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ADENOSINE A_{2A} RECEPTOR OCCUPANCY STIMULATES COLLAGEN EXPRESSION BY HEPATIC STELLATE CELLS VIA PATHWAYS INVOLVING PKA, src AND erk 1/2 SIGNALING CASCADE OR P38 MAPK SIGNALING PATHWAY

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RUNNING TITLE: Adenosine A_{2A} Receptor Signaling in LX2 Stellate Cells

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NON-STANDARD ABBREVIATIONS:
HSCs: hepatic stellate cells; siRNA: small interfering RNA; ECM: extracellular matrix; PKA: protein kinase A; PKAI: PKA peptide inhibitor; srcI: src kinase inhibitor II.

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ABSTRACT

Prior studies indicate that adenosine and the adenosine A_{2A} receptor play a role in hepatic fibrosis by a mechanism which has been proposed to involve direct stimulation of hepatic stellate cells (HSCs). The objective of this study was to determine whether primary hepatic stellate cells produce collagen in response to adenosine (via activation of adenosine A_{2A} receptors) and to further determine the signaling mechanisms involved in adenosine A_{2A} receptor-mediated promotion of collagen production. Cultured primary HSCs increase their collagen production following stimulation of the adenosine A_{2A} receptor in a dose-dependent fashion. Similarly, LX-2 cells, a human HSC line, increases expression of precollagen αI and precollagen αIII mRNA and their translational proteins, collagen type I and type III in response to pharmacologic stimulation of adenosine A_{2A} receptors. Based on the use of pharmacologic inhibitors of signal transduction, adenosine A_{2A} receptor-mediated stimulation of precollagen αI mRNA and collagen type I collagen expression were regulated by signal transduction involving protein kinase A, src and MEK/erk but surprisingly adenosine A_{2A} receptor-mediated stimulation of precollagen αIII mRNA and collagen type III protein expression depend upon activation of p38 MAPK, findings confirmed by siRNA-mediated knockdown of src, erk1, erk2 and p38 MAPK. These results indicate that adenosine A_{2A} receptors signal for increased collagen production by multiple signaling pathways. These results provide strong evidence in support of the hypothesis that adenosine receptors promote hepatic fibrosis, at least in part, via direct stimulation of collagen expression and that signaling for collagen production proceeds via multiple pathways.

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INTRODUCTION

HSCs play a central role in the pathogenesis of hepatic fibrosis/cirrhosis(Friedman, 2000; Li and Friedman, 1999). Following hepatic injury HSCs become activated and, like other matrix-producing cells, take on a myofibroblastic phenotype.(Rockey et al., 1993) After activation HSCs synthesize and deposit markedly greater levels of collagen, predominantly collagen type I and type III, and other matrix proteins in the ECM(Geerts et al., 1989). HSCs have been established as the source of collagen type I and type III in the liver. These activated HSCs secrete collagen type I and type III, the principal matrix proteins responsible for the development of liver fibrosis and cirrhosis. Since collagen type I and type III are major components to be responsible for the exuberant and unbalanced wound-healing response in liver fibrosis, their selective removal would be a potential mechanism to attenuate liver fibrosis.

Based on the prior observation that adenosine, acting at adenosine A_{2A} receptors, plays a role in wound healing (Montesinos et al., 2002; Montesinos et al., 1997; Victor-Vega et al., 2002) we hypothesized that adenosine and adenosine A_{2A} receptors might play a role in excess fibrosis in the skin and hepatic fibrosis. Indeed, endogenously released adenosine and adenosine A_{2A} receptors play a critical role in the development of liver and skin fibrosis following toxic insults based on studies in animal models(Chan et al., 2006b). Thus, both adenosine A_{2A} receptor antagonists and adenosine A_{2A} receptor deletion protect mice from developing skin and liver fibrosis (Chan et al., 2006a; Chan et al., 2006b).

Signaling for enhanced collagen production following adenosine A_{2A} receptor activation has been explored only superficially and inhibition of protein kinase A and erk1/2 all partially inhibit overall collagen production by the LX-2 hepatic stellate cell line and primary dermal fibroblasts (Arslan and Fredholm, 2000; Chan et al., 2006a; Chan et al., 2006b; Chern et al., 1995; Mori et al., 2004; Seidel et al., 1999). Adenosine A_{2A} receptors may signal through other

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pathways as well including src kinase(Lee and Chao, 2001; Rajagopal and Chao, 2006; Schulte and Fredholm, 2003).

The aim of the present study is to determine whether collagen production by HSCs is regulated by adenosine A_{2A} receptors and better characterize the signal-transduction mechanisms involved in adenosine A_{2A} receptor-mediated regulation of matrix production. We present evidence here that occupancy of adenosine A_{2A} receptors regulates the transcription and translation of collagen type I via activation of PKA, src kinase and erk1/2 MAPK and collagen type III via p38 MAPK signaling pathway.

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MATERIALS AND METHODS

Reagents and drugs

Adenosine A_{2A} receptor agonist, CGS21680 (2-(p-(2-carbonylethyl) phenylethylamino)-5-N-ethylcarboxamido adenosine) was purchased from Research Biochemicals (Natick, MA, USA). Adenosine A_{2A} receptor antagonist 4-(2-[7-amino-2-(2-furyl)[1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-yl amino]ethyl)phenol (ZM241385) was from Tocris (Ellisville, MI, USA). PKAI, a PKA inhibitor, which corresponds to the amino acids 5–24 of the naturally occurring PKA inhibitor(Kemp et al., 1991), was from Promega (Madison, WI, USA). srcI,, MEK/erk MAPK inhibitors, U0126 and PD98059, and p38 MAPK inhibitor, SB202190 were from Calbiochem (San Diego, CA, USA) and their structures are described in the manufacturer's catalogue. CellTiter 96 proliferation assay kit was from Promega (Madison, WI, USA). Sirius red F3B and Fast Green FCF were from Sigma (St. Louis, MO, USA). Sircol collagen assay kit was from Accurate Chemical (Westbury, NY, USA). TRIZOL was from Invitrogen (Carlsbad, CA, USA). SuperScript™ III RT kit was from Invitrogen (Carlsbad, CA, USA). Brilliant® SYBR® Green QPCR Master Mix was from Stratagene (Cedar Creek, TX, USA).

Cell culture

Cultured normal human hepatic primary HSCs isolated from single donor human liver were purchased from ScienCell Research Laboratories, (San Diego, CA, USA). To activate hepatic primary HSCs, cells were cultured in Stellate Cell Medium (SteCM, ScienCell Research Laboratories, San Diego, CA, USA) supplemented with 2% fetal bovine serum (FBS, GIBCO, Invitrogen Corporation, CA, USA), 1% of stellate cell growth supplement (ScienCell Research Laboratories, CA, USA), 100 U/ml penicillin (GIBCO, NJ, USA) and 100 U/ml streptomycin (GIBCO, NJ, USA) on uncoated plastic flasks (BD Biosciences, MA, USA) for 14 days(Ikeda et al., 1999; Marra et al., 2000) and were then used for biological studies.

The LX-2 human hepatic stellate cell line was previously characterized by and kindly provided by Dr. S.L. Friedman, (Mount Sinai School of Medicine, NY,

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USA)(Xu et al., 2005). LX-2 cells were grown and maintained in Dulbecco's Modified Eagle Medium (, GIBCO, NJ, USA) supplemented with 10% calf serum, 2 mM L-glutamine (GIBCO, Invitrogen Corporation, CA, USA), 100 U/ml penicillin (GIBCO, Invitrogen Corporation, CA, USA) and 100 U/ml streptomycin (GIBCO, Invitrogen Corporation, CA, USA). Cells were cultured in 75 cm^2 flasks until 80-90% confluence and were then used for biological studies.

Cell proliferation assay

The effect of CGS21680 and ZM241385 on proliferation of the LX-2 cells was analyzed by morphologic analysis and CellTiter 96 proliferation assay kit according to the manufacturer's instructions. Briefly, the cells were seeded into 96-well plates (5×10^3 cells per well). Six wells were used for each concentration of each compound (*n*=5 for each concentration). The CellTiter 96 Aqueous One Solution [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) and phenazine ethosulfate] was added to the wells, and the MTS was reduced into a colored formazan product by the living cells. The quantity of formazan product was measured 3 hours later at a wavelength of 490 nm, as the optical density of the dye at 490 nm is directly proportional to the number of living cells(Zolnai et al., 1998). The results were confirmed by three independent experiments.

Sircol collagen assay

The collagen content in cold methanol-fixed primary HSCs and LX-2 cells was determined by the differential binding of Sirius red F3B and Fast Green FCF to collagen and non-collagenous proteins, respectively, in the presence of picric acid(Jimenez et al., 1985; Lopez-De Leon and Rojkind, 1985). Collagen content of primary HSCs and LX-2 cells was determined by a modification of the original method(Bennett et al., 2003; Freeman et al., 2002). The cells were plated in 24-well plates at a density that resulted in 80-90% confluence after a 24 hr incubation in medium containing 2% (for primary HSCs) or 10% FBS (for LX-2 cells). Culture medium was then replaced with serum-free medium supplemented with 1% of stellate cell growth supplement, 2 mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin for 24 hours and then cells were

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treated with various adenosine receptor agonists and/or antagonists for 24 hours. After treatment, the medium was harvested, and the cells were washed and fixed in cold methanol for 15 minutes. Cells were stained at room temperature as follows: (I) 15 minutes in 0.5 ml of a solution containing 0.01% Fast Green FCF, 0.5% picric acid in distilled water; (II) 15 minutes in 0.5 ml of a solution containing 0.04% Fast Green FCF, 0.1% Sirius red, 0.5% picric acid in distilled water; and (III) washing in tap water for 5 minutes until the elution fluid was completely clear. Cells were allowed to dry before elution of the bound dyes with 1 ml of 50% methanol containing 50 mM NaOH. After a spectrum scan to establish the peak absorbance for each dye, the absorbance of the solution at 630 and 540 nm was determined by spectrophotometer (SmartSpec™ Plus Spectrophotometer, Bio-Rad, Hercules, CA, USA). Calculation of collagen and protein content was performed, as described(Jimenez et al., 1985), and the data are expressed as the change in the content of soluble collagen production (µg/mg total proteins) over the treatment interval to correct for collagen present in the cultures without the addition of the test compounds.

Total soluble collagen concentration in the cell supernatants was also measured using the Sircol collagen assay kit. Briefly, a 200-µl aliquot of supernatant was added to 1 ml of Sircol dye and incubated at room temperature for 30 minutes, then centrifuged to pack the collagen-dye complex at the bottom of the tube. The pellet obtained was dissolved in alkali reagent (0.5M NaOH solution) to release the collagen-dye complex whose absorbance was measured at 540 nm. The concentration of collagen production was determined from a collagen standard curve provided with the assay.

Real-time quantitative reverse transcription-polymerase chain reaction (Real-time RT-PCR)

Total RNA was isolated from $3X10^8$ LX-2 cells using the TRIZOL method. RNA (1.0 µg) was reverse transcribed in 20 µl buffer containing 50 µM oligo(dT)20, 25 mM $MgCl_2$, 0.1 M DTT, 40 U/µl RNaseOUT and 200 U/µl SuperScript™ III RT for 50 minutes at 50°C. The reaction was stopped by incubating the samples at 85°C for 5 minutes, and 40 µl of nuclease-free water

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was added. Real-time PCR was performed by using the Brilliant® SYBR® Green QPCR Master Mix. The PCR template source was either 30 ng first-strand complementary DNA (cDNA) or purified DNA standard. Primer sequences used to amplify the desired cDNA are detailed in Table I. Amplification was performed with a spectrofluorometric thermal cycler (Stratagene, Cedar Creek, TX, USA). After an initial denaturation step at 95°C for 10 min, amplification was performed using 40 cycles of denaturation (95°C for 30 seconds), annealing (56°C for 1 minute) and extension (72°C for 1 minute). For each run, a standard curve was generated from purified DNA ranging from 10^6 to 10^{12} copies. To standardise mRNA levels, we amplified GAPDH, a housekeeping gene, as an internal control. Gene expression was normalized by calculating the ratio between the number of cDNA copies of collagen type I, type III and that of GAPDH. After amplification, a final melting curve was recorded by cooling the PCR mixture to 65°C for 30 seconds and then slowly heating it to 95°C at 30 seconds. Fluorescence was measured continuously during the slow temperature rise to monitor the dissociation of double stranded DNA. Specificity of the expected Lightcycler products was demonstrated by melting curve analysis. Amplification products formed in the Light Cycler were checked by electrophoresis on 1.5% ethidium bromide stained agarose gel. The estimated size of the amplified fragments matched the calculated size.

Western blot analysis

Cells were rinsed with ice-cold PBS, and total cell protein extracts were prepared using a cell lysis buffer (Cell Signaling Technology™, Danvers, MA, USA) containing 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1% Protease Inhibitor Cocktail (Sigma, St. Louis, MO, USA). Protein concentrations were measured by a BCA assay (PIERCE, Rockford, IL, USA). Ten micrograms protein extracts were boiled in Laemmli sample buffer and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were transferred to a nitrocellulose membrane (0.2 μm, Bio-Rad, Hercules, CA, USA) using a Bio-Rad gel-blotting apparatus. Membranes were stained with Ponceau red to confirm bands existed on the

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membranes, followed by blocking of the membranes in 5% non-fat milk in TBST (10 mM Tris HCl [pH 7.5], 150 mM NaCl, 0.1% Tween 20) for 1 hour. Blots were probed with monoclonal antibodies directed against mouse collagen type I (Chemicon, Temecula, CA, USA), type III (Abcam, Cambridge, MA, USA) and phosphorylated and total src (P-Y(416), activated src, Upstate, Charlottesville, VA, USA) diluted 1:500 with 0.05% TTBS or polyclonal antibodies directed against rabbit total erk1 (Chemicon, Temecula, CA, USA), total erk2 (Upstate, Charlottesville, VA, USA), phosphorylated erk1/2 and phosphorylated and total p38 MAPK (Biosource, Camarillo, CA, USA) diluted 1:1000, and incubated overnight at 4°C. After 3 × 10 min wash, blots were incubated for 1 hour at room temperature with alkaline phosphatase-conjugated anti-mouse IgG (Amersham, GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) or alkaline phosphatase-conjugated anti-rabbit IgG secondary antibody (Sigma, St. Louis, MO, USA) diluted 1:1000 with TBST for 1 hour at room temperature. After each incubation step, membranes were washed in TBST for 20 minutes. Proteins of interest were visualized by ECF substrate (ECF; Amersham, Braunschweig, Germany) and scanned on fluorescent scanning instrument (Storm 860 Molecular Imager, GMI, Ramsey, Minnesota) for appropriate times.

RNA interference

siRNA sequences against src, erk1, erk2 and p38 were designed using criteria previously described by Elbashir et al.(Elbashir et al., 2002) and the sequences are shown in table 2. Parallel Scrambled siRNAs as the negative control were also designed differing from the siRNAs by 3 nucleotides. These sequences did not match human genes as checked via the NCBI standard nucleotide-nucleotide BLAST program. Sequences were purchased from Sigma (St. Louis, MO, USA). Transfection of siRNAs into LX2 cells was performed 24 hours after plating. Briefly, LX-2 cells were seeded at a density of 1×10^7 cells/100mm Petri dishes the day before transfection to achieve 50-60% confluence. Transfections were carried out with 50 nmol of siRNA duplex using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the instructions of the manufacturer. After 6 hours incubation, the transfection

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medium was removed and cells were changed to serum-free medium. After 48 hours, transfected cells were treated with stimuli or medium for 24 hours followed by lysis, harvest of proteins Western blot analysis. For real time PCR, stimulation by CGS21680 for 12 hours was carried out 60 hours after transfection and then cells were collected for extraction of total RNA.

Statistical Analysis

All experiments were performed at least 3 times and results were analyzed by analysis of variance (ANOVA) with repeated measures and post-hoc analysis using Bonferroni correction.

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RESULTS

Proliferation of LX-2 cells was not regulated by adenosine A_{2A} receptor agonists and antagonists

To exclude the effect of adenosine agonists and antagonists on cell viability, the morphology of LX2 cells as well as the proliferation of LX-2 cells was examined after 24 hour treatment by microscope and CellTiter 96 proliferation assay, respectively. Following treatment with CGS21680 (1nM-1μM) and ZM241385 (1nM-1μM), the morphology of the LX2 cells appeared similar to that of the cells without the treatment (data not shown). Similarly, proliferation of LX2 cells was unaffected by either the adenosine A_{2A} receptor agonist or antagonist at these concentrations (data not shown).

Adenosine A_{2A} receptor occupancy increases total soluble collagen production by LX-2 cells

In our initial studies we assessed the effect of varying doses of CGS21680 on collagen production by LX-2 cells and compared this to a known stimulus for collagen production, TGFβ. The adenosine A_{2A} receptor agonist CGS21680 significantly increased both soluble and cell-associated collagen production by as much as 2-fold in a dose-dependent fashion (EC_{50}=79nM for supernatant total soluble collagen production; EC_{50}=82nM for cell-associated total soluble collagen production). The magnitude of the CGS21680-induced increase was similar to that induced by TGFβ (Figure 1). The A_{2A} receptor antagonist ZM241385 completely abrogated the effect of CGS21680 on collagen production (Figure 2). These results are consistent with the hypothesis that adenosine A_{2A} receptors, when occupied, promote collagen production.

Adenosine A_{2A} receptors regulate collagen production by primary human hepatic stellate cells

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Because LX-2 cells are an immortal cell line and may differ from primary hepatic stellate cells we determined the effect of adenosine A_{2A} receptor agonists and antagonists on collagen production by primary cells. As shown in Figure 3, the effect of CGS21680 and ZM21385 on collagen production was nearly identical in the LX-2 cells and primary hepatic stellate cells.

Effect of CGS24680 and ZM241385 on precollagen αI and precollagen αIII mRNA expression

In response to liver injury, HSCs undergo an activation process in which they proliferate and synthesize a fibrotic matrix rich in collagens type I and type III. We therefore determined whether adenosine A_{2A} receptor occupancy stimulates production of only one or both types of collagen. As shown in Figure 4, CGS21680 stimulated an increase in expression of precollagen αI mRNA by as much as 70% over baseline ($P<0.01$), an increase that was blocked by the A_{2A} antagonist ZM241385 (figure 4A). Similarly, CGS21680 also increased precollagen αIII mRNA by as much as 44% ($P<0.01$) and ZM241385 abrogated the CGS21680-induced increase in precollagen αIII mRNA (Figure 4B, $P<0.01$). As with total soluble collagen production, TGF-β stimulated an increase in precollagen αI and precollagen αIII mRNA similar to that stimulated by CGS21680 (Figure 5). CGS21680 increased collagen type I and type III protein expression and, as with mRNA, ZM241385 prevented the adenosine A_{2A} receptor agonist from stimulating an increase in collagens type I and type III levels (figure 5).

Involvement of protein kinases in CGS21680-stimulated soluble collagen production

To better understand how adenosine A_{2A} receptor activation regulates collagen production we examined the signaling mechanisms downstream of receptor activation that might affect collagen production using a series of kinase inhibitors. Surprisingly, the inhibitors of PKA (PKAI), src kinase (srcl), MEK/erk (UO126 and PD98059)and p38 MAPK (SB202190) inhibited the adenosine A_{2A} receptor-mediated increase in supernatant and cell-associated total soluble collagen production (figure 6A). Because a number of signaling mechanisms

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were implicated in these studies we further dissected the effect of protein kinase inhibitors on precollagen αI and precollagen αIII mRNA expression by real-time RT-PCR. Treatment of the cells with PKAI, srcI, U0126 and PD98059 significantly attenuated precollagen αI mRNA up-regulation by CGS21680 but SB202190 did not. In contrast, only SB202190 completely abolished CGS21680-induced precollagen αIII mRNA expression (figure 6B). In a similar fashion, inhibition of PKA, src kinase and MEK/erk MAPK but not p38 MAPK reversed the effect of adenosine A_{2A} receptor occupancy on collagen type I but not collagen type III protein expression (figure 6 C-1) and inhibition of p38 MAPK reversed the effect of receptor occupancy on collagen type III but not collagen type I (figure 6 C-2). These findings suggest that adenosine A_{2A} receptors regulate both collagen type I and type III production but do so by different signaling mechanisms.

Adenosine A_{2A} receptor activation stimulates signaling events by distinct pathways

Because inhibitors of protein kinases may have other effects on cellular function we determined whether the agents studied modulated the signaling events which appeared to be required for adenosine A_{2A} receptors to stimulate collagen production. CGS21680 stimulated the phosphorylation of erk1/2 and src and these signaling events were inhibited by PKA, src kinase and MEK/erk MAPK but not p38 MAPK inhibitors, findings that parallel the effects of these agents on collagen I synthesis (Figure 6). Similarly, CGS21680 stimulates erk1/2 phosphorylation which is blocked by inhibitors of PKA, src kinase and erk1/2. In contrast the p38MAPK inhibitor did not interfere with any of these signaling events. The adenosine A_{2A} receptor agonist also stimulated the phosphorylation of p38MAPK which was blocked by SB20190 but not by the inhibitors of PKA, src and erk1,2 kinase (Figure 7). Inhibitors of p38MAPK and erk1/2 activation did not prevent phosphorylation of src although a src kinase inhibitor blocked activation of src by adenosine A_{2A} receptor occupancy (Figure 7) and forskolin, a direct activator of adenylate cyclase, stimulates phosphorylation of erk1/2 and src (Supplemental figures 1 and 2), suggesting that the sequence

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of signaling events is cAMP-PKA-src-erk1/2. Again, these observations on the events of signal transduction are consistent with the functional effects of adenosine A_{2A} receptors on collagen type I and type III production.

siRNA-mediated knockdown of src, erk1, erk2 and p38 decreases CGS21680-stimulated collagen type I and type III

To confirm that the signaling events indicated by the pharmacologic inhibitors were indeed responsible for adenosine A_{2A} receptor-mediated stimulation of collagens I and III we diminished expression of src, erk1, erk2 and p38MAPK by use of siRNA. Appropriately sequenced siRNA diminished expression of the corresponding kinases (Figure 8) and reversed the CGS21680-mediated increase in phosphorylated src (figure 8A), erk1 (figure 8B), erk2 (figure 8C) and p38 MAPK (figure 8D). More importantly, knockdown of src and erk1 significantly diminished precollagen αI mRNA and reversed the CGS21680-mediated increase in precollagen αI (figure 9A, 9B). Knockdown of erk2 also diminished precollagen αI mRNA and reversed CGS21680-mediated increase in precollagen αI mRNA but the differences observed did not achieve statistical significance (figure 9C). Knockdown of both erk1 and erk2 by siRNA led to greater reversal of the effect of CGS21680 on the expression of precollagen than knockdown of either kinase alone (figure 9D). Knockdown of p38 MAPK diminished precollagen αIII mRNA and reversed CGS21680-mediated precollagen αIII mRNA (figure 9E). hese results parallel and confirm the effects of the pharmacologic inhibitors on adenosine A_{2A} receptor signaling events involved in promoting collagen production.

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DISCUSSION

We report here evidence that adenosine A_{2A} receptor occupancy directly stimulates collagen production by both primary human hepatic stellate cells and the human hepatic stellate cell line LX-2. More strikingly we have observed that adenosine A_{2A} receptors stimulate both collagen type I and type III expression but by distinct signaling pathways; via PKA-src-erk1/2 for collagen type I and via p38 MAPK for collagen type III.

One important limitation in studying hepatic stellate cell function and the regulation of this function by receptor ligands or other agents has been the difficulty in obtaining these cells in a consistent state of activation. To obviate this problem Xu and colleagues established the human hepatic cell line LX-2 and extensively characterized them, demonstrating that they retain the key features of stellate cells including cytokine signalling, neuronal gene expression, retinoid metabolism, and fibrogenesis, making them highly suitable for culture based studies of human hepatic fibrosis as a valuable tool(Xu et al., 2005). Because the effects of adenosine A_{2A} receptor occupation on collagen production were identical in primary hepatic cells and cultured LX-2 cells we concluded that LX-2 cells were an excellent model for studying signaling at adenosine A_{2A} receptors leading to collagen production by hepatic stellate cells.

Topical application of adenosine A_{2A} receptor agonists have previously been shown to promote wound healing in part by stimulating production of matrix in the wound. More recent results indicate that adenosine A_{2A} receptors stimulate collagen type I and type III production by primary human dermal fibroblasts as well and that this stimulation plays a role in dermal fibrosis in response to bleomycin treatment(Chan et al., 2006a). These findings are consistent with those reported here and previously(Chan et al., 2006b).

Classical signal transduction at adenosine A_{2A} receptors involves coupling of the receptor to Gsα signaling proteins to activate adenylate cyclase, thereby elevating the level of cAMP and activating PKA in response to ligand binding (Klinger et al., 2002a). It has also been reported that activation of adenosine A_{2A} receptor increases the activity of erk1/2 MAPK (Arslan and Fredholm, 2000;

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Chern et al., 1995; Schulte and Fredholm, 2003; Seidel et al., 1999). In probing signal transduction at adenosine A_{2A} receptors we used a series of kinase inhibitors previously shown to disrupt signal transduction via inhibition of protein phosphorylation of appropriate substrates. The results indicate that in LX-2 cells adenosine A_{2A} receptor-stimulated of increased production of collagen type I proceeds via cAMP-dependent PKA-mediated activation of src kinase and erk1/2 MAPK. Further evidence for involvement of this pathway in signal transduction at adenosine A_{2A} receptors is provided by the demonstration that adenosine A_{2A} receptor occupation leads to phosphorylation (activation) of erk1/2. Previous studies demonstrate that the well-characterized cAMP/PKA and MAPK pathways exhibit cross-talk (Stork and Schmitt, 2002) although, in general, emphasis has been placed on the ability of PKA-dependent phosphorylation to disrupt the interaction between $p21^{ras}$ and c-RAF, which results in cAMP-mediated suppression of the MAPK pathway (Burgering et al., 1993). Nonetheless, stimulation of $G_{\alpha S}$-coupled receptors may also result in both increased cellular cAMP and with stimulation of MAPK (Daaka et al., 1997; Keiper et al., 2004) as we have observed in LX-2 cells.

Prior experiments carried out in CHO cells indicate that adenosine A_{2A} receptors activate erk1/2 in a cAMP-dependent fashion that relies on src or a src-like kinase downstream of PKA for MAPK activation (Seidel et al., 1999) (Klinger et al., 2002b). This sequence of signaling events is also most consistent with the our results since forskolin, which directly stimulates cAMP production, treatment of the cells leads to phosphorylation of src and erk (Supplemental Figures 1 and 2) and treatment of LX-2 cells with the src inhibitor prevented phosphorylation of erk1/2 (Figure 7B) but inhibitors of erk activation did not affect src activation. Thus, the sequence of signaling most consistent with our results is cAMP-PKA-src-erk1/2.

We were surprised to find that adenosine A_{2A} receptor activation mediates an increase in collagen type III production expression via activation of p38 MAPK but independent of PKA, src, MEK and erk MAPK. There are few reports that link adenosine A_{2A} receptors to p38 MAPK and fewer clues as to which mediator

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is involved between receptors and p38 MAPK activation. A recent report (Rahman et al., 2004) demonstrated that cAMP inhibits p38 MAPK activation in human umbilical vein endothelial cells. In contrast, PKA was found to activate p38 MAPK in macrophages (Chio et al., 2004). Furthermore, the signaling pathways up- and downstream of p38 MAPK pathway are diverse which may explain why p38 MAPK pathway can be activated by various stimuli (Craxton et al., 1998; Han et al., 1994; Moriguchi et al., 1996; Pietersma et al., 1997; Wang et al., 1997).

We also demonstrated that RNAi-mediated knockdown of src, erk1, erk2 and erk1/2 was sufficient to inhibit baseline as well as CGS21680-stimulated precollagen αI mRNA expression. Also RNAi-mediated p38 knockdown significantly downregulated baseline and CGS21680-stimulated precollagen αIII mRNA expression. The results of the studies with siRNA-mediated knockdown of relevant signaling molecules were clearly consistent with the results with pharmacologic inhibitors of the same kinases providing a second level of confidence in the results.

Based on studies in knockout mice as well as pharmacologic evidence we have previously reported that endogenously released adenosine and adenosine A_{2A} receptors play a major role in the development of hepatic fibrosis in experimental murine models. These observations may shed particular light on alcohol-induced hepatic fibrosis/cirrhosis. Prior studies demonstrate that ethanol treatment increases extracellular adenosine by inhibiting adenosine uptake via the nucleoside transporter (Nagy et al., 1990). Acute treatment with ethanol increases extracellular adenosine concentrations *in vitro* (Nagy et al., 1989) which causes release of adenosine from rat cerebellar synaptosomes (Clark and Dar, 1989). Ethanol-induced accumulation of extracellular adenosine activates adenosine A_{2A} receptor to stimulate cAMP production(Nagy et al., 1989). Similarly, treatment of mice with hepatotoxins leads to increased adenosine release *ex vivo* (Chan et al., 2006b). Because ethanol promotes purine release in humans as well(Puig and Fox, 1984) the prior studies suggest that ethanol-induced increases in extracellular adenosine levels promote hepatic fibrosis by

stimulating enhanced production of collagen by otherwise activated hepatic stellate cells. Moreover, these observations suggest a mechanism for the observation that coffee-drinking protects against death from hepatic cirrhosis in that caffeine, contained in coffee in pharmacologically relevant concentrations, is a well-known adenosine receptor antagonist(Corrao et al., 1994; Corrao et al., 2001; Gallus et al., 2002; Klatsky et al., 1993; Ruhl and Everhart, 2005; Sharp et al., 1999).

We conclude that in hepatic stellate cells signaling for collagen production via adenosine A_{2A} receptors is complex. Although generally thought of as nearly exclusively signaling via G_s/cAMP/PKA and their related downstream signaling events these results indicate that stimulation of adenosine A_{2A} receptors activates p38 MAPK as well. Moreover, both of these signals contribute to stimulating expression of collagen by hepatic stellate cells. Although it is likely that other events also contribute, adenosine, released as a result of hepatic injury or exposure to agents like ethanol, contributes to the development of hepatic cirrhosis via stimulating hepatic stellate cell production of collagen.

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REFERENCES:

Arslan G and Fredholm BB (2000) Stimulatory and inhibitory effects of adenosine A(2A) receptors on nerve growth factor-induced phosphorylation of extracellular regulated kinases 1/2 in PC12 cells. *Neurosci Lett* **292**(3):183-186.

Bennett RG, Kharbanda KK and Tuma DJ (2003) Inhibition of markers of hepatic stellate cell activation by the hormone relaxin. *Biochem Pharmacol* **66**(5):867-874.

Burgering BM, Pronk GJ, van Weeren PC, Chardin P and Bos JL (1993) cAMP antagonizes p21ras-directed activation of extracellular signal-regulated kinase 2 and phosphorylation of mSos nucleotide exchange factor. *Embo J* **12**(11):4211-4220.

Chan ES, Fernandez P, Merchant AA, Montesinos MC, Trzaska S, Desai A, Tung CF, Khoa DN, Pillinger MH, Reiss AB, Tomic-Canic M, Chen JF, Schwarzschild MA and Cronstein BN (2006a) Adenosine A(2A) receptors in diffuse dermal fibrosis: Pathogenic role in human dermal fibroblasts and in a murine model of scleroderma. *Arthritis Rheum* **54**(8):2632-2642.

Chan ES, Montesinos MC, Fernandez P, Desai A, Delano DL, Yee H, Reiss AB, Pillinger MH, Chen JF, Schwarzschild MA, Friedman SL and Cronstein BN (2006b) Adenosine A(2A) receptors play a role in the pathogenesis of hepatic cirrhosis. *Br J Pharmacol*.

Chern Y, Chiou JY, Lai HL and Tsai MH (1995) Regulation of adenylyl cyclase type VI activity during desensitization of the A2a adenosine receptor-mediated cyclic AMP response: role for protein phosphatase 2A. *Mol Pharmacol* **48**(1):1-8.

Chio CC, Chang YH, Hsu YW, Chi KH and Lin WW (2004) PKA-dependent activation of PKC, p38 MAPK and IKK in macrophage: implication in the induction of inducible nitric oxide synthase and interleukin-6 by dibutyryl cAMP. *Cell Signal* **16**(5):565-575.

Clark M and Dar MS (1989) Effect of acute ethanol on release of endogenous adenosine from rat cerebellar synaptosomes. *J Neurochem* **52**(6):1859-1865.

Corrao G, Lepore AR, Torchio P, Valenti M, Galatola G, D'Amicis A, Arico S and di Orio F (1994) The effect of drinking coffee and smoking cigarettes on the risk of cirrhosis associated with alcohol consumption. A case-control study. Provincial Group for the Study of Chronic Liver Disease. *Eur J Epidemiol* **10**(6):657-664.

Corrao G, Zambon A, Bagnardi V, D'Amicis A and Klatsky A (2001) Coffee, caffeine, and the risk of liver cirrhosis. *Ann Epidemiol* **11**(7):458-465.

Craxton A, Shu G, Graves JD, Saklatvala J, Krebs EG and Clark EA (1998) p38 MAPK is required for CD40-induced gene expression and proliferation in B lymphocytes. *J Immunol* **161**(7):3225-3236.

Daaka Y, Luttrell LM and Lefkowitz RJ (1997) Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. *Nature* **390**(6655):88-91.

Elbashir SM, Harborth J, Weber K and Tuschl T (2002) Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods* **26**(2):199-213.

Freeman TL, Kharbanda KK, Tuma DJ and Mailliard ME (2002) Inhibition of hepatic stellate cell collagen synthesis by N-(methylamino)isobutyric acid. *Biochem Pharmacol* **63**(4):697-706.

Friedman SL (2000) Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. *J Biol Chem* **275**(4):2247-2250.

Gallus S, Tavani A, Negri E and La Vecchia C (2002) Does coffee protect against liver cirrhosis? *Ann Epidemiol* **12**(3):202-205.

Molecular Pharmacology Fast Forward. Published on September 14, 2007 as DOI: 10.1124/mol.107.038760
This article has not been copyedited and formatted. The final version may differ from this version.

Geerts A, Vrijsen R, Rauterberg J, Burt A, Schellinck P and Wisse E (1989) In vitro differentiation of fat-storing cells parallels marked increase of collagen synthesis and secretion. *J Hepatol* **9**(1):59-68.

Han J, Lee JD, Bibbs L and Ulevitch RJ (1994) A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* **265**(5173):808-811.

Ikeda K, Wakahara T, Wang YQ, Kadoya H, Kawada N and Kaneda K (1999) In vitro migratory potential of rat quiescent hepatic stellate cells and its augmentation by cell activation. *Hepatology* **29**(6):1760-1767.

Jimenez W, Pares A, Caballeria J, Heredia D, Bruguera M, Torres M, Rojkind M and Rodes J (1985) Measurement of fibrosis in needle liver biopsies: evaluation of a colorimetric method. *Hepatology* **5**(5):815-818.

Keiper M, Stope MB, Szatkowski D, Bohm A, Tysack K, Vom Dorp F, Saur O, Oude Weernink PA, Evellin S, Jakobs KH and Schmidt M (2004) Epac- and Ca2+ -controlled activation of Ras and extracellular signal-regulated kinases by Gs-coupled receptors. *J Biol Chem* **279**(45):46497-46508.

Kemp BE, Pearson RB and House CM (1991) Pseudosubstrate-based peptide inhibitors. *Methods Enzymol* **201**:287-304.

Klatsky AL, Armstrong MA and Friedman GD (1993) Coffee, tea, and mortality. *Ann Epidemiol* **3**(4):375-381.

Klinger M, Freissmuth M and Nanoff C (2002a) Adenosine receptors: G protein-mediated signalling and the role of accessory proteins. *Cell Signal* **14**(2):99-108.

Klinger M, Kudlacek O, Seidel MG, Freissmuth M and Sexl V (2002b) MAP kinase stimulation by cAMP does not require RAP1 but SRC family kinases. *J Biol Chem* **277**(36):32490-32497.

Lee FS and Chao MV (2001) Activation of Trk neurotrophin receptors in the absence of neurotrophins. *Proc Natl Acad Sci U S A* **98**(6):3555-3560.

Li D and Friedman SL (1999) Liver fibrogenesis and the role of hepatic stellate cells: new insights and prospects for therapy. *J Gastroenterol Hepatol* **14**(7):618-633.

Lopez-De Leon A and Rojkind M (1985) A simple micromethod for collagen and total protein determination in formalin-fixed paraffin-embedded sections. *J Histochem Cytochem* **33**(8):737-743.

Marra F, Efsen E, Romanelli RG, Caligiuri A, Pastacaldi S, Batignani G, Bonacchi A, Caporale R, Laffi G, Pinzani M and Gentilini P (2000) Ligands of peroxisome proliferator-activated receptor gamma modulate profibrogenic and proinflammatory actions in hepatic stellate cells. *Gastroenterology* **119**(2):466-478.

Montesinos MC, Desai A, Chen JF, Yee H, Schwarzschild MA, Fink JS and Cronstein BN (2002) Adenosine promotes wound healing and mediates angiogenesis in response to tissue injury via occupancy of A(2A) receptors. *Am J Pathol* **160**(6):2009-2018.

Montesinos MC, Gadangi P, Longaker M, Sung J, Levine J, Nilsen D, Reibman J, Li M, Jiang CK, Hirschhorn R, Recht PA, Ostad E, Levin RI and Cronstein BN (1997) Wound healing is accelerated by agonists of adenosine A2 (G alpha s-linked) receptors. *J Exp Med* **186**(9):1615-1620.

Mori Y, Higuchi M, Masuyama N and Gotoh Y (2004) Adenosine A2A receptor facilitates calcium-dependent protein secretion through the activation of protein kinase A and phosphatidylinositol-3 kinase in PC12 cells. *Cell Struct Funct* **29**(4):101-110.

Moriguchi T, Kuroyanagi N, Yamaguchi K, Gotoh Y, Irie K, Kano T, Shirakabe K, Muro Y, Shibuya H, Matsumoto K, Nishida E and Hagiwara M (1996) A novel kinase cascade mediated by mitogen-activated protein kinase kinase 6 and MKK3. *J Biol Chem* **271**(23):13675-13679.

Nagy LE, Diamond I, Casso DJ, Franklin C and Gordon AS (1990) Ethanol increases extracellular adenosine by inhibiting adenosine uptake via the nucleoside transporter. *J Biol Chem* **265**(4):1946-1951.

Nagy LE, Diamond I, Collier K, Lopez L, Ullman B and Gordon AS (1989) Adenosine is required for ethanol-induced heterologous desensitization. *Mol Pharmacol* **36**(5):744-748.

Pietersma A, Tilly BC, Gaestel M, de Jong N, Lee JC, Koster JF and Sluiter W (1997) p38 mitogen activated protein kinase regulates endothelial VCAM-1 expression at the post-transcriptional level. *Biochem Biophys Res Commun* **230**(1):44-48.

Puig JG and Fox IH (1984) Ethanol-induced activation of adenine nucleotide turnover. Evidence for a role of acetate. *J Clin Invest* **74**(3):936-941.

Rahman A, Anwar KN, Minhajuddin M, Bijli KM, Javaid K, True AL and Malik AB (2004) cAMP targeting of p38 MAP kinase inhibits thrombin-induced NF-kappaB activation and ICAM-1 expression in endothelial cells. *Am J Physiol Lung Cell Mol Physiol* **287**(5):L1017-1024.

Rajagopal R and Chao MV (2006) A role for Fyn in Trk receptor transactivation by G-protein-coupled receptor signaling. *Mol Cell Neurosci*.

Rockey DC, Housset CN and Friedman SL (1993) Activation-dependent contractility of rat hepatic lipocytes in culture and in vivo. *J Clin Invest* **92**(4):1795-1804.

Ruhl CE and Everhart JE (2005) Coffee and tea consumption are associated with a lower incidence of chronic liver disease in the United States. *Gastroenterology* **129**(6):1928-1936.

Schulte G and Fredholm BB (2003) Signalling from adenosine receptors to mitogen-activated protein kinases. *Cell Signal* **15**(9):813-827.

Seidel MG, Klinger M, Freissmuth M and Holler C (1999) Activation of mitogen-activated protein kinase by the A(2A)-adenosine receptor via a rap1-dependent and via a p21(ras)-dependent pathway. *J Biol Chem* **274**(36):25833-25841.

Sharp DS, Everhart JE and Benowitz NL (1999) Coffee, alcohol, and the liver. *Ann Epidemiol* **9**(7):391-393.

Stork PJ and Schmitt JM (2002) Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. *Trends Cell Biol* **12**(6):258-266.

Victor-Vega C, Desai A, Montesinos MC and Cronstein BN (2002) Adenosine A2A receptor agonists promote more rapid wound healing than recombinant human platelet-derived growth factor (Becaplermin gel). *Inflammation* **26**(1):19-24.

Wang W, Zhou G, Hu MC, Yao Z and Tan TH (1997) Activation of the hematopoietic progenitor kinase-1 (HPK1)-dependent, stress-activated c-Jun N-terminal kinase (JNK) pathway by transforming growth factor beta (TGF-beta)-activated kinase (TAK1), a kinase mediator of TGF beta signal transduction. *J Biol Chem* **272**(36):22771-22775.

Xu L, Hui AY, Albanis E, Arthur MJ, O'Byrne SM, Blaner WS, Mukherjee P, Friedman SL and Eng FJ (2005) Human hepatic stellate cell lines, LX-1 and LX-2: new tools for analysis of hepatic fibrosis. *Gut* **54**(1):142-151.

Zolnai A, Toth EB, Wilson RA and Frenyo VL (1998) Comparison of 3H-thymidine incorporation and CellTiter 96 aqueous colorimetric assays in cell proliferation of bovine mononuclear cells. *Acta Vet Hung* **46**(2):191-197.

Molecular Pharmacology Fast Forward. Published on September 14, 2007 as DOI: 10.1124/mol.107.038760
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FOOTNOTES

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CONFLICT OF INTEREST: Dr. Che has nothing to disclose. Dr. Cronstein holds intellectual property (Patents on use of adenosine A_{2A} receptor agonists to promote wound healing and use of A_{2A} receptor antagonists to inhibit fibrosis. Patent on testing for Single nucleotide polymorphisms in the adenosine A_1 receptor in patients with fibromyalgia. Patent on use of adenosine A_1 receptor antagonists to treat osteoporosis and other diseases of bone). Dr. Cronstein has served as a consultant to the following companies: King Pharmaceutical (licensee of patents above), CanFite Biopharmaceuticals, Bristol-Myers Squibb, Cellzome, Tap Pharmaceuticals, Prometheus Laboratories, Regeneron (Westat, DSMB), Sepracor, Amgen, Endocyte. In addition, Dr. Cronstein has received honoraria for speaking from Tap Pharmaceuticals and Amgen. He holds stock in CanFite Biopharmaceuticals and receives research grants from King Pharmaceuticals.

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FIGURE LEGENDS

Figure 1. Determination of increased total soluble collagen production both in supernatant and LX-2 cells. LX-2 cells were treated with the indicated concentration of adenosine A_{2A} receptor agonist, CGS21680, as described, for 24 hours before measurement of collagen. CGS-21680 increased collagen production measured both in the cells and supernatant ($P < 0.01$, 1-way ANOVA). The cells were also treated with 40 ng/ml TGF-β as a positive control. A, CGS21680-induced collagen production in supernatant; B, CGS21680-induced collagen production in LX-2 cells.

* P<0.01 *vs* cells alone. *n*=3

Figure 2. ZM241385 inhibited CGS21680-induced total soluble collagen production. LX-2 cells were treated with TGF-β (40 ng/ml, positive control), 1 μM CGS21680, 1 μM ZM241385 or 1 μM CGS21680 plus 1 μM ZM241385 for 24 h before harvesting and measurement of collagen in supernatant and cells, as described. The data are presented as mean ± SD of triplicate cultures in four separate experiments.

*P<0.01 *vs* cells alone

**P<0.01 vs CGS21680 treated

Figure 3. Correspondence of collagen production between primary HSCs and LX-2 cells. Primary HSCs and LX-2 cells were treated with TGF-β (40 ng/ml, positive control), 1 μM CGS21680, 1 μM ZM241385 or 1 μM CGS21680 plus 1 μM ZM241385 for 24 h before harvesting and measurement of collagen production in supernatant and cells, as described. The CGS21680-induced increase in collagen production measured in primary cells did not differ from that observed in LX-2 cells. The data are presented as mean ± SD of triplicate cultures carried out in duplicate in six separate experiments. A, CGS21680-induced collagen production in supernatant; B, CGS21680-induced collagen production in LX-2 cells.

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Figure 4. Effect of CGS21680 and ZM241385 on the expression of procollagen αI and procollagen αIII mRNA in LX-2 cells. LX-2 cells were cultured in serum-free DMEM containing stellate cell growth supplement and treated with TGF-β, (40ng/ml), 1 μM CGS21680, 1 μM ZM241385 or 1 μM CGS21680 plus 1 μM ZM241385 for the indicated periods of time before the RNA was harvested. Following reverse transcription the number of copies of mRNA for the two transcripts was determined by real time PCR. Data were normalized to GAPDH mRNA and are expressed as the mean ± SD (*n* = 4). A. procollagen αI mRNA; B. procollagen αIII mRNA.
**P* < 0.01, ***P* < 0.05, vs cells alone.
† P<0.01, †† P<0.05 vs CGS21680 treated

Figure 5. Western blot analyses of LX-2 cell lysates for collagen type I and type III. LX-2 cells were treated with 40 ng/ml TGF-β, 1 μM CGS21680, 1 μM ZM241385 or 1 μM CGS21680 plus 1 μM ZM241385 for 24 h before the proteins were harvested. Lysates were subject to SDS-PAGE and immunoblotting. Data are expressed as the mean ± SD (*n* = 3). A. collagen type I; B. collagen type III.
**P* < 0.01 vs cells alone. ***P* < 0.01 vs CGS21680 treated.

Figure 6. Effects of PKI, srcI, U0126, PD98059 and SB202198 on CGS21680-stimulated collagen expression in LX-2 cells. LX-2 cells were treated with 1 μM CGS21680 alone, or in combination with 1 μM PKI, 1 μM srcI, 1 μM U0126, 1 μM PD98059 or SB202190 respectively for 24 h. Collagen production, precollagen αI and precollagen αIII mRNA and collagen type I and type III were measured as described. Data are expressed as the mean ± SD. A. collagen production; B. precollagen and collagen type I (C-1), collagen type III (C-2).
**P* < 0.01, **P<0.05 vs cells alone.
† P<0.01, †† P<0.05 vs CGS21680 treated

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Figure 7. Effects of PKI, srcI, U0126, PD98059 and SB202198 on the phosphorylation of src, erk1/2 and P38 MAPK in LX-2 cells treated with CGS21680. LX-2 cells were treated with 1 μM CGS21680 alone, or in combination with 1μM PKI, 1μM srcI, μM U0126, 1μM PD98059 or SB202190 for 10 min. Phosphorylated (tyr416) and total src, erk1/2 and p-38 MAPK were detected by immunoblot of lysates, as described. Data are expressed as the mean ± SD (*n* = 3). A. src kinase; B. erk1/2 MAPK; C. p-38 MAPK.

**P* < 0.01 vs cells alone.

† P<0.01 vs CGS21680 treated.

Figure 8. siRNA-mediated knockdown of src, erk1, erk2 and p38 decreased total and phosphoryled src, erk1, erk2 and p38 expression in non- and CGS21680-stimulate LX2 cells. LX2 cells were transiently transfected with src, erk1, erk2 and p38 siRNAs (50 nM) or scrambled siRNAs for 48 hours and then treated with or without CGS21680 (1 μM) for 24 h. siRNA significantly reduced the level of corresponding total and phosphorylated src, erk1, erk2 and p38 proteins. Results are expressed as comparisons between siRNA transfected cells and wild type or cells transfected with corresponded scrambled siRNAs (n=3 separate experiments). A. src siRNA transfected; B. erk1 siRNA transfected; C. erk2 siRNA transfected; D. p38 siRNA transfected.

***P* < 0.05. *P<0.01.

Figure 9. siRNA-mediated knockdown of src, erk1, erk2 and p38 downregulated the mRNA levels of precollagen αI or precollagen αIII in LX-2 cells. LX-2 cells were transiently transfected with src, erk1, erk2, erk1/2 and p38 siRNAs (50 nM) or scrambled siRNAs (50 nM) individually. At 60 hour after transfection cells were treated with or without CGS21680 (1 μM) for 12 hours. Total RNA was extracted, reverse transcribed and real time PCR was performed to determine precollagen αI and precollagen αIII mRNA levels. src (A), erk1 (B),

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erk2 (C) and erk1/2 (D) siRNAs significantly reduced the expression of both basal and CGS21680-stimulated precollagen αI mRNA. In contrast, p38 siRNA significantly reduced the expression of precollagen αIII mRNA (E), Results are expressed as comparisons between siRNA transfected cells and wild type or corresponded scrambled siRNAs and represent the means (± SD) of three separate determinations.

** P <0.05; * P < 0.01

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Table 1. Sequences of primers for the real-time RT-PCR assays used in this study

	Forward primer 5′-3′	Reverse primer 5-3′	amplicon size (bp)
precollagen αI	TGTTCAGCTTTGTGGACCT	CCGTTCTGTACGCAGGTGAT	130
precollagen αIII	GAAGATGTCCTTGATGTGC	AGCCTTGCGTGTTCGATATT	260
GAPDH	GCTGCCCAGAACATCATCC	GTCAGATCCACGACGGACAC	134

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Table 2. sequences of siRNA of src, erk1, erk2 and p38 used in this study

		sense sequence 5'-3'	antisense sequence 5'-3'
	1	GGACCAUGGGUAGCAACAA	UUGUUGCUACCCAUGGUCG
src	2	CCUUCCUGGAGGACUACUU	AAGUAGUCCUCCAGGAAGG
	3	GCAUUCGAGAUGGCAGAUU	AAUCUGCCAUCUCGAAUGC
	1	GCAGCUGAGCAAUGACCAU	AUGGUCAUUGCUCAGCUGC
erk1	2	GCUGAACUCCAAGGGCUAU	AUAGCCCUUGGAGUUCAGC
	3	CCAUAUCUGGAGCAGUAUU	AAUACUGCUCCAGAUAUGG
	1	GGACCUCAUGGAAACAGAU	AUCUGUUUCCAUGAGGUCC
erk2	2	GCUGCAUUCUGGCAGAAAU	AUUUCUGCCAGAAUGCAGC
	3	CCAUAUCUGGAGCAGUAUU	AAUACUGCUCCAGAUAUGG
	1	CCAGACCAUUUCAGUCCAU	AUGGACUGAAAUGGUCUGG
p38	2	GAAGCUUACAGAUGACCAU	AUGGUCAUCUGUAAGCUUC
	3	GGCACAUAGUAGAGACAAU	AUUGUCUCUACUAUGUGCC

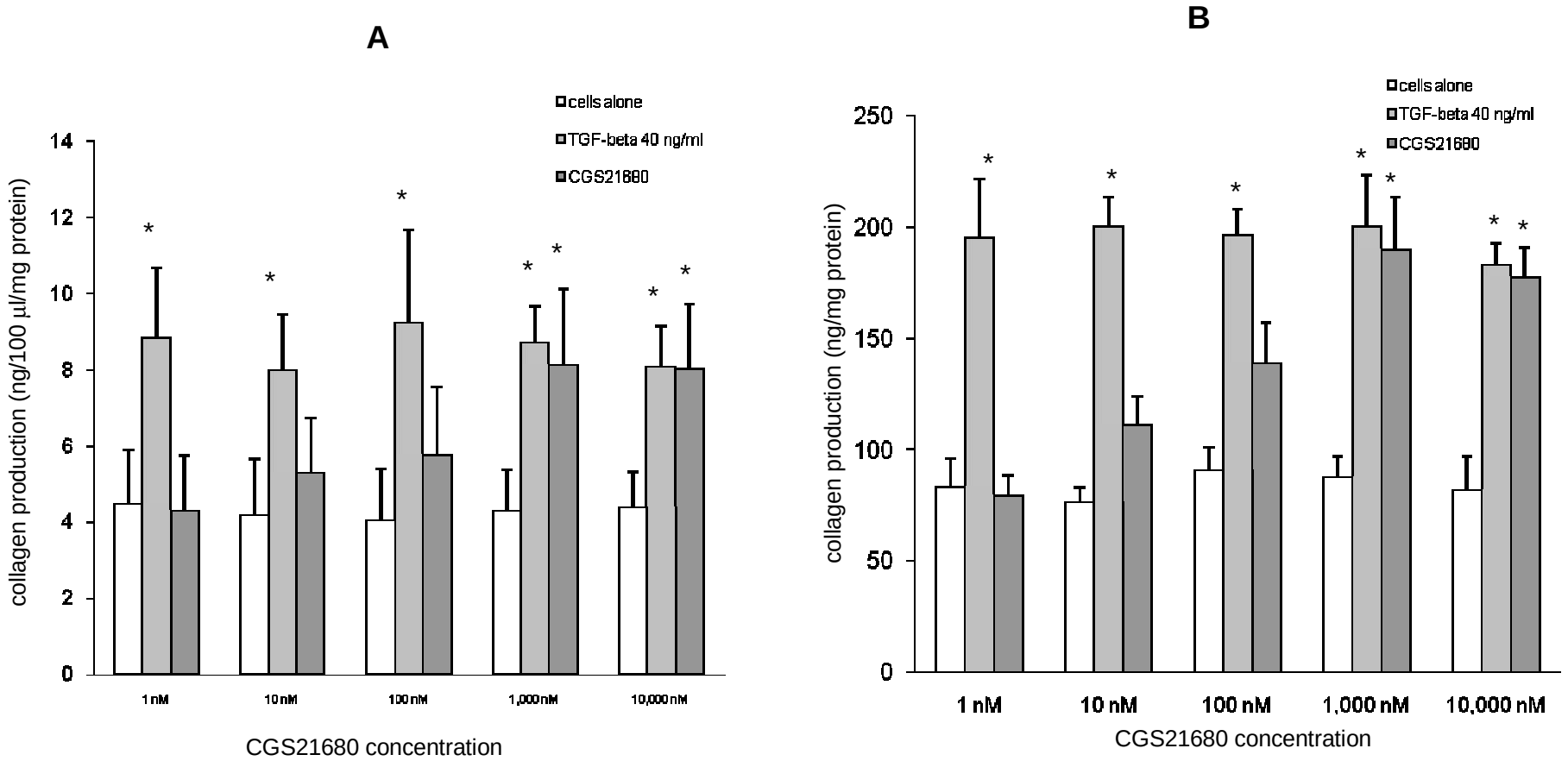


Figure 1

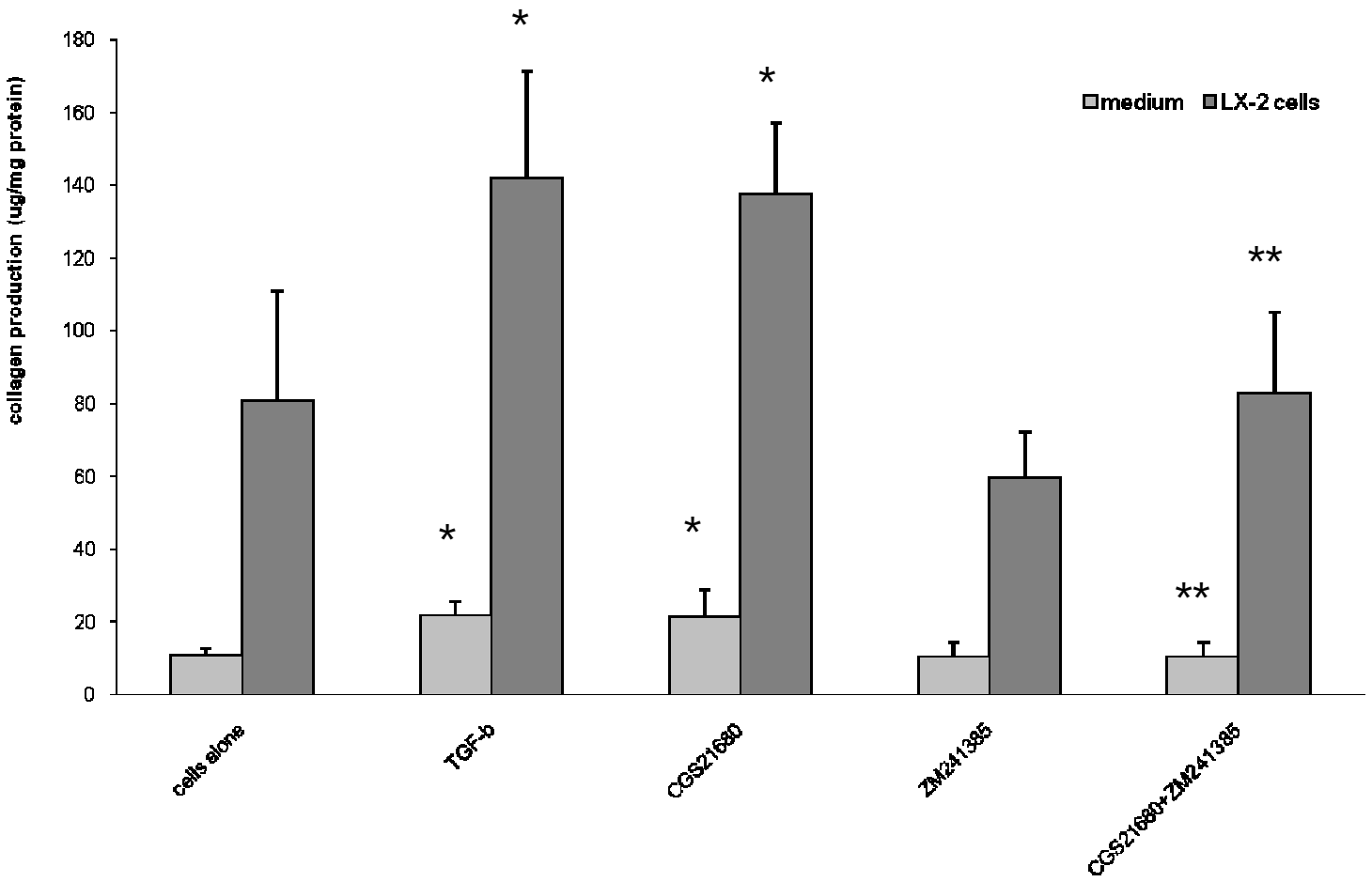
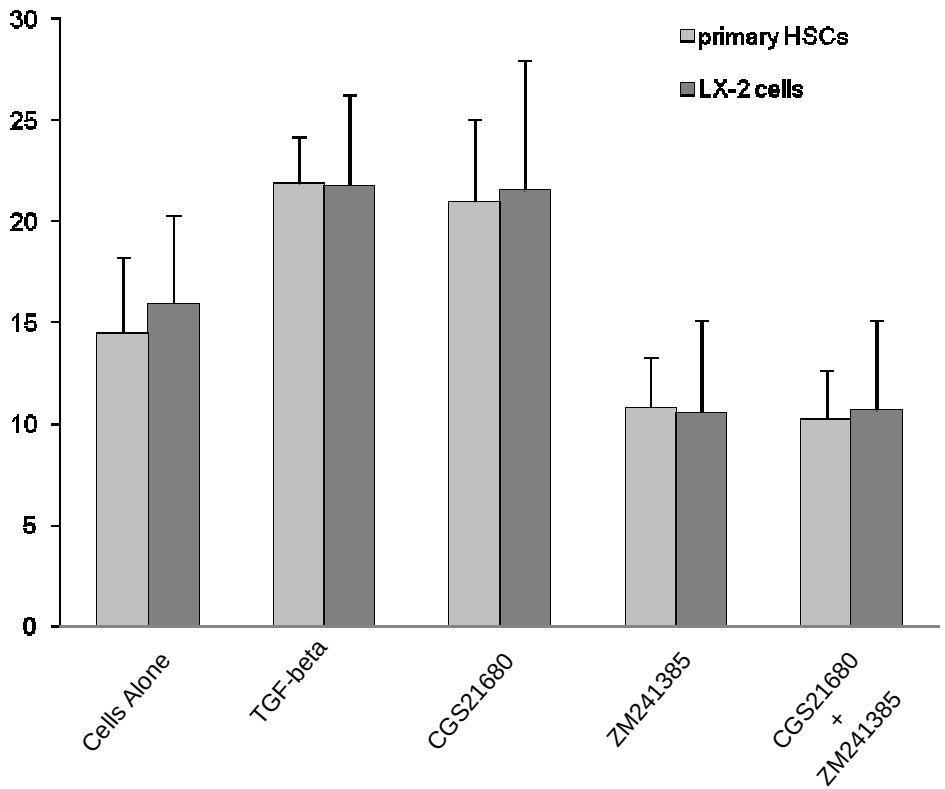


Figure 2



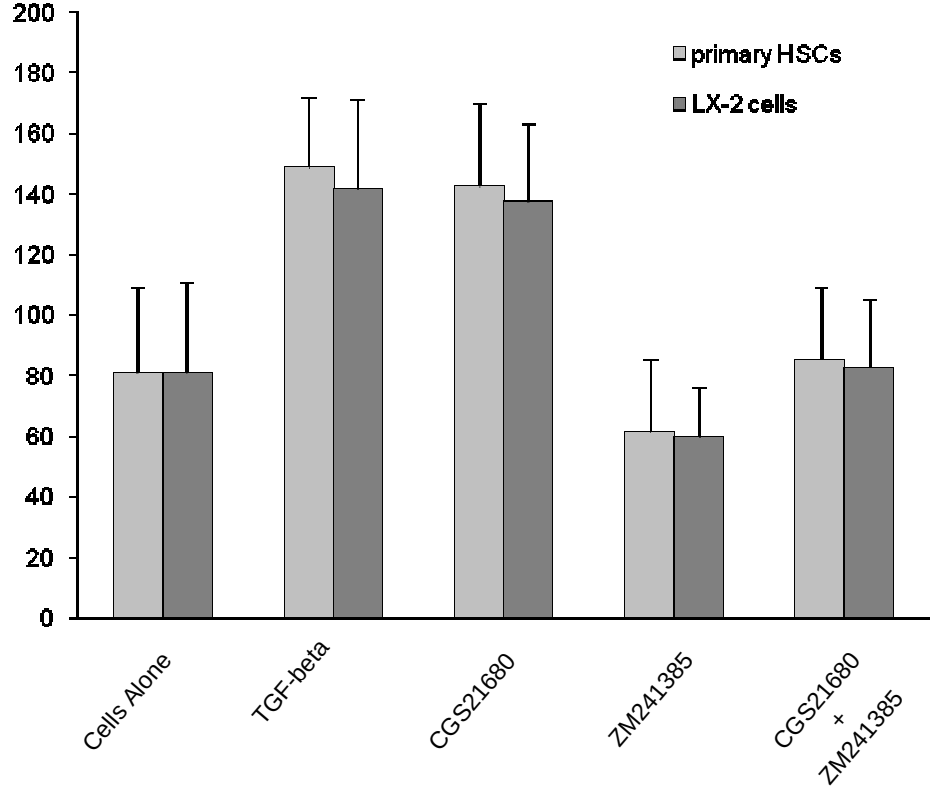


Figure 3

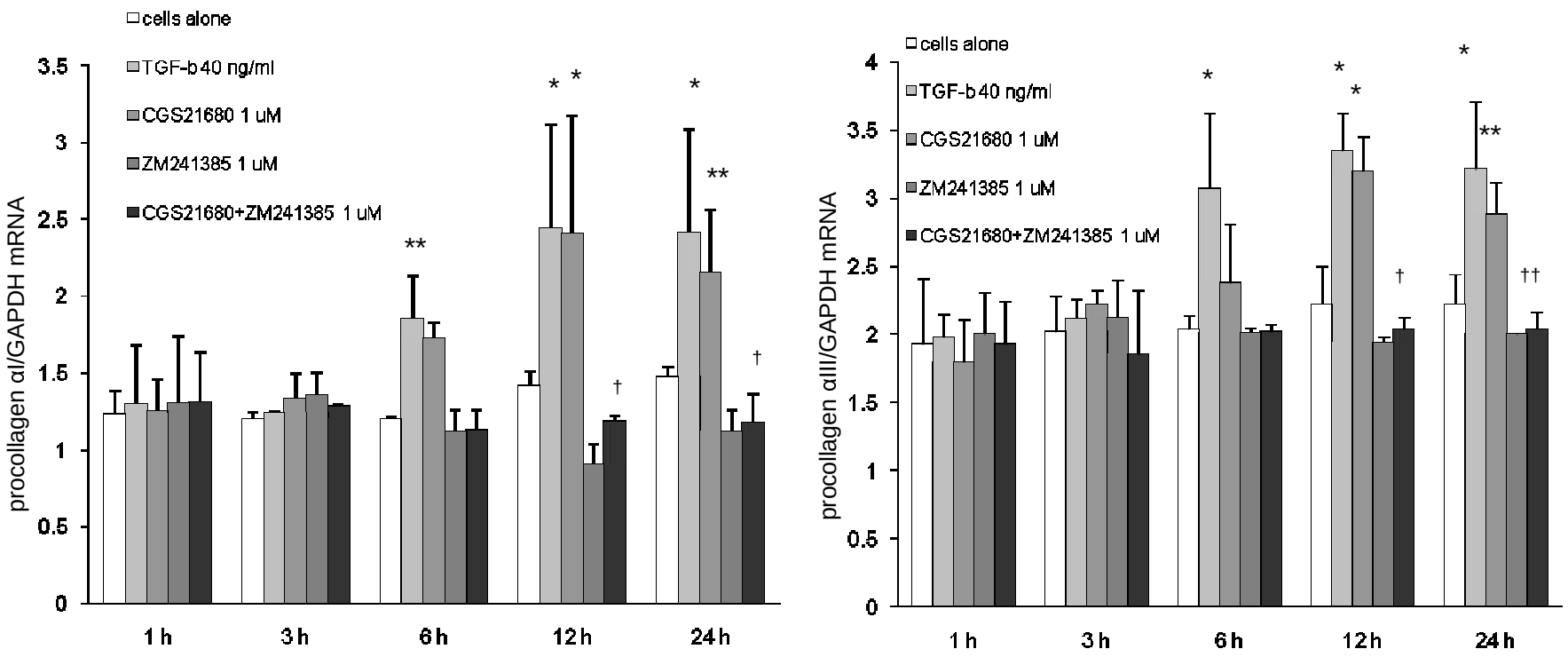


Figure 4

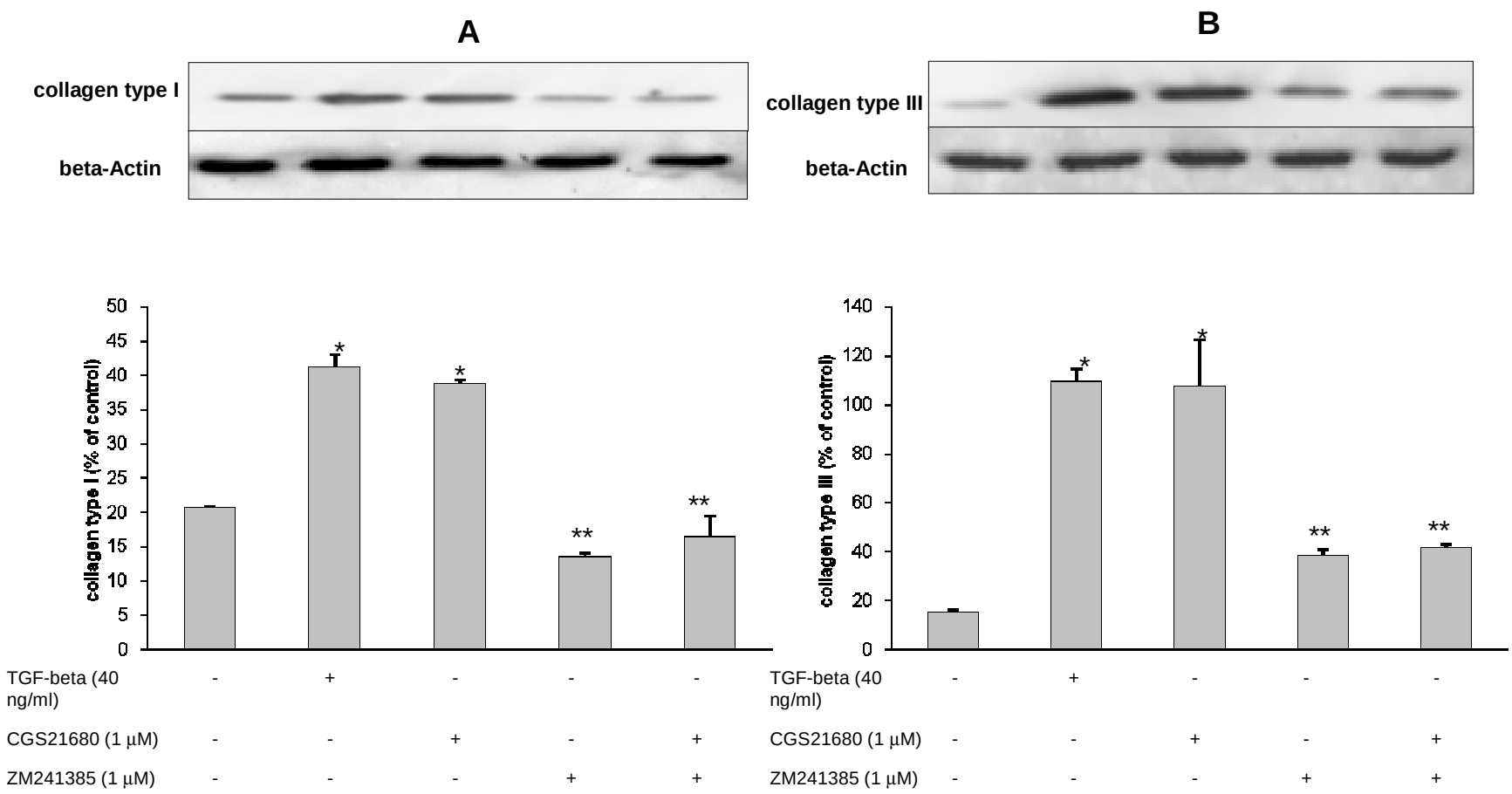


Figure 5

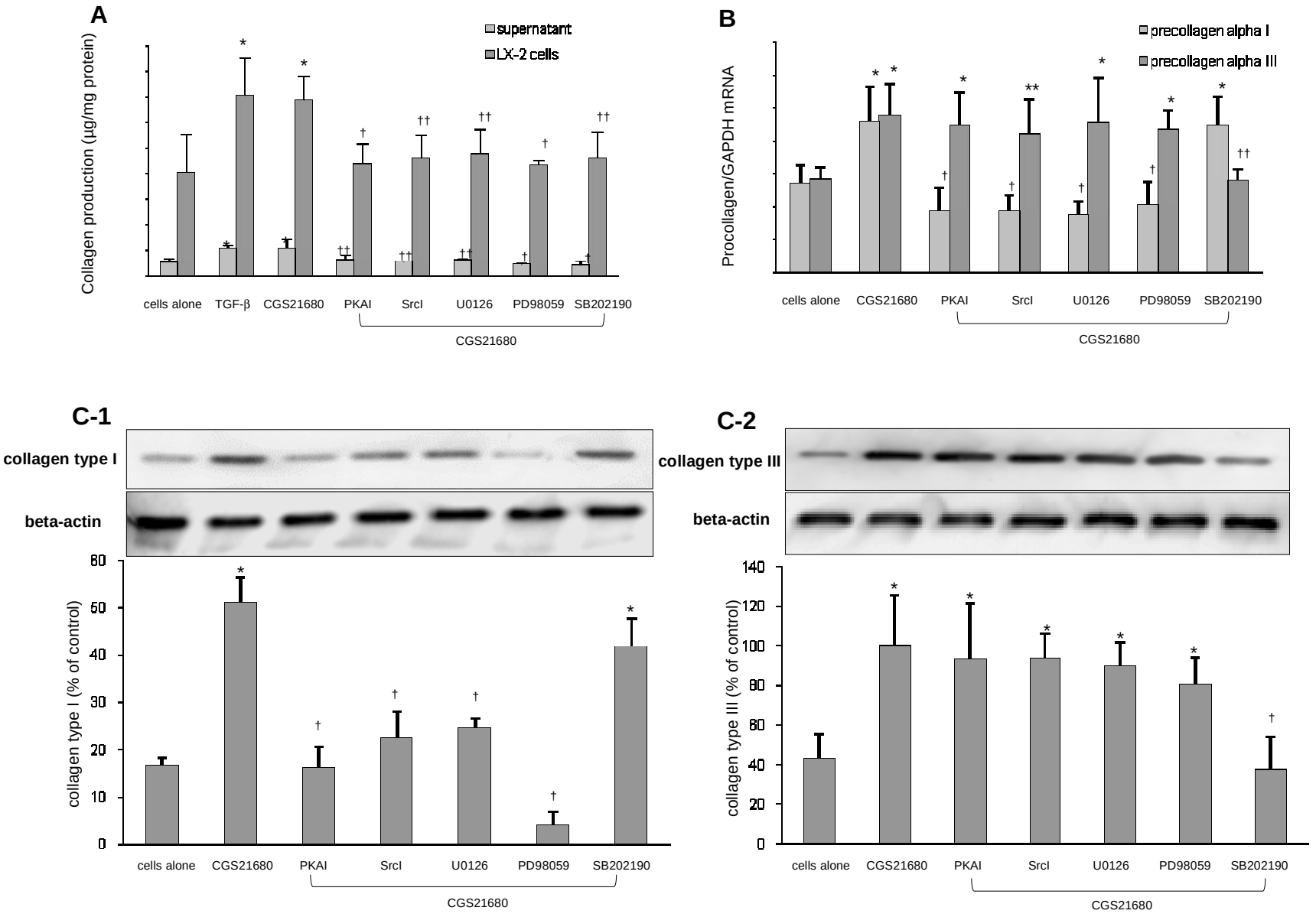


Figure 6

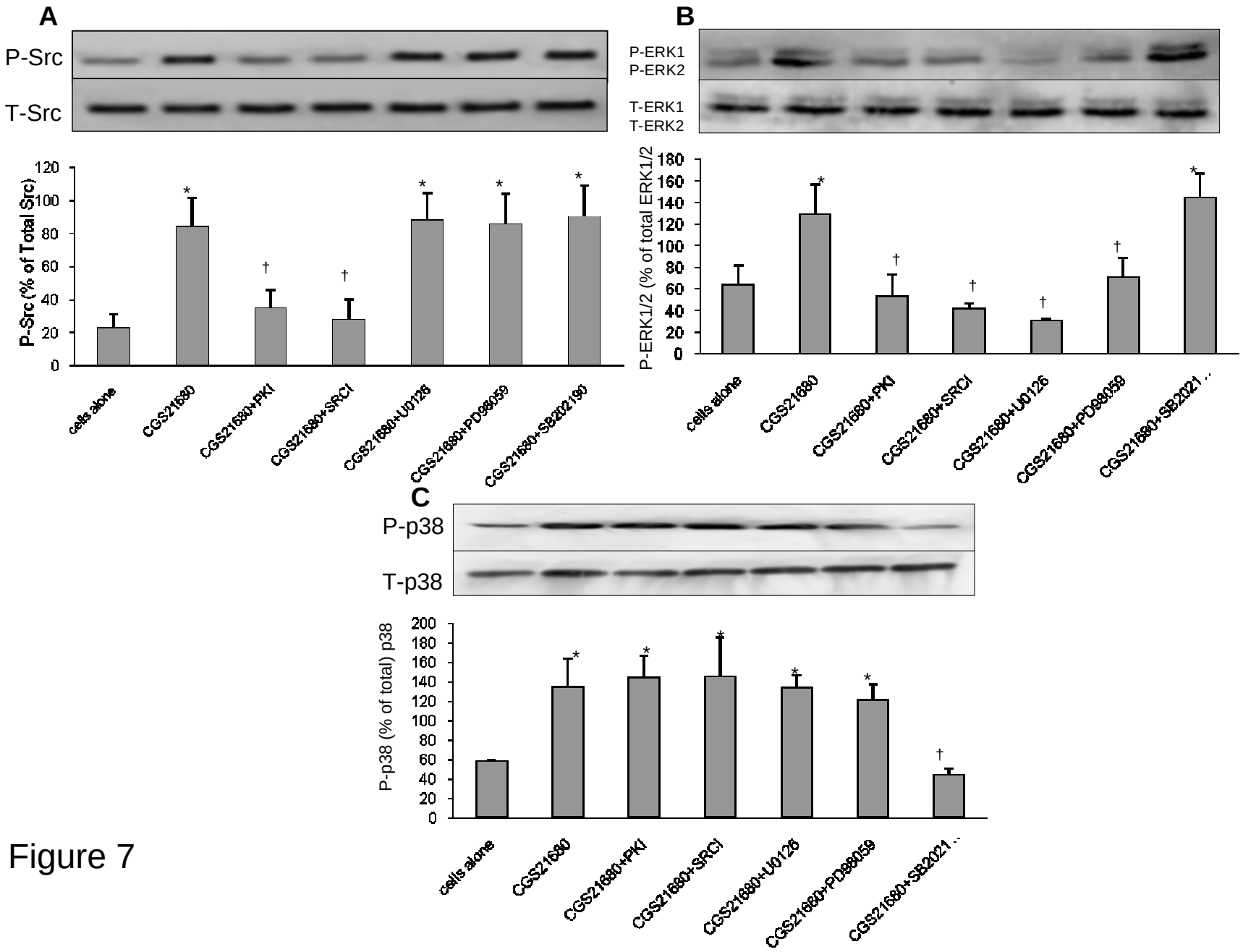


Figure 7

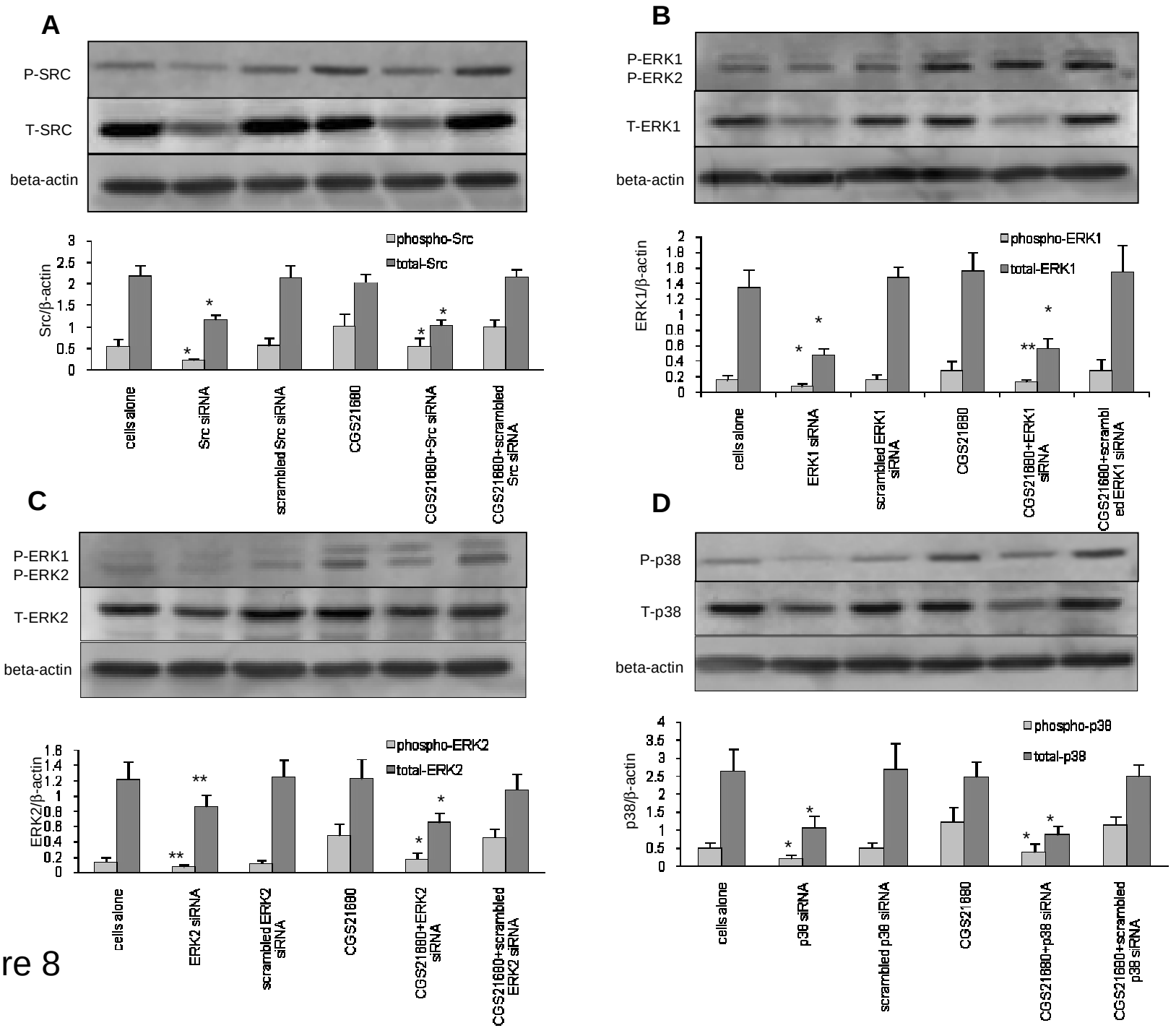


Figure 8

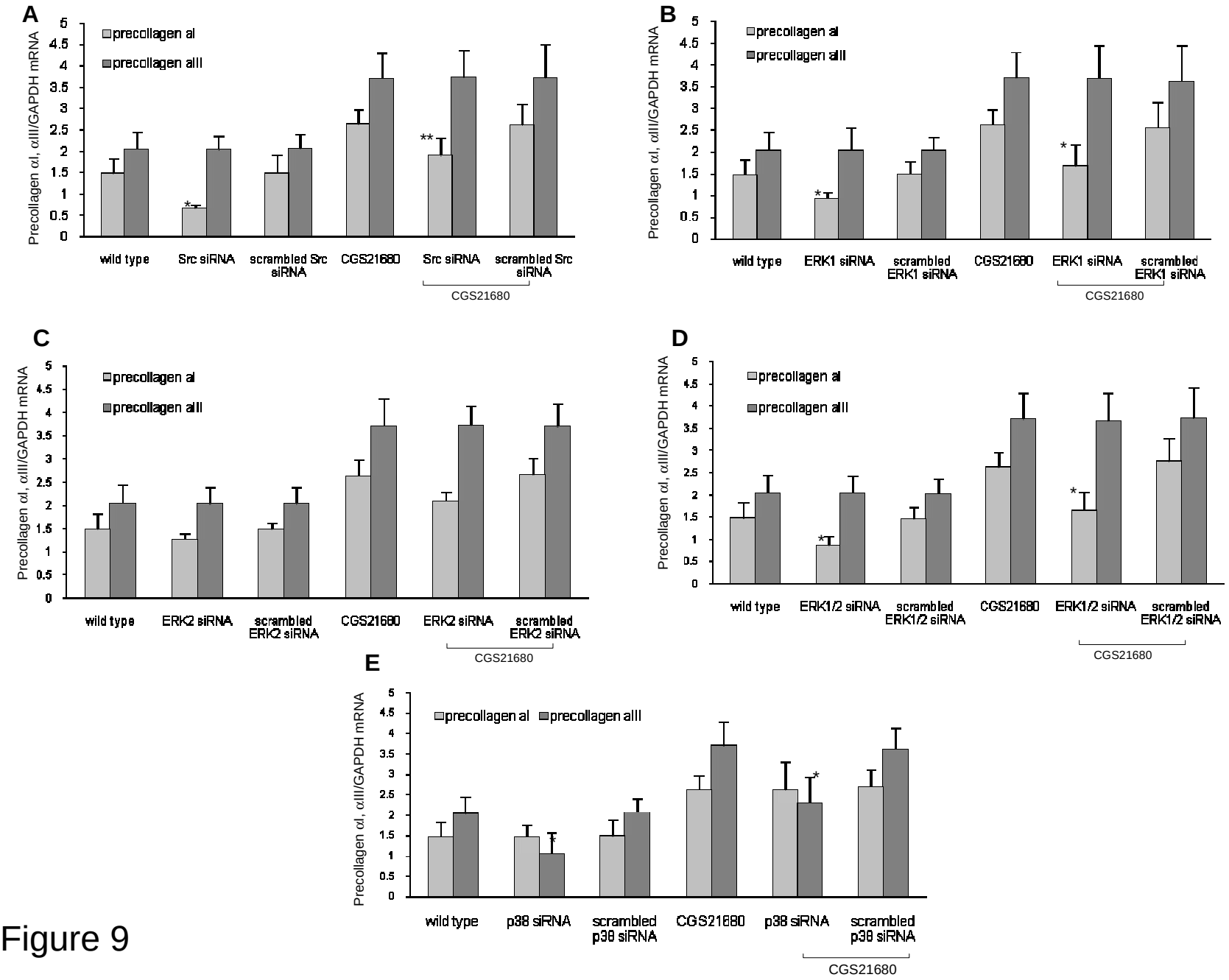


Figure 9