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Increase in cellular cyclic AMP concentrations reverses the pro-fibrogenic phenotype of cardiac myofibroblasts: A novel therapeutic approach for cardiac fibrosis

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Running title: cAMP reverses the cardiac myofibroblast phenotype

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Abbreviations: AC, adenylyl cyclase; ADM, adrenomedullin; α-SMA, α-smooth muscle actin; Bera, beraprost; cAMP, cyclic adenosine monophosphate; CF, cardiac fibroblast; ColIα1, collagen Iα1; Epac, Exchange protein activated by cAMP; ECM, extracellular matrix; Iso, isoproterenol; Fsk, forskolin; GPCR, G protein-coupled receptor; IMBX, 1-methyl-3-(2-methylpropyl)-7H-purine-2,6-dione; N6-Phe-cAMP, N^6-phenyladenosine-3', 5'-cyclic monophosphate; PAI-1, plasminogen activator inhibitor-1; PDE, phosphodiesterase; PKA, protein kinase A

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Abstract

Tissue fibrosis is characterized by excessive production, deposition and contraction of the extracellular matrix (ECM). The second messenger cyclic AMP (cAMP) has anti-fibrotic effects in fibroblasts from several tissues, including cardiac fibroblasts (CFs): Increased cellular cAMP levels can prevent the transformation of CFs into pro-fibrogenic myofibroblasts, a critical step that precedes increased ECM deposition and tissue fibrosis. Here we tested two hypotheses: 1) myofibroblasts have a decreased ability to accumulate cAMP in response to G protein-coupled receptor (GPCR) agonists and 2) increasing cAMP will not only prevent, but also reverse, the myofibroblast phenotype. We found that myofibroblasts produce less cAMP in response to GPCR agonists or forskolin and have decreased expression of several adenylyl cyclase (AC) isoforms and increased expression of multiple cyclic nucleotide phosphodiesterases (PDEs). Furthermore, we find that forskolin-promoted increases in cAMP or N6-Phe-cAMP, a PKA-selective analog, reverses the myofibroblast phenotype, as assessed by the expression of collagen Iα1, α-smooth muscle actin, plasminogen activator inhibitor-1 and cellular contractile abilities, all hallmarks of a fibrogenic state. These results indicate that: 1) altered expression of AC and PDE isoforms yield a decrease in cAMP concentrations of cardiac myofibroblasts (relative to CFs) that likely contribute to their pro-fibrotic state, and 2) approaches to increase cAMP concentrations not only prevent fibroblast-to-myofibroblast transformation but also can reverse the pro-fibrotic myofibroblastic phenotype. We conclude that therapeutic strategies designed to enhance cellular cAMP concentrations in CFs may provide a means to reverse excessive scar formation following injury and to treat cardiac fibrosis.

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Introduction

The lack of drugs that can treat fibrosis of the heart (Fan et al., 2012), lung (van der Vliet and Bove, 2011), liver (Kisseleva et al., 2012), kidney (Kriz et al., 2011) and other tissues (Insel et al., 2012) represent an important unmet medical need. Fibrosis is characterized by the excessive deposition of collagens and extracellular matrix (ECM) proteins that lead to impaired organ function (Goldsmith et al., 2013). Fibroblasts are the predominant cell type responsible for the homeostatic maintenance of tissue ECM, healing after injury and age-associated remodeling (Tomasek et al., 2002, Wynn, 2008, van den Borne et al., 2010). In the heart, pathologic transformation of cardiac fibroblasts (CFs) to activated (pro-fibrogenic) myofibroblasts leads to decreased myocardial compliance, diastolic dysfunction and accompanying heart failure (Wynn, 2008, Creemers and Pinto, 2011, Fan et al., 2012). Myofibroblasts are characterized by increased protein synthesis, including of collagens (in particular, types I and III), other ECM proteins, certain cytokines and α-smooth muscle actin (α-SMA), a contractile protein and marker of pro-fibrogenic CF activation (Hinz et al., 2001, Swaney et al., 2005, Hinz, 2007).

Proper wound healing requires the resolution of inflammatory responses and a decrease in fibroblast activity (Kisseleva et al., 2012, van den Borne et al., 2010, Hinz, 2007, Tomasek et al., 2002). The precise mechanisms responsible for such resolution are not well defined, especially in terms of the decrease in the activity of fibroblasts. While apoptosis of fibroblasts can contribute to the attenuation of pro-fibrotic remodeling (Insel et al., 2012, Huang et al., 2009), other processes likely also contribute to the “reversal” of pro-fibrotic phenotypes (Kisseleva et al., 2012). One potential mediator is the second messenger cyclic AMP (cAMP). Activation of cAMP-dependent processes is known to inhibit CF-to-myofibroblast conversion (Miller et al., 2011, Swaney et al., 2005) and overexpression of adenylyl cyclase (AC) and

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activation of Gs-coupled G protein-coupled receptors (GPCRs), which stimulate cAMP synthesis, decrease collagen synthesis and α-SMA expression in CFs (Yokoyama et al., 2008, Swaney et al., 2005, Davaille et al., 2000, Heusinger-Ribeiro et al., 2001, Schiller et al., 2010). However, the contribution of cAMP to maintain, and potentially to reverse, the pro-fibrotic state is not known. We thus undertook the current study using primary isolates of adult CFs in order to test the hypotheses that: 1) myofibroblasts have a decreased ability to generate cAMP in response to Gs-coupled GPCR agonists and 2) increasing cAMP will not only prevent but also reverse the myofibroblast phenotype. The results shown here provide evidence in support of both of these hypotheses.

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Materials and Methods

Cardiac fibroblast isolation and culture

Approval for the ethical care and use of animals for this study was obtained from the UCSD Institutional Animal Care and Use Committee in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. CFs were isolated from the hearts of 3-month-old male Sprague Dawley rats, as previously described (Yokoyama et al., 2008). Briefly, hearts were removed and retrograde-perfused with collagenase type II (200 units/mL) (Worthington Biochemical Corp., NJ). Cardiac myocytes and CFs were separated via gravity sedimentation; CFs were then cultured in DMEM containing 10% FBS, 1% penicillin/streptomycin and incubated at 37 °C, 10% CO_2. CFs were grown to confluency, split to appropriate sized culture dishes and serum-starved for 24 h prior to use in experiments.

cAMP radioimmunoassay

CFs were plated in 24-well plates (30,000 cells/well) and serum-starved for 48 h prior to cAMP quantification by radioimmunoassay (RIA), as previously described (Swaney et al., 2005). After serum starvation, CFs were incubated with 200 µM isobutylmethylxanthine (IBMX) for 20 min (when applicable) and then with GPCR agonists of interest, forskolin (Fsk, Ascent, Cambridge, MA),Isoproternol, Beraprost, or Adrenomedullin (Iso, Bera, or ADM, Sigma, St. Louis, MO),for 10 min. Incubations were terminated by addition of 7.5% trichloroacetic acid (TCA). RIA (Liu et al., 2004) was used to quantify cellular cAMP as compared to a standard curve and normalized to total protein content per sample, as determined by a Bradford assay (Bio-Rad).

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Quantitative real-time PCR (qPCR)

Total RNA was extracted from CFs using the RNeasy Mini kit (Qiagen, Alameda, CA) according to the manufacturer's instructions. cDNA was synthesized using the Superscript III reverse transcriptase kit (Invitrogen), per the manufacturer's instructions. qPCR was performed using 8 ng cDNA, 0.5 μM forward and reverse primers, and qPCR Mastermix Plus enzyme kit (Eurogentec, San Diego, CA). Primers for PCR amplification (Table 1) were designed based on the nucleotide sequences of the respective gene targets using Primer3Plus software. When possible, each forward and reverse primer set was designed between multiple exons. Primers for rat ACs were designed by QuantiTect Primer Assays (Qiagen). Amplification efficiency of each primer pair was tested prior to analysis, and relative gene expression levels were determined using the ΔΔCT method with 18S as the reference gene (Pfaffl, 2001).

Immunofluorescence microscopy for α-SMA protein

The abundance and organization of α-SMA into stress fibers was assessed by immunofluorescence microscopy. CFs were cultured in a 3-dimensional gel consisting of rat tail collagen type I (BD Bioscience). After treatment, CFs were fixed in 10% buffered formalin for 15 min and permeabilized with 0.3% Triton X/PBS. Primary antibodies for α-SMA (diluted 1:200; Sigma-Aldrich) and phalloidin (diluted 1:100; Life Technologies) were used followed by either ALEXA Fluor 488- or 555-conjugated secondary antibody (Life Technologies). Coverslips were mounted in gelvatol and images captured using a Zeiss LSM510 confocal microscope.

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Immunoblotting

Whole cell lysates were prepared in 150 mM Na_2CO_3 buffer (pH 11) and homogenized by sonication. Equal amounts of protein (Bradford assay) were separated by SDS/PAGE using 10% polyacrylamide precast gels (Invitrogen) and transferred to a poly(vinylidenedifluoride) membrane with the iBlot system (Invitrogen). Membranes were blocked in PBS Tween (1%) containing 5% nonfat dry milk and incubated with primary antibody (α-SMA, Sigma-Aldrich; PAI-1, BD Bioscience; AC5/6, Santa Cruz; PDE8A, Fabgennix; or GAPDH, Abcam) overnight at 4°C. Bound antibodies were visualized using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and ECL reagent (Amersham Pharmacia, Pittsburg, PA). Bands were compared to molecular weight standards to confirm migration of proteins at the appropriate size.

3-D collagen gel contraction assay

CFs were suspended at 100,000 cells/gel in a solution containing 2.5 mg/mL collagen type I (BD Biosciences) and cast into a 24 well plate. Collagen gel disks were detached from the sides of the well, and 0.5 mL of serum-free media was added to the well in the presence or absence of 10 ng/mL TGF-β1. Drug treatments were added at the concentrations and times indicated. Images of collagen gel contraction were taken at day 0, 2, 4 and 6 with a Sensicam QE imager and analyzed by ImageJ.

Statistical analysis

Data were analyzed using Graph-Pad Prism 4.0 (GraphPad Software, La Jolla, CA). Numerical values are presented as mean ± S.E.M. Analysis of numerical data were done using

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ANOVA with Tukey's test or Student's t test, where appropriate. $P<0.05$ was considered significant.

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Results

cAMP accumulation is decreased in cardiac myofibroblasts compared to CFs

Treatment of adult rat ventricular CFs with several different GPCR agonists or with the AC activator Fsk increased cellular cAMP levels in a concentration-dependent manner (Fig. 1A). The rank order of the maximal responses generated by those stimulants of cAMP formation were as follows: Fsk > isoproterenol (Iso, a β-adrenergic receptor agonist) > beraprost (Bera, a prostaglandin I_2 receptor agonist) = adrenomedullin (ADM, a calcitonin/adrenomedullin receptor agonist). Butaprost, a prostaglandin E2 receptor (EP_2) agonist, did not increase cAMP accumulation in CFs.

We next generated pro-fibrogenic myofibroblasts in order to compare their cAMP accumulation with that of CFs. To generate myofibroblasts, we incubated the adult rat ventricular CFs with 10 ng/mL TGF-ß1 for 48 h, a treatment protocol that transforms CFs to myofibroblasts (Bujak and Frangogiannis, 2007, Leask and Abraham, 2004, Yokoyama et al., 2008). CFs and myofibroblasts were then incubated with 200 µM IBMX, a non-selective cyclic nucleotide phosphodiesterase (PDE) inhibitor, prior to stimulation with Fsk or the GPCR agonists (Iso, Bera or ADM, each at 10 µM, a saturating concentration for each agonist) (Fig. 1B). Fsk and Iso increased cAMP accumulation in CFs by ~55- and 70-fold, respectively, but had diminished responses in myofibroblasts in which cAMP accumulation only increased by 22- and 15- fold, respectively. Decreased cAMP accumulation by myofibroblasts also occurred in response to Bera and ADM. The potential ability of IBMX to inhibit adenosine receptors did not contribute to these responses as rolipram, a PDE4-selective antagonist that does not inhibit adenosine receptors, elicited similar results (data not shown), indicating that the enhanced cAMP

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with addition of IBMX occurs via PDE inhibition. Thus, myofibroblasts accumulate substantially less cAMP in response to incubation with Fsk and Gs-coupled GPCR agonists.

Myofibroblasts have altered profiles of expression of AC and PDE isoforms

To assess the basis for the decreased cAMP accumulation in myofibroblasts, we quantified the expression of AC and PDE isoforms (using real-time qPCR) by CFs and myofibroblasts. The rank-order of expression of AC isoforms was as follows: AC6 > AC5 > AC3 > AC4 > AC7 > AC9 > AC8 with AC1 and AC2 not being detected. Compared to CFs, myofibroblasts had a statistically significant decrease in expression of AC6 and AC5, the two highest expressed AC isoforms, and also of AC9 (decreases of 28%, $p<0.01$; 27%, $p<0.05$; and 57%, $p<0.05$, respectively) (Fig. 2A).

The cyclic nucleotide PDE profile of CFs revealed the following rank-order of expression of the 6 highest expressed isoforms: PDE 8A, 1A, 4D, 3B, 4A and 10A with mRNA for several other PDEs (PDEs 2A, 3A, 5A and 7A) expressed at lower levels (Fig. 2B). Compared to CFs, myofibroblasts had increased expression of multiple PDE isoforms. PDE8A, the most abundant PDE isoform in adult rat CFs, increased by 13% ($p<0.01$) in myofibroblasts. Expression of other abundant isoforms, PDE4D, 4A and 10A, was increased relative to CFs by 22%, 38% and 45%, respectively ($p<0.05$). One exception was PDE1A, whose expression was decreased by 45% ($p<0.01$) in myofibroblasts. PDEs 1B, 1C, 7B, 9A and 11A were detected at very low levels and their expression was unchanged in myofibroblasts, while expression of PDE8B was not detected.

Protein expression of AC5/6 and PDE8A, the most abundant of their respective isoforms, were assessed via immunoblotting (Fig. 2C). Upon TGF-ß stimulation, AC5/6 protein expression decreased by 90% ($p<0.05$), while PDE8A protein expression increased by 2.7 fold ($p<0.05$).

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TGF-ß treatment also increased CF α-SMA protein expression by 2.5 fold ($p<0.01$). Overall, the decrease in expression of the most abundant ACs (AC5/6) in myofibroblasts, accompanied by an increase in expression of several highly abundant PDEs likely accounts for the decreased cAMP accumulation of myofibroblasts in response to Fsk or Gs-coupled GPCR stimulation (Fig. 1). Since increased cellular concentrations of cAMP can blunt the transformation of CFs to myofibroblasts (Miller et al., 2011, Yokoyama et al., 2008, Swaney et al., 2005), we infer that the decrease in the ability of myofibroblasts to generate cAMP and increased ability to hydrolyze cAMP contribute to the myofibroblastic phenotype.

Incubation with forskolin can reverse the enhanced synthesis of collagen, collagen gel contraction and expression of α-SMA and PAI-1 of cardiac myofibroblasts

Previous data have indicated that incubation of CFs with agents that increase cellular cAMP levels when cells are also incubated with TGF-ß1 blocks the conversion of CFs to myofibroblasts (Miller et al., 2011, Yokoyama et al., 2008, Swaney et al., 2005). To test whether raising cAMP levels in myofibroblasts can reverse an already-developed pro-fibrotic state, we treated myofibroblasts (CFs that were incubated with TGF-ß1 for 4 or 6 days) (Fig. 3A) with Fsk (10 µM) for 48 h prior to quantifying the expression of collagen Iα1 (ColIα1) mRNA expression. Incubation with Fsk reversed the increases in ColIα1 expression in cells that had been incubated with TGF-ß1 for either 4- or 6-days (Fig. 3B). The most dramatic effect of the ability of Fsk to reverse ColIa1 expression occurred in cells incubated with TGF-ß1 for 6 days: Fsk reduced expression of ColIα1 44% ($p<0.001$), i.e., to a level akin to that of untreated CFs.

To test whether this reversal in ColIα1 expression could be promoted by activation of protein kinase A, we used a similar protocol and treated the myofibroblasts with the selective

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PKA agonist, N6-Phe-cAMP (from Biolog/Axxora, Farmingdale, NY). Treatment with N6-Phe-cAMP inhibited ColIα1 mRNA expression ($p<0.001$; Fig. 3C). Together, these findings imply that the anti-fibrotic effects of cAMP can occur via PKA activation.

As a second approach to assess reversal of the myofibroblastic phenotype, we seeded CFs into a deformable 3-D collagen gel in order to quantify their contractile abilities. Within 2 days of incubation with TGF-ß1, the cells had a statistically significant increase in gel contraction (Fig 4A). This increase progressed over the 6-day period of the experiment. By day 6, gels containing TGF-ß1-treated CFs had a 20% ($p<0.01$) smaller diameter than did CFs in gels without TGF-ß1. Incubation with Fsk for the last 48 h of the 6-day incubation with treatment with TGF-ß1 significantly ($p<0.01$) reversed the extent of collagen gel contraction (Fig. 4A).

The expression of α-SMA confers the contractile phenotype to CFs (Hinz et al., 2001). We used immunostaining to assess the expression of α-SMA in CFs cultured in the 3-D collagen gels. The 6-day incubation with TGF-ß1stimulated α-SMA expression and converted the CFs to a myofibroblastic morphology (Fig. 4B). Treatment with Fsk during the final 2 days of the 6-day incubation with TGF-ß1 reversed this morphologic appearance and reduced the content of α-SMA-positive stress fibers. Consistent with these findings, Fsk treatment decreased the TGF-ß1-stimulated expression of α-SMA protein and that of PAI-1, a pro-fibrotic marker that inhibits the activation of plasmin and matrix metalloproteinases (Ghosh and Vaughan, 2012) (Fig. 4C).

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Discussion

The data presented here provide an explanation for the lower cAMP accumulation of CFs that undergo conversion to cardiac myofibroblasts: Altered expression of AC isoforms (with decreased expression of major AC isoforms) and of PDE isoforms (with increased expression of several isoforms known to hydrolyze cAMP, i.e., PDE 8A, 4A, 4D and 10A (Bender and Beavo, 2006)). We found that several GPCR agonists and Fsk have a reduced ability to increase cellular cAMP concentrations in myofibroblasts compared to CFs. Consistent with the role of cAMP in attenuating the myofibroblastic phenotype, deceased AC5/6 expression has been noted in rat CFs following myocardial infarction (Swaney et al., 2007), and overexpression of these isoforms can prevent the conversion of CFs (Swaney et al., 2005) and pulmonary fibroblasts (Dunkern et al., 2007, Kolodsick et al., 2003, Liu et al., 2004) to myofibroblasts. Anti-fibrotic roles for cAMP have also been noted in studies of hepatic (Davaille et al., 2000, Windmeier and Gressner, 1997), renal (Heusinger-Ribeiro et al., 2001) and dermal (Parekh et al., 2007) fibroblasts. Thus, in addition to the heart, the anti-fibrotic action of cAMP may occur in fibroblasts of other organs prone to fibrosis. We and others have reported that anti-fibrotic effects of cAMP can occur via actions of both PKA and Epac and have observed an inhibition of Epac expression by incubation of cardiac fibroblasts with pro-fibrotic agonists (Yokoyama et al., 2008, Huang et al., 2008).

In contrast to the data shown here, Miller et al. have reported that PDE1A is the major isoform increased in cardiac myofibroblasts and that its inhibition can limit fibrosis following myocardial infarction (Miller et al., 2011). However, the analysis by those authors of expression of PDE isoforms was conducted in neonatal rat CFs stimulated with angiotensin II (Ang II) while our studies were conducted with adult rat CFs. Explanations for the different results that we and Miller et al. find may reflect differences in PDE isoform expression between neonatal and adult

CFs or perhaps differences in myofibroblasts that are generated by CFs incubated with Ang II rather than TGF-ß1. Regardless of the explanation, our data suggest that multiple PDE isoforms are potentially attractive therapeutic targets to blunt cardiac fibrosis.

An important result of the current study is that treatment of TGF-ß1-generated cardiac myofibroblasts with Fsk reverses multiple features of the myofibroblastic phenotype and that treatment with N6-Phe-cAMP has similar effects to Fsk to decrease ColIα1 expression. Such phenotypic reversal is thus a potential mechanism for attenuation of abnormal wound healing responses and tissue fibrosis and that occurs independent of (or in parallel with) myofibroblast apoptosis (Insel et al., 2012, Huang et al., 2009). While other reports describe the ability of cAMP to inhibit the conversion of fibroblasts to myofibroblasts (Schiller et al., 2010), the current findings indicate that cAMP-dependent signaling can reverse fibrotic activity by decreasing the expression of collagen, α-SMA and PAI-1, and reducing contractility of myofibroblasts stimulated by TGF-ß1. These results thus identify a cAMP-dependent action to reverse fibrosis and are consistent with recent observations noted in pulmonary myofibroblasts treated with prostaglandin E2, which likely acts via Gs-linked GPCRs (Garrison et al., 2013). Therefore, agents targeted to GPCRs, ACs and PDEs preferentially expressed by CFs and myofibroblasts (Snead and Insel, 2012) and to the activation of post-cAMP-dependent signaling pathways (Schiller et al., 2010, Yokoyama et al., 2008) have the potential to be novel treatment strategies to prevent and potentially reverse cardiac fibrosis, and perhaps fibrosis in other tissues.

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Authorship Contributions

Participated in research design: Yokoyama, Patel, Insel

Conducted experiments: Aroonsakool, Yokoyama, Lu

Performed data analysis: Yokoyama, Lu, Aroonsakool

Wrote or contributed to the writing of this manuscript: All the authors

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Footnotes

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[1]These authors (D.L., N.A. and U.Y.) contributed equally to this study.

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Figure Legends

Figure 1: cAMP accumulation is decreased in myofibroblasts compared to CFs. (A) CFs were incubated with increasing concentrations of forskolin (Fsk) or GPCR agonists (isoproterenol (Iso), beraprost (Bera), adrenomedullin (ADM) and butaprost). Cellular concentrations of cAMP increased in a concentration-dependent manner in response to Fsk, Iso, Bera and ADM. (B) CFs (open bars) or myofibroblasts (CFs stimulated with 10 ng/mL TGF-ß1 for 48 h; solid bars) were incubated with saturating concentrations of GPCR agonists or Fsk (10 µM) in the presence of 200 µM IBMX. Myofibroblasts generated significantly less cAMP compared to CFs: Fsk increased cAMP concentrations 22-fold in myofibroblasts vs. 55-fold in CFs, while Iso increased cAMP concentrations 15-fold in myofibroblasts vs. 70-fold in CFs. Data are presented as mean +/- SEM of 4 independent experiments; **p<0.01.

Figure 2: TGF-ß1-induced myofibroblasts have altered profiles of AC and PDE isoform expression. (A) Myofibroblasts (CFs incubated with 10 ng/mL TGF-β1 for 48 h; solid bars) decreased their expression of several AC isoforms. AC6, AC5 and AC9 mRNA expression were decreased in myofibroblasts compared to CFs (open bars) by 28%, 27%, and 57% respectively. (B) Four of the six most highly expressed PDE isoforms in CFs increase expression following myofibroblast transformation: PDE 8A, 4D, 4A and 10A by 13%, 22%, 38% and 45%, respectively. In contrast, PDE1A decreased in expression by 45% in myofibroblasts. (C) AC5/6 protein expression decreased by 90%, while PDE8A and α-SMA expression increased by 2.7- and 2.5-fold, respectively, in myofibroblasts incubated with 10 ng/mL TGF-ß for 72 h. Data are presented as mean +/- SEM of at least 3 independent experiments; *p<0.05, **p<0.01.

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Figure 3: cAMP, acting via PKA, reverses TGF-β1-induced myofibroblast differentiation. (A) Treatment regime showing the duration of TGF-ß1 (10 ng/mL), Fsk (10 µM) or N6-Phe-cAMP (50 µM) treatment. (B) Incubation of CF with TGF-ß1 for 6 days increased collagen Iα1 (ColIα1) mRNA expression by 38%. ColIα1 expression decreased 44% following Fsk treatment for the last 48 h of the 6-day treatment regime. (C) N6-Phe-cAMP, a PKA-selective analog, reversed ColIα1 mRNA expression stimulated by both 4- and 6-day incubation with TGF-ß1. Data are presented as mean +/- SEM of 4 independent experiments; *p<0.05, ***p<0.001.

Figure 4: cAMP reverses α-SMA and PAI-1 expression and contractility in 3-D collagen gels of TGF-β1-induced myofibroblasts. (A) TGF-ß1 incubation of CF for 2-6 days increased the contraction of collagen gels. Gels at day 6 had 20% less surface diameter with TGF-ß treatment. Addition of Fsk for the final 48 h significantly reversed the contractile phenotype. (B) Incubation with TGF-ß1 for 6 days increased expression of α-SMA-positive stress fibers of CFs grown in 3-D culture. Incubation with Fsk for the last 48 h diminished α-SMA expression (red: F-actin, green: α-SMA, blue: DAPI; 40X magnification). (C) Incubation with Fsk reduced expression of α-SMA and PAI-1 protein in CFs incubated in the absence (control/Fsk) or presence (TGF-β1, TGF-β1 + Fsk) for 4 days and are shown for triplicate samples. Data are presented as mean +/- SEM of 3 independent experiments; *p<0.05, **p<0.01.

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Tables

Table 1: Primer sequences for qPCR

Gene	Forward 5'-3'	Reverse 5'-3'
PDE1A	tttgtgatcggaagtcaacg	agctgcttgccccatagtta
PDE1B	cagcccttgacagatgatga	caatggacatctggttggtg
PDE1C	ggcccttgaagttggttaca	gcagccgagaagattattgc
PDE2A	gagagaaagtgctgggagaaga	ccgtgcctgtgtagtggaa
PDE3A	aagacatggggctctttgaa	gaatcggctgtgttgtgaga
PDE3B	cagtggcaagatgttcagga	aaggcttgggtcaatcagaa
PDE4A	gcaacttctcactcttaaccaatgt	aggcaccaatccagctctt
PDE4B	caagcagaacgatgtggaaa	tgactccaaagcgtgagatg
PDE4D	ggaggacaatcgtgagtggt	tcagtgtctgactcgccatc
PDE5A	agtttgctttgcacgcctat	ccaagccacagaagatgaca
PDE7B	ctcctacccgttcattgactt	tgtcaaagtcccacattcca
PDE8A	agttggcatttggagaggtg	tttgcatactggatgatgtgg
PDE8B	atcacaggcagacccgtaac	ctgcatggagaagaggaagg
PDE9A	cctttgatgtctggctttgg	gagtgtgattgggttgatgct
PDE10A	agcgatgaagtccctcaa	cccctcgaattaccttctcc
PDE11A	aactgatgtccccaaagtgc	tcgctgacattcacaggaag
Collagen Iα1	ctggcaagaacggagatgat	caccatccaaaccactgaaa
18S	gtaacccgttgaaccccatt	ccatccaatcggtagtagcg

Figure 1

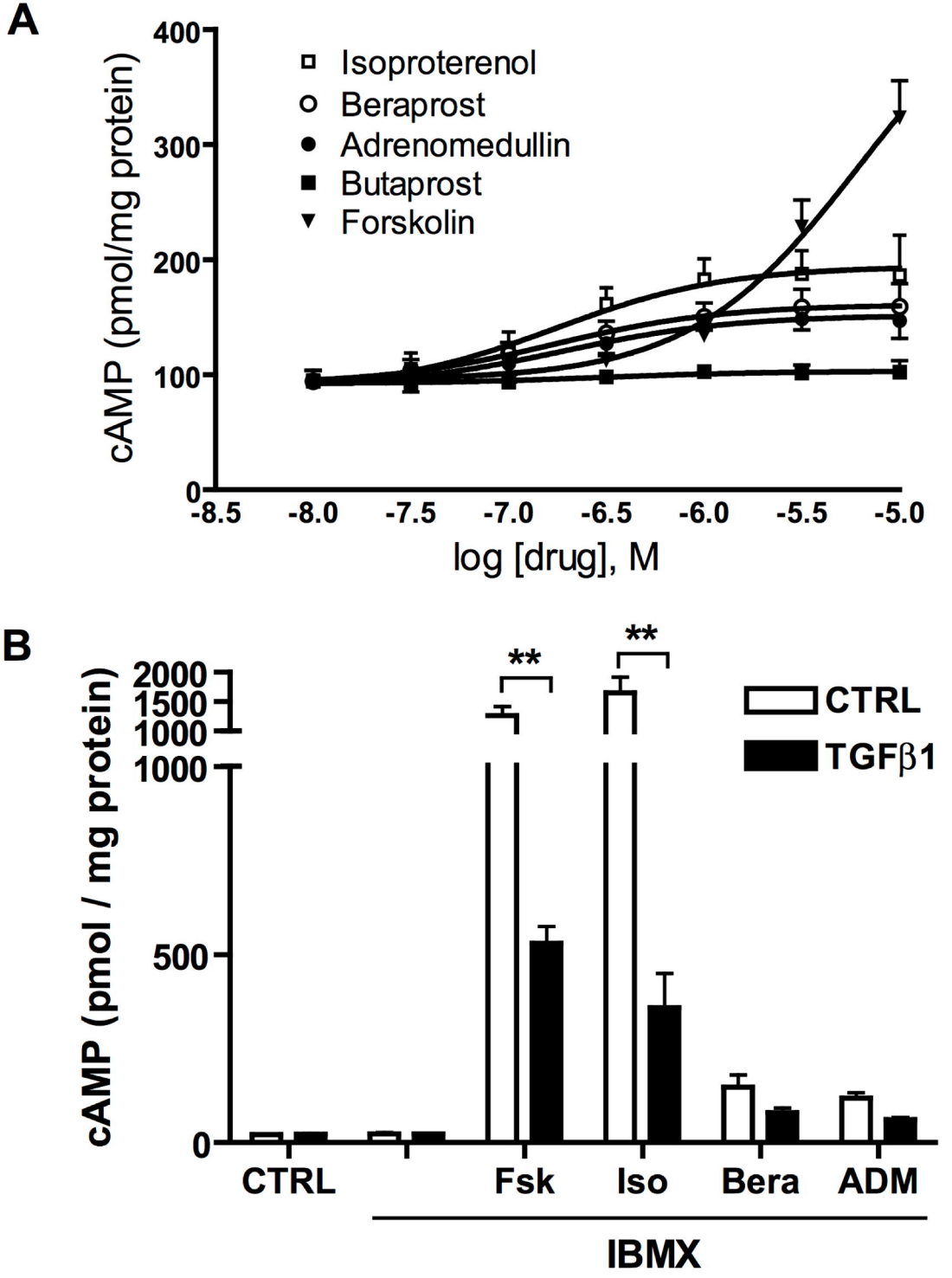
A
400
300
200
100
0
cAMP (pmol/mg protein)
Isoproterenol
Beraprost
Adrenomedullin
Butaprost
Forskolin
-8.5
-8.0
-7.5
-7.0
-6.5
-6.0
-5.5
-5.0
log [drug], M
B
2000
1500
1000
1000
500
0
cAMP (pmol / mg protein)
**
**
CTRL
TGFβ1
CTRL
Fsk
Iso
Bera
ADM
IBMX

Figure 2

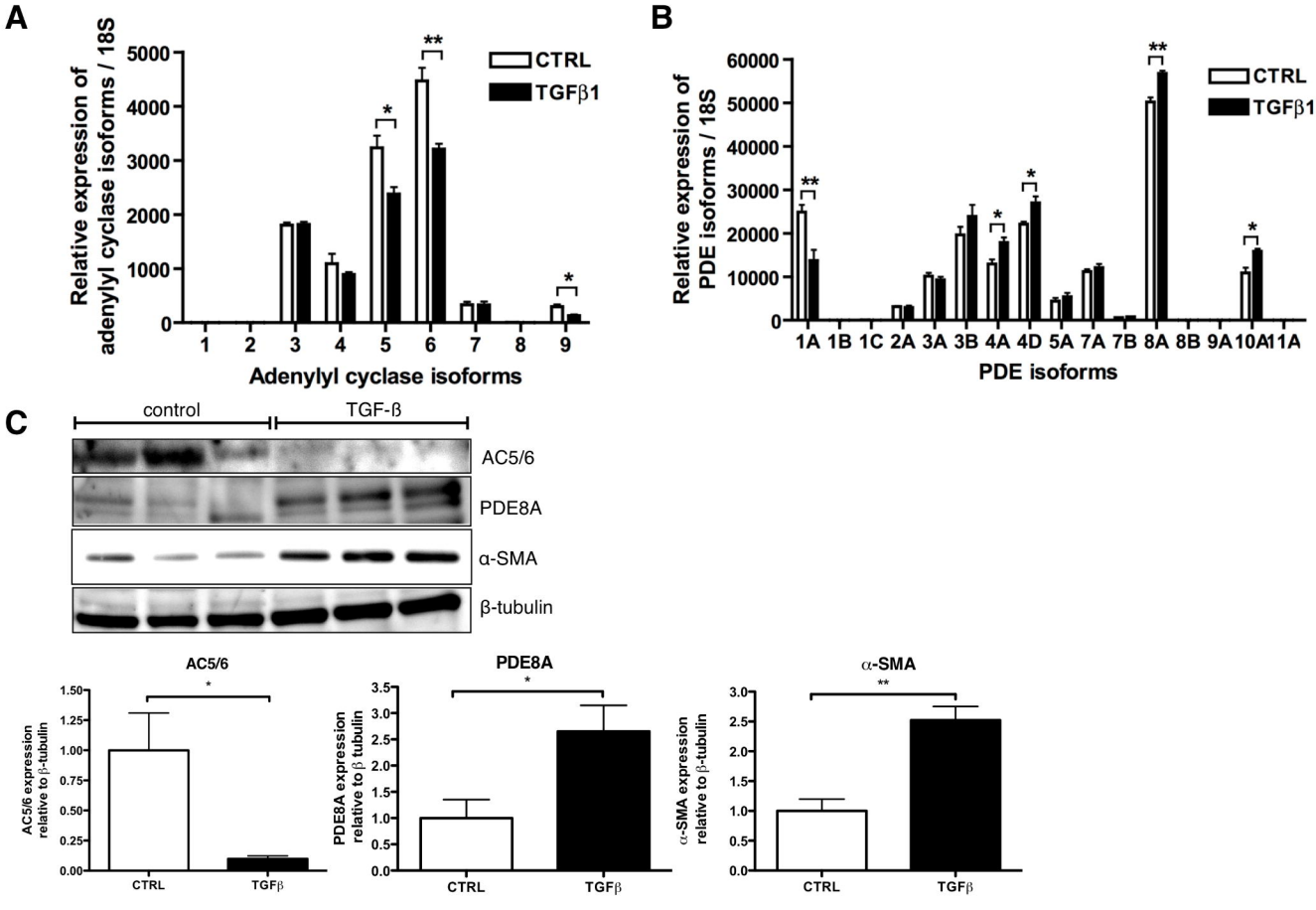
A
Relative expression of adenylyl cyclase isoforms / 18S
5000
4000
3000
2000
1000
0
CTRL
TGFβ1
*
**
*
1 2 3 4 5 6 7 8 9
Adenylyl cyclase isoforms
B
Relative expression of PDE isoforms / 18S
60000
50000
40000
30000
20000
10000
0
CTRL
TGFβ1
**
*
*
**
*
1A 1B 1C 2A 3A 3B 4A 4D 5A 7A 7B 8A 8B 9A 10A 11A
PDE isoforms
C
control
TGF-ß
AC5/6
PDE8A
α-SMA
β-tubulin
AC5/6
AC5/6 expression relative to β-tubulin
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
CTRL
TGFβ
PDE8A
PDE8A expression relative to β tubulin
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
CTRL
TGFβ
α-SMA
α-SMA expression relative to β-tubulin
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
CTRL
TGFβ

Figure 3

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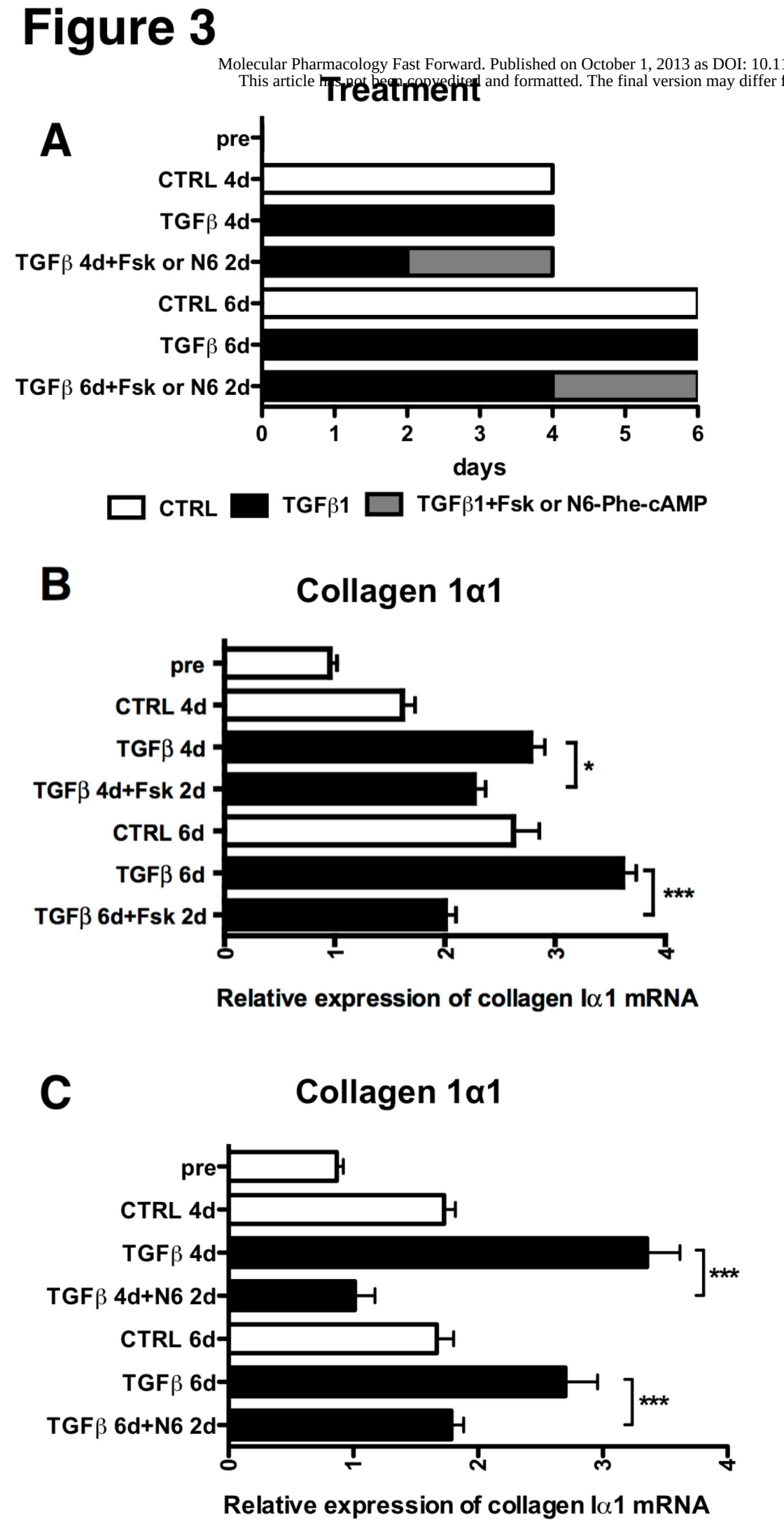
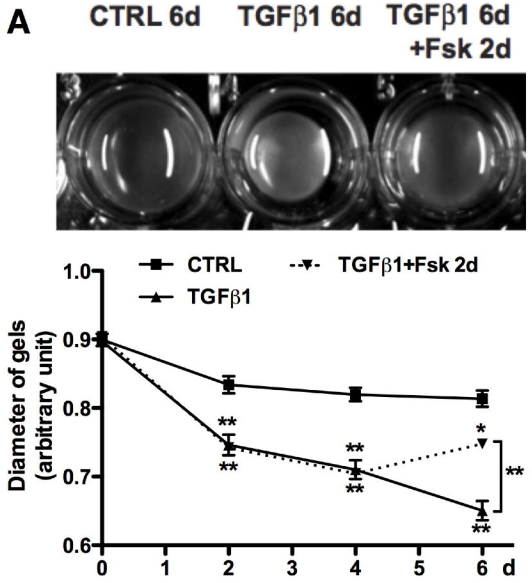
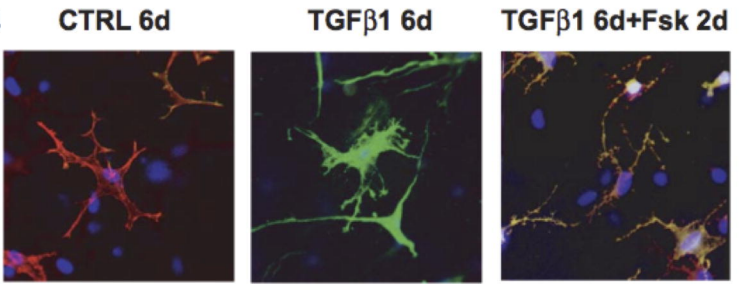
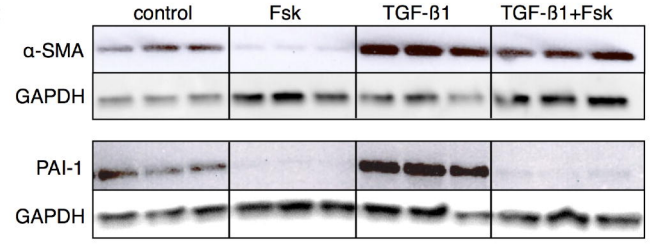


Figure 4


A
CTRL 6d
TGFβ1 6d
TGFβ1 6d
+Fsk 2d
CTRL
TGFβ1
TGFβ1+Fsk 2d
Diameter of gels
(arbitrary unit)
1.0
0.9
0.8
0.7
0.6
0
1
2
3
4
5
6
d
**
*


B
CTRL 6d
TGFβ1 6d
TGFβ1 6d+Fsk 2d


C
control
Fsk
TGF-ß1
TGF-ß1+Fsk
α-SMA
GAPDH
PAI-1
GAPDH