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TITLE PAGE

Focal adhesion kinase regulates collagen I-induced airway smooth muscle phenotype switching

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Running head: FAK regulates collagen I-induced ASM phenotype switching

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Nonstandard abbreviations: AHR, airway hyperresponsiveness; ASM, airway smooth muscle; BTSM, bovine tracheal smooth muscle; DMEM, dulbecco's modified eagle's medium; ECM, extracellular matrix; FAK, focal adhesion kinase; FAT, focal adhesion targeting; FBS, fetal bovine serum; FRNK, FAK-related non-kinase; GFP, green fluorescent protein; ILK, integrin linked kinase; KH, Krebs Henseleit; MAPK, mitogen-activated protein kinase; MEK, mitogen activated protein kinase kinase; PDGF, platelet-derived growth factor; PI3-kinase, phosphatidylinositol-3-kinase;

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Abstract

Increased extracellular matrix (ECM) deposition and airway smooth muscle (ASM) mass are major contributors to airway remodeling in asthma. Recently, we have demonstrated that the ECM protein collagen I, which is increased surrounding asthmatic ASM, induces a proliferative, hypocontractile ASM phenotype. Little is known, however, on the signaling pathways involved. Using bovine tracheal smooth muscle, we investigated the role of focal adhesion kinase (FAK) and downstream signaling pathways in collagen I-induced ASM phenotype modulation. Phosphorylation of FAK was increased during adhesion to both uncoated and collagen I-coated culture dishes, without differences between these matrices. No differences in cellular adhesion were found either. Inhibition of FAK activity by overexpression of the FAK deletion mutants FAT (focal adhesion targeting domain) and FRNK (FAK-related non-kinase) attenuated adhesion. After attachment, FAK phosphorylation was time-dependently increased in cells cultured on collagen I, whereas no activation was found on an uncoated plastic matrix. In addition, collagen I time- and concentration-dependently increased cell proliferation, which was fully inhibited by FAT and FRNK. Similarly, the specific pharmacological FAK inhibitor PF-573,228 as well as specific inhibitors of p38 MAPK and Src also fully inhibited collagen I-induced proliferation, whereas partial inhibition was observed by inhibition of PI3-kinase and MEK. The inhibition of cell proliferation by these inhibitors was associated with attenuation of the collagen I-induced hypocontractility. Collectively, the results indicate that induction of a proliferative, hypocontractile ASM phenotype by collagen I is mediated by FAK and downstream signaling pathways.

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Introduction

Airway hyperresponsiveness, persistent airway obstruction and decline in lung function are characteristic features of chronic asthma (Bousquet et al., 2000). Airway remodelling, including increased airway smooth muscle (ASM) mass and altered extracellular matrix (ECM) deposition, is considered to contribute to these features (Bousquet et al., 2000; Jeffery, 2001; Dekkers et al., 2009a). Increased ASM mass may comprise hyperplasia as well as hypertrophy (Ebina et al., 1993) and in keeping with hyperplasia ASM cells display phenotype plasticity and may re-enter the cell-cycle (Halayko et al., 2008). Thus, exposure of ASM cells to mitogenic stimuli results in the induction of a proliferative phenotype associated with a decreased contractile function (Hirst et al., 2000b; Dekkers et al., 2007; Gosens et al., 2002; Dekkers et al., 2012). Phenotype plasticity is a dynamic and reversible process and removal of mitogenic stimuli, for example by serum deprivation in the presence of insulin, results in the reintroduction of a (hyper)contractile ASM phenotype (Schaafsma et al., 2007; Dekkers et al., 2009b; Ma et al., 1998).

From biopsy studies, it has become apparent that ECM deposition is increased beneath the epithelial basement membrane in the airways of asthmatics (Roche et al., 1989). In addition, the total amount of ECM in the microenvironment of the ASM is increased as well (Bai et al., 2000), and may involve deposition of various ECM proteins, including collagen type I (Roberts and Burke, 1998). The ECM in the ASM layer plays a key role in determining its physical and mechanical properties. In addition, ECM proteins may affect ASM phenotype switching. Growth factor-induced phenotype switching of ASM cells is inhibited by culturing the cells on laminin-111 resulting in the maintenance of a contractile ASM phenotype (Dekkers et al., 2007; Hirst et al., 2000a; Dekkers et al., 2010). Conversely, culturing of ASM cells on monomeric collagen type I enhances growth factor-induced proliferation (Hirst et al., 2000a; Nguyen et al., 2005; Dekkers et al., 2010). Moreover, collagen I induces a hypocontractile, proliferative ASM phenotype

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in intact human and bovine tracheal smooth muscle preparations in the absence of other mitogens (Dekkers et al., 2007; Dekkers et al., 2012). ASM cells obtained from asthmatics produce more collagen I compared to cells obtained from healthy subjects (Johnson et al., 2004). In addition, nonasthmatic ASM cells cultured on an ECM laid down by asthmatic ASM cells proliferate more rapidly and vice versa (Johnson et al., 2004), suggesting that changes in the ECM profile may contribute to enhanced asthmatic ASM growth in situ.

Integrins are a group of heterodimeric transmembrane glycoproteins linking the ECM to the intracellular compartment (Giancotti and Ruoslahti, 1999). The collagen-binding integrin α2β1 is the main integrin involved in collagen I-induced ASM cell attachment, ASM cell proliferation, cytokine production and glucocorticosteroid resistance (Nguyen et al., 2005; Fernandes et al., 2006). In addition, the fibronectin-binding integrins α4β1 and α5β1 appeared important in the enhancement of platelet-derived growth factor (PDGF)-induced proliferation by collagen I, whereas the fibronectin-binding integrin αvβ3 was also required for attachment to collagen I (Nguyen et al., 2005). Recently, we have demonstrated that the α5β1 integrin is also of major importance in collagen I-induced increase of basal proliferation (Dekkers et al., 2010). No information is yet available on the signalling pathways of ECM-integrin interactions in ASM cells. From other cell types it is known that most integrins activate focal adhesion kinase (FAK), resulting in autophosphorylation at Tyr397 and generating a binding site for Src, which then phosphorylates a number of other tyrosine residues on FAK (Giancotti and Ruoslahti, 1999; Hynes, 2002; Cox et al., 2006). FAK may subsequently activate downstream signalling cascades, including the phosphatidylinositol-3-kinase (PI3-kinase) and mitogen activated protein kinase (MAPK) pathways (Giancotti and Ruoslahti, 1999)

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The aim of the present study was to explore the role of FAK and downstream signalling pathways in collagen I-induced ASM phenotype switching. Using bovine tracheal smooth muscle (BTSM) cells, we examined the effects of monomeric collagen I on FAK phosphorylation during adhesion and proliferation. The role of FAK in these processes was assessed by overexpression of FAK and of the FAK deletion mutants FAT (derived from the focal adhesion targeting (FAT) domain of FAK) and FRNK (FAK-related non-kinase), which inhibit FAK localization to the focal adhesions and FAK activation (Richardson and Parsons, 1996; Hildebrand et al., 1993). In addition, by pharmacological inhibition of FAK, Src, mitogen activated protein kinase kinase (MEK), PI3-kinase and p38 MAPK, we investigated the contribution of these pathways to collagen I-induced BTSM proliferation and hypocontractility.

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Materials and methods

Tissue preparation and organ-culture procedure.

Bovine tracheae were obtained from local slaughterhouses and BTSM strips of macroscopically identical length (1 cm) and width (2 mm) were prepared as described previously (Dekkers et al., 2007). Muscle strips were washed in Medium Zero (sterile DMEM, supplemented with sodium pyruvate (1 mM), non-essential amino-acid mixture (1:100), gentamicin (45 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (1.5 µg/ml), apo-transferrin (5 µg/ml, human) and ascorbic acid (0.1 mM)) and transferred into suspension culture flasks. Strips were maintained in culture in Medium Zero using an Innova 4000 incubator shaker (37°C, 55 rpm) for 4 days. When applied, monomeric collagen type I (50 µg/ml), PF-573,228 (FAK inhibitor II, 100 nM), PP2 (10 µM), U0126 (3 µM), LY294002 (10 µM) and/or SB203580 (10 µM) were present during the entire incubation period.

Isometric tension measurements.

Isometric tension measurements were performed as described (Dekkers et al., 2007). In short, BTSM strips were washed with Krebs Henseleit (KH) buffer (composition (mM): NaCl 117.5, KCl 5.60, $MgSO_4$ 1.18, $CaCl_2$ 2.50, NaH_2PO_4 1.28, $NaHCO_3$ 25.00 and glucose 5.50, pregassed with 5% CO_2 and 95% O_2; pH 7.4 at 37°C). Subsequently, strips were mounted for isometric recording in organ baths. During a 90-min equilibration period resting tension was gradually adjusted to 3 g. Subsequently, BTSM strips were precontracted with 20 and 40 mM KCl solutions. Collectively, the total washout period before the start of the isometric tension experiments was at least 3 h. After washing, maximal relaxation was established by the addition of (-)-isoproterenol (0.1 µM; Sigma). Tension was readjusted to 3 g and after another equilibration period of 30 min

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cumulative concentration response curves were constructed to methacholine. When maximal tension was reached, the strips were washed and maximal relaxation was established using isoproterenol (10 µM).

Isolation of bovine tracheal smooth muscle cells.

BTSM cells were isolated as described (Dekkers et al., 2007). In short, BTSM tissue was chopped and tissue fragments were washed in Medium Plus (Dulbecco's modified eagle's medium (DMEM) supplemented with sodium pyruvate (1 mM), non-essential amino-acid mixture (1:100), gentamicin (45 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (1.5 µg/ml) and fetal bovine serum (FBS, 0.5%)). Enzymatic digestion was performed in Medium Plus, supplemented with collagenase P (0.75 mg/ml), papain (1 mg/ml) and soybean trypsin inhibitor (1 mg/ml). The suspension was incubated in an incubator shaker (Innova 4000) at 37°C, 55 rpm for 20 min, followed by a 10 min period of shaking at 70 rpm. After filtration of the obtained suspension over a 50 µm gauze, the cells were washed in Medium Plus, supplemented with 10% FBS. For all protocols, cells were used in passage 1-2.

Transfection of BTSM cells with GFP expression vectors.

BTSM cells were plated at a density of 30.000 cells/well in 24-well culture plates and allowed to attach overnight, or grown to 95% confluency in 100 mm culture dishes. Subsequently, cells were washed twice with phosphate-buffered saline (PBS). Transfections in 24-well culture plates were performed using a mixture of 2 µl lipofectamine 2000 and 0.1 µg expression vector (green fluorescent protein (GFP) or GFP-FAK) or 0.8 µg expression vector (GFP, GFP-FAT or GFP-FRNK) for 6 h in 120 µl plain DMEM without serum and antibiotics. For transfections in the 100 mm dishes, a mixture of 60 µl lipofectamine 2000 and 3 µg or 24 µg of expression vector, respectively,

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in 3.6 ml of DMEM were used. After 6 h, cells were washed twice with PBS and medium was replaced by DMEM Zero supplemented with 0.1% FBS. Preliminary results indicated that transfection efficiency for GFP reached 30±4% (n=3).

Cell adhesion assay.

Collagen-coated (50 µg/ml) culture plates were prepared as described (Dekkers et al., 2007). The method for measurement of cell adhesion was adapted from (Orian-Rousseau et al., 1998). Untransfected, GFP-, GFP-FAK-, GFP-FAT- or GFP-FRNK-transfected BTSM cells were harvested from 100 mm dishes by trypsinization. Cells were washed, resuspended in Medium Plus and transferred into uncoated or collagen-coated 24-well culture plates at a density of 50.000 cells/well and placed back in the incubator. At varying time intervals, plates were removed from the incubator and overlying medium was removed by gentle aspiration. After washing with 0.5 ml PBS at 37 °C, cells were fixed with 70% ethanol for 15 minutes at 4 °C. Subsequently, the plates were air dried for at least 30 min at 37 °C and stained for 25 minutes at room temperature using 0.1% crystal violet in water (0.3 ml/well). Cells were rinsed briefly with water and air dried. The stain was solubilized at room temperature using 10% acetic acid in water (0.5 ml/well) and quantified by colorimetric analysis (550 nm, Biorad 680 plate reader).

Western analysis.

For the measurement of the phosphorylation of FAK, BTSM cells were cultured on uncoated or collagen I (50 µg/ml)-coated surfaces. At varying periods of time, culture medium - also containing non-adhered cells - was removed gently and the attached cells were lysed in homogenization buffer (composition in mM: Tris-HCl 50.0, NaCl 150.0, EDTA 1.0, PMSF 1.0, Na_3VO_4 1.0, NaF 1.0, pH 7.4, supplemented with leupeptin 10

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µg/ml, aprotinin 10 µg/ml, pepstatin 10 µg/ml, Na-deoxycholate 0.25 % and Igepal 1% (NP-40)). Protein content was determined and equal amounts of protein were subjected to electrophoresis and transferred onto PVDF membranes. Membranes were subsequently blocked in blocking buffer (composition: Tris-HCl 50.0 mM; NaCl 150.0 mM; Tween-20 0.1%, dried milk powder 5% (FAK) or BSA 5% (pFAK)) for 60 min at room temperature. Next, membranes were incubated overnight at 4 °C with primary antibodies (rabbit anti-FAK 1:2000 and rabbit anti-pFAK 1:1000, dilutions in blocking buffer containing BSA 5% or BSA 3%, respectively). After three washes with TBS-Tween 20 (TBST 0.1%, containing Tris-HCl 50.0 mM, NaCl 150.0 mM and Tween 20 0.1%) of 10 min each, membranes were incubated with horseradish peroxidase-labelled secondary anti-rabbit antibodies (dilution 1:2000 in blocking buffer containing 5% or 3% BSA, respectively) at room temperature for 90 min, followed by another three washes with TBST 0.1%. Antibodies were then visualized on film using enhanced chemiluminescence reagents and analyzed by densitometry (Totallab^{TM}). Bands for pFAK were normalized to total FAK expression. Data are expressed as percentage of the maximal FAK phosphorylation of matched samples run on the same gels.

Alamar blue proliferation assay.

Alamar blue conversion was used to determine changes in cell number. Previous studies have shown that changes in conversion of Alamar blue closely match changes in absolute cell number (Dekkers et al., 2012). BTSM cells were plated on uncoated or collagen I (1-100 µg/ml)-coated 24-well culture plates at a density of 30,000 cells/well and were allowed to attach overnight in Medium Plus, containing 10% FBS. The next day, cells were washed twice with PBS and made quiescent by incubation in Medium Zero, supplemented with 0.1% FBS for 3 days. Cells were then incubated with or without PDGF-AB (10 ng/ml) for 4 days in Medium Zero. Thereafter, cells were washed two

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times with PBS and incubated with HBSS containing 5% (vol/vol) Alamar blue solution. Conversion of Alamar blue into its reduced form by mitochondrial cytochromes was quantified by fluorimetric analysis, as indicated by the manufacturer. Data were expressed as % of Alamar blue conversion by unstimulated, vehicle-treated BTSM cells. When applied, PF-573,228 (10-1000 nM), PP2 (10 µM), U0126 (3 µM), LY294002 (10 µM) or SB203580 (10 µM) were present during the entire incubation period. For overexpression of GFP, GFP-FAK, GFP-FAT or GFP-FRNK, BTSM cells were transfected with the vectors after attachment, and subsequently cells were made quiescent as described above.

[^{3}H]-thymidine-incorporation.

[^{3}H]-Thymidine-incorporation was performed as described previously (Dekkers et al., 2007; Dekkers et al., 2009b). BTSM cells were plated on uncoated or collagen I-coated 24-well culture plates at a density of 30,000 cells/well and allowed to attach overnight in Medium Plus. The next day, cells were transfected with the GFP, GFP-FAK, GFP-FAT or GFP-FRNK, washed with PBS and made quiescent by incubation in Medium Zero, supplemented with 0.1% FBS for 72 h. Subsequently, cells were washed and incubated in the absence or presence of PDGF (10 ng/ml) in Medium Zero for 28 h, the last 24 h in the presence of [methyl-^{3}H]-thymidine (0.25 µCi/ml). After incubation, the cells were washed with PBS at room temperature. Subsequently, the cells were treated with ice-cold 5% trichloroacetic acid on ice for 30 min, and the acid-insoluble fraction was dissolved in NaOH (1 M). Incorporated [^{3}H]-thymidine was quantified by liquid-scintillation counting using a Beckman LS1701 β-counter.

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Materials.

DMEM, FBS, sodium pyruvate solution, non-essential amino acid mixture, gentamicin solution, penicillin/streptomycin solution and amphotericin B solution (Fungizone) were obtained from Gibco BRL Life Technologies (Paisley, U.K.). Bovine serum albumin, apo-transferrin (human), leupeptin, aprotinin, pepstatin, soybean trypsin inhibitor, insulin (bovine pancreas) and (-)-isoproterenol hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). PDGF-AB (human) was from Bachem (Weil am Rhein, Germany). Methacholine was obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Anti-FAK was from Cell Signalling (Boston, MA, USA). Anti-FAK [pY397] and Alamar blue were from Biosource (Camarillo, CA, USA). Collagenase P and papain were from Boehringer (Mannheim, Germany). Monomeric collagen type I (calf skin) was from Fluka (Buchs, Switzerland). Lipofectamine was from Invitrogen (Paisley, UK). PF-573,228 6-((4-((3-(Methanesulfonyl)benzyl)amino)-5-trifluoromethylpyrimidin-2-yl) amino)-3,4-dihydro-1H-quinolin-2-one and L(+)-ascorbic acid were from Merck (Darmstadt, Germany). SB203580 (4-[5-(4-Fluorophenyl)-2-[4-methylsulphonyl)phenyl]-1H-imidazol-4-yl]pyridine), LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), U0126 (1,4-diamino-2,3-dicyano-1,4-bis [2-aminophenylthio]butadiene) and PP2 (4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine) were obtained from Tocris Cookson (Bristol, UK). pEGFP expression plasmids (Clontech) encoding FAK, and the FAK deletion mutants FAT and FRNK coupled to GFP were kindly provided by Dr. B. van de Water and Dr. S.E. Le Dévédec from the Division of Toxicology, Leiden Amsterdam Center for Drug Research (Van de Water et al., 2001; Ilic et al., 1998). All used chemicals were of analytical grade.

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Data analysis.

Data represent means±SEM, from *n* separate experiments. Statistical significance of differences between means were calculated using one-way ANOVA for repeated measurements, followed by a Student-Newman-Keuls multiple comparisons test. Differences were considered to be statistically significant when $P<0.05$.

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Results

Role of focal adhesion kinase in bovine tracheal smooth muscle cell adhesion.

To investigate the effects of collagen I on ASM cell adhesion, BTSM cells were removed from the culture dish by trypsinization and replated onto uncoated plastic or monomeric collagen I (50 µg/ml)-coated culture plates. BTSM cells adhered to both substrates within 8 hours, without differences between plastic or collagen I (Figure 1A). To assess changes in FAK activation during adhesion, BTSM cells were plated and after 1, 2, 4, 6 and 24 h non-adhered cells were removed, adhered cells were lysed and FAK phosphorylation was determined. A significant increase ($P<0.05$) in FAK phosphorylation was observed in the cells adhered to plastic and collagen I compared to cells in suspension (Figure 1B). No differences in FAK phosphorylation were observed between both substrates. No FAK phosphorylation was observed in the non-adhered cells (data not shown).

To investigate the role of FAK in ASM cell adhesion, BTSM cells were transfected with GFP expression vectors encoding GFP (control), GFP-FAK or the FAK deletion mutants GFP-FAT and GFP-FRNK. In successfully transfected cells, expression of GFP-FAK, GFP-FAT and GFP-FRNK was detected in the focal adhesion sites (Figure 2), which is in correspondence with previous findings in rabbit primary synovial fibroblasts (Ilic et al., 1998). By contrast, expression of GFP was observed diffusely throughout the cytoplasm. Cells expressing GFP, GFP-FAT and GFP-FRNK remained elongated, whereas cells overexpressing GFP-FAK lost their elongated appearance. To assess whether FAK activation was required for ASM cell adhesion, BTSM cells were transfected with the expression vectors, trypsinized and replated onto plastic. No effects of overexpression of GFP-FAK on cell adhesion were observed, whereas overexpression of GFP-FRNK or GFP-FAT significantly reduced cell adhesion (Figure 3).

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Cell adhesion was maximally reduced at t=24 h, reaching 62±8% (P<0.001) in GFP-FRNK transfected cells and 67±12% (P<0.01) in GFP-FAT transfected cells compared to GFP transfected cells. Of note, the inhibition of BTSM cell adhesion was comparable to the transfection efficiency (30±4%). Collectively, these results indicate that endogenously expressed FAK is sufficient for BTSM cell adhesion, which can be inhibited by overexpression of GFP-FAT or GFP-FRNK.

Role of focal adhesion kinase in collagen I-induced bovine tracheal smooth muscle phenotype switching.

Culturing of BTSM cells on collagen I concentration-dependently increased cell number (Figure 4A). The concentration of collagen required for 50% increase (EC_{50}) in cell number was 14.1±1.8 µg/ml. The collagen I-induced increase in BTSM proliferation was also time-dependent, reaching 153±12% at day 4 (P<0.001, Figure 4B). To assess whether increases in cell number were associated with FAK activation, BTSM cells were cultured on uncoated plastic or collagen I (50 µg/ml) and cells were lysed after 1, 2, 3 or 4 days of culture and analyzed for FAK phosphorylation. Culturing on collagen I increased FAK phosphorylation at days 2, 3 and 4 (P<0.05, Figure 4C). No significant changes in total FAK expression were observed during the treatment period. In agreement with the findings for FAK phosphorylation during cell adhesion, no significant increase in phosphorylation of FAK was observed on a collagen I matrix after 1 day.

To investigate the role of FAK in BTSM cell proliferation, cells were plated on plastic or collagen I (50 µg/ml), allowed to attached overnight and transfected with GFP, GFP-FAK, GFP-FAT and GFP-FRNK expression vectors. As previous studies (Hirst et al., 2000a; Nguyen et al., 2005; Dekkers et al., 2010) have shown that collagen I enhances growth factor-induced proliferation, cells were subsequently were made quiescent and then stimulated with vehicle or PDGF-AB (10 ng/ml) for 4 days. Although

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not statistically significant, overexpression of GFP-FAK tended to decrease proliferation induced by both PDGF and collagen I (Figure 5A). Moreover, no effects of GFP-FAK overexpression were observed on DNA synthesis induced by PDGF, collagen I or the combination of both (Figure 5B). Overexpression of GFP-FAT or GFP-FRNK fully inhibited the increase in BTSM cell number induced by collagen I, whereas no significant effects of the inhibitory proteins were observed on PDGF-induced proliferation (Figure 5C). PDGF-induced proliferation on collagen I was normalized to the level observed for PDGF-induced proliferation in cells cultured on plastic. No significant effects of overexpression of GFP-FAT or GFP-FRNK were observed on cell number in the absence of collagen I or PDGF. Similar effects were observed for GFP-FAT and GFP-FRNK on collagen I-induced DNA synthesis (Figure 5D). Fully in line with these findings, collagen I-induced BTSM cell proliferation was also concentration-dependently inhibited by the specific pharmacological FAK inhibitor PF-573,228 (FAK inhibitor II) at concentrations that were specific for FAK inhibition (IC_{50}=65±16 nM, Figure 6A) (Slack-Davis et al., 2007).

To assess whether activation of FAK was also required for the induction of a hypocontractile phenotype by collagen I, BTSM strips were cultured in the absence and presence of collagen I (50 µg/ml) and/or PF-573,228 (100 nM) for 4 days. As described previously (Dekkers et al., 2007; Dekkers et al., 2012), culturing of BTSM strips in the presence of collagen I for 4 days significantly ($P<0.05$) reduced maximal methacholine-induced contractile force compared to vehicle-treated control strips (Figure 6B, Table 1). Combined treatment with PF-573,228 prevented the induction of a hypocontractile phenotype by collagen I, whereas the inhibitor by itself did not affect BTSM contractility. The sensitivity (pEC_{50}) for methacholine was unaffected by all treatments (Table 1).

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Role of Src, MEK, PI3-kinase and p38 MAPK in the induction of a proliferative, hypocontractile ASM phenotype by collagen I.

To determine the contribution of downstream signalling pathways of FAK in the induction of a proliferative, hypocontractile ASM phenotype by collagen I, BTSM cells were cultured on plastic or collagen I in the absence and presence of specific pharmacological inhibitors of Src (PP2, 10 μM), MEK (U0126, 3 μM), PI3-kinase (LY294002, 10 μM) or p38 MAPK (SB203580, 10 μM). Collagen I-induced proliferation was inhibited by all inhibitors investigated (Figure 7A). To investigate whether these pathways were involved in collagen I-induced hypocontractility as well, BTSM strips were cultured in the absence and presence of collagen I (50 μg/ml) and the inhibitors for 4 days. As observed for proliferation, collagen I-induced hypocontractility was normalized by all inhibitors investigated (Figure 7B, Table 2). The sensitivity for methacholine was unaffected by all treatments (Table 2).

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Discussion

In the current study, we demonstrate that the induction of a proliferative, hypocontractile ASM phenotype by monomeric collagen type I is dependent on the activation of FAK and downstream signalling pathways. Our results indicate that FAK is activated during BTSM cell adhesion and that overexpression of the two FAK deletion mutants FAT and FRNK, which compete with endogenous FAK for localization to the focal adhesions, inhibits cell adhesion. Moreover, FAK was activated during and required for collagen I-induced BTSM cell proliferation and phenotype switching. Pharmacological inhibition of Src, MEK, PI3-kinase and p38 MAPK signalling pathways, which may be activated downstream of FAK, inhibited the induction of a proliferative and hypocontractile phenotype induced by collagen I as well.

Airway hyperresponsiveness (AHR) is a characteristic feature of asthma and is defined by an exaggerated airway narrowing in response to either direct (histamine, methacholine) or indirect (exercise, cold air, hyperventilation) stimuli (Postma and Kerstjens, 1998). Variable AHR is observed after allergen exposure and is considered to reflect airway inflammation, whereas persistent AHR is considered to relate to structural changes in the airway wall, collectively termed airway remodelling (Cockcroft and Davis, 2006; Meurs et al., 2008). Increased ASM mass, as a feature of airway remodelling, is considered to be the most important factor contributing to AHR and decline in lung function in asthmatics (Ebina et al., 1993; Lambert et al., 1993; Oliver et al., 2007). Previously, we and others have shown that changes in the ECM environment surrounding the ASM may contribute to ASM accumulation (Dekkers et al., 2010; Dekkers et al., 2007; Hirst et al., 2000a; Johnson et al., 2004). Thus, culturing of ASM cells on collagen I matrices increased proliferative responses, which was associated with a decreased contractile function of intact ASM tissue by this ECM protein, indicating that collagen I modulates the ASM phenotype from a contractile to a proliferative,

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hypocontractile phenotype (Dekkers et al., 2007; Hirst et al., 2000a; Dekkers et al., 2012; Dekkers et al., 2010). Little is known, however, on the signalling pathways involved in this process. In the current study, we found that culturing of BTSM cells on collagen I time-dependently increased phosphorylation of FAK, a cytoplasmic protein tyrosine kinase activated by most integrins (Giancotti and Ruoslahti, 1999). Activation of FAK was found to be essential in collagen I-induced BTSM cell proliferation as overexpression of FAT and FRNK, which inhibit FAK translocation to the focal adhesions and subsequent activation of the enzyme (Richardson and Parsons, 1996; Hildebrand et al., 1993), fully inhibited collagen I-induced proliferation. Accordingly, collagen I-induced proliferation was also inhibited by the specific pharmacological inhibitor PF-573,228 (FAK inhibitor II) at concentrations which have previously been found to be specific for FAK (Slack-Davis et al., 2007). In addition, this inhibitor fully reversed collagen I-induced hypocontractility, suggesting a key role of FAK in collagen I-induced ASM phenotype switching. Activation of FAK was also observed during adhesion of BTSM cells to uncoated plastic and collagen I matrices, without differences between the two matrices. In addition, FAK activation was also required for BTSM cell adhesion, as indicated by its inhibition in FAT and FRNK overexpressing cells. The effects of overexpression of FAT and FRNK on collagen I-induced changes in BTSM cell number as mentioned above are unlikely to be due to changes in cell adhesion, as overexpression of these proteins only inhibited the collagen I-induced proliferative responses, whereas no effects were observed on basal and PDGF-induced increases in cell number. In addition, no effects of overexpression of FAK itself were observed on the parameters assessed, suggesting that the endogenous expression of this kinase is sufficient and not the limiting factor in the activation of downstream signalling pathways.

Changes in FAK activation during the proliferative phase only became apparent after 2 days of culturing on collagen I, suggesting that FAK is not directly activated by

collagen I-binding integrins, but that additional processes may be required. Indeed, studies in vascular smooth muscle cells have indicated that culturing on monomeric collagen type I increased the expression of other ECM proteins, including fibronectin (Ichii et al., 2001), suggesting that the activation of FAK could be due to autocrine ECM deposition. This notion is also supported by previous findings showing that collagen I-induced increases in basal and growth factor-induced ASM proliferation required not only the collagen-binding integrin α2β1, but also the fibronectin binding integrins α4β1 and α5β1 (Dekkers et al., 2010; Nguyen et al., 2005).

No effects of FAT or FRNK were observed on PDGF-induced proliferation, although in fibroblasts activation of the PDGF receptor has been shown to induce FAK phosphorylation (Sieg et al., 2000). Also in BTSM cells, PDGF time-dependently increased FAK phosphorylation (data not shown). The lack of effect of the deletion mutants, however, may be explained by the fact that activation of FAK by PDGF requires interaction of the receptor with the FERM domain, which is localized at the N-terminus of the kinase (Cox et al., 2006; Sieg et al., 2000). Both deletion mutants, however, are derived from the C-terminal domain and inhibit FAK localization to the focal adhesions, which is required for FAK activation by integrins (Richardson and Parsons, 1996; Hildebrand et al., 1993), but do interfere with the activation of FAK via the FERM domain, providing a potential explanation for the lack of effect on PDGF-induced proliferation.

Phosphorylation of FAK at Tyr397 generates a high affinity binding site for Src, which then in turn fully activates FAK by phosphorylating Tyr576 and Tyr577 in the kinase domain (Hynes, 2002; Cox et al., 2006). Previous studies have found a critical role for Src in growth factor-induced ASM proliferation (Krymskaya et al., 2005). The essential role of Src in collagen I-induced BTSM phenotype modulation was indicated by the full inhibition of collagen I-induced proliferation and hypocontractility by the pharmacological inhibitor PP2. Upon full activation by Src, FAK initiates a number of

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other signalling pathways, including the PI3-kinase and MAPK signalling pathways (Giancotti and Ruoslahti, 1999). Activation of these pathways has been found to be important in the response of ASM cells to growth factors. PI3-kinase activation has been associated with transcriptional activation and protein synthesis leading to ASM cell proliferation, hypertrophy, and both hypo- and hypercontractility (Walker et al., 1998; Halayko et al., 2004; Schaafsma et al., 2007; Dekkers et al., 2009b). Integrins may not only activate PI3-kinase through FAK, but also via integrin linked kinase (ILK), another cytoplasmic protein tyrosine kinase, which is activated by the β1 subunit of integrins (Liu et al., 2000). ILK has also been shown to be important in the regulation of contractile protein expression by human ASM cells. Knock-down of ILK increased mRNA and protein expression of smooth muscle-specific myosin heavy chain (sm-MHC), via regulation of Akt, which is downstream of PI3-kinase (Wu et al., 2008). In the present study, inhibition of both PI3-kinase and FAK prevented collagen I-induced proliferation and hypocontractility, indicating the involvement of the both pathways in collagen I-induced BTSM proliferation. p42/p44 MAPK transfers growth promoting signals to the nucleus and subsequently increase ASM proliferation (Gosens et al., 2008). In addition, p38 MAPK is involved in the regulation of growth factor-induced proliferation in ASM as well (Fernandes et al., 2004). Inhibition of the MAPK signalling pathways, either directly (p38 MAPK) or by inhibiting MEK, which is upstream of p42/p44 MAPK, also inhibited collagen I-induced BTSM proliferation and hypocontractility. Collectively, these findings suggest that collagen I-induced activation of FAK results in activation of Src and, subsequently, of PI3-kinase and MAPK signalling pathways downstream, which are all involved in collagen I-induced BTSM cell proliferation and hypocontractility.

Next to its important role in ECM-induced phenotype switching, FAK has also shown to be involved in acute ASM contractile responses. Thus, phosphorylation and membrane localization of the kinase is increased by mechanical strain and by contractile

agonists (Smith et al., 1998; Tang et al., 1999; Gunst et al., 2003). Knock-out of FAK in human tracheal smooth muscle strips decreased tension development, myosin light chain phosphorylation and calcium signalling in response to the muscarinic receptor agonist acetylcholine and the membrane depolarizing stimulus KCl (Tang and Gunst, 2001), suggesting an important role of FAK in smooth muscle contraction. These findings and our current findings suggest that modulation of FAK activity in asthma may be an important new target in the treatment of ASM responsiveness and proliferation.

In conclusion, the present study provides new insights in the signalling events leading to ASM phenotype modulation by collagen I. These signalling pathways involve activation of FAK as well as Src, MEK, PI3-kinase and p38 MAPK downstream. Moreover, our results indicate that modulation of FAK activity may be a new target in the treatment of both variable and persistent AHR in asthmatics.

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Authorship contributions

Participated in research design: BGJD, AIRS, RDvdS, WJK, JZ, HM.

Conducted experiments: BGJD, AIRS, RDvdS, WJK.

Performed data analysis: BGJD, AIRS, RDvdS, WJK, JZ, HM.

Wrote or contributed to the writing of the manuscript: BGJD, AIRS, RDvdS, WJK, JZ, HM.

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Footnotes

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Figure legends

Figure 1: FAK is activated during BTSM cell adhesion to plastic and collagen I. (A) Adhesion of BTSM cells to plastic and collagen I. BTSM cells were allowed to adhere either directly onto the plastic surface of culture plates or to collagen I (50 µg/ml)-coated surfaces. Data are expressed as percentage of adhesion to uncoated plates at t=24 hours and represent means±SEM of 4 experiments, each performed in triplicate. (B) FAK is activated during adhesion, without differences between plastic and collagen I matrices. Data are expressed as percentage of the maximal FAK phosphorylation observed between 1-24 h. Data represent means±SEM of 4 experiments after densitometric analysis. Representative immunoblots of Y397 pFAK (upper panel) and total FAK (lower panel) are shown. *P<0.05, **P<0.01, ***P<0.001 compared to cells in suspension.

Figure 2: Cellular expression of GFP fusion proteins in BTSM cells transfected with expression vectors encoding for GFP (A), GFP-FAK (B) and the FAK deletion mutants GFP-FAT (C) and GFP-FRNK (D).

Figure 3: FAK is required for BTSM cell adhesion. (A) Overexpression of GFP-FAK did not affect BTSM cell adhesion. BTSM cells were cultured in 100 mm dishes and transfected with expression constructs for GFP (control) or GFP-FAK. After 6 hours, medium was replaced by Medium Zero + 0.1% FBS and cells were allowed to express fusion proteins for 3 days, after which cells were trypsinized and replated onto plastic. Data are expressed as percentage of adhesion for GFP expressing cells at t=24 hours and represent means±SEM of 5 experiments, each performed in triplicate. (B) Overexpression of the FAK deletion mutants GFP-FAT and GFP-FRNK decreases

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BTSM cell adhesion to plastic. Data are expressed as percentage of adhesion for GFP expressing cells at t=24 hours and represent means±SEM of 5 experiments, each performed in triplicate. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ compared to GFP controls

Figure 4: Collagen I increases BTSM proliferation and FAK phosphorylation in a concentration- and time-dependent fashion. (A) BTSM cell number after culturing for 4 days on increasing concentrations of collagen I (0-100 µg/ml). Data represent means±SEM of 4 experiments, each performed in triplicate. (B) BTSM cell number after culturing on uncoated plastic or collagen I (50 µg/ml) for 2 or 4 days. Data represent means±SEM of 6 experiments, each performed in triplicate (C) Phosphorylation of FAK is time-dependently increased by culturing on collagen I (50 µg/ml), but not on uncoated plastic. Data represent means±SEM of 4 experiments after densitometric analysis. Representative immunoblots of Y397 pFAK (upper panel) and total FAK (lower panel) are shown. *P<0.05, ***P<0.001 compared to cells cultured on plastic.

Figure 5: Activation of FAK is required for collagen I-induced BTSM cell proliferation. (A) Overexpression of FAK tends do decrease BTSM cell number. BTSM cells were plated in 24-well culture plates and allowed to adhere. After adhesion, cells were transfected with GFP or GFP-FAK and subsequently serum deprived in Medium Zero + 0.1% FBS for 3 days. Cells were stimulated with or without PDGF-AB (10 ng/ml) for 4 days and cell number was assessed. (B) No effects of GFP-FAK overexpression were observed on DNA synthesis in BTSM cells. (C) Overexpression of GFP-FAT and GFP-FRNK decreases collagen I-induced increases in BTSM cell number. (D) Overexpression of GFP-FAT and GFP-FRNK decreased collagen I-induced DNA synthesis. Data represent means±SEM of 7 experiments, each performed in triplicate. *P<0.05, **P<0.01, ***P<0.001 compared to GFP-transfected control cells cultured on

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uncoated plastic. #P<0.05 compared to GFP-transfected cells grown on collagen I and/or stimulated with PDGF.

Figure 6: Pharmacological inhibition of FAK inhibits collagen I-induced BTSM phenotype switching. (A) Effect of the specific FAK inhibitor PF-573,228 (FAK inhibitor II, 0-1000 nM) on basal and collagen I (50 µg/ml)-induced changes in BTSM cell number. Data represent means±SEM of 6 experiments performed in triplicate. (B) Effect of PF-573,228 (100 nM) on collagen I-induced hypocontractility. Data represent means±SEM of 6 experiments, each performed in duplicate. *P<0.05, **P<0.01 compared to vehicle-treated control. #P<0.05, ##P<0.01 compared to collagen I-treated control.

Figure 7: Src, MEK, PI3-kinase and p38 MAPK are required for collagen I-induced BTSM cell proliferation and hypocontractility. (A) Effects of pharmacological inhibitors of Src (PP2, 10 µM), MEK (U0126, 3 µM), PI3-kinase (LY294002, 10 µM) and p38 MAPK (SB203580, 10 µM) on basal and collagen I (50 µg/ml)-induced changes in cell number. Data represent means±SEM of 6 experiments, each performed in triplicate. ***P<0.01 compared to untreated control cells cultured on plastic. ###P<0.001 compared to cells grown on collagen I in the absence of inhibitors. (B) Effects of these inhibitors on collagen I-induced hypocontractility. Data represent means±SEM of 5-10 experiments, each performed in duplicate. *P<0.05 compared to vehicle-treated control. #P<0.05, ##P<0.01, ###P<0.001 compared to collagen I-treated control.

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Tables

Table 1: Contractile responses of BTSM strips to methacholine after 4 days of culturing in the absence or presence of collagen I, with or without the specific FAK inhibitor PF-573,288.

	Control		*Collagen I (50 µg/ml)*	
	E_{max} (g)	*pEC_{50} (- log M)*	*E_{max} (g)*	*pEC_{50} (- log M)*
Vehicle	35.0±3.4	6.54±0.09	24.6±2.8*	6.33±0.03
PF-573,228 (100 nM)	32.6±2.2	6.53±0.13	32.9±1.7$^{\#}$	6.53±0.15

Data represent means±SEM of 6 independent experiments, each performed in duplicate. Abbreviations: E_{max}: maximal contraction; EC_{50}: concentration of agonist eliciting half-maximal response; pEC_{50}: negative logarithm of the EC_{50} value. *P<0.05 compared to vehicle-treated control. $^{\#}$P<0.05 compared to collagen I-treated control (vehicle).

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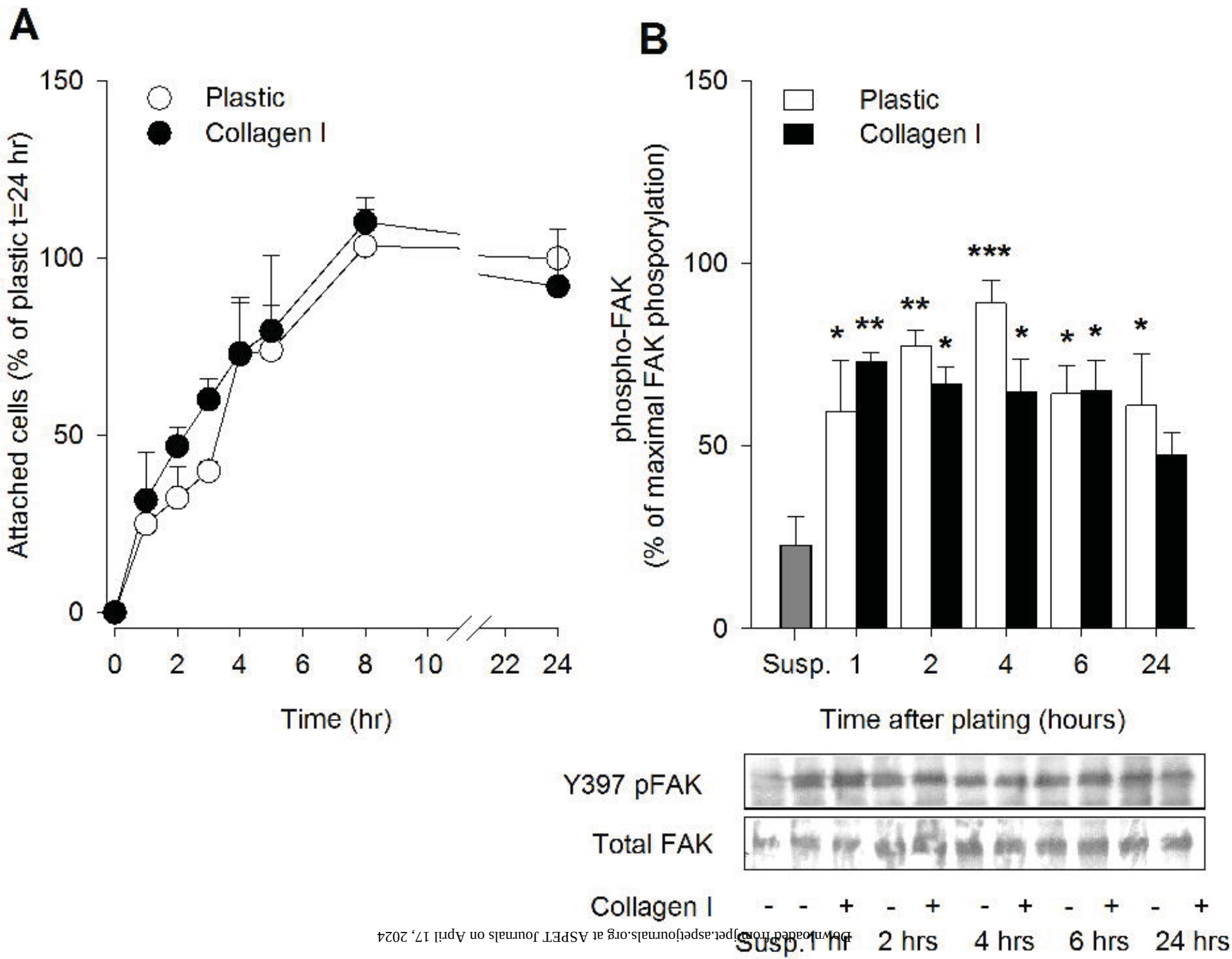
Table 2: Contractile responses of BTSM strips to methacholine after 4 days of culturing in the absence or presence of collagen I, with or without specific inhibitors of Src (PP2), MEK (U0126), PI3-kinase (LY294002) or p38 MAPK (SB203580).

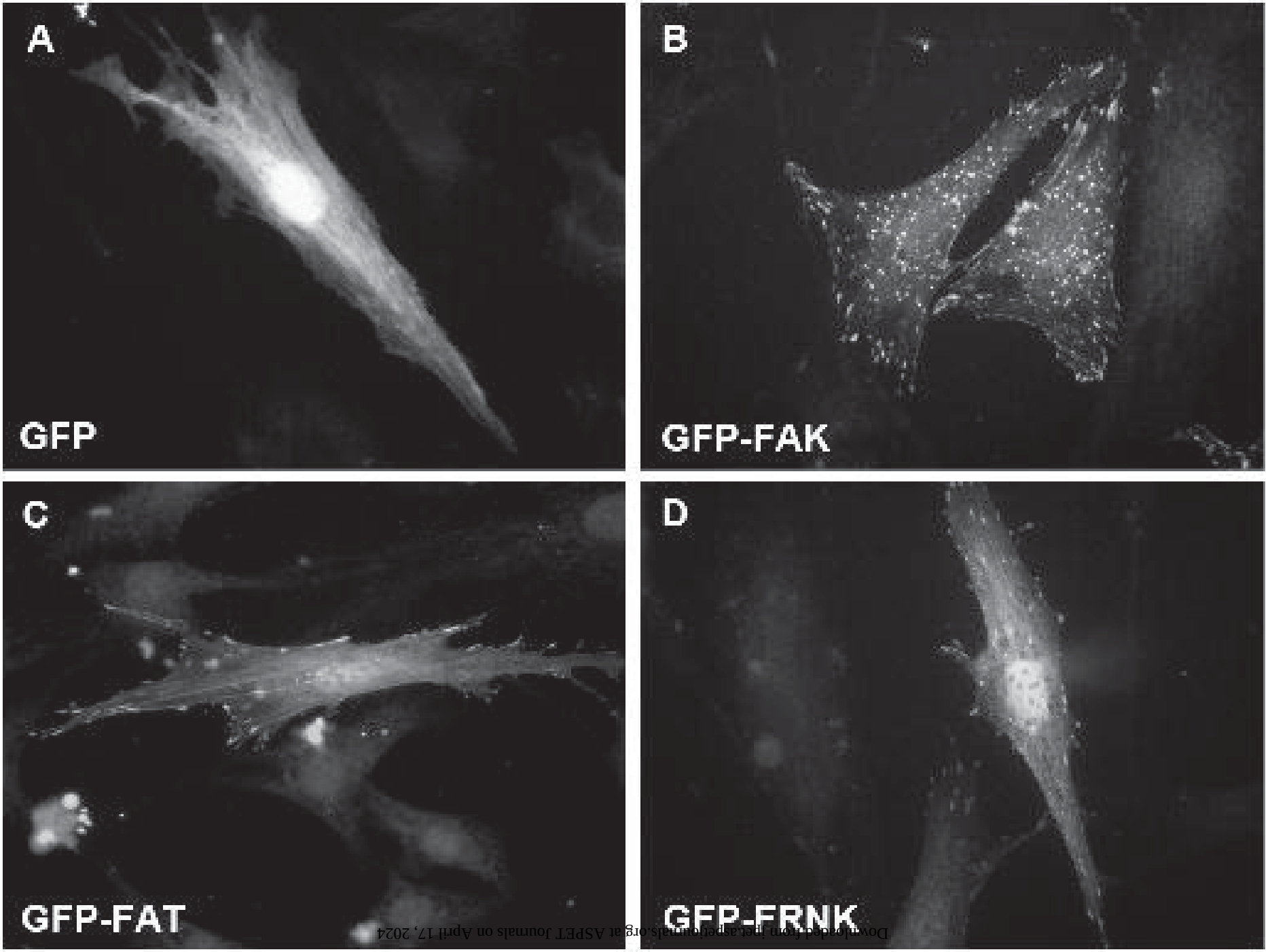
	Control		*Collagen I (50 µg/ml)*	
	E_{max} *(% of control)*	pEC_{50} *(- log M)*	E_{max} *(% of control)*	pEC_{50} *(- log M)*
Vehicle	100±0	6.82±0.12	77±4*	6.72±0.11
PP2 (10 µM)	91±11	6.75±0.23	111±13$^{\#}$	6.72±0.15
U0126 (3 µM)	99±8	6.76±0.11	94±5$^{\#}$	6.94±0.12
LY294002 (10 µM)	85±6	7.21±0.14	114±2$^{\#\#}$	7.04±0.16
SB203580 (10 µM)	104±5	6.83±0.18	101±5$^{\#\#\#}$	6.84±0.24

Data represent means±SEM of 5-10 independent experiments, each performed in duplicate. Abbreviations: E_{max}: maximal contraction; EC_{50}: concentration of agonist eliciting half-maximal response; pEC_{50}: negative logarithm of the EC_{50} value. *P<0.05 compared to vehicle-treated control. $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 compared to collagen I-treated control (vehicle).

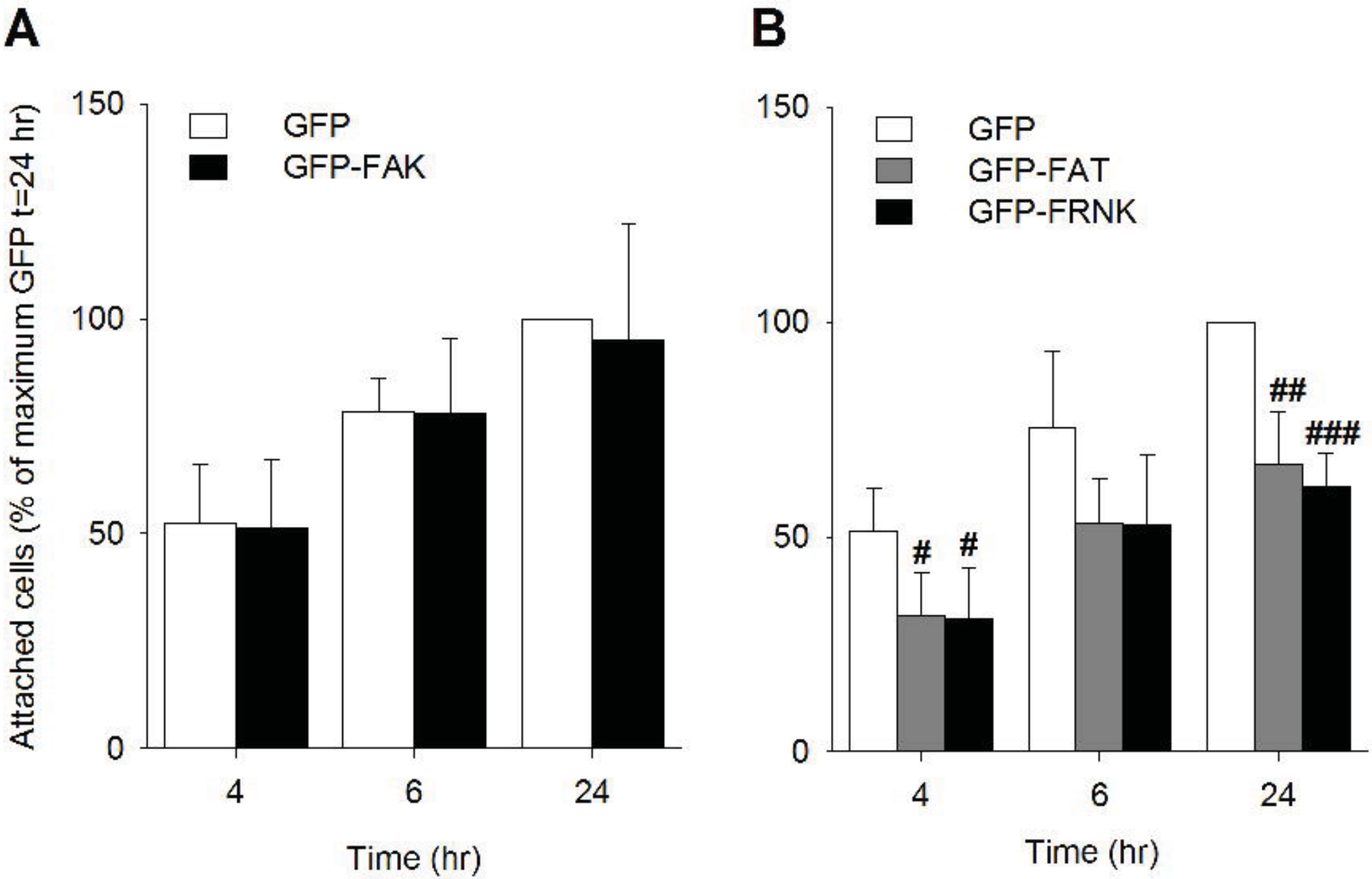
Dekkers et al, JPET #203042 Figure 1



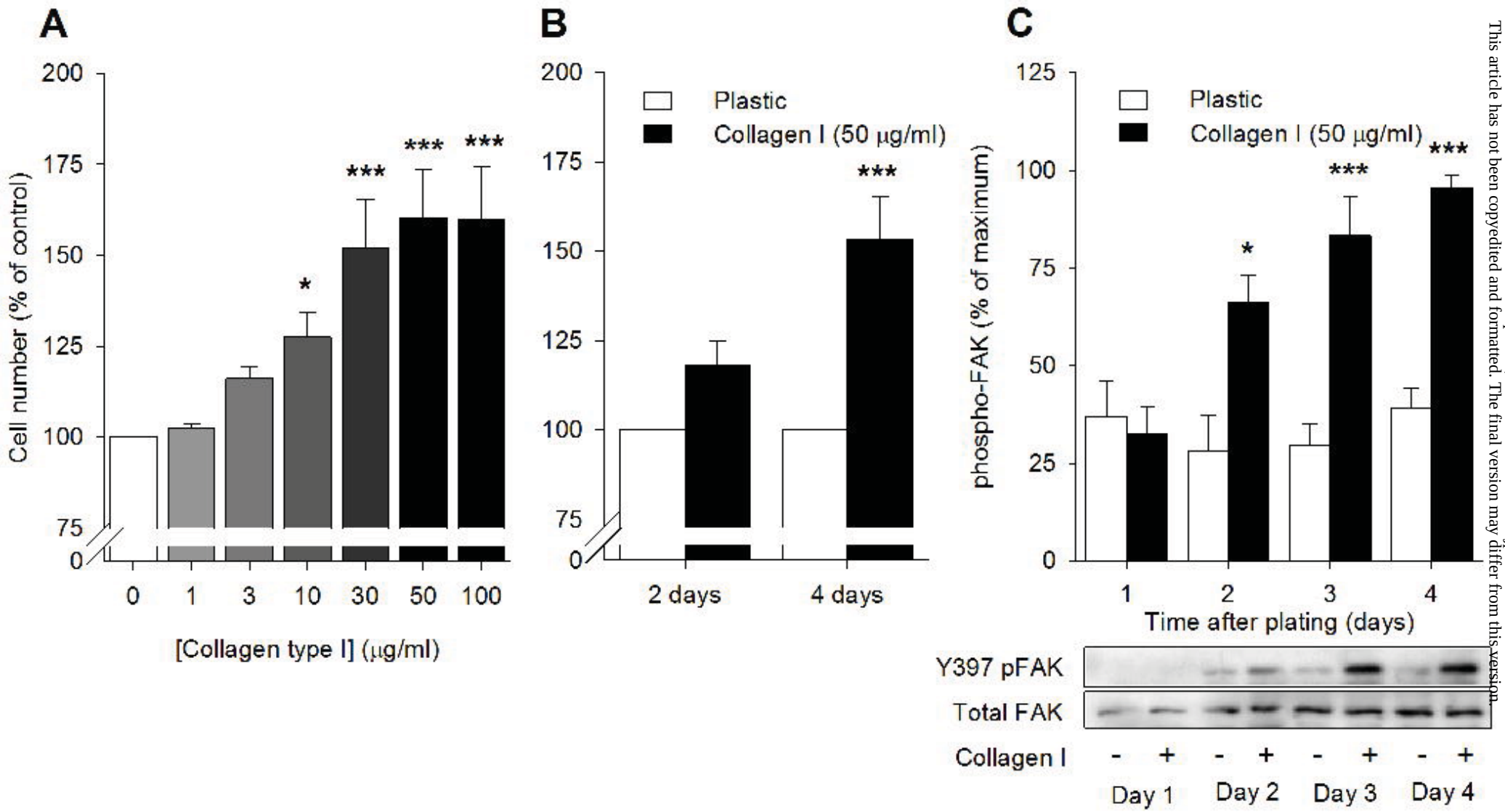
Dekkers et al, JPET #20304 Figure 2



Dekkers et al, JPET #203042 Figure 3



Dekkers et al, JPET #203042 Figure 4



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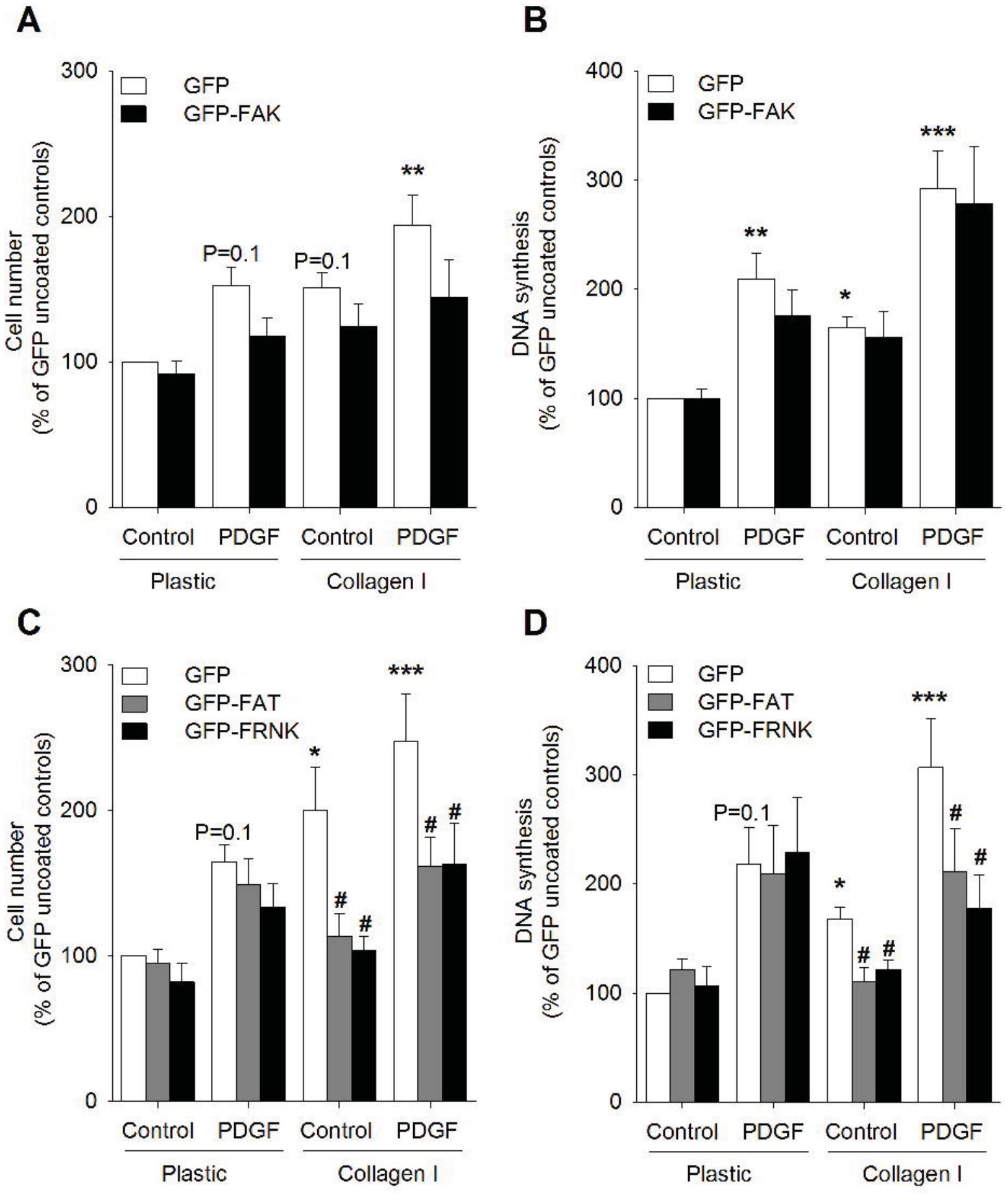
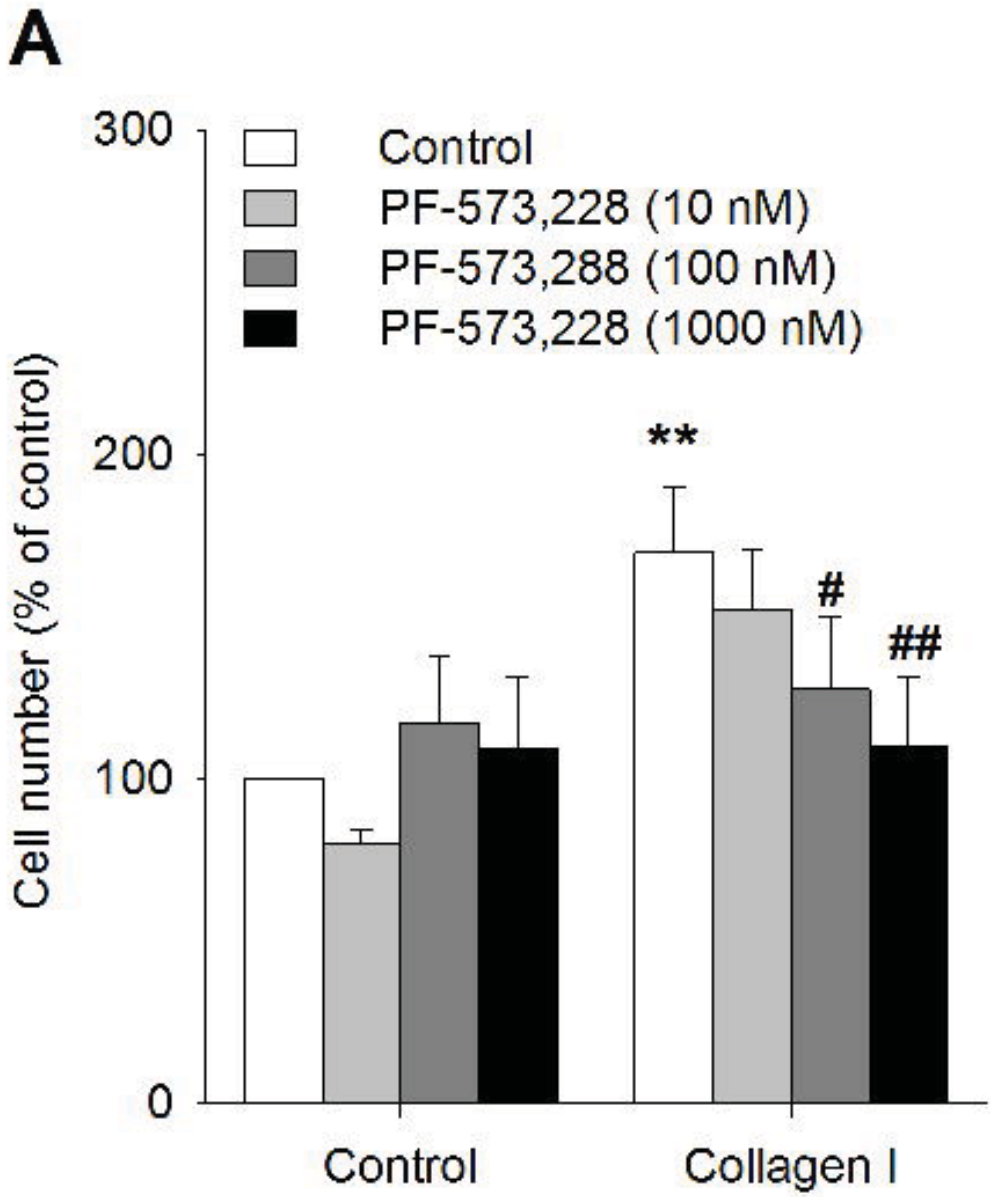
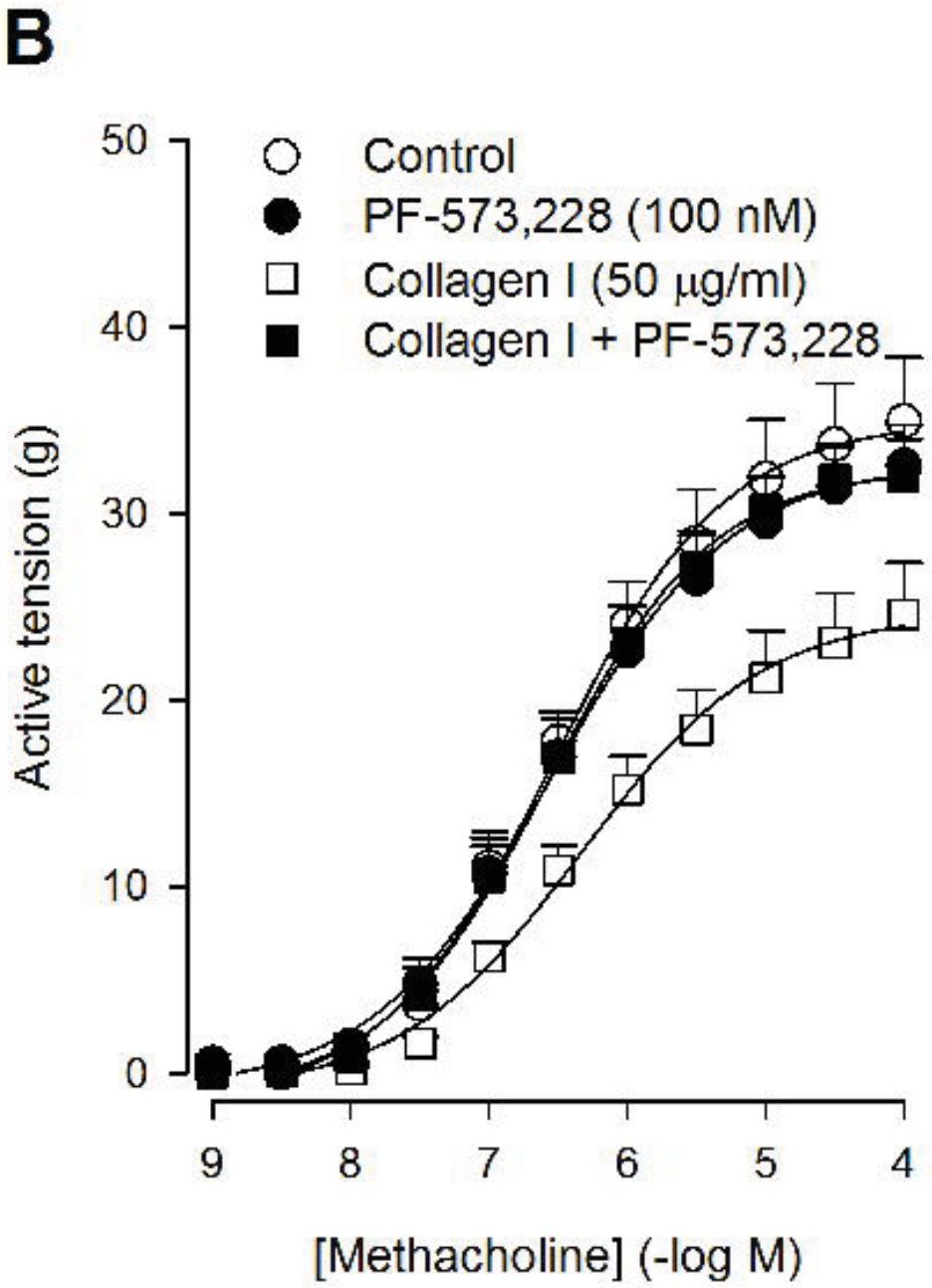
Dekkers et al, JPET #203042 Figure 5



Dekkers et al, JPET #203042 Figure 6





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