-γ agonist 15d-PGJ2 collaborated with prednisolone to modulate pro-fibrotic gene expression. (A) PPAR-γ levels were analyzed by Western blot in synovial fibroblasts stimulated as previously indicated. The left panel shows images obtained from a representative experiment and the quantification analysis of 6 performed Western blots is on the right. The values were normalized to heat shock protein 90 (HSP90) levels and the control 1-day conditions were used as a reference. (B) Synovial cells were pretreated with the PPAR-γ agonist 15d-PGJ2 and/or prednisolone (pred) for 6 h before TGF-β stimulation at the indicated times. Total RNAs were isolated and analyzed by quantitative RT-PCR to determine relative gene expression of α-smooth muscle actin 2 (ACTA2), connective transforming growth factor (CTGF), collagen I (COL1A1), collagen III (COL3A1), thrombospondin 1 (THBS1), fibronectin 1(FN1), metalloproteinase-13 (MMP13), and tissue inhibitor of metalloproteinases 1 (TIMP1). The values were normalized to hypoxanthine guanine phosphoribosyltransferase (HPRT) expression ($n = 6$) and the control 1-day conditions were used as a reference. Whisker lines indicate the maximum and minimum values detected. Significance was set at $*=P \leq 0.05$.

At the protein level, 15d-PGJ2 per se did not decrease TGF-β-induced α-sma expression or did not synergize the effects of prednisolone on the decrease in TGF-β-induced α-sma expression (Fig. 4A). However, 15d-PGJ2 per se significantly attenuated the TGF-β-induced expression of collagen III and cell migration (Fig. 4B and D), although it failed to reduce the intracellular collagen accumulation assayed by PSR. In contrast, a significant decrease in cell proliferation or migration was observed when 15d-PGJ2 was combined with prednisolone + TGF-β (Fig. 4C and D).

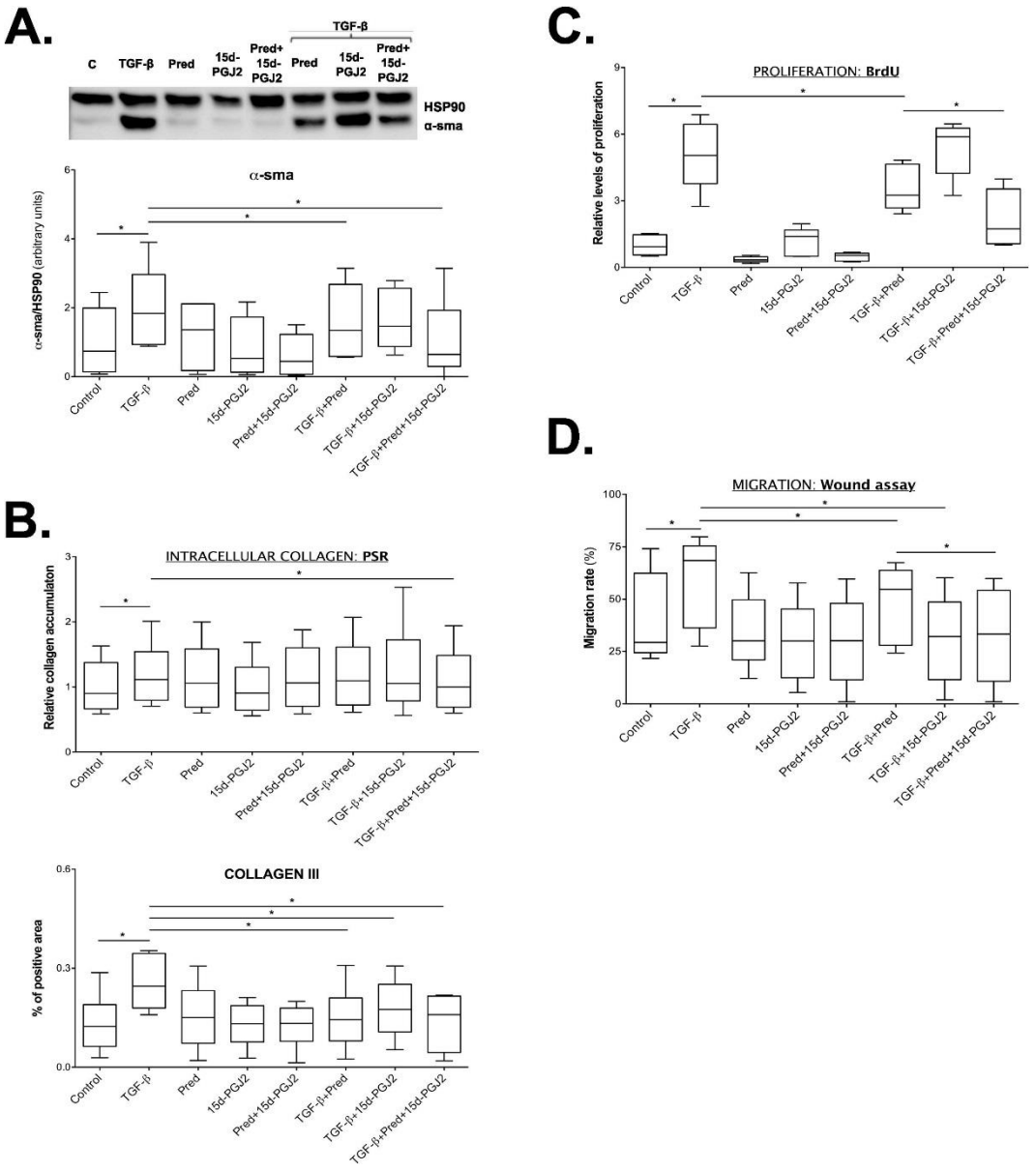

Fig. 4. 15d-PGJ2 collaborates with prednisolone to modulate TGF-ß-induced pro-fibrotic processes. (A) Synovial cells were pretreated with the PPAR-γ agonist 15d-PGJ2 and/or prednisolone (pred) for 6 h before TGF-β stimulation at the indicated times. The protein expression of α-smooth muscle actin 2 (α-sma) was analyzed by Western blot. The upper panel shows images obtained from a representative experiment and below are the quantification results for 6 performed Western blots. The values were normalized to heat shock protein 90 (HSP90) levels and the control conditions were used as a reference (n = 6). (B) Total intracellular collagen (upper panel) and collagen type III (lower panel) accumulation were quantified by picrosirius red (PSR) and immunocytochemistry, respectively (n = 6). (C) Proliferation analysis was performed by BrdU assays (n = 5). (D) Measurement of the cell migratory capacity was assayed by wound assay. The represented data are the percentage of migration compared to the respective conditions at day 0 (n = 5). Whisker lines indicate the maximum and minimum values detected. Significance was set at *=P ≤ 0.05.

3.4. Smad signaling is involved in prednisolone modulation of pro-fibrotic pathways

Smad signaling is recognized as a major pathway in TGF-β signaling of fibrosis [3], [7]. It principally includes two intracellular pathways involving the phosphorylation of different cytoplasmic R-Smads. Here, we evaluated the activation of both pathways by measuring pSmad2 or pSmad1/5 levels at an early (1 h) or longer period (24 h) of stimulation. As shown in Fig. 5, TGF-β induced Smad2 phosphorylation, which was slightly inhibited by prednisolone at 1 h of stimulation and only by prednisolone plus 15d-PGJ2 pretreatment over time (Fig. 5A and B). TGF-β significantly increased the pSmad1/5 levels after 1 h of incubation (Fig. 5A), whereas no difference was observed between TGF-β and the control at 24 h. In contrast, prednisolone significantly upregulated pSmad1/5 levels after the longer period of stimulation (Fig. 5B). 15d-PGJ2 treatment attenuated the effects of prednisolone (Fig. 5B). To clarify whether Smad signaling activation could participate in the effects of prednisolone on TGF-β-induced fibrosis, we pretreated synoviocytes with 10 μM SB-431542, a potent and specific inhibitor of ALK-5, an upstream effector of Smad2/3. As expected, SB-431542 efficiently blocked Smad2 phosphorylation in all the tested conditions (Fig. 6A). Additionally, it did not modify pSmad1/5 values in the control or TGF-β alone-treated cells (Fig. 6A). ALK-5 inhibitor decreased cell proliferation and α-sma levels induced by TGF-β alone or in combination with prednisolone in absence or presence of 15d-PGJ2 (Fig. 6B,C). Pretreatment with SB-431542 also attenuated intracellular and type III collagen in TGF-β-treated synoviocytes, but it failed to modulate collagen production in the presence of prednisolone (Fig. 6D,E).

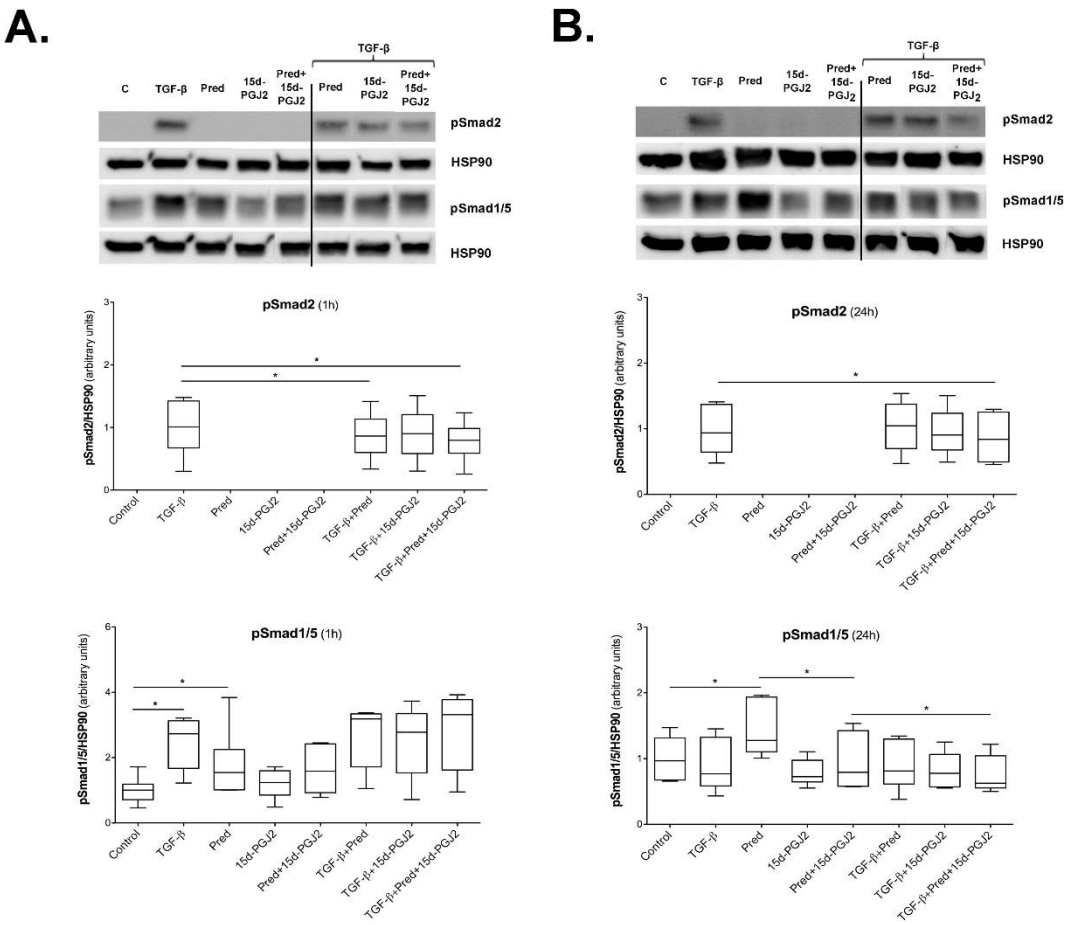

Fig. 5. Smad phosphorylation is modulated under prednisolone and TGF-ß stimulation. Synovial fibroblasts were pretreated with 15d-PGJ2 and/or prednisolone (pred) for 6 h before TGF-β stimulation at the indicated times. pSmad2 or pSmad1/5 levels at (A) an early (1 h) or (B) longer (24 h) period of stimulation were analyzed by Western blot. The upper panels show images of a representative experiment from 6 independent experiments and below are the respective Western blot quantification results (n = 6). The values were normalized to heat shock protein 90 (HSP90) levels and TGF-β (in pSmad2 panels) or the control conditions (in pSmad1/5 panels) were used as a reference. Whisker lines indicate the maximum and minimum values detected. Significance was set at *=$P \leq 0.05$.

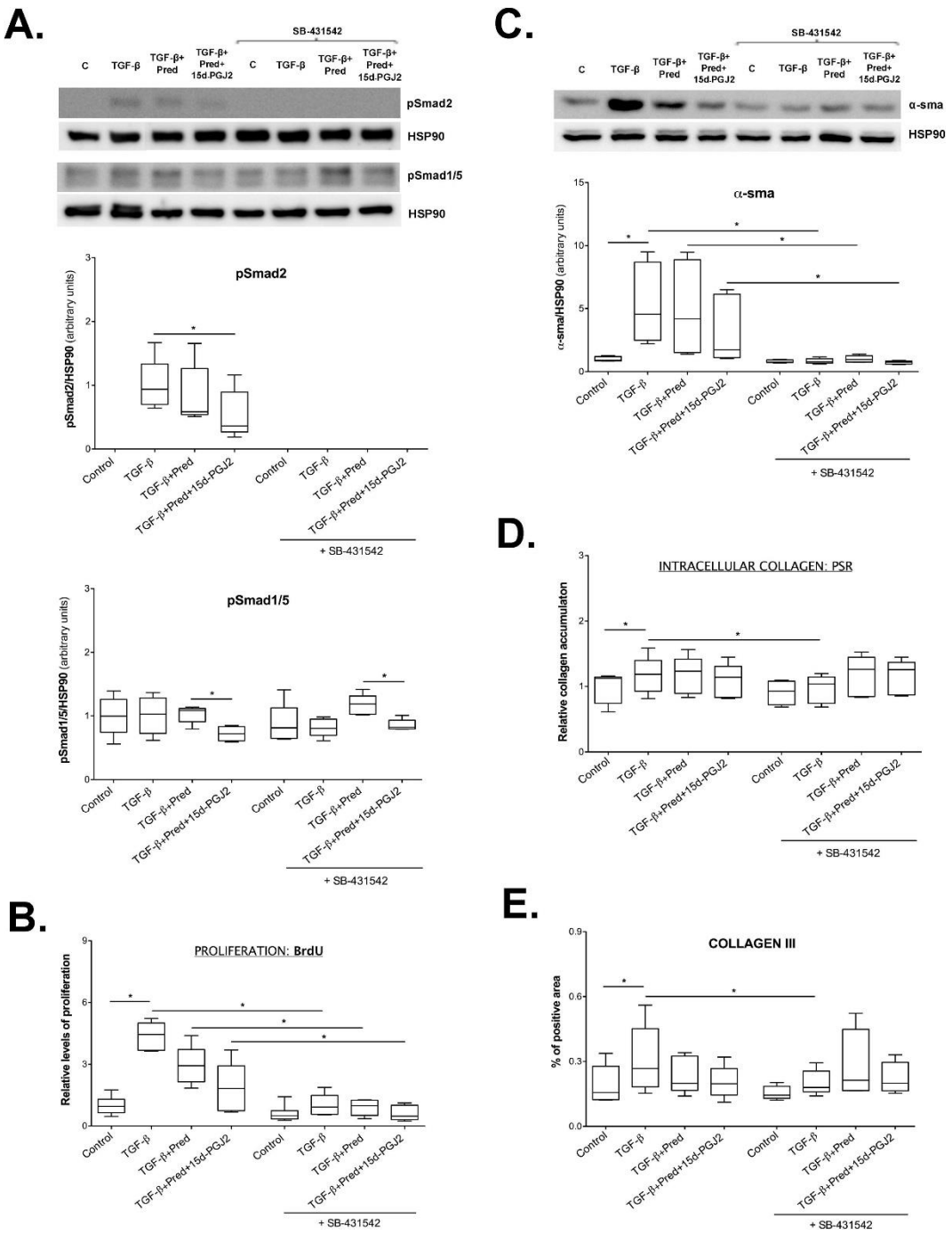

Fig. 6. ALK5-Smad2/3 signaling is involved in prednisolone modulation of pro-fibrotic pathways. Synovial fibroblasts were treated with an ALK-5 inhibitor (SB-431542) and incubated as previously indicated. Then, (A) pSmad2 and pSmad1/5 and (C) α-smooth muscle actin 2 (α-sma) levels were quantified by Western blot. The values were normalized to heat shock protein 90 (HSP90) levels and TGF-β (in pSmad2 panel) or the control conditions (in pSmad1/5 and α-sma panels) were used as a reference. (B) Proliferation analysis was performed by BrdU assays. (D) Intracellular collagen and (E) collagen type III were quantified by picrosirius red (PSR) and immunocytochemistry, respectively. Whisker lines indicate the maximum and minimum values detected (n = 5). Significance was set at *=$P \leq 0.05$.

3.5. Prednisolone modulates TGF-β-induced β-catenin levels

Sustained activation of the Wnt/β-catenin signaling appears to contribute to fibrogenesis [11]. To elucidate whether β-catenin signaling could be involved in TGF-β-induced pro-fibrotic pathways, we assessed β-catenin levels by Western blot. TGF-β significantly upregulated the expression of β-catenin in synovial fibroblasts stimulated for 1 or 7 days (Fig. 7A). After 7 days of stimulation, 15d-PGJ2 and prednisolone significantly reduced the β-catenin expression induced by TGF-β (Fig. 7A). Interestingly, only cells pretreated with prednisolone plus 15d-PGJ2 showed a strong decrease in β-catenin levels after TGF-β stimulation at both tested times (Fig. 7A). Likewise, treatment with ALK-5 inhibitor blocked β-catenin accumulation in TGF-β-treated cells both in the presence and absence of prednisolone (Fig. 7B). These results suggest that agonists of PPAR-γ or inhibition of ALK5 (Smad2/3) activation from the TGF-β pathway can modulate Wnt signaling by decreasing β-catenin expression levels.

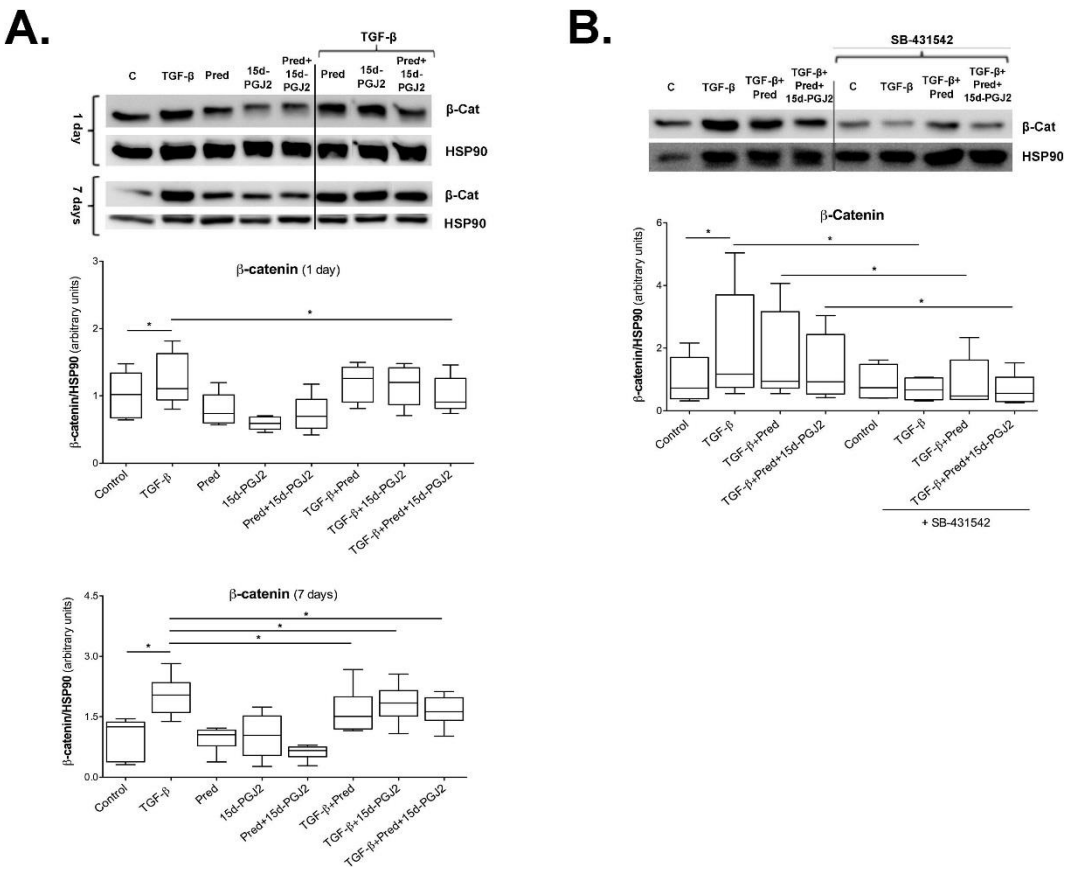

Fig. 7. TGF-β-induced β-catenin accumulation is modulated by prednisolone and is dependent on ALK5 signaling. (A) Synovial fibroblasts were pretreated with the PPAR-γ agonist 15d-PGJ2 and/or prednisolone (pred) for 6 h previous to TGF-β stimulation at the indicated times. β-catenin levels were visualized by Western blot. The upper panel shows the representative images of 1 experiment from 6 independent experiments performed and below are the quantification analysis (n = 6). (B) In 5 additional experiments, cells were treated with the ALK-5 inhibitor (SB-431542) and incubated as previously indicated. Then, β-catenin levels were assayed by Western blot (n = 5). The values were normalized to heat shock protein 90 (HSP90) levels and the control conditions were used as a reference. Significance was set at *=$P \leq 0.05$.

4. Discussion

Synovial fibrosis is a pathological event characterized by the development of an intra-articular excess of fibrous tissue, which results in pain and the loss of joint function in musculoskeletal disorders such as OA [2], [3]. To date, no specific pharmacological or nonsurgical therapy can cure this condition or provide significant long-term benefits. In this study, the impact of GCs on the activation of pathological pathways leading to synovial fibrosis was evaluated. We observed a potential therapeutic effect of prednisolone against pro-fibrotic processes induced by TGF-β in OA synovial fibroblasts. The use of PPAR-γ agonists, such as 15d-PGJ2, further improved the anti-fibrotic actions of prednisolone.

GCs are used in the management of OA, rheumatoid arthritis, and other joint pathologies. However, long-term or high-dose administration of GCs habitually results in side effects in the joint such as osteoporosis, chondrocyte apoptosis [35], [36], and induction of the pro-inflammatory adipokine leptin [15], [18], [19]. Regarding fibrosis, GCs were demonstrated to promote lung fibroblasts to myofibroblast differentiation and pro-fibrotic pathways in kidney cells [16], [37]. However, other findings indicate a beneficial effect of GCs on pro-fibrotic signaling in bone and the peritoneal membrane [17], [38]. Thus, the inconsistent impact of GCs suggests the possibility of cell/tissue-dependent GCs action and raises the necessity of cell/tissue type-specific studies.

In this study, we evaluated the effects of prednisolone on the expression of markers involved in pro-fibrotic signaling in fibroblast-like synoviocytes after an early or late period of treatment. Although we observed an initial pro-fibrotic impact (i.e., decrease of CDH1 expression and increase of ACTA2, FN1, THSB1 and CTGF), the anti-fibrotic properties of prednisolone predominated over time, as previous findings seem to suggest [38]. Pathological situations (such as hypertrophic scars) differ from normal healing by the persistence of myofibroblasts [1], [39]. Prednisolone initially increased the expression of the myofibroblastic marker α-sma [40]; however, we failed to observe any modulation at the protein level or any morphological changes in the synoviocytes. We also detected that GC treatment upregulated CTGF and early extracellular matrix genes expression. Linking these results, Lee et al. described that CTGF prompted the differentiation of MSCs into extracellular protein-positive but α-sma-negative fibroblasts, suggesting that activated fibroblast by CTGF after prednisolone treatment could participate to normal wound healing rather than fibrosis [42]. In agreement, Kydd et al (2005) described how glucocorticoid treatment induced the transient induction of pro-fibrotic genes in an animal model of articular damage [13] and Remst et al. (2013) observed in a murine model with adenoviral expression of CTGF, that this growth factor induced weak but finally resolved fibrosis in the joint. In contrast, activation of TGF-β signaling can lead to permanent synovial fibrosis [29]. Thus, in agreement with previous studies [6], [28], we observed that TGF-β elicited a potent pro-fibrotic response in OA synovial fibroblasts.

To shed light on the discrepant effects of GCs on TGF-β inducing pro-fibrotic pathways [16], [17], we tested the effects of prednisolone on TGF-β-induced fibrosis markers. Prednisolone treatment significantly attenuated α-sma protein levels, type III collagen as well as cell proliferation and migration after TGF-β stimulation. Moreover, GC preincubation reduced TGF-β-induced gene expression of TIMP1 and extracellular matrix components. In contrast, prednisolone upregulated CTGF gene levels and failed to modulate the observed THBS-1 overexpression and intracellular collagen accumulation elicited under TGF-β treatment [2], [9], [28]. Altogether, our results indicate that prednisolone presents a predominant anti-fibrotic effect on OA synovial fibroblasts over a long time period, though a secondary and transitory pro-fibrotic impact could take place.

GCs are commonly used to treat chronic inflammatory diseases that present fibrosis; however, they usually show low anti-fibrotic effectiveness [14], [43]. Because prednisolone failed to achieve a fully suitable anti-fibrotic impact in our model, we tested whether co-treatment with a PPARγ agonist could improve the beneficial effects, as a great number of studies appeared to suggest [26], [44], [45]. It is widely accepted that PPARγ can participate in controlling fibrogenesis by inhibiting the TGF-β pathway [20]. In our study, we detected that TGF-β treatment reduced PPAR-γ levels in OA synovial fibroblasts. Hence, we tested the effects of the PPAR-γ agonist 15d-PGJ2, an endogenous ligand. 15d-PGJ2 alone presented a moderate effect on pro-fibrotic pathways activated by TGF-β. Interestingly, the combination of prednisolone with 15d-PGJ2 further improved the anti-fibrotic effects of both treatments separately. In agreement, a growing number of studies indicate the existence of cooperative actions between PPAR-γ agonists and glucocorticoid receptor signaling [46], [47]. Accordingly, others and we have recently described that 15d-PGJ2 modulates glucocorticoid signaling by alleviating activated pathways controlled by the glucocorticoid receptor [27], [31].

Intracellular TGF-β signaling is primarily mediated through the canonical Smad pathway. In our work, the chemical inhibition of ALK5-Smad2/3 signaling attenuated all the TGF-β-induced pro-fibrotic markers tested, indicating that these pathways are mainly controlled by Smad2/3 signaling. We observed that prednisolone upregulated pSmad1/5 levels and inhibited early TGF-β-induced pSmad2, suggesting a Smad signaling switch after glucocorticoid treatment as previously published [16], [18]. However, prednisolone was only able to maintain the inhibition of Smad2 phosphorylation over time in the presence of 15d-PGJ2. Taken together, our findings advise the inhibition of Smad2 signaling as a mechanism of action for the anti-fibrotic activity of the combined treatment. Additionally, 15d-PGJ2 inhibited most of the pro-fibrotic markers that prednisolone induced itself, and was also able to attenuate prednisolone-elicited Smad1/5 phosphorylation.

Activation of the canonical Wnt pathway, which involves regulation of the protein β-catenin, appears to be involved in fibrotic disease [11], [48]. Evidence indicates cross-talk between the Wnt/β-catenin and TGF-β/Smad pathways for promoting pro-fibrotic processes through the coregulation of fibrogenic gene targets [11], [12]. In our study, we observed that prednisolone reduced the accumulation of β-catenin induced by TGF-β. Once again, the addition of 15d-PGJ2 further improved the GC inhibition. We also observed that the inhibition of ALK5-Smad2/3 signaling attenuated β-catenin accumulation. These findings highlight the existence of interactions between β-catenin/Smad2/3 and suggest that their modulation could be responsible of the anti-fibrotic responses of prednisolone and 15d-PGJ2. Accordingly, mice lacking Smad3 displayed less β-catenin stabilization and activation [49], and knockdown of cytosolic β-catenin in epithelial cells attenuated TGF-β1-induced epithelial-mesenchymal transition through the inhibition of β-catenin/pSmad3 [50]. Nonetheless, a great number of studies indicate that PPAR-γ agonists could modulate fibrosis independent of the PPAR-γ and/or Smad signaling pathways [20], [32], [51]. Thus, more studies will be required to specifically address the inhibition of TGF-β-activated pro-fibrotic pathways by PPAR-γ agonists.

In conclusion, prednisolone modulates pro-fibrotic pathways commonly activated by TGF-β in fibroblast-like synoviocytes. These modulations are characterized by predominant anti-fibrotic impacts; however, secondary and transitory pro-fibrotic effects should not be discarded. Interestingly, the anti-fibrotic actions of GCs are reinforced in the presence of the PPAR-γ agonist 15d-PGJ2. This effect is likely mediated by the attenuation of Smad2/3 signaling and subsequent activation of Wnt signaling by β-catenin accumulation. Nonetheless, Smad1/5 signaling also appears to participate in the control of GC-induced pro-fibrotic pathways. These findings shed light on the potential therapeutic effects of GC treatment combined with PPAR-γ agonists against synovial fibrosis, though future studies are warranted to further evaluate this concern.

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All authors were involved in drafting the article or revising it critically for important intellectual content, and they read and approved the final version to be published. Study design and conception were performed by CV-G, DS, MM, RM. Acquisition of the data was collected by CV-G and SN. Analysis and interpretation of the data were carried out by CV-G, OM, EC, CD, DS. Synovial tissues were provided from PG and WK.

The institutional review boards (Research Ethics Committee) of CHU de Liège, Belgium, approved the study protocol and the use of verbal informed consent to allow research procedures on the tissues collected.

Declaration of interest

None.

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