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Development of a simple osteoarthritis model useful to predict *in vitro* the anti-hypertrophic action of drugs

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Running title: Development of an innovative OA model in vitro

Abstract

Osteoarthritis (OA) is characterized by cartilage degradation, inflammation and hypertrophy. Therapies are mainly symptomatic and aim to manage pain. Consequently, medical community is waiting for new treatments able to reduce OA process. This study aims to develop an *in vitro* simple OA model useful to predict drug ability to reduce cartilage hypertrophy.

Human primary OA chondrocytes were incubated with transforming growth factor beta 1 (TGF-β1). Hypertrophy was evaluated by Runx2, type X collagen, MMP13 and VEGF expression. Cartilage anabolism was investigated by Sox9, aggrecan, type II collagen and glycosaminoglycan expression.

In chondrocytes, TGF-β1 increased expression of hypertrophic genes and activated canonical WNT pathway, while it decreased dramatically cartilage anabolism, suggesting that this treatment could mimic some OA features *in vitro*. Additionally, EZH2 inhibition, that has been previously reported to decrease cartilage hypertrophy and reduce OA development *in vivo*, attenuated COL10A1 and MMP13 upregulation and SOX9 downregulation induced by TGF-β1 treatment. Similarly, pterosin B (an inhibitor of Sik3), and DMOG (a hypoxia-inducible factor prolyl hydroxylase which mimicks hypoxia), repressed the expression of hypertrophy markers in TGF-β stimulated chondrocytes.

In conclusion, we established an innovative OA model *in vitro*. This cheap and simple model will be useful to quickly screen new drugs with potential anti-arthritic effects, in complementary to current inflammatory models, and should permit to accelerate development of efficient treatments against OA able to reduce cartilage hypertrophy.

Introduction

Osteoarthritis (OA) is the most common rheumatologic disease worldwide, affecting more than 151 million of persons worlwide (1). Disability and pain resulting from OA usually affects life quality of patients. Furthermore, it is highly onerous, costing up to 2.5% of gross domestic product in the western world (2).

OA is characterized by loss of articular cartilage, osteophyte formation, subchondral bone changes, fibrosis, and in many patients by inflammation. This disease is challenging to manage because of the absence of cartilage self-repair. Currently, treatments are symptomatic and mainly aim to reduce pain and inflammatory eruptions. At advance stages, surgical procedures are inevitable, and prosthesis are employed to replace damaged joints. However, in about twenty percent of the patients, this procedure does not result in a pain free joint (3). Consequently, medical community is waiting for treatments able to reduce OA process. However, the development of new anti-arthritic drugs is challenging in particular due to the absence of *in vitro* model able to mimic all OA hallmarks.

Chondrocytes in OA cartilage show an aberrant phenotype and actively produce cartilage-degrading enzymes, such metallopeptidases (MMPs) and aggrecanases. Age and activation of inflammatory cytokines play a role in these changes. Additionally, during OA, articular chondrocytes taking a differentiation route resembling that of growth plate chondrocytes, expressing hypertrophy-like changes. They express type X collagen and MMP-13. These phenotype changes having similarities to terminal differentiating chondrocytes, are accompanied by acquisition of an autolytic phenotype resulting in destruction of the surrounding cartilage (4).

Human articular chondrocytes (HAC) treatment with interleukin-1 (IL-1)-β is the most approved *in vitro* OA model (5–7). Its advantage is to mimic the process of inflammation, but

it does not take into account the other components of the disease, especially cartilage hypertrophy.

Clinical studies have shown that TGF-β levels are very low or absent in normal articular joints, while they are elevated in OA patients synovium and cartilage (8–13). These elevated levels of TGF-β in OA joints activate cells that are not normally exposed to high levels of TGF-β, leading to an altered cellular differentiation which may contribute to OA pathogenesis (3). Experimental OA models in mice confirm the involvement of TGF-β, and particularly TGF-β1, in development of this disabling disease. For instance, bolus injections of TGF-β1 or adenoviral overexpression of TGF-β1 in mouse knee joints induce the formation of osteophytes with structure and localization which are similar to osteophytes found in spontaneous murine OA (14,15). Additionally, the inhibition of TGF-β signaling pathways significantly decreases osteophyte formation in OA mice (16,17). These results indicate, that at least in experimental models, TGF-β is the main driver of osteophyte formation and chondrocyte hypertrophy (3). Beside its effect on osteophyte formation, TGF-β is also involved in inflammation. Intra-articular injection of rat knee joints with TGF-β results in swelling and erythema within one day (18). This TGF-β-induced inflammation contribute to joint damage in OA. These clinical and preclinical data conduct us to hypothesis that TGF-β1 may be used *in vitro* to mimic some aspects of OA process.

In this study, we investigated the effect of TGF-β1 in human OA articular chondrocytes, and evaluated the ability of three drugs known to reduce cartilage hypertrophy *in vivo* to counteract TGF-β effects *in vitro*. Together, our data permit to validate that human OA articular chondrocytes treated with TGF-β1 could be used as an *in vitro* OA model to easily screen the ability of drugs to slow down disease progression and to reduce cartilage hypertrophy.

Materials and Methods

Cell culture and treatments

Human OA cartilage was obtained from the femoral heads of 20 patients undergoing hip replacement surgery (ages 49–85 years; median 73.5 years). The experimental protocol was approved by local ethical committee, named "Comité de Protection des Personnes Nord Ouest III" (agreement #A13-D46-VOL.19). All methods were performed in accordance with the relevant guidelines and regulations. Informed consent was obtained from all participants. They all signed agreement forms before the surgery, rendering to local legislation.

Chondrocytes obtained as previously described (19). Briefly, cells were released by cartilage digestion with type XIV pronase (2 mg/ml for 30 minutes; Sigma-Aldrich) and type II collagenase (2 mg/ml for 15 hours; Thermofisher). The cells were then washed with PBS and seeded at 4 x 10^4 cells/cm^2 in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 IU/ml of penicillin, 100 µg/ml of erythromycin and then incubated at 37°C in a humidified atmosphere containing 5% CO_2 until they reached 80% confluency. At this stage (about one week), cells were treated with TGF-β1 (R&D Biosystems; 5 ng/ml) for 1 or 2 days in the presence or absence of EPZ-6438 (Selleckchem), an EZH2 inhibitor also called tazemetostat, PterosinB (AOBIOUS), a Sik-3 inhibitor, and DMOG (Echelon Biosciences Inc), a drug mimicking hypoxia.

Metalloproteinase 13 (MMP13) release assay

MMP13 release into conditioned media was quantified using commercially available enzyme immunoassay kit (R&D Biosystems). Absorbance was determined at 450 nm with a wavelength correction set at 540 nm (Multiskan GO spectrophotometer, Thermo Scientific, France).

GAG release assay

GAG release into the medium was measured using a sulfated glycosaminoglycan assay kit (Blyscan™, Biocolor) according manufacturer's instructions.

RNA isolation and real-time reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted with NucleoSpin® RNA (Macherey-Nagel) according to manufacturer's protocol, and reverse transcribed into cDNA as previously described (5). Amplification of the generated cDNA was performed by real-time PCR using Step One Plus Real Time PCR system (Applied Biosystems) with appropriate primers. The relative mRNA level was calculated with the $2^{-\Delta\Delta CT}$ method. *RPL13a* expression was used as reference.

Protein extraction and western blotting

Cells were lysed and western blotting performed as previously described (5). The following antibodies were used: type I, II, and X type collagen (ab34710, ab182563, ab34712) were purchased from Abcam, aggrecan (AB10031) from Millipore, Sox9 (LS-C368527) from LSBio and β-actin (sc-47778), β-catenin (sc-7963), goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP from Santa Cruz Biotechnology.

Immunocytochemistry

Cells were washed once with PBS and fixed in 4% PFA during 10 min. Then, cells were washed three times with PBS and incubated overnight at 4°C with a solution composed by

PBS, 0.25% Triton, 1% BSA and 1/100^{e} dilution of H3K27me3 antibody (C36B11, Cell signaling). Next, cells were washed and incubated with a solution composed by PBS, 0.25% triton, 1% BSA, 1/1000^{e} Hoechst solution and 1/400^{e} dilution of Alexa Fluor 594 Goat IgG Anti Rabbit IgG (111-585-003, Jackson). Fluorescence was evaluated using EVOS FL Auto 2 Cell Imaging System (ThermoFisher Scientific).

Statistical analysis

Statistical significance was determined by Student's t-test. P-values less than 0.05 were considered significant.

Results

TGF-β1 increased the expression of genes associated to cartilage hypertrophy in OA chondrocytes

First, we tested whether TGF-β1 treatment is able to regulate the expression of genes associated to cartilage hypertrophy in human OA articular chondrocytes *in vitro*. We incubated cells with 5 ng/ml TGF-β1 for 24h and 48h, and next observed cell morphology and analyzed the expression of several genes known to be upregulated in hypertrophic OA cartilage. We investigated the expression of type X collagen (COL10A1) and Matrix Metallopeptidase 13 (MMP-13), the two most widely used markers of hypertrophic chondrocytes, as well as the expression of type I collagen (COL1A1), Runt Related Transcription Factor 2 (RUNX2), and Vascular endothelial growth factor (VEGF), which have also been associated to hypertrophy chondrocytes (4).

Microscopically, we did not observe any major difference in term of morphology between untreated cells and TGF-β treated-cells. It seems however, that TGF-β increased cell number (figure 1A). Concerning gene expression, TGF-β treatment strongly increased the level of mRNA of hypertrophic genes (COL10A1, MMP13, COL1A1, RUNX2 and VEGF) (figure 1B). This effect was also observed at protein level, at least for type X collagen and MMP-13 (figure 1B and C).

TGF-β1 activated β-catenin/WNT pathway in OA chondrocytes

To confirm that TGF-β1 treatment is able to induce hypertrophy in OA chondrocytes, we investigated Wnt pathways. Indeed, this signaling pathway is the major regulator of chondrocyte hypertrophy. Elevated canonical WNT signaling drives OA development by increasing RUNX2 activity thereby boosting hypertrophic differentiation of chondrocytes (4,20).

To investigate canonical WNT signaling, we analyzed the expression of β-catenin, and secreted frizzled-related protein 1 precursor (SFRP1). We found that in OA chondrocytes, TGF-β1 increased protein level of β-catenin (figure 2A), and down-regulated the WNT antagonist SFRP1 (figure 2B). This is coherent with an activation of canonical WNT signaling.

TGF-β1 decreased cartilage anabolism in OA chondrocytes

Cartilage hypertrophy is known to be also associated to a loss of the differentiated phenotype of chondrocytes, which is correlated to a decreased expression of SRY-Box 9 (Sox9), a crucial factor for chondrocytes, and a reduced deposit of cartilage matrix composed of type II

collagen (COL2A1), aggrecan (ACAN) and glycoaminoglycans (GAG). So, we investigated the expression of this cartilage markers in OA chondrocytes treated with TGF-β1 for 24h and 48h. In these experimental conditions, TGF-β1 reduced COL2A1, ACAN and SOX9 expression at both mRNA and protein levels (figure 3A and B). Additionally, TGF-β1 decreased GAG production (figure 3C).

Together, these results showed that TGF-β1 treatment in OA chondrocytes is able to mimic *in vitro* some OA traits, and to induce phenotype resembling to hypertrophic OA chondrocytes, suggesting that this model could be used to test the effects of putative anti-arthritic drugs on chondrocyte hypertrophy.

EZH2 inhibition counteracted the increase of H3K27 trimethylation induced by TGF-β1, and reduced hypertrophy *in vitro*

Recently, it has been shown that the inhibition of Enhancer of zeste homolog 2 (EZH2), an histone methyltransferase, ameliorates osteoarthritis development in mouse OA model, through the reduction of cartilage hypertrophy (21). Consequently, an EZH2 inhibitor should be a good tool to test the ability of our *in vitro* model to predict ability of drugs to reduce cartilage hypertrophy *in vivo*.

Herein, we investigated whether EPZ6438 (also named tazemetostat) counteracts TGF-β1-induced hypertrophy phenotype in cultures of human OA chondrocytes. First, we showed that EPZ6438 reduced, as expected, the trimethylation of H3K27 (H3K27me3) in chondrocytes, proving that the drug has efficiency inhibited the histone methylase EZH2 (figure 4). In addition, we observed that TGF-β1 increased the proportion of H3K27me3 positive cells. This effect was counteracted by the presence of EPZ6438.

Next, we investigated EPZ6438 effect on the expression of genes associated to hypertrophic or differentiated articular chondrocytes. In human OA chondrocytes treated with TGF-β1, EZH2 inhibitor reduced the expression of hypertrophic genes, namely MMP13 and type X collagen, while it increased Sox9, type II collagen and aggrecan expression (figure 5).

Pterosin B and DMOG reduced the expression of MMP13 and type X collagen in TGF-β stimulated chondrocytes

Next, we investigated the effect of two others drugs which are have been described as candidate therapeutic for osteoarthritis due to their ability to reduce hypertrophy (figure 6). First, we tested pterosin B, a Sik3 pathway inhibitor, which delay chondrocyte hypertrophy and mineralization (22). As expected, we observed that pterosin B reduces COL10A1 expression in TGF−β stimulated chondrocytes. Next, we studied the effects of dimethyloxalylglycine (DMOG), a hypoxia-inducible factor (HIF) prolyl hydroxylase, which mimics hypoxia in stabilizing HIF factors and enhancing their nuclear localization. Hypoxia and DMOG are known to reduce the expression of markers associated with chondrocyte hypertrophy, in particular type X collagen and MMP13 (23,24). Herein, we showed that in our *in vitro* model, DMOG reduced the expression of these two markers of hypertrophy. Together, these experiments confirms that human OA articular chondrocytes treated with TGF-β1 can be used as an *in vitro* OA model to easily screen the ability of drugs to reduce cartilage hypertrophy.

Discussion

In this paper, we established a simple model of culture able to mimic some features of osteoarthritis process, and particularly the shift of articular chondrocytes toward a

hypertrophic phenotype. This *in vitro* model could be useful to predict the ability of drugs to reduce cartilage hypertrophy and thus to slow down OA disease *in vivo*.

Osteoarthritis is a complex disease, difficult to reproduce *in vitro.* At cartilage level, OA is characterized by a tissue erosion due mainly to inflammation, but also by the formation of osteophytes due to terminal differentiation of articular chondrocytes which become hypertrophic. Current *in vitro* models used to study molecular OA mechanisms or to test the anti-arthritic effects of drugs consist usually to mimic inflammation observed during OA, by treated chondrocytes with IL-1β or TNF-α. Indeed, these cytokines have been found at an elevated level in synovial fluid of OA patients. These models permit to evaluate the ability of drugs to reduce inflammation as well as to decrease the expression of enzymes involved in cartilage breakdown. However, they fail to reproduce the induction of hypertrophic phenotype of chondrocytes observed during OA pathology. Herein, we propose another model based on the incubation of OA chondrocytes with TGF-β1. This model could be very useful to screen new drugs against OA, in complement to inflammatory models (based on IL-1β or TNF-α), before performing preclinical trials in rodents.

As IL-1β and TNF-α, TGF-β1 level has been found increased in joints of OA patients (3,4), and numerous studies show its involvement in OA process. Its role on cartilage is different in an OA joint than in a normal joint (3). It would be a protective effect in normal chondrocytes, whereas it would be harmful in aged and OA chondrocytes. Herein, in this study, we only used aged OA chondrocytes. It is possible that different results would be observed in young normal chondrocytes. In human OA chondrocytes, we observed that TGF-β1 treatment (5 ng/ml) increased hypertrophic markers, notably MMP13 and type X collagen. Type X collagen is considered as the standard marker for chondrocyte hypertrophy. MMP13, as called collagenase-3, is one of the major enzymes involved in the degradation of OA cartilage. Although accepted as a hypertrophy marker, its synthesis can be induced in chondrocytes by

alternative routes, such as inflammation or mechanical stress (4). So, it can be also considered as a marker of inflammation and cartilage breakdown.

Additionally, we showed that TGF-β1 activates the β-catenin/WNT pathway, and increases the expression of other hypertrophic markers, such as RUNX2 that is a master transcription factor involved in the control of chondrocyte hypertrophic differentiation, and that directly regulates the expression of type X collagen and MMP-13 (20,25–27).

Contrary to hypertrophic genes, the markers of differentiated chondrocytes (SOX9, COL2A1, ACAN) were down-regulated by TGF-β1 treatment. This is coherent with observation of the reduced synthesis of cartilage during advanced stage of osteoarthritis. Consequently, treatment of human OA chondrocytes with TGF-β1 mimics numerous features observed during osteoarthritis. This culture method could thus be useful to reproduce chondrocyte hypertrophy *in vitro*, permitting to study molecular mechanisms involved in this process and to test putative new treatments against OA before animal experimentation.

To confirm the efficiency of this culture model to predict drug ability to reduce cartilage hypertrophy during OA, we investigated the effect of three drugs known for the ability to reduce cartilage hypertrophy. First, we tested an epidrug on the expression of a panel of gene regulated by TGF-β1 in human OA chondrocytes. We found that EZH2 inhibition by EPZ-6438 opposites to the major effects of TGF-β1: it increases COL2A1, ACAN as well as SOX9 expression and decreases MMP13 and COL10A1 synthesis. These results are fully in agreement with published data from *in vivo* experiments showing that intra-articular injections of an inhibitor of histone methyltransferase enhancer of zeste homolog 2 (EZH2), a chromatin modifier that activates Wnt/β-catenin signaling, reduced cartilage hypertrophy and also decreased articular cartilage degradation in surgically induced osteoarthritis (21). We also validate our model using two others drugs, pterosin B (a Sik-3 inhibitor) and DMOG (a mimic of hypoxia), which are known to reduce hypertrophy cartilage *in vivo* (22,24).

In conclusion, we established a model able to induce chondrocyte hypertrophy *in vitro*, which permits to evaluate the ability of drugs to reduce cartilage hypertrophy. This tool should be useful to identify more quickly new drugs having anti-arthritic action. In addition, this study confirms that TGF-β1 induces harmful effects in OA chondrocytes, suggesting that therapeutic strategy aiming to favor TGF-β1 signaling to treat OA should be used with important precaution, and be very well controlled.

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Conflict of interest

Authors declare no conflict of interests

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Figure legends

Figure 1: TGF-β1 stimulates chondrocytes hypertrophy.

Human primary OA chondrocytes were treated with TGF-β1 (5 ng/ml) for 24h or 48 h. After treatment, RNA and proteins were extracted.

(A) Photography of cells after treatments.

(B) Relative mRNA expression of hypertrophy genes (type I and X collagen, Runx2, VEGF and MMP-13) was determined by real-time RT-PCR. Values were expressed as relative gene expression compared to untreated cells. Histograms represent means ± SEM (n=5). *: p-value ≤ 0.05; **: p-value ≤ 0.01.

(C) Protein expression of type I and X collagen was analyzed by Western-blot. Figure represents an image representative of results from 3 independent experiments.

(D) MMP-13 release in medium was determined by ELISA assay. Data are expressed as means ± SEM (n=3). *: p-value ≤ 0.05.

Figure 2: TGF-β1 activates β-catenin/WNT pathway.

Primary OA chondrocytes were treated with TGF-β1 (5 ng/ml) for 24h and 48h.

(A) After treatment, mRNA was extracted, and SIRP1 mRNA expression was determined by real-time RT-PCR. Data are expressed as means ± SEM (n=5). **: p-value ≤ 0.01.

(B) Proteins were extracted and β-catenin expression was evaluated by western blot (n=3).

Figure 3: TGF-β1 reduces the expression of genes involved in cartilage anabolism

Primary OA chondrocytes were treated as previously.

(A) COL2A1, ACAN and Sox9 expression were determined at mRNA level by RT-PCR. Data are expressed relative to untreated cells. Histograms represent means ± SEM (n=5). **: p-value ≤ 0.01.

(B) Protein expression were evaluated by western blot (n=3).

(C) GAG release into the medium was measured by Blyscan assay. Data are expressed as means ± SEM (n=3). *: p-value ≤ 0.05; **: p-value ≤ 0.01.

Figure 4: EZH2 inhibition counteracts the increase of H3K27 trimethylation induced by TGF-β1

Primary chondrocytes were treated with TGF-β1 (5 ng/ml) in the presence or absence of EPZ-6438 (10 μM) for 48 h.

(A) After treatment, H3K27me3 levels (red) were evaluated by immunohistology. Nucleus was marked by Hoescht staining (blue)

(B) H3K27me3 positive cells were quantified in three independent experiments. For each experiments, at least twenty cells were counted. Data are expressed as means ± SEM (n=3). *: p-value ≤ 0.05; **: p-value ≤ 0.01.

Figure 5: EPZ6438 counteracts the effects of TGF-β1 in OA chondrocytes.

Primary chondrocytes were treated with TGF-β1 (5 ng/ml) in the presence or absence of EPZ-6438 (10 μM) for 48 h. After treatment, RNA and proteins were extracted.

(A) mRNA relative expression of type X collagen, MMP-13, Sox9, COL2A1 and ACAN was determined by real-time RT-PCR. Data are expressed as means ± SEM relative to gene

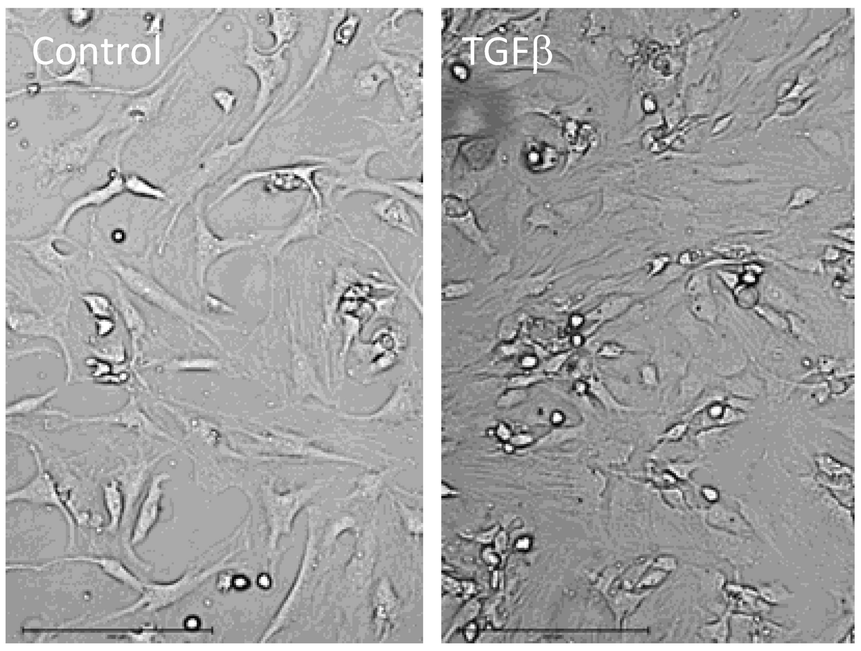
expression in TGF-β1-treated cells (n=4). Histograms represents. *: p-value ≤ 0.05; **: p-value ≤ 0.01.

(B) Protein expression of collagen type X was determined by western blot (n=3).

(C) MMP13 release in medium was determined by ELISA assay. Data are expressed as means ± SEM (n=3). *: p-value ≤ 0.05.

Figure 6: Pterosin B and DMOG reduced the expression of MMP13 and type X collagen in TGF-β stimulated chondrocytes

Primary chondrocytes were treated with TGF-β1 (5 ng/ml) in the presence or absence of PterosinB (300µM) or DMOG (500µM) for 48 h. After treatment, RNA were extracted. mRNA relative expression of type X collagen and MMP-13 was determined by real-time RT-PCR. Data are expressed as means ± SEM relative to gene expression in TGF-β1-treated cells (n=3). Histograms represents. *: p-value ≤ 0.05; **: p-value ≤ 0.01.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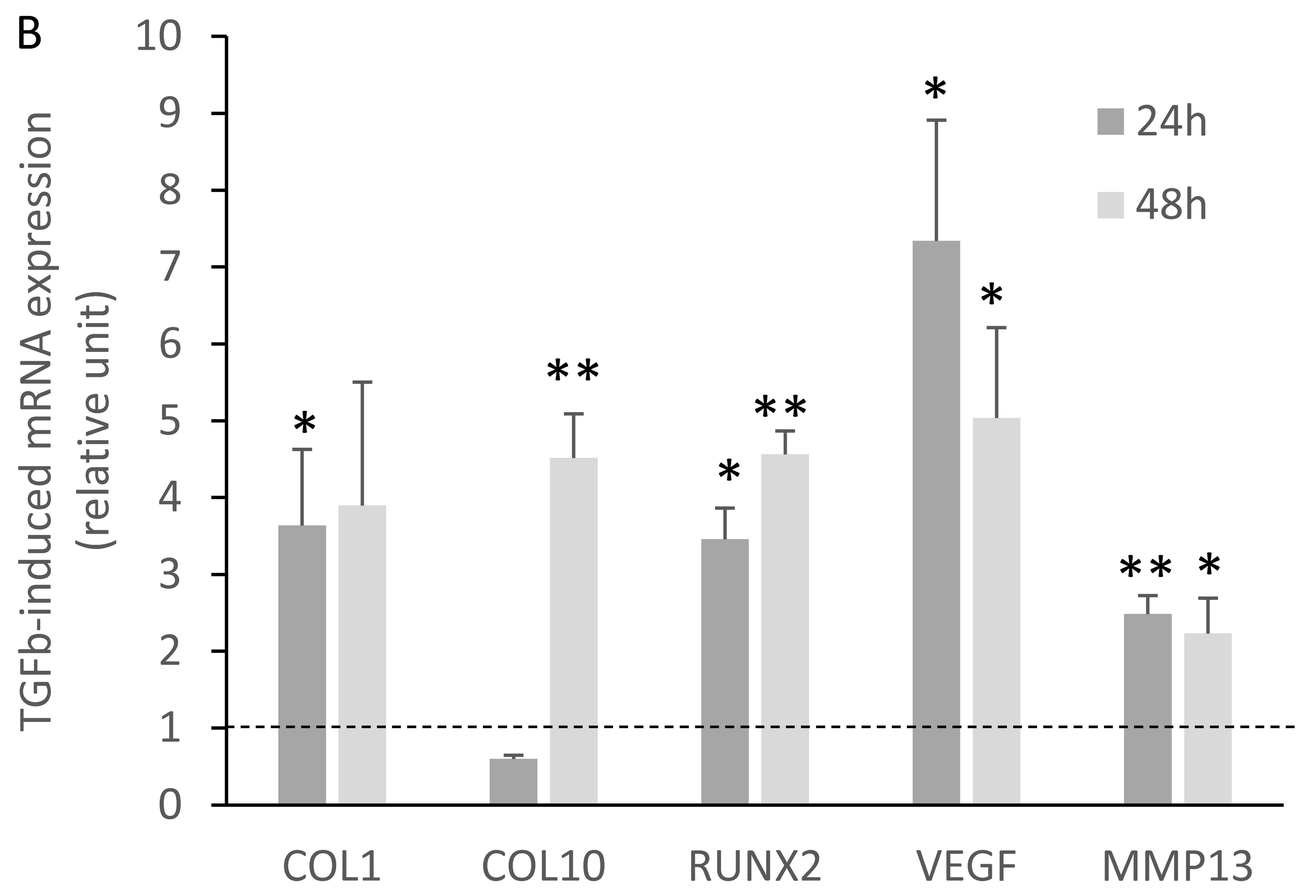
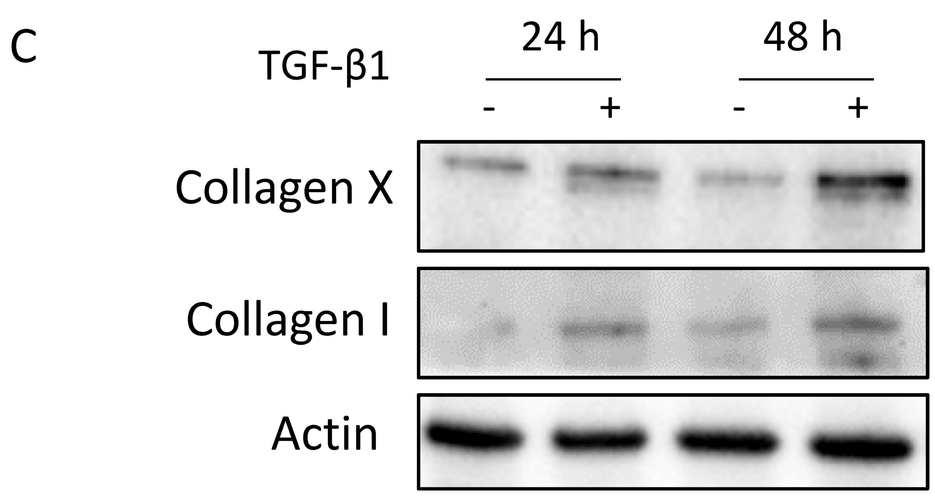
Control
TGFβ


B
TGFb-induced mRNA expression (relative unit)
10
9
8
7
6
5
4
3
2
1
0
24h
48h
COL1
COL10
RUNX2
VEGF
MMP13


C
TGF-β1
24 h
48 h
-
+
-
+
Collagen X
Collagen I
Actin

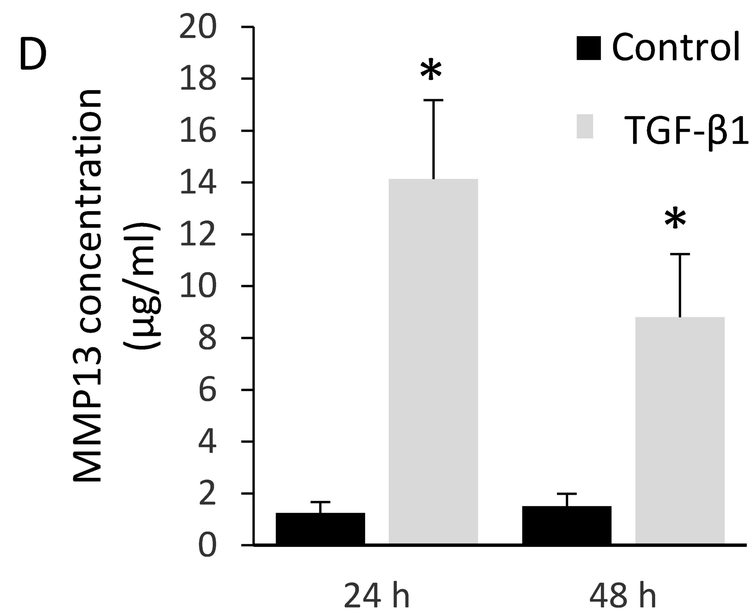

D
MMP13 concentration (µg/ml)
20
18
16
14
12
10
8
6
4
2
0
Control
TGF-β1
24 h
48 h

Figure 1

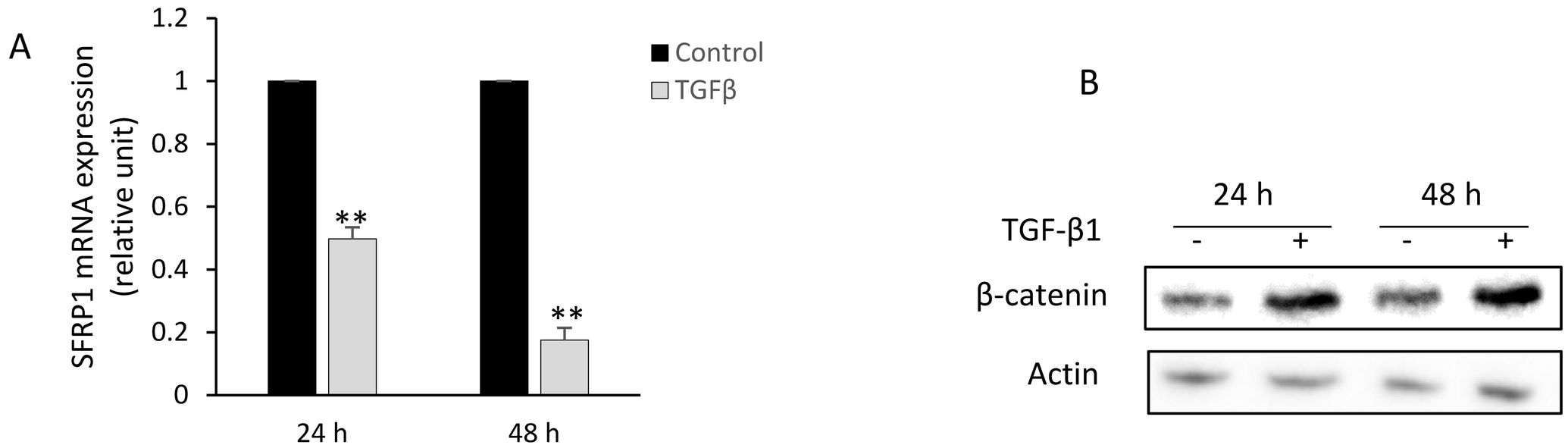
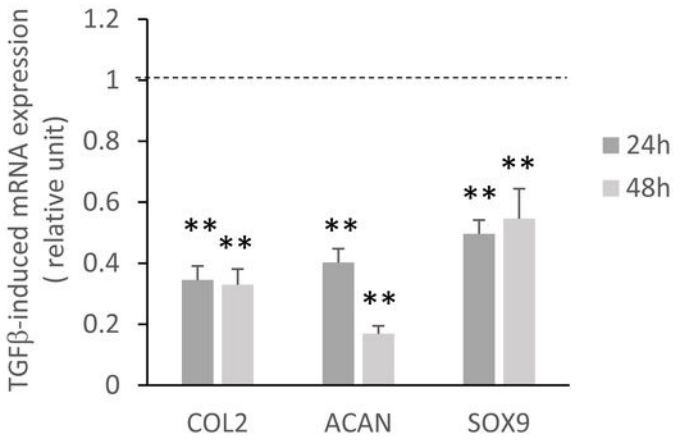
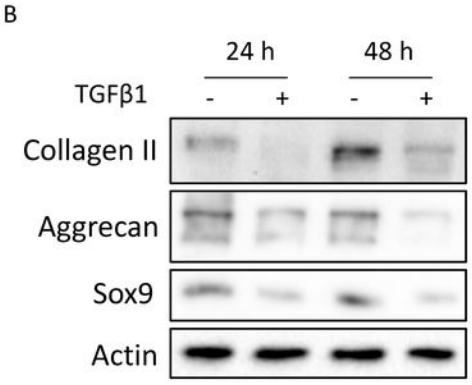

A
SFRP1 mRNA expression (relative unit)
1.2
1
0.8
0.6
0.4
0.2
0
Control
TGFβ
**
**
24 h
48 h
B
24 h
48 h
TGF-β1
-
+
-
+
β-catenin
Actin

Figure 2


TGFβ-induced mRNA expression
(relative unit)
1.2
1
0.8
0.6
0.4
0.2
0
24h
48h
COL2
ACAN
SOX9


B
24 h
48 h
TGFβ1
-
+
-
+
Collagen II
Aggrecan
Sox9
Actin

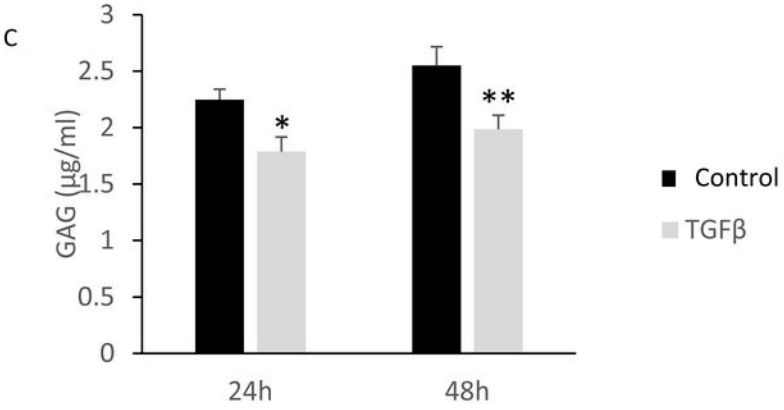

C
GAG (μg/ml)
3
2.5
2
1.5
1
0.5
0
24h
48h
Control
TGFβ

Figure 3

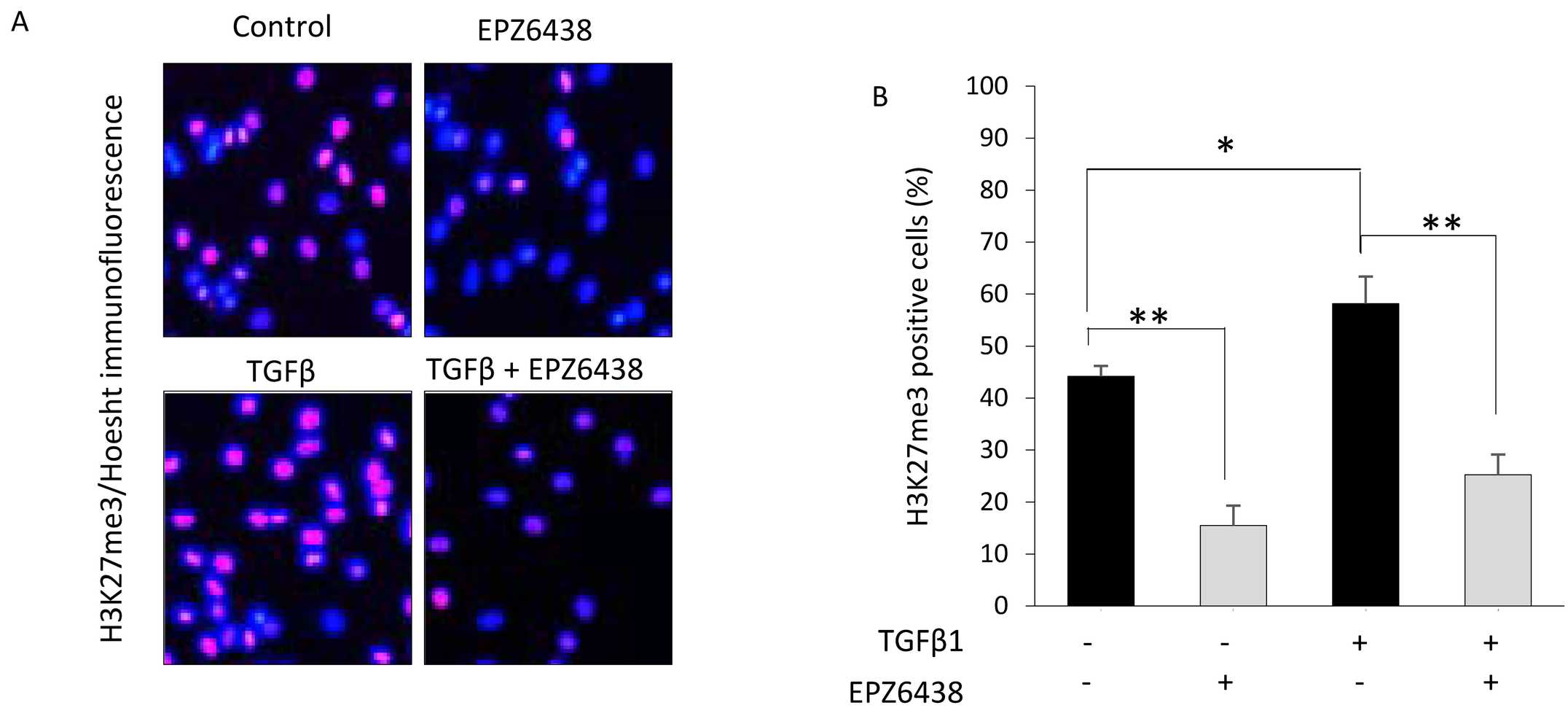
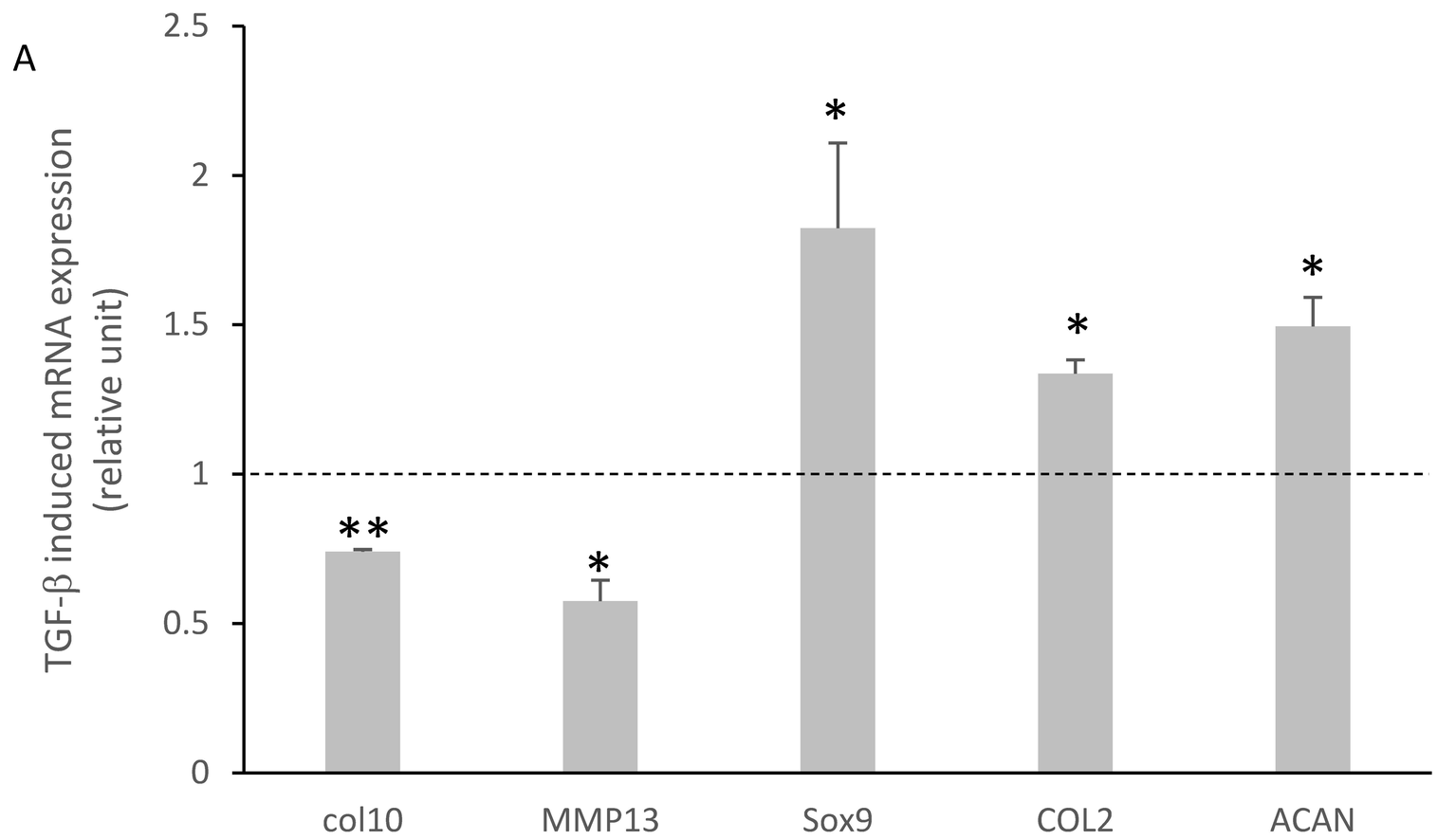
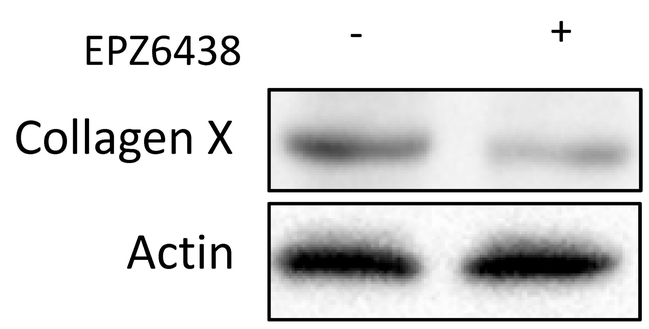

A
Control
EPZ6438
TGFβ
TGFβ + EPZ6438
H3K27me3/Hoesht immunofluorescence
B
H3K27me3 positive cells (%)
100
90
80
70
60
50
40
30
20
10
0
*
**
**
TGFβ1
EPZ6438
- - + +
- + - +

Figure 4


A
TGF-β induced mRNA expression
(relative unit)
2.5
2
1.5
1
0.5
0
**
*
*
*
*
col10
MMP13
Sox9
COL2
ACAN


B
EPZ6438
-
+
Collagen X
Actin

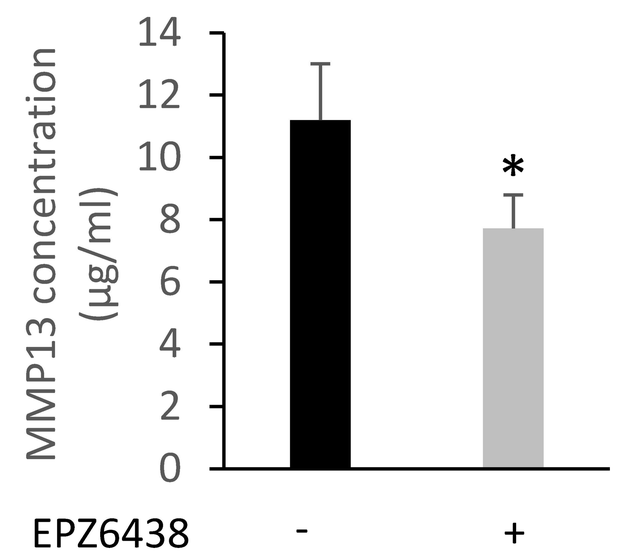
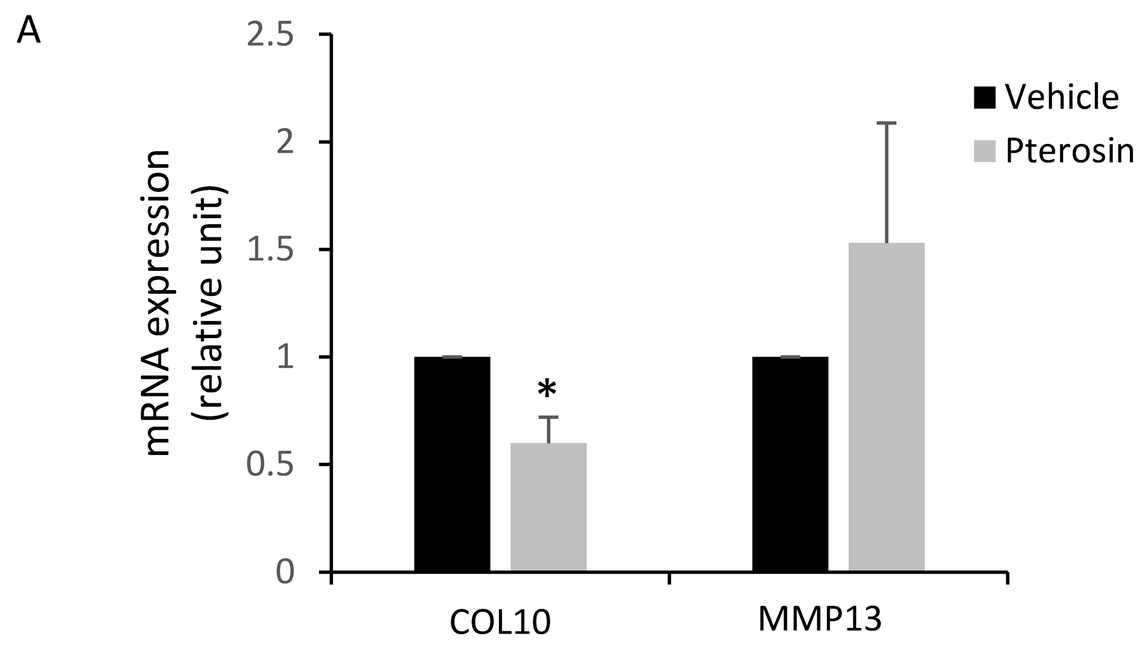

C
MMP13 concentration
(μg/ml)
14
12
10
8
6
4
2
0
*
EPZ6438
-
+

Figure 5


A
2.5
2
1.5
1
0.5
0
mRNA expression
(relative unit)
Vehicle
Pterosin
*
COL10
MMP13

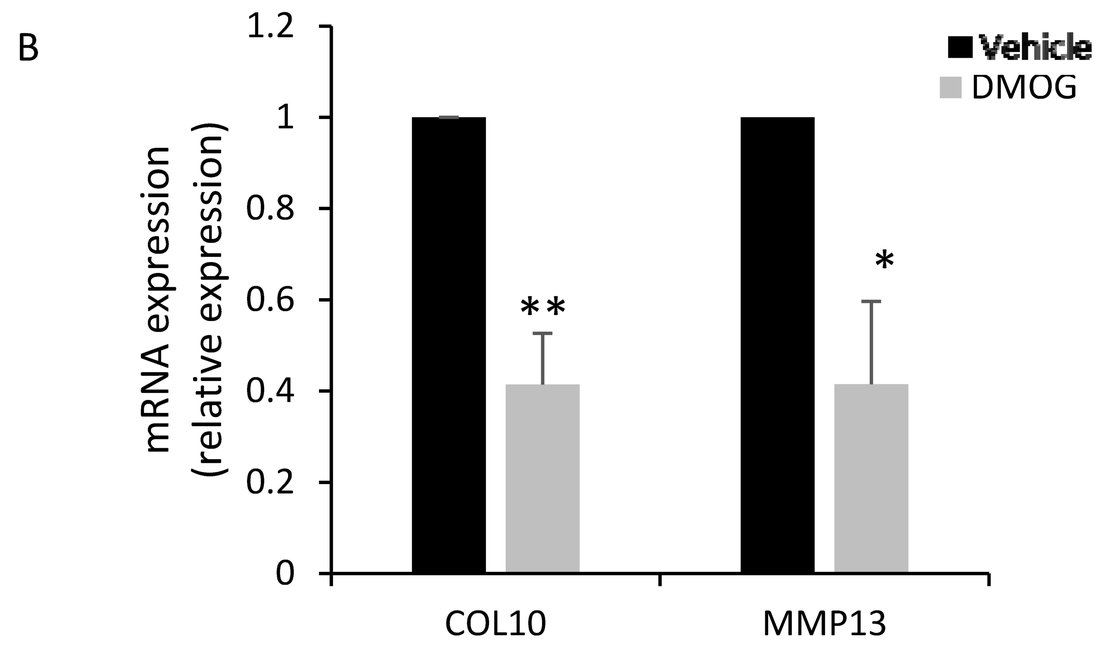

B
1.2
1
0.8
0.6
0.4
0.2
0
mRNA expression
(relative expression)
Vehicle
DMOG
**
*
COL10
MMP13

Figure 6