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ABSTRACT

Osteoarthritis (OA) is characterized by synovitis and synovial fibrosis. Synoviocytes are fibroblast-like resident cells of the synovium that are activated by TGF-β to proliferate, migrate and produce extracellular matrix. Synoviocytes secrete hyaluronan (HA) and proteoglycan-4 (PRG4). HA reduced synovial fibrosis *in vivo* and the *Prg4*$^{-/-}$ mouse exhibits synovial hyperplasia. We investigated the antifibrotic effects of increased intracellular cAMP in TGF-β stimulated human OA synoviocytes. TGF-β1 stimulated collagen I (COL1A1), α-SMA, TIMP-1, PLOD2 expression and procollagen I, α-SMA, HA and PRG4 production, migration and proliferation of OA synoviocytes were measured. Treatment of OA synoviocytes with forskolin (10μM) increased intracellular cAMP levels and reduced TGF-β1 stimulated COL1A1, α-SMA and TIMP-1 expression, with no change in PLOD2 expression. Forskolin also reduced TGF-β1 stimulated procollagen I and α-SMA content, as well as synoviocyte migration and proliferation. Forskolin (10μM) increased HA secretion, PRG4 expression and production. A cell permeable cAMP analog reduced COL1A1 and α-SMA expression and enhanced HA and PRG4 secretion by OA synoviocytes. HA and PRG4 reduced α-SMA expression and content and PRG4 reduced COL1A1 expression and procollagen I content in OA synoviocytes. *Prg4*$^{-/-}$ synovium exhibited increased α-SMA, COL1A1 and TIMP-1 expression as compared to *Prg4*$^{+/+}$ synovium. *Prg4*$^{-/-}$ synoviocytes demonstrated strong α-SMA and collagen type I staining while these were undetected in *Prg4*$^{+/+}$ synoviocytes, and was reduced with PRG4 treatment. We conclude that increasing intracellular cAMP levels in synoviocytes mitigates synovial fibrosis through enhanced production of HA and PRG4, possibly representing a novel approach for treatment of OA synovial fibrosis.

Keywords: cAMP, fibrosis, PRG4, HA, Osteoarthritis.

INTRODUCTION

Hallmarks of osteoarthritis (OA) include cartilage degeneration, subchondral bone remodeling, and synovitis (26, 27, 41). Major abnormalities in the OA synovium include synovial hyperplasia, inflammatory cell infiltration, angiogenesis and fibrosis (4, 45, 47, 55). Synovial fibrosis is a common feature in advanced OA that contributes to joint pain and stiffness (13, 24). The TGF-β1 family and its associated signaling pathways play an essential role in maintaining homeostasis in healthy joints (52). However, TGF-β1 switches to a pathologic role in OA joints that drives synovial fibrosis (36). TGF-β1 upregulates the expression of synovial collagen type 1, tissue inhibitor of metalloproteinase 1 (TIMP-1), and procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2) (35, 36). The net result is an increase in collagen I formation, an increase in collagen crosslinks and a reduction in collagen turnover (35, 36).

The normal synovium contains two types of intimal cells: type A macrophages and type B fibroblasts, or synoviocytes (48). In synoviocytes, TGF-β1 induces fibrotic changes characterized by cell proliferation and collagen type I accumulation (46). Additionally, TGF-β1 promotes the differentiation of OA synoviocytes into a myofibroblast-like phenotype, characterized by the expression of alpha smooth muscle actin (α-SMA) (28). Synoviocytes produce hyaluronan (HA), a glycosaminoglycan synthesized by membrane-bound hyaluronan synthase (HAS) with three isoforms identified to date (HAS1, HAS2 and HAS3) (54). Synoviocytes also produce proteoglycan-4 (PRG4), a heavily glycosylated mucinous glycoprotein (9, 17). HA and PRG4 play important roles in joint lubrication (25). HA and PRG4 may also play a role in joint fibrosis as HA treatment reduced synovial fibrosis *in vivo* and findings in synovial tissues from *Prg4*$^{-/-}$ mice include increased synovial thickening and a proliferative capacity for *Prg4*$^{-/-}$ synoviocytes under basal and cytokine stimulated conditions (2, 32, 37).

Cyclic adenosine monophosphate (cAMP) is a pleiotropic intracellular second messenger generated by adenylyl cyclase (AC) enzymes in response to G-protein coupled receptor (GPCR) activation (16). The antifibrotic effect of cAMP has been described in fibroblasts

from multiple origins and include inhibition of fibroblast proliferation, reduction in fibroblast migration and reduced synthesis of extracellular matrix components (12, 38, 44, 56). The role of cAMP in regulating TGF-β1's fibrotic response in synoviocytes is unknown. Our aim was to study the impact of forskolin, an AC activator, on the expression and production of α-SMA, collagen type I, and expression of *TIMP-1*, *PLOD2*, *HAS* isoforms, *PRG4* and production of HA and PRG4 in a model of TGF-β1 stimulated OA synoviocytes. Given the potential involvement of HA and PRG4 in synovial fibrosis, we also studied the antifibrotic effect of HA and PRG4 in human and murine synoviocytes. We hypothesized that increasing intracellular cAMP exerts an antifibrotic effect in OA synoviocytes and promotes HA and PRG4 production.

MATERIALS AND METHODS

Ethical approvals: Animal breeding and tissue harvest was approved by the IACUC committee at Rhode Island Hospital.

Patient characteristics and experimental approach

OA synoviocytes (500,000 cells per vial; Cell Applications, USA) were isolated from synovial tissues from de-identified OA patients undergoing knee replacement (n=10; median age = 63; range: 54 to 69). Six patients were female. Five patients were Caucasian, and the other five were unspecified. Synoviocytes were received in their second passage. OA Synoviocytes were cultured in 75 cm^2 culture flasks in DMEM media supplemented with 10% FBS and were used between the third and sixth passages to avoid alterations in patterns of gene expression (10, 30). Experimental data are represented as the mean ± S.D. of 3-6 biological replicates.

We initially studied cAMP generation in OA synoviocytes using a 0.01 to 10μM forskolin concentration range. The 10μM was selected based on previous studies (21, 50). Following confirmation of cAMP accumulation by forskolin, we determined its effect on α-SMA, collagen type I, TIMP-1 and PLOD2 expression in TGF-ß1 stimulated OA synoviocytes. The antifibrotic effect of intracellular cAMP accumulation in OA synoviocytes was further

confirmed using a cell permeable cAMP analog, 8-bromo cAMP. To further appreciate the antifibrotic effect of forskolin, we evaluated the impact of cAMP generation on HAS isoform expression and HA production, as well as PRG4 expression and production. Based on forskolin's observed effect on HA and PRG4, we studied the antifibrotic effect of HA and PRG4 in human OA synoviocytes, murine *Prg4*$^{-/-}$ synoviocytes as well as fibrosis markers in the *Prg4*$^{-/-}$ synovium. Finally, we studied the efficacy of forskolin in mitigating TGF-β1 stimulated OA synoviocyte migration and proliferation.

cAMP generation in OA synoviocytes by forskolin

cAMP levels were measured in OA synoviocytes using the cADDis assay (Montana Molecular, USA). The assay utilizes a fluorescent cAMP sensor that measures changes in intracellular cAMP levels. An increase in intracellular cAMP levels results in a reduction in green fluorescence. OA synoviocytes (10,000 cells per well) were cultured overnight in sterile 96-well clear-bottom black plates in the presence of a recombinant mammalianized baclovirus expressing the cAMP sensor according to manufacturer's recommendations. Subsequently, media supernatants were replaced with Dulbecco's phosphate-buffered saline (DPBS; Thermo Fisher Scientific, USA) (200μL per well). Forskolin (Sigma-Aldrich, USA) at a final concentration of 0.01, 0.1, 1, and 10μM, vehicle and a positive control were added and fluorescence intensity using 494/522 nm wavelengths was measured every 30 seconds over 30 minutes. Data is presented as the ratio of fluorescence intensity reduction at each time point to fluorescence intensity at baseline.

Gene expression studies

OA synoviocytes (300,000 cells per well) were treated with TGF-β1 (1ng/mL; R&D systems, USA) in serum-free DMEM ± forskolin (0.1, 1, or 10μM), 8-bromo cAMP (Sigma Aldrich) (100 and 500μM), human synoviocyte PRG4 (apparent MW 280 kDa as a monomer; 100μg/mL) (18) and/or high molecular weight HA (MW >950 kDa; R&D Systems) (100 μg/mL) for 24 hours followed by RNA extraction, cDNA synthesis and qPCR as previously described (1). The cycle threshold (Ct) value of target genes were normalized to the Ct value

of *GAPDH* in the same sample, and the relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (23). Target genes included α-SMA (*ACTA2*), collagen type I (*COL1A1*), *TIMP-1*, *PLOD2*, *HAS1*, *HAS2*, *HAS3*, and *PRG4* (primers and probes were obtained from Thermo Fisher Scientific). Data are presented as fold expression of target genes in the different experimental groups compared to untreated controls.

α-SMA and procollagen type I quantitation

OA synoviocytes were seeded in cell culture dishes (20.8 cm^2) at 1.0 x 10^6 cells per dish until confluence. OA synoviocytes in serum-free DMEM media (5 ml per dish) were treated with TGF-β1 (1ng/mL) ± forskolin (10μM), PRG4 (100μg/mL) or HA (100μg/mL) for 24 hours. Cell protein extraction was performed using M-PER reagent supplemented with protease and phosphatase cocktail inhibitor (Thermo Fisher Scientific) and quantified using micro BCA assay (Thermo Fisher Scientific).

Gel electrophoresis was performed using 10% PAGE gels (Bio-Rad) and 10μg protein per well. Following transfer, membranes were blocked with 5% non-fat dry milk for 2 hours at room temperature. Membranes were probed with anti-α-SMA (1:1,000 dilution; ab5694) and anti-GAPDH (1:5,000 dilution; ab9485) (Abcam) overnight in tris-buffered saline tween 20 (TBS-T). Following washing with TBS-T, membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit (1:5,000 dilution; ab6721) antibody for 1 hour at room temperature (Abcam). Protein bands were developed using Lumigen ECL Ultra reagent (Lumigen, USA) and visualized using Bio-Rad ChemiDoc XRS+ system (Bio-Rad). Bands of interest were selected and quantified using Image J software. The ratio of α-SMA band intensities to corresponding GAPDH band intensities of the different experimental groups were calculated and normalized to controls.

Procollagen I content in OA synoviocytes was determined using an ELISA (Abcam and R&D Systems). A total of 5 μg protein in 100 μL buffer was used in each experimental group and procollagen I concentrations (pg/mL) were divided by 50 and expressed as procollagen I protein (pg) per μg protein.

Immunocytostaining of α-SMA in OA synoviocytes

OA synoviocytes (200,000 cells per well) were cultured on collagen type I-coated 12 mm glass coverslips for 48 hours in DMEM supplemented with 10% FBS. Subsequently, cells were treated with TGF-β1 (1 ng/ml) ± forskolin (10μM) for 48 hours in serum-free DMEM. Synoviocytes were fixed in 10% neutral buffered formalin for 10 min followed by washing twice with PBS. Cells were permeabilized for 5 min using 0.01% Triton X100 in PBS and blocked using 2%BSA for 1 hour at room temperature. Probing was performed using FITC-conjugated anti α-SMA antibody (1:100; ab8211; Abcam) and Alexa Fluor 594 conjugated anti-alpha tubulin antibody (1:500; ab195889; Abcam) overnight at 4^{o}C. Following washing with PBS, cells were mounted with DAPI mounting medium (Abcam) for 1 hour and viewed under a confocal microscope

HA, PRG4 and HAS1 quantitation

HA concentrations: OA synoviocytes (300,000 cells per well) in serum-free DMEM were treated with TGF-β1 (1ng/mL) ± forskolin (10μM) for 24 hours. Media supernatants were collected and assayed for HA using a quantitative assay kit (R&D systems). In a separate set of experiments, OA synoviocytes (300,000 cells per well) in serum-free DMEM were treated with TGF-β1 (1ng/mL) ± 8-bromo cAMP (100 and 500μM) for 24 hours. HA concentrations were determined as described above.

PRG4 concentrations: OA synoviocytes (20,000 cells per well) were seeded in sterile 96 well plates for 48 hours followed by treatment with TGF-β1 (1ng/mL) ± forskolin (10μM) for 48 hours. PRG4 concentrations, normalized to cell density, were determined in media supernatants as previously described (1). In a separate set of experiments, OA synoviocytes (300,000 cells per well) in serum-free DMEM were treated with TGF-β1 (1ng/mL) ± 8-bromo cAMP (100 and 500μM) for 24 hours. PRG4 concentrations were determined as described above.

HAS1 content: OA synoviocytes were seeded and treated as described for α-SMA and procollagen type I. HAS1 content in protein isolates were determined using an ELISA (MyBioSource, USA). A total of 1μg total protein in 100μL volume was added to the wells of the ELISA plate. HAS1 concentrations (ng/mL) were divided by 10 and expressed as HAS1 content (ng) per μg total protein.

HAS1 knockdown and its impact on forskolin-induced HA secretion in OA synoviocytes

OA synoviocytes (300,000 cells per well) in Opti-MEM reduced serum medium (Thermo Fisher Scientific) were treated with a *HAS1* small interfering RNA (siRNA) (Thermo Fisher Scientific) (25 pmoles per well) or a non-targeted negative control (NC) siRNA (25 pmoles per well) (Thermo Fisher Scientific) for 48 hours. Transfection was performed using Lipofectamine RNAiMAX (Thermo Fisher Scientific) per manufacturer's recommendations. To confirm *HAS1* knockdown, *HAS1* expression was determined as described above. In a separate set of experiments, *HAS1* knockdown in OA synoviocytes was performed followed by media change to serum-free DMEM and stimulation with TGF-β1 (1ng/mL) ± forskolin (10μM) for 24 hours. Subsequently, HA concentrations were determined in media supernatants as described above.

Gene expression studies in $Prg4^{+/+}$ and $Prg4^{-/-}$ synovial tissues and immunocytostaining of murine synoviocytes

The phenotype of the $Prg4^{-/-}$ mouse has been previously reported (37), and is characterized by cartilage degeneration and a hyperplastic synovium contributing to joint failure (37). The $Prg4^{-/-}$ and $Prg4^{+/+}$ mouse colonies are maintained by Dr. Jay at Rhode Island Hospital. $Prg4^{-/-}$ mouse is also commercially available (stock #025737; The Jackson Laboratory, Maine, USA). Synovial tissues were isolated from male $Prg4^{-/-}$ and $Prg4^{+/+}$ mice (8-10 weeks old). The skin and surrounding tissues of the knee joints were removed. The joint capsule was cut open along both sides of the patella under a stereo microscope and the synovium from the lateral and medial sides was carefully isolated. A total of 15 $Prg4^{+/+}$ and 15 $Prg4^{-/-}$ mice were used in this study. Synovial tissues were harvested from every mouse

and tissues from 3 mice were randomly pooled into one sample, generating 5 pooled samples in each genotype. RNA isolation, cDNA synthesis and qPCR were performed as previously described (53). Genes of interest included *ACTA2, COL1A1, TIMP-1 and PLOD2* with *GAPDH* as an internal reference gene (Thermo Fisher Scientific).

Prg4$^{+/+}$ and *Prg4*$^{-/-}$ synoviocytes were isolated as previously described (2). Synovial tissues from male *Prg4*$^{+/+}$ and *Prg4*$^{-/-}$ mice (15 animals per genotype) were used to isolate the synoviocytes. Synoviocytes were plated onto sterile chamber slides (Thermo Fisher Scientific) at a density of 1.0 x 10^6 cells per well and allowed to adhere for 24 hrs. Synoviocytes were incubated with human synoviocyte PRG4 (100 μg/mL) in serum-free DMEM for 24 hours followed by washing with PBS and cell fixation with 4% formalin. Probing was performed using anti-α-SMA antibody (1:100 dilution; ab5694) or anti-collagen type I antibody (1:200 dilution; ab34710) (Abcam) at 4^{o}C overnight. Following washing with PBS, cells were incubated with Cy3 goat anti-rabbit IgG antibody (1:200 dilution; A10520; Thermo Fisher Scientific) for 1 hr at room temperature in the dark. Following washing with PBS, Alexa Fluor 488-conjugated phalloidin, a filamentous actin (F-actin) probe (1:125 dilution; A12379; Thermo Fisher Scientific), was added for 20 min in the dark. Cells were subsequently mounted with DAPI mounting medium (Vector Labs) and viewed under a fluorescent microscope (Nikon E 800).

Basal and TGF-β1 induced OA synoviocytes proliferation and migration

In sterile 96 well plates, OA synoviocytes (10,000 cells per well) were cultured in serum-free DMEM media and incubated with forskolin (3, 10 and 30μM) ± TGF-β1 (1ng/mL) for 48 hours at 37^{o}C. Cell proliferation was determined using the MTT reagent (Sigma). OA synoviocytes (100,000 cells per well) were seeded in 24-well culture plates in DMEM+10% FBS for 72 hours. A 1,000 μL pipette tip was used to perform a scratch in the confluent synoviocyte monolayer. TGF-β1 (1ng/mL) stimulation was performed in serum-free DMEM ± forskolin (10μM) for 48 hours. Subsequently, media was aspirated and cells were stained (Cell Biolabs, USA) followed by imaging using all-in-one fluorescence microscope (Keyence, USA). A region of interest (ROI) was defined and the scratch width was

measured at multiple locations in the ROI. The mean scratch width was calculated and used to estimate the mean scratch area. Data is presented as the ratio of the scratch areas of the different experimental groups to the scratch area at baseline.

Statistical Analyses

Variables were initially tested for normality. Normally distributed variables were compared using Student's *t*-test for two groups or analysis of variance (ANOVA) with Tukey's post-hoc test for more than two groups. Variables that did not satisfy the normality assumption were tested using ANOVA on the ranks. Statistical analysis of gene expression data was performed using ΔCt values (C_t target gene-C_t GAPDH) for each gene of interest. Significance level was set at 0.05.

RESULTS

Forskolin treatment increased intracellular cAMP, reduced *ACTA2, COL1A1, TIMP-1* expression and reduced α-SMA and procollagen type I in TGF-β1 stimulated OA synoviocytes

A representative dose-response of forskolin is shown in figure 1A. Treatment with forskolin (0.01μM) did not increase intracellular cAMP while the 0.1, 1 and 10μM forskolin treatments resulted in detectable increases in cAMP. Forskolin (10μM) increased intracellular cAMP compared to vehicle (*fig. 1B; p<0.001*) (n=3 patients). TGF-β1 induced *ACTA2*, *COL1A1*, *TIMP-1* and *PLOD2* expression (*fig. 1C through F*; *p<0.001 versus control for the 4 genes*) (n=4 patients). Forskolin treatment reduced *ACTA2 (fig. 1C; p<0.001)*, *COL1A1* (*fig. 1D; p<0.01*), and *TIMP-1* (*fig. 1E; p<0.001*) expression compared to TGF-β1 alone. Forskolin treatment did not alter TGF-β1 stimulated *PLOD2* expression (*fig. 1F; p=0.833*). Forskolin did not alter basal *COL1A1* (*p=0.623*), *TIMP-1* (*p=0.802*) or *PLOD2* (*p=0.752*) expression. In contrast, forskolin reduced basal *ACTA2* expression (*p=0.018*).

A Western Blot and semi-quantitative analysis of α-SMA using GAPDH as a loading control are shown in fig. 1G and fig. 1H, *respectively*. TGF-β1 increased α-SMA protein in OA synoviocytes compared to control (*fig. 1H; p<0.001*) (n=6 patients). Forskolin reduced TGF-β1 stimulated α-SMA production (*p=0.013*). Forskolin alone did not alter basal α-SMA content (*p=0.660*). Representative confocal images of TGF-β1-treated OA synoviocytes ± forskolin is shown in figure 1J. Control OA synoviocytes exhibited a positive α-SMA staining, and the appearance of a myofibroblast-like phenotype in a number of cells. TGF-β1 treatment resulted in stronger α-SMA staining and the appearance of myofibrils and this was markedly reduced with forskolin co-treatment. TGF-β1 increased procollagen type I protein compared to control (*fig. 1I; p<0.01*) (n=3 patients). Forskolin reduced TGF-β1 linked procollagen type I production (*p=0.015*), and did not alter basal procollagen type I (*p=0.991*).

Forskolin treatment enhanced HA secretion and modulated HAS isoform gene expression and *HAS1* knockdown attenuated forskolin's effect on HA production in TGF-β1 stimulated OA synoviocytes

HA concentrations were higher in TGF-β1 treated OA synoviocytes and forskolin-treated OA synoviocytes compared to untreated controls (*fig. 2A; p<0.001 for both comparisons*) (n=4 patients). HA concentrations in the TGF-β1 + forskolin group were higher than HA concentrations in the TGF-β1 or forskolin alone groups (*p<0.001 for both comparisons*). TGF-β1 induced *HAS1* (*fig. 2B; p<0.001*) and *HAS2* (*fig. 2C; p<0.001*) with no effect on *HAS3* (*fig. 2D; p=0.719*) expression in OA synoviocytes (n=4 patients). Forskolin treatment upregulated basal *HAS1*, *HAS2* and *HAS3* expression (*p<0.001 for all comparisons*). Combined treatment of TGF-β1 and forskolin increased *HAS1* expression over TGF-β1 alone (*fig. 2B; p=0.026*). In contrast, *HAS2* expression in the TGF-β1 + forskolin group was lower than *HAS2* expression in the TGF-β1 group (*fig. 2C; p=0.024*).

The total cellular HAS1 content was higher in TGF-β1 (*p=0.021*) and TGF-β1 + forskolin (*p<0.01*) treatments compared to control (fig. 2E) (n=4 patients). HAS1 content was not different between TGF-β1 + forskolin and TGF-β1 alone groups (*p=0.971*). Similarly, there was no difference in HAS1 between forskolin and control groups (*p=0.303*). *HAS1*

expression was reduced by approximately 68% in OA synoviocytes transfected with *HAS1* siRNA (*fig. 2F; p<0.01*) (n=4 OA patients). HA concentrations in TGF-β1 stimulated *HAS1* knockdown OA synoviocytes were not different from unstimulated *HAS1* knockdown OA synoviocytes (*fig. 2G; p=0.962*) (n=4 patients). Similarly, HA concentrations in TGF-β1 + forskolin treated *HAS1* knockdown OA synoviocytes were not different from HA concentrations in TGF-β1 treated *HAS1* knockdown OA synoviocytes (*p=0.514*). Finally, HA concentrations in TGF-β1 + forskolin treated OA synoviocytes were higher than HA concentrations in TGF-β1 + forskolin treated *HAS1* knockdown OA synoviocytes (*p<0.001*).

Forskolin treatment enhanced *PRG4* expression and secretion in TGF-β1 stimulated OA synoviocytes

TGF-β1 induced *PRG4* expression (*fig. 3A; p<0.001*) and increased PRG4 production by OA synoviocytes (*fig. 3B; p<0.01*) (n=4 patients). Forskolin did not alter basal *PRG4* expression (*fig. 3A; p=0.063*) or PRG4 production (*fig. 3B; p=0.996*) in OA synoviocytes. *PRG4* expression in the TGF-β1 + forskolin group was higher than TGF-β1 alone (*fig. 3A; p=0.037*). Correspondingly, PRG4 concentrations were higher in the TGF-β1 + forskolin group compared to the TGF-β1 group (*fig. 3B; p=0.031*).

Impact of PRG4 and HA treatments on *ACTA2* and *COL1A1* expression and α-SMA and procollagen type I in TGF-β1 stimulated OA synoviocytes

ACTA2 expression was lower in the TGF-β1 + PRG4 group compared to TGF-β1 alone (*fig. 3C; p<0.001*) (n=4 patients). Similarly, *ACTA2* expression in the TGF-β1+ HA and TGF-β 1 + PRG4 + HA groups was lower than *ACTA2* expression in TGF-β1 alone (*p<0.001* for both comparisons). *COL1A1* expression was lower in the TGF-β1 + PRG4 group compared to TGF-β1 + HA (*p<0.01*) or TGF-β1 alone (*p<0.001*) (fig. 3D) (n=4 OA patients). In contrast, HA treatment did not alter TGF-β1 induced *COL1A1* expression (*p=0.897*). *COL1A1* expression in the TGF-β1 + PRG4 + HA group was lower than TGF-β1 (*p<0.001*) and TGF-β1 + HA (*p<0.01*) groups.

A Western Blot and semi-quantitative analysis of α-SMA using GAPDH as a loading control are shown in 3E and 3F, respectively. α-SMA content was lower in TGF-β1 + PRG4 (*fig. 3F; $p<0.01$*) and in TGF-β1 + HA (*$p<0.01$*) compared to TGF-β1 alone (n=4 patients). Procollagen type I content was lower in TGF-β1 + PRG4 compared to TGF-ß1 alone (*fig. 3G; $p<0.01$*) (n=4 patients). There was no difference in procollagen type I content between TGF-β1 + HA and TGF-β1 groups (*$p=0.059$*).

A cell permeable cAMP analog treatment reduced *ACTA2* and *COL1A1 expression and enhanced HA and PRG4 secretion in* TGF-β1 stimulated OA synoviocytes

TGF-β1 induced *ACTA2* and *COL1A1* expression in OA synoviocytes (fig. 4A and 4B; *$p<0.001$* against control for both genes). 8-bromo cAMP (100μM) treatment did not significantly alter TGF-β1 induced *ACTA2* and *COL1A1* expression (*$p>0.05$* for both comparisons). In contrast, 8-bromo cAMP (500μM) treatment reduced ACTA2 (*$p<0.001$*) and COL1A1 (*$p<0.001$*) expression in TGF-β1 stimulated OA synoviocytes (n=3 OA patients). HA and PRG4 media concentrations in TGF-β1 + 8-bromo cAMP (500μM) group were significantly higher than corresponding concentrations in TGF-β1 only group (fig. 4C and 4D; *$p<0.001$* for both comparisons) (n=3 OA patients).

***ACTA2, COL1A1* and *TIMP-1* expression was higher in *Prg4*$^{-/-}$ synovial tissues and human synoviocyte PRG4 treatment reduced α-SMA and collagen type I staining in *Prg4*$^{-/-}$ synoviocytes**

Expression of *ACTA2* (*$p=0.021$*), *COL1A1* (*$p<0.001$*) and *TIMP-1* (*$p<0.01$*) was higher in *Prg4*$^{-/-}$ synovia compared to *Prg4*$^{+/+}$ synovia (fig. 5A). In contrast, *PLOD2* expression was lower in *Prg4*$^{-/-}$ tissues compared to *Prg4*$^{+/+}$ tissues (*$p<0.01$*).

Merged images of α-SMA and collagen type I stained *Prg4*$^{+/+}$ and *Prg4*$^{-/-}$ synoviocytes is shown in figure 5B. We observed strong α-SMA and collagen type I staining in *Prg4*$^{-/-}$ synoviocytes. α-SMA staining co-localized with F-actin staining. In contrast, there was no detected α-SMA or collagen type I staining in *Prg4*$^{+/+}$ synoviocytes. PRG4 treatment reduced α-SMA and collagen type I staining in *Prg4*$^{-/-}$ synoviocytes.

Forskolin reduced TGF-β1 induced OA synoviocyte proliferation and migration

Forskolin (3 μM and 10μM) treatments did not alter basal OA synoviocyte proliferation (*fig. 6A; p=0.891 and p=0.117*) (n=4 patients). In contrast, the 30μM treatment increased basal OA synoviocyte proliferation compared to untreated control (*p<0.01*). TGF-β1 stimulated OA synoviocyte proliferation (fig. 6B; *p<0.001*) (n=4 patients). OA synoviocyte proliferation in the TGF-β1 + forskolin (30μM) group was lower than TGF-β1 alone (*p<0.001*), TGF-β1 + forskolin (3μM) (*p<0.01*) or TGF-β1 + forskolin (10μM) (*p<0.01*). There was no difference in cell proliferation between TGF-β1 + forskolin (10μM) and TGF-β1 alone (*p=0.063*). Representative wound scratch images are show in figure 6C. TGF-β1 enhanced OA synoviocyte migration (*fig. 6D; p<0.01*) (n=4 patients). OA synoviocyte migration in the TGF-β1 + forskolin (10μM) group was lower than in the TGF-β1 only group (*p<0.01*). There was no difference in cell migration between forskolin treated and untreated OA synoviocytes (*p=0.887*).

DISCUSSION

In this paper, we show that TGF- β1 resulted in excess collagen type I production, induction of *TIMP-1* and *PLOD2* expression and α-SMA upregulation, together with stimulating OA synoviocyte migration and proliferation. Forskolin, by virtue of its ability to generate cAMP, reduced collagen production and blunted TIMP-1 expression while inhibiting synoviocyte proliferation and migration. *PLOD2* induction in osteoarthritic synoviocytes is consistent with its established role in mediating synovial collagen crosslinking (3, 29). Forskolin did not alter *PLOD2* expression, which may be related to the TGF-β1's signaling pathways. Remst *et al* have shown that in OA synoviocytes, an ALK 1/2/3/6 inhibitor completely blocked TGF-β1 induced collagen type I expression whereas TGF-β1 induced *PLOD2* expression was only slightly reduced (34).

OA synoviocytes proliferate in response to various mitogenic stimuli (1, 5, 15). In our experiments, TGF-β1 induced cell proliferation with a magnitude comparable to what has

been previously reported (5). Forskolin, at the treatment level that inhibited migration, exhibited a marginal antiproliferative effect and a higher concentration was needed to observe significant antagonism of TGF-β1's mitogenic effect. This might be due to a low proliferative capacity of OA synoviocytes. In the absence of TGF-β1, forskolin acted as a mitogen to produce a low, yet significant, stimulation of proliferation. Forskolin directly binds to AC and generates cAMP from ATP (43). The increase in cAMP results in activation of protein kinase A (PKA) dependent and independent pathways (39, 43). In the presence of growth factors, forskolin activates cAMP-dependent PKA which interferes with Raf-1 activation and signaling to blunt cell proliferation (11). PKA also activates CREB, which can compete for cofactors with SMAD-mediated transcription stimulated by TGF-β1 (22). In the absence of growth factor, PKA-dependent and independent pathways stimulate cAMP-mediated cell proliferation (6).

We measured α-SMA in OA synoviocytes from human patients and forskolin addition consistently attenuated TGF-β1-stimulated α-SMA expression. α-SMA is a specific marker of myofibroblasts (7, 8). Myofibroblasts are effector cells in fibrosis that possess enhanced ability to produce collagen, proliferate and migrate (7, 8). We have shown that forskolin treatment appeared to markedly reduce TGF-β1's induced myofibroblast-like phenotype in OA synoviocytes. The causal role that myofibroblasts may play in synovial fibrosis is understudied and unclear. Steenvoorden *et al* reported that α-SMA staining was only found in blood vessels in synovia from healthy individuals (49). Interestingly, TGF- β1 stimulation of normal synoviocytes increased collagen type I expression with no effect on α-SMA expression or production (49). Mattey *et al* have shown that TGF-β1 or IL-4 treatments trigger differentiation of OA synoviocytes into myofibroblast-like cells, characterized by α-SMA expression *in vitro* (28). Evidence relating myofibroblasts to changes occurring in joint fibrosis was reported by Sasabe *et al* (40). Using a rat knee contracture model, myofibroblasts expressing α-SMA were detected as early as 1 week from joint immobilization and this was associated with increased collagen type I expression and joint capsule fibrosis (40). We have also detected α-SMA protein in *Prg4*$^{-/-}$ synoviocytes with no α-SMA signal in normal murine synoviocytes. The positive α-SMA signal in *Prg4*$^{-/-}$

knockout synoviocytes is associated with phenotypical changes in the synovium including synovial lining thickening and enhanced synoviocyte proliferation (37).

TGF-β1 induced *HAS1* and *HAS2* expression with no effect on *HAS3*, and enhanced HA secretion by OA synoviocytes. Synoviocytes contain higher levels of *HAS1* message compared to *HAS2* with *HAS3* being the least abundant (33). Earlier reports are in agreement with our finding that TGF-β1 stimulation of arthritic synoviocytes resulted in a higher HAS1/HAS2 transcript ratio above control level and that in turn resulted in higher extracellular HA levels (5, 33). Our data suggests that the majority of TGF-β1 linked HA secretion is mediated by HAS1, as HAS1 knockdown diminished extracellular HA concentrations. Co-treatment with forskolin increased the HAS1/HAS2 transcript ratio above the corresponding TGF-ß1 ratio with greater HA secretion over 24 hours. The increase in HAS1 mRNA in forskolin-treated synoviocytes did not translate to increased HAS1 cellular pool. This might be related to the rate at which the message is being translated. Furthermore, the majority of HAS1 cellular fraction is inactive and is found in the cytoplasm either diffused or partially co-localized with the Golgi apparatus, whereas the plasma membrane-bound fraction is small and is catalytically active (51). Therefore, the possibility that the newly synthesized HAS1 enzyme, in response to forskolin treatment, could have been trafficked to the membrane resulting in increasing the rate of HA synthesis could not be ruled out. Assessing the impact of forskolin on membrane-bound HAS1 level and activity was technically challenging and was not feasible to perform. Our data should also be considered in the context that other factors *e.g.* post-translational modifications, availability of precursors and regulation of HAS activity will likely contribute to the amount of HA secreted by the OA synoviocytes (33).

Increasing intracellular cAMP resulted in increasing PRG4 expression and production by OA synoviocytes in the setting of TGF-β1 stimulation. This contextual effect is due to CREB stimulation which was previously shown to enhance PRG4 production by superficial zone articular chondrocytes (31). PRG4 is a mucin-like glycoprotein synthesized by synoviocytes and superficial zone articular chondrocytes with a heavily glycosylated central domain and an *N*- and *C*-termini (19). The mouse Prg4 gene is highly homologous to the human Prg4

gene (14). Human and mouse Prg4 genes each consist of 12 exons and the N- and C-termini are highly conserved across species (14). PRG4 exists in the synovial fluid in monomeric or multimeric forms and functions as a boundary lubricant (19, 42). PRG4 binds to CD44, the HA receptor, and exerts an anti-inflammatory effect in OA synoviocytes (1, 2). PRG4 also acts in an autocrine manner to regulate OA synoviocyte proliferation (1). We have found that PRG4 and HA had equivalent efficacy in reducing α-SMA content in osteoarthritis synoviocytes. However, PRG4, at a physiologically-relevant concentration (20), was more efficacious than HA in reducing collagen I expression and production, indicative of a potential role in antagonizing profibrotic alterations in synovial tissues. This function is likely related to its interaction with the CD44 receptor, given that HA reduced synovial fibrosis in a CD44-mediated manner (32). PRG4 reduced collagen I and α-SMA staining in *Prg4*$^{-/-}$ synoviocytes. The link between PRG4 expression and synovial fibrosis is further illustrated by the upregulation of collagen type I, TIMP-1 and α-SMA in the *Prg4*$^{-/-}$ synovium. As laying excess collagen type I is a prominent feature in synovial fibrosis, the strong immunocytostaining for collagen type I in *Prg4*$^{-/-}$ synoviocytes, coupled with other synovial changes, support a fibrotic *Prg4*$^{-/-}$ synovium. We did not include human normal synoviocytes in our study design. Furthermore, we did not examine the efficacy of forskolin or PRG4 in an *in-vivo* model of synovial fibrosis.

In summary, our data demonstrate that forskolin; a diterpene produced by the roots of the Indian plant Coleus forskohili (43), increases intracellular cAMP levels and produces an antifibrotic effect in OA synoviocytes. Increasing intracellular cAMP levels directly via treatment with a cell permeable cAMP analog recapitulated the antifibrotic effect of forskolin. Forskolin reduces collagen type I expression and procollagen type I production and inhibits TGF-β1 linked fibroblast migration and proliferation. Forskolin also increased HA and PRG4 secretion by OA synoviocytes; an effect that may contribute to its overall antifibrotic efficacy. Approaches that increase cAMP levels in synoviocytes can promote an antifibrotic phenotype and may be a novel approach for slowing the progression of synovial fibrosis in OA.

LIST OF ABBREVIATIONS

α-SMA: Alpha smooth muscle actin**; AC:** Adenylyl cyclase**;** ***ACTA2****:* Alpha smooth muscle actin gene; **ALK:** Anaplastic Lymphoma Kinase; **ANOVA:** Analysis of variance; **cAMP:** Cyclic adenosine monophosphate; **cDNA:** Complementary deoxyribonucleic acid; **CD44:** Cluster of differentiation 44; ***COL1A1***: Collagen type I gene; **C_t:** Threshold cycle**; DMEM:** Dulbecco's Modified Eagle's Medium; **DPBS:** Dulbecco's Phosphate-Buffered Saline; **ELISA:** Enzyme linked immunosorbent assay; **FITC:** Fluorescein Isothiocyanate; **FsK:** Forskolin; **GAPDH:** Glyceraldehyde-3-Phosphate Dehydrogenase**; GPCR:** G-protein coupled receptor; **HA:** Hyaluronan; **HAS:** Hyaluronan synthase; **IACUC:** Institutional Animal Care and Use Committee; **kDa:** Kilodaltons; **MTT:** 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide; **MW:** Molecular Weight; **OA:** Osteoarthritis; **PAGE:** Polyacrylamide Gel Electrophoresis; **PKA:** Protein kinase A; **PLOD2:** Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2; **PRG4:** Proteoglycan-4; **qPCR:** Quantitative polymerase chain reaction; **RNA:** Ribonucleic Acid; **ROI:** Region of interest; **S.D.:** Standard deviation; **siRNA:** Small interfering ribonucleic acid; **TBS-T:** Tris buffered saline + 0.05% Tween 20; **TGF-β1:** Transforming growth factor beta-1; **TIMP-1:** Tissue Inhibitor of Metalloproteinase 1.

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DISCLOSURES

Authors MQ, LZ and RO have nothing to disclose. GJ authored patents related to use of recombinant PRG4 and holds equity in Lubris LLC, MA, USA. KE co-authored patent applications related to use of recombinant PRG4.

AUTHOR CONTRIBUTIONS

Authors MQ, LZ and KE carried out the experiments and participated in data analysis. GJ participated in study design and critical interpretation of results. KE and RO conceived the study and participated in data analysis and interpretation. All authors participated in drafting

and critical evaluation of the manuscript. All authors have read and approved the final version of the manuscript.

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Legends

Fig. 1 Impact of forskolin (FsK) treatment on intracellular cyclic adenosine monophosphate (cAMP) levels, basal and transforming growth factor beta 1 (TGF-β1)-induced alpha smooth muscle actin (*ACTA2*), collagen I (*COL1A1*), tissue inhibitor of metalloproteinase 1 (*TIMP-1*) and procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (*PLOD2*) gene expression and alpha smooth muscle action (α-SMA) and procollagen type I production in osteoarthritic (OA) synoviocytes. TGF-β1 (1ng/ml) stimulation of OA-FLS was performed for 24 hours in all experiments except α-SMA immunocytostaining (stimulation was performed using 1 ng/ml TGF-β1 for 48 hours). Data is presented as the mean ± S.D. of experiments utilizing OA synoviocytes from different patients. **p<0.001; **p<0.01; ***p<0.05*.

A. Representative dynamic change in intracellular cAMP levels in OA synoviocytes following treatment with FsK (0.01, 0.1, 1 and 10μM). FsK treatment (0.1, 1 and 10μM) resulted in detectable cAMP levels in OA synoviocytes. The cAMP signal was detected using a cAMP-specific sensor. **B.** cAMP levels were elevated in FsK (10μM)-treated OA synoviocytes (n=3 patients). **C.** FsK treatment (1 and 10μM) reduced TGF-β1 induced *ACTA2* expression (n=4 patients). **D.** FsK treatment (10μM) reduced TGF-β1 induced *COL1A1* expression (n=4 patients). **E.** FsK treatment (10μM) reduced TGF-β1 induced *TIMP-1* expression (n=4 patients). **F.** Fsk treatment (10μM) did not alter TGF-β1 induced *PLOD2* expression (n=4 patients). **G.** Western Blot of α-SMA (predicted MW: 42 kDa) in control, TGF-β1, TGF-β1 + FsK and FsK-treated OA synoviocytes. GAPDH (predicted MW: 40 kDa) was used as loading control. **H.** Semi-quantitative densitometry analysis of α-SMA normalized to GAPDH and expressed as ratio to control in cell extracts of control, TGF-β1, TGF-β1 + FsK and FsK-treated OA synoviocytes. FsK (10μM) treatment reduced TGF-β1 linked increase in α-SMA in OA synoviocytes (n=6 patients). **I.** Procollagen type I content in cell extracts of control, TGF-β1, TGF-β1 + FsK and FsK-treated OA synoviocytes. Data was normalized to total protein content. FsK (10μM) treatment reduced TGF-β1 linked increase in procollagen type I content in OA synoviocytes (n=3 patients). **J.** FsK (10μM) treatment reduced α-SMA staining and myofibroblast-like phenotype in TGF-β1 stimulated OA synoviocytes.

Fig. 2 Impact of forskolin (FsK; 10μM) treatment on basal and transforming growth factor beta 1 (TGF-β1)-induced hyaluronan (HA) production, expression of hyaluronan synthase isoforms 1, 2, and 3 (*HAS1*, *HAS2* and *HAS3*) and the role of HAS1 in mediating TGF-β1 and FsK-linked HA production in osteoarthritic (OA) synoviocytes. Data is presented as the mean ± S.D. of experiments utilizing OA synoviocytes from 4 patients. **p<0.001; **p<0.01; ***p<0.05; n.s.: non significant.*

A. Hyaluronan concentrations in control, TGF-β1, TGF-β1 + FsK, and FsK-treated OA synoviocytes. **B.** FsK treatment enhanced TGF-β1 induced *HAS1* expression. **C.** FsK treatment reduced TGF-β1 induced *HAS2* expression. **D.** FsK treatment increased basal *HAS3* expression in OA synoviocytes. **E.** HAS1 protein content in cell extracts of control, TGF-β1, TGF-β1 + FsK and FsK-treated OA synoviocytes. Data was normalized to total protein content. There was no difference in HAS1 protein between TGF-β1 and TGF-β1 +

FsK treatments. **F.** HAS1 expression was reduced in HAS1 siRNA-treated OA synoviocytes compared to untreated control and negative control siRNA (NC siRNA)-treated OA synoviocytes. **G.** FsK and/or TGF-β1 treatments did not significantly change hyaluronan production following *HAS1* knockdown in OA synoviocytes.

Fig. 3 Impact of forskolin (FsK; 10μM) treatment on basal and transforming growth factor beta 1 (TGF-β1) induced proteoglycan-4 (PRG4) gene expression and production by osteoarthritic (OA) synoviocytes and efficacy of human synoviocyte PRG4 (100μg/ml) and hyaluronan (HA) (100μg/ml) in modulating TGF-β1 induced expression and production of alpha smooth muscle actin and collagen type I in OA synoviocytes. Data is presented as the mean ± S.D. of experiments utilizing OA synoviocytes from 4 patients. *$p<0.001$; **$p<0.01$; ***$p<0.05$.

A. TGF-β1 increased PRG4 expression and FsK treatment enhanced TGF-ß1's effect. **B.** FsK treatment enhanced TGF-β1 linked PRG4 production by OA synoviocytes. **C.** PRG4 and/or HA treatments reduced TGF-β1 induced alpha smooth muscle actin gene (*ACTA2*) expression in OA synoviocytes. **D.** PRG4 treatment reduced TGF-β1 induced collagen type I (*COL1A1)* gene expression in OA synoviocytes. **E.** Western Blot of α-SMA (predicted MW: 42 kDa) in control, TGF-β1, TGF-β1 + PRG4 (100μg/ml) and TGF-β1 + HA (100μg/ml) treated OA synoviocytes. GAPDH (predicted MW: 40 kDa) was used as loading control. **F.** Semi-quantitative densitometry analysis of α-SMA normalized to GAPDH and expressed as ratio to control in cell extracts of control, TGF-β1, TGF-β1 + PRG4 and TGF-β1 + HA treated OA synoviocytes. PRG4 and HA treatments reduced TGF-β1 linked increase in α-SMA in OA synoviocytes. **G.** Procollagen type I content in cell extracts of control, TGF-β1, TGF-β1 + PRG4 and TGF-β1 + HA-treated OA synoviocytes. Data was normalized to total protein content. PRG4 treatment reduced TGF-β1 linked increase in procollagen type I content in OA synoviocytes.

Fig. 4 Impact of 8-bromo cAMP (8-Br-cAMP; 100 and 500μM) treatment on basal and transforming growth factor beta 1 (TGF-β1)-induced alpha smooth muscle actin (*ACTA2*) and collagen I (*COL1A1*) expression and hyaluronan (HA) and proteoglycan-4 (PRG4) secretion in osteoarthritic synoviocytes. Data is presented as the mean ± S.D. of experiments utilizing OA synoviocytes from 3 patients. *$p<0.001$. **A.** 8-Br-cAMP (500μM) reduced TGF-β1 linked *ACTA2* expression in OA synoviocytes. **B.** 8-Br-cAMP (500μM) reduced TGF-β1 linked *COL1A1* expression in OA synoviocytes. **C.** 8-Br-cAMP (500μM) increased HA secretion in TGF- β1stimulated OA synoviocytes. **D.** 8-Br-cAMP (500μM) increased PRG4 secretion in TGF- β1stimulated OA synoviocytes.

Fig. 5 Gene expression of alpha smooth muscle actin (*ACTA2*), collagen type I (*COL1A1*), tissue-inhibitor of metalloproteinase-1 (*TIMP-1*) and procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (*PLOD2*) in synovial tissues isolated from $Prg4^{+/+}$ and $Prg^{-/-}$ mice and immunocytostaining of alpha smooth muscle actin (α-SMA) and collagen type I in $Prg^{+/+}$ and $Prg4^{-/-}$ synoviocytes and impact of human synoviocyte PRG4 treatment. *$p<0.001$; **$p<0.01$; ***$p<0.05$.

A. *ACTA2*, *COL1A1* and *TIMP-1* expression in *Prg4*$^{-/-}$ synovial tissues was higher than *Prg4*$^{+/+}$ synovial tissues. *PLOD2* expression in *Prg4*$^{-/-}$ synovial tissues was lower than *Prg4*$^{+/+}$ synovial tissues. Each group contained 5 samples with each sample generated by pooling synovial tissues from 3 mice.

B. Merged images depicting α-SMA and collagen type I protein immunostaining in isolated *Prg4*$^{+/+}$ synoviocytes and *Prg4*$^{-/-}$ synoviocytes (bright orange) and counterstained with F-actin (green) and DAPI (blue). α-SMA and collagen type I staining was detected in *Prg4*$^{-/-}$ synoviocytes (white arrows) and no staining was detected in *Prg4*$^{+/+}$ synoviocytes. α-SMA and collagen type I staining intensities were reduced by human synoviocyte PRG4 treatment for 24 hours. Scale = 50μm.

Fig. 6 Impact of forskolin (FsK) treatment on basal and transforming growth factor beta 1 (TGF-β1) induced proliferation and migration of osteoarthritic (OA) synoviocytes. Data is presented as the mean ± S.D. of experiments utilizing OA synoviocytes from 4 patients. **p<0.001; **p<0.01; ***p<0.05*. Scale = 1,000 μM. **A.** FsK (30μM) treatment enhanced basal OA synoviocyte proliferation. **B.** FsK (30μM) treatment reduced TGF-β1-induced OA synoviocyte proliferation. **C.** Representative images showing baseline and 48-hour basal, TGF-β1, TGF-β1 + FsK and FsK alone induced OA synoviocyte migration using an *in vitro* scratch assay. **D.** FSK (10μM) treatment reduced TGF-β1 stimulated OA synoviocyte migration.

Fig. 1

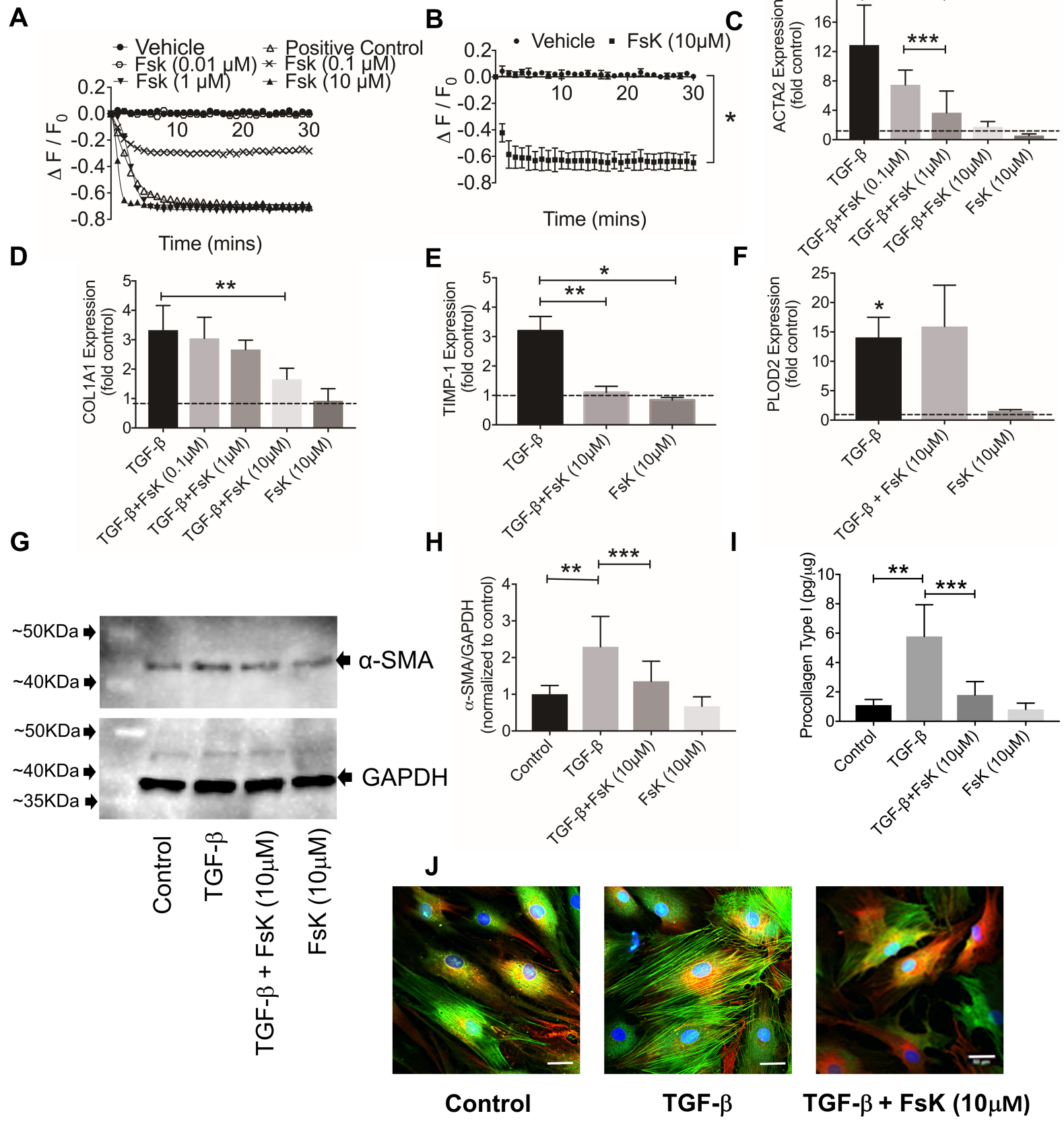
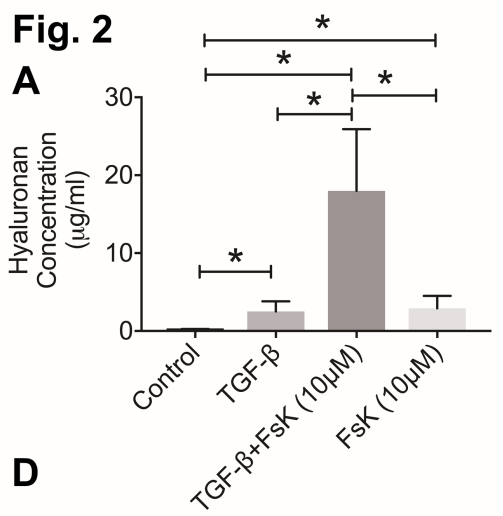
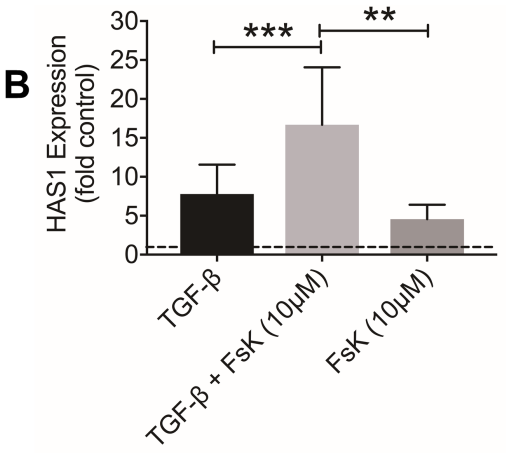
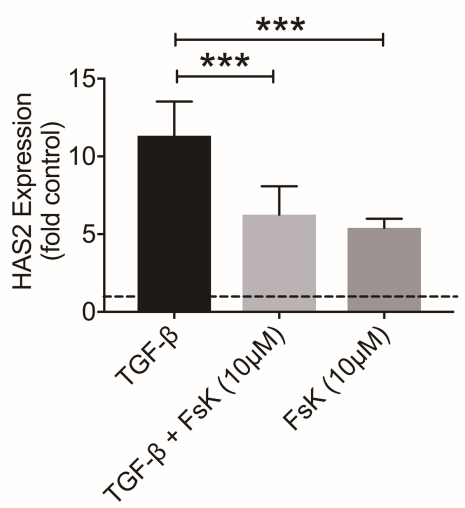
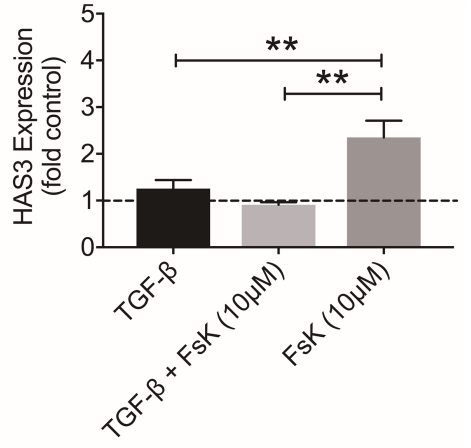

A
Vehicle
Fsk (0.01 μM)
Fsk (1 μM)
Positive Control
Fsk (0.1 μM)
Fsk (10 μM)
Δ F / F0
Time (mins)
B
Vehicle
FsK (10μM)
Δ F / F0
Time (mins)
C
ACTA2 Expression (fold control)
TGF-β
TGF-β+FsK (0.1μM)
TGF-β+FsK (1μM)
TGF-β+FsK (10μM)
FsK (10μM)
D
COL1A1 Expression (fold control)
TGF-β
TGF-β+FsK (0.1μM)
TGF-β+FsK (1μM)
TGF-β+FsK (10μM)
FsK (10μM)
E
TIMP-1 Expression (fold control)
TGF-β
TGF-β+FsK (10μM)
FsK (10μM)
F
PLOD2 Expression (fold control)
TGF-β
TGF-β + FsK (10μM)
FsK (10μM)
G
~50KDa
~40KDa
α-SMA
~50KDa
~40KDa
~35KDa
GAPDH
Control
TGF-β
TGF-β + FsK (10μM)
FsK (10μM)
H
α-SMA/GAPDH (normalized to control)
Control
TGF-β
TGF-β+FsK (10μM)
FsK (10μM)
I
Procollagen Type I (pg/μg)
Control
TGF-β
TGF-β+FsK (10μM)
FsK (10μM)
J
Control
TGF-β
TGF-β + FsK (10μM)

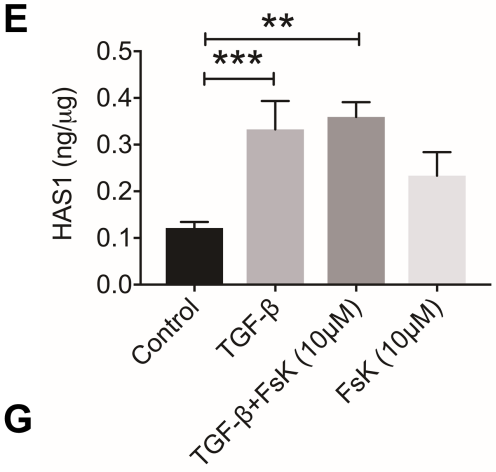
Fig. 2

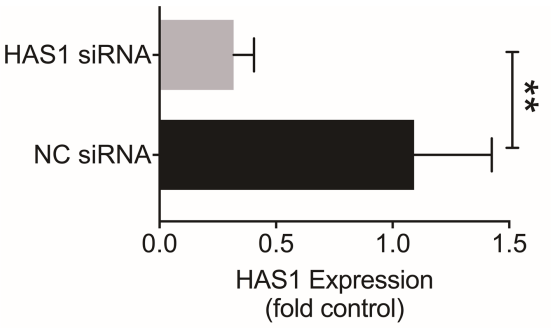

A
Hyaluronan Concentration (μg/ml)
Control
TGF-β
TGF-β+FsK (10μM)
FsK (10μM)


B
HAS1 Expression (fold control)
TGF-β
TGF-β + FsK (10μM)
FsK (10μM)


C
HAS2 Expression (fold control)
TGF-β
TGF-β + FsK (10μM)
FsK (10μM)


D
HAS3 Expression (fold control)
TGF-β
TGF-β + FsK (10μM)
FsK (10μM)


E
HAS1 (ng/μg)
Control
TGF-β
TGF-β+FsK (10μM)
FsK (10μM)


F
HAS1 siRNA
NC siRNA
HAS1 Expression (fold control)

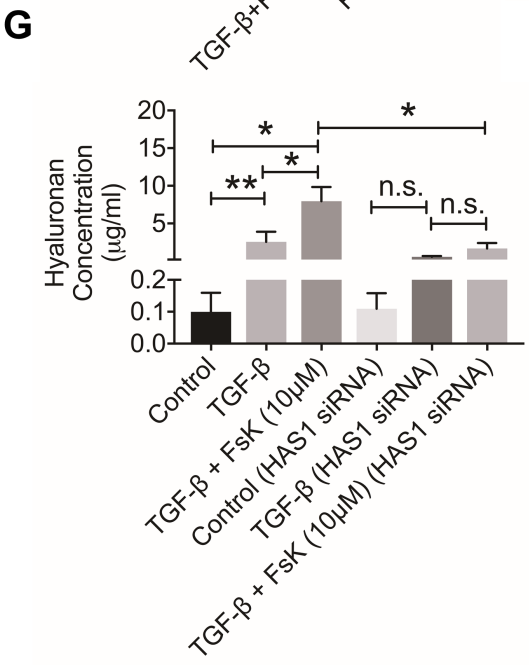

G
Hyaluronan Concentration (μg/ml)
n.s.
n.s.
Control
TGF-β
TGF-β + FsK (10μM)
Control (HAS1 siRNA)
TGF-β (HAS1 siRNA)
TGF-β + FsK (10μM) (HAS1 siRNA)

Fig. 3

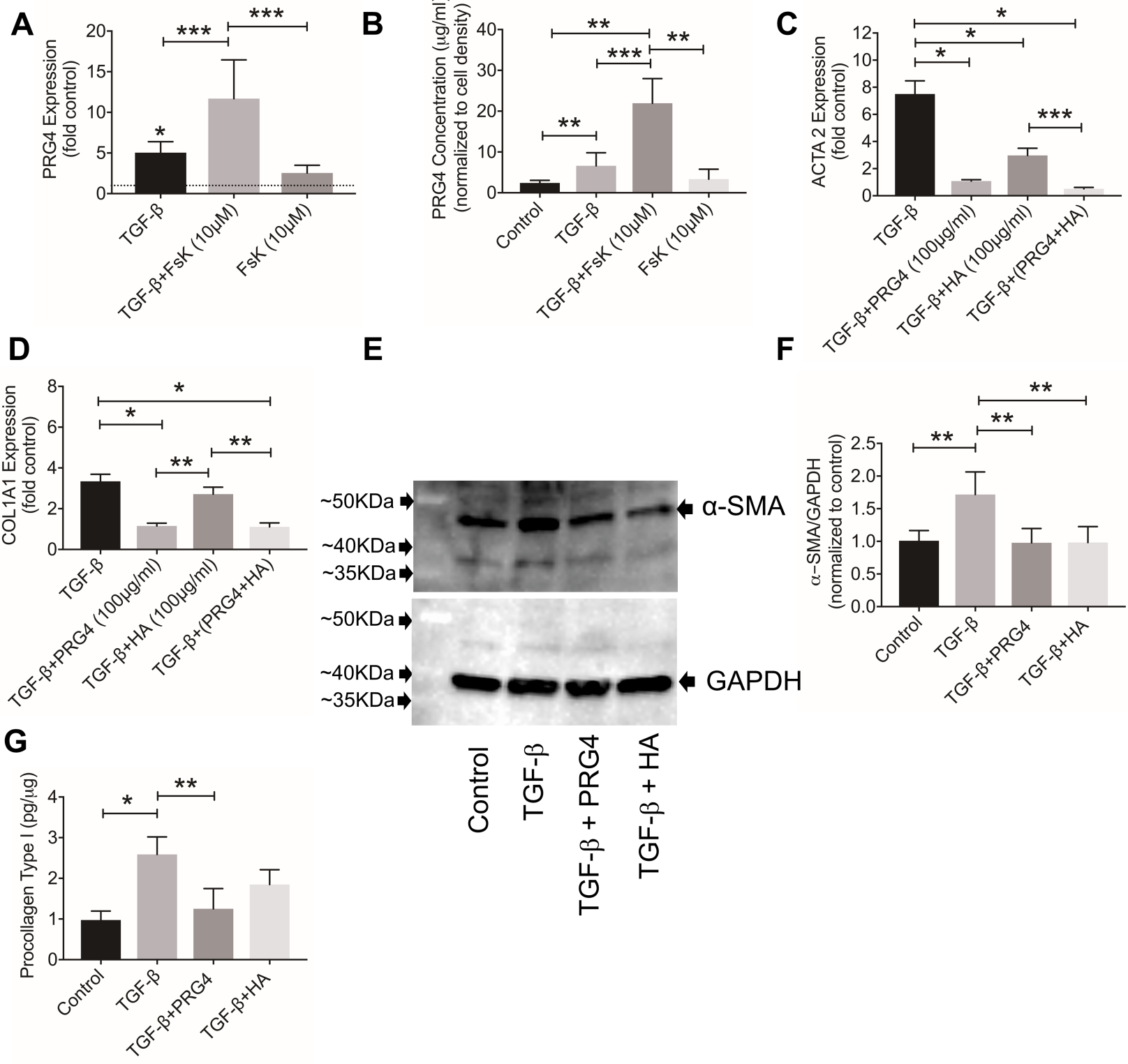

A
PRG4 Expression (fold control)
TGF-β
TGF-β+FsK (10μM)
FsK (10μM)
B
PRG4 Concentration (μg/ml) (normalized to cell density)
Control
TGF-β
TGF-β+FsK (10μM)
FsK (10μM)
C
ACTA 2 Expression (fold control)
TGF-β
TGF-β+PRG4 (100μg/ml)
TGF-β+HA (100μg/ml)
TGF-β+(PRG4+HA)
D
COL1A1 Expression (fold control)
TGF-β
TGF-β+PRG4 (100μg/ml)
TGF-β+HA (100μg/ml)
TGF-β+(PRG4+HA)
E
~50KDa
~40KDa
~35KDa
α-SMA
~50KDa
~40KDa
~35KDa
GAPDH
Control
TGF-β
TGF-β + PRG4
TGF-β + HA
F
α−SMA/GAPDH (normalized to control)
Control
TGF-β
TGF-β+PRG4
TGF-β+HA
G
Procollagen Type I (pg/μg)
Control
TGF-β
TGF-β+PRG4
TGF-β+HA

Fig. 4

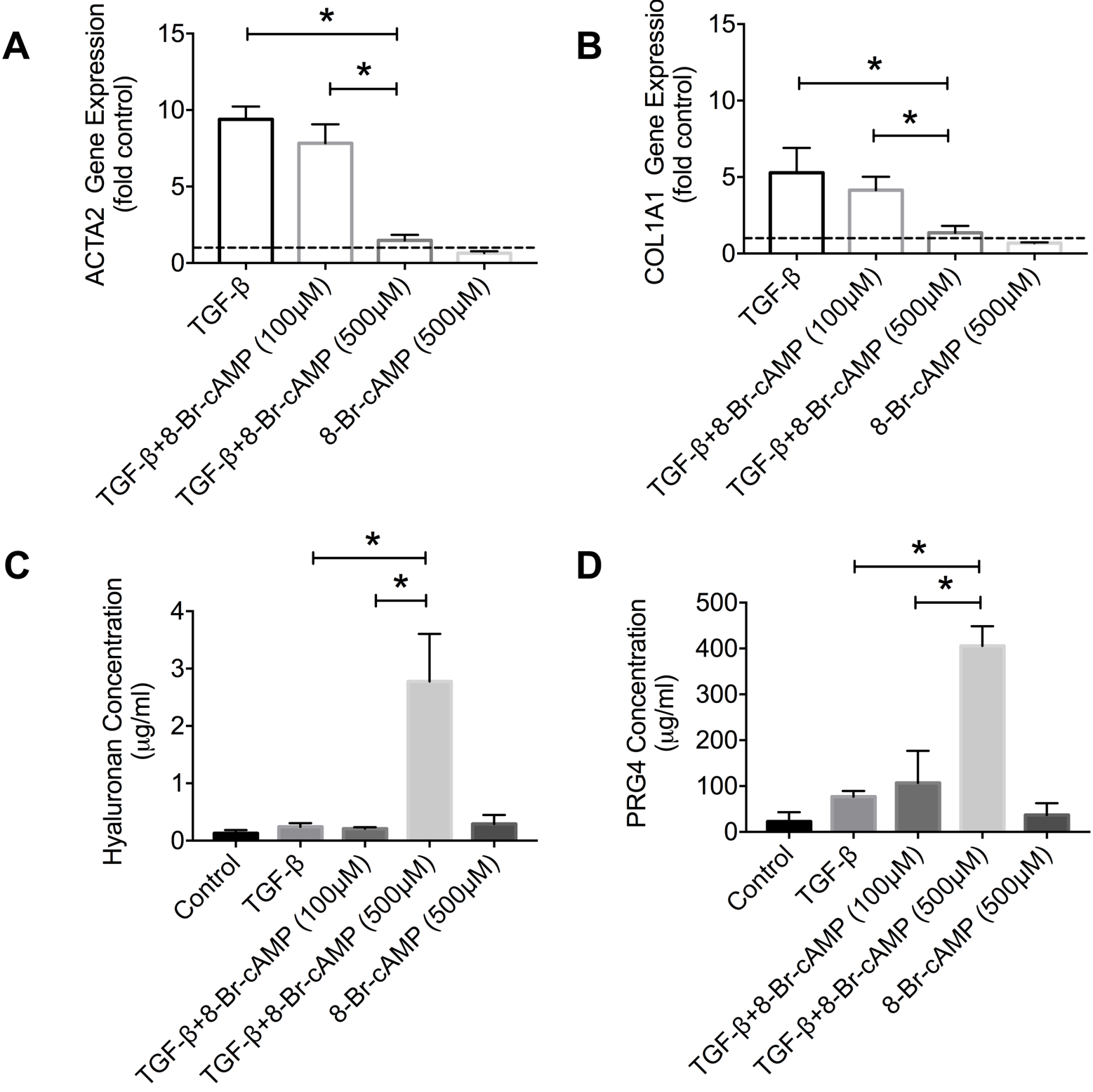
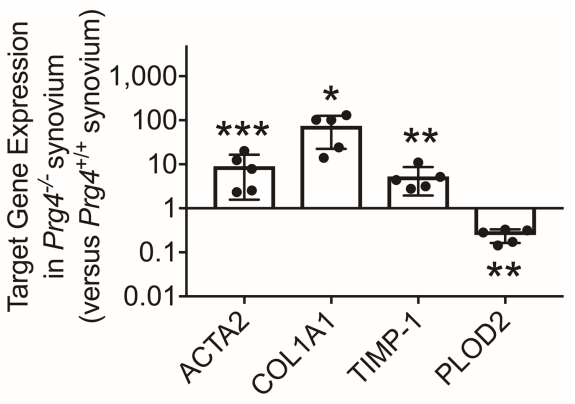

A
ACTA2 Gene Expression (fold control)
15
10
5
0
*
*
TGF-β
TGF-β+8-Br-cAMP (100μM)
TGF-β+8-Br-cAMP (500μM)
8-Br-cAMP (500μM)
B
COL1A1 Gene Expression (fold control)
15
10
5
0
*
*
TGF-β
TGF-β+8-Br-cAMP (100μM)
TGF-β+8-Br-cAMP (500μM)
8-Br-cAMP (500μM)
C
Hyaluronan Concentration (μg/ml)
4
3
2
1
0
*
*
Control
TGF-β
TGF-β+8-Br-cAMP (100μM)
TGF-β+8-Br-cAMP (500μM)
8-Br-cAMP (500μM)
D
PRG4 Concentration (μg/ml)
500
400
300
200
100
0
*
*
Control
TGF-β
TGF-β+8-Br-cAMP (100μM)
TGF-β+8-Br-cAMP (500μM)
8-Br-cAMP (500μM)

Fig. 5

A


Target Gene Expression in Prg4-/- synovium (versus Prg4+/+ synovium)
1,000
100
10
1
0.1
0.01
***
*
**
**
ACTA2
COL1A1
TIMP-1
PLOD2

B

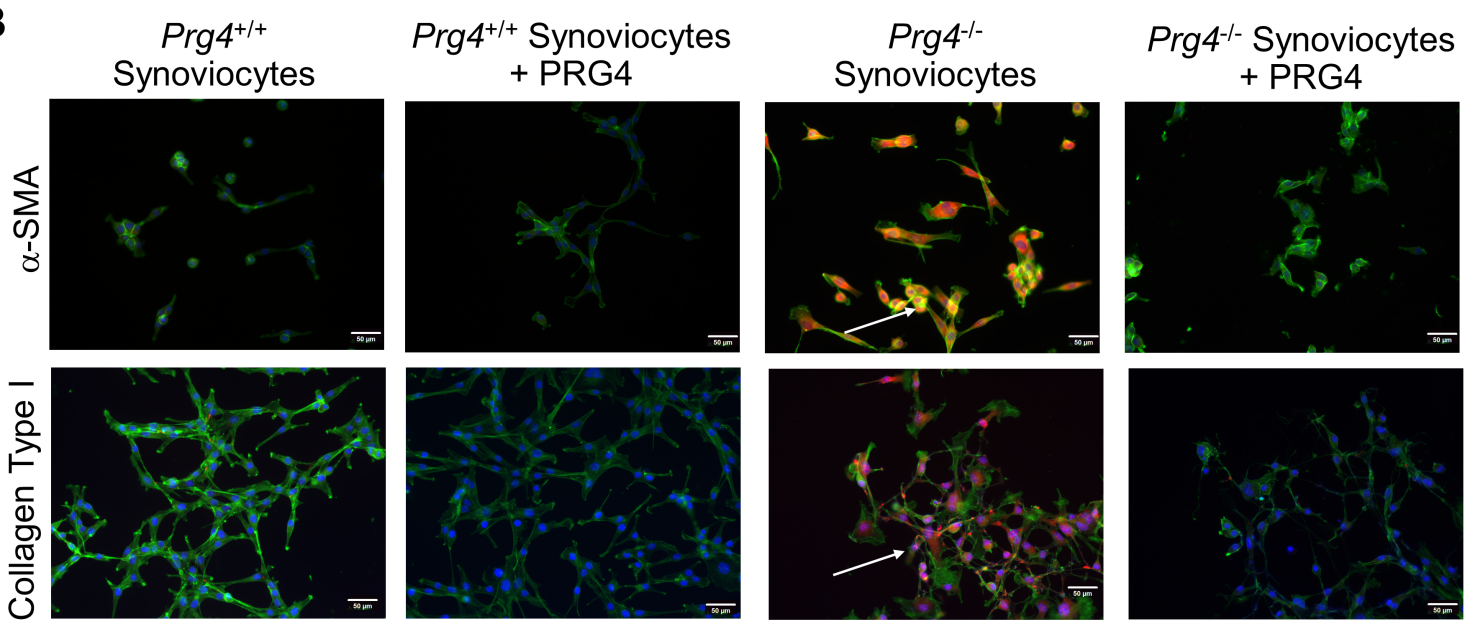
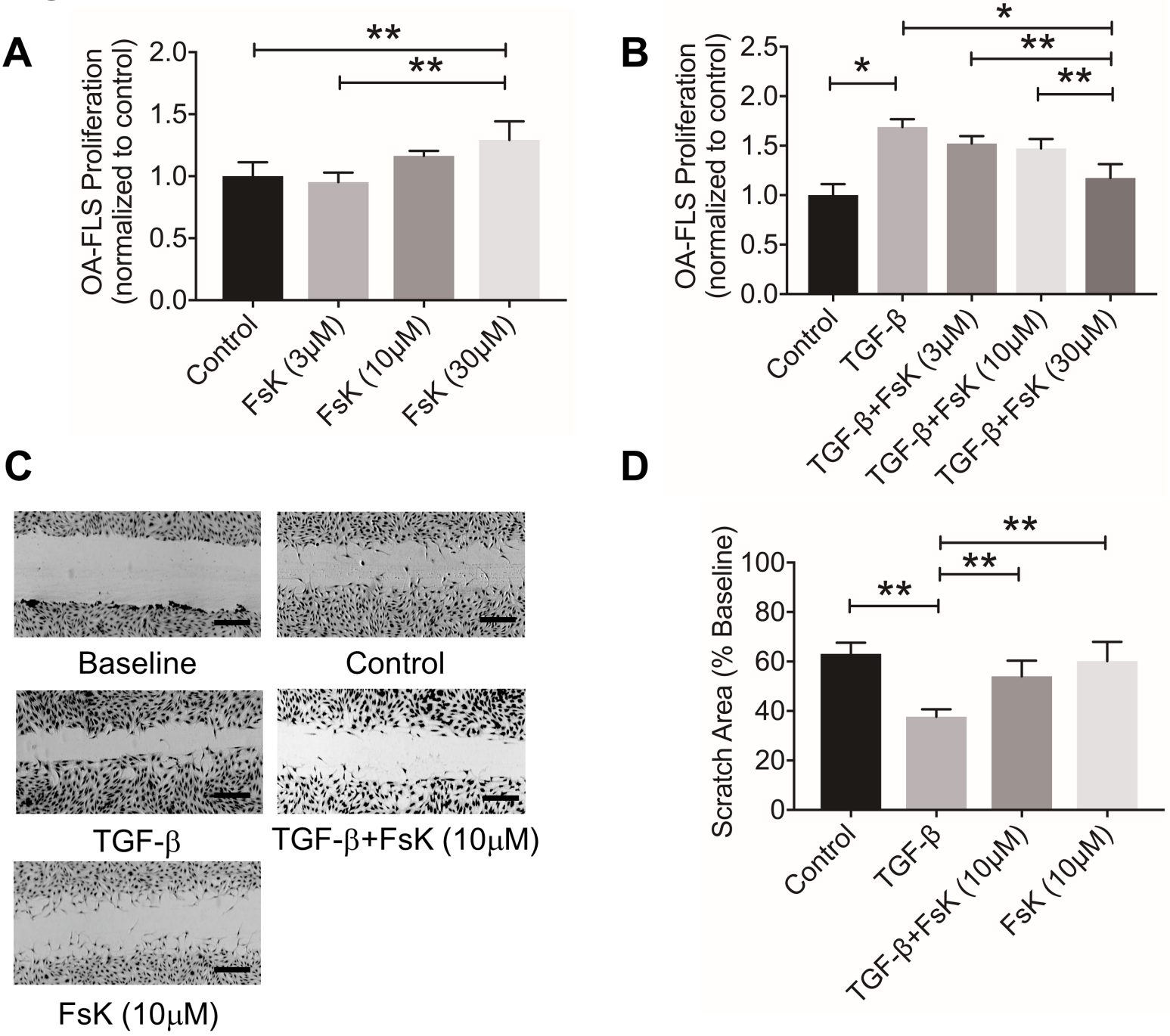

Prg4+/+ Synoviocytes
Prg4+/+ Synoviocytes + PRG4
Prg4-/- Synoviocytes
Prg4-/- Synoviocytes + PRG4
α-SMA
Collagen Type I

Fig. 6


A
OA-FLS Proliferation (normalized to control)
2.0
1.5
1.0
0.5
0.0
**
**
Control
FsK (3μM)
FsK (10μM)
FsK (30μM)
B
OA-FLS Proliferation (normalized to control)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
**
**
Control
TGF-β
TGF-β+FsK (3μM)
TGF-β+FsK (10μM)
TGF-β+FsK (30μM)
C
Baseline
Control
TGF-β
TGF-β+FsK (10μM)
FsK (10μM)
D
Scratch Area (% Baseline)
100
80
60
40
20
0
**
**
**
Control
TGF-β
TGF-β+FsK (10μM)
FsK (10μM)