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The Different Roles of Myosin IIA and Myosin IIB in Contraction of 3D Collagen Matrices by Human Fibroblasts

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Abstract

Contraction of 3D collagen matrices by fibroblasts frequently is used as an in vitro model of wound closure. Different iterations of the model -- all conventionally referred to as “contraction” -- involve different morphological patterns. During floating matrix contraction, cells initially are round without stress fibers and subsequently undergo spreading. During stressed matrix contraction, cells initially are spread with stress fibers and subsequently undergo shortening. In the current studies, we used siRNA silencing of myosin IIA (MyoIIA) and myosin IIB (MyoIIB) to test the role of myosin II isoforms in fibroblast interactions with 3D collagen matrices and collagen matrix contraction. We found that MyoIIA but not MyoIIB was required for cellular global inward contractile force, formation of actin stress fibers, and morphogenic cell clustering. Stressed matrix contraction required MyoIIA but not MyoIIB. Either MyoIIA or MyoIIB was sufficient for floating matrix contraction (FMC) stimulated by platelet-derived growth factor. Neither MyoIIA or MyoIIB was necessary for FMC stimulated by serum. Our findings suggest that myosin II-dependent motor mechanisms for collagen translocation during extracellular matrix remodeling differ depending on cell tension and growth factor stimulation.

Keywords

Myosin; 3D Collagen Matrix; Cell Contraction; Morphogenic Cell Clustering; Wound Repair

INTRODUCTION

Two different mechanisms of wound closure have been described. One depends on smooth muscle-like fibroblasts (myofibroblasts) undergoing contraction within newly synthesized wound fibrous connective tissue known as granulation tissue [1, 2]. The other depends on the motile activity of fibroblasts at wound margins and occurs independently of granulation tissue [3]. Myofibroblasts are activated by mechanical tension [4] and have been implicated in pathological wound repair conditions such as wound contracture and hypertrophic scar

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[5-7]. However, myofibroblasts often appear late relative to the timing of wound closure [8-11] and are not required for fetal wounds to close [12]. Similarly, in a mouse model of hypertrophic scarring, the mechanical tension sensor focal adhesion kinase (FAK) [13-15] was required for the fibrotic response but not for incisional wound closure [16]. The above findings are consistent with the idea that wound closure can occur by different mechanisms depending on mechanical tension within the wound [17].

Collagen matrix contraction by fibroblasts was introduced as an in vitro model of wound closure [18]. Different iterations of this model -- all conventionally referred to as "contraction" -- involve different cellular mechanisms. That is, contraction refers to the change that occurs in the collagen matrix but not to the cellular mechanism responsible for collagen translocation leading to matrix remodeling. In the case of floating matrix contraction assays, cells initially are round without stress fibers and become spread during matrix contraction. In the case of stressed matrix contraction, cells initially are spread with stress fibers and subsequently undergo shortening and stress fiber disruption during matrix contraction [17].

Non-muscle myosin II plays central roles in cell adhesion, migration and contraction with different isoforms exhibiting different functional roles [e.g., reviewed in 19, 20, 21]. The myosin II inhibitor blebbistatin inhibited stressed collagen matrix contraction by human fibroblasts but was less effective in blocking floating matrix contraction depending on growth factor conditions [22-24]. These observations raised the possibility that the role of myosin II in collagen matrix contraction was tension and growth factor dependent. In the current studies, we tested the effects of siRNA silencing of MyoIIA and MyoIIB on stressed and floating collagen matrix contraction to determine what roles the different isoforms play in the cellular mechanisms involved. Details are reported herein.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's Medium (DMEM), 0.25% trypsin/EDTA and antibiotic-antimycotic solutions were purchased from GIBCO (Grand Island, NY). PureCol (Bovine Collagen Solution, Type I) was purchased from Advanced BioMatrix (San Diego, CA). Rat tail Type I collagen was obtained from BD Biosciences (Bedford, MA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). Platelet-derived growth factor (PDGF) was obtained from Upstate Biotechnology (Lake Placid, NY). Fatty acid-free bovine serum albumin (BSA), focal adhesion kinase inhibitor PF-228, and monoclonal anti-actin antibody were obtained from Sigma (St. Louis, MO). BSA (fraction V) was obtained from Equitech (Kerrville, TX). Blebbistatin was obtained from TRC (North York, Ontario, Canada). Polyclonal anti-MyoIIA and anti-MyoIIB were purchased from Covance (Alice, TX). Hoechst 33342, Alexa Fluor 488 phalloidin, Alexa Fluor 594 phalloidin, and Alexa 488 Fluor and 568 Fluor conjugated antibodies against mouse and rabbit IgG (H+L) were obtained from Invitrogen-Molecular Probes (Eugene, OR). Polyclonal anti-FN and horseradish peroxidase-conjugated goat anti-mouse IgG were obtained from Abcam (Cambridge, MA). Fluoromount-G was purchased from Southern Biotechnology (Birmingham, AL). Horseradish peroxidase-conjugated goat anti-rabbit IgG

was obtained from Thermo Scientific (Pittsburgh, PA). Pierce ECL Western blotting substrate was purchased from Thermo Scientific (Rockford, IL). ON-TARGETplus siRNA for human MYH9 and MYH10 were purchased from Dharmacon (Chicago, IL). Oligofectamine solution was purchased from Invitrogen (Gaithersburg, MD)

Cell culture

Use of human foreskin fibroblasts was approved by the University Institutional Review Board (Exemption #4). BR5 cells (early passage, hTERT immortalized, human foreskin fibroblasts) [25] were cultured in DMEM supplemented with 10% FBS in a 37°C and 5% CO₂ humidified incubator. Experimental incubation media consisted of DMEM supplemented with 5 mg/ml BSA (fatty-acid-free) (DMEM/BSA), 5 mg/ml BSA and 50 ng/ml PDGF (DMEM/PDGF), and 10% FBS (DMEM/FBS).

siRNA transfection

siRNA transfection was accomplished beginning with trypsin rounded cells as described previously [25, 26]. MyoIIA and MyoIIB silencing incubations contained 800 µl of DMEM (serum and antibiotics-free) plus 200 µl of a mixed solution containing 100nM ONTARGETplus siRNAs for human MYH9 and MYH10 respectively, Opti-MEM (Invitrogen, Carlsbad, CA), and 0.2mM oligofectamine. After an initial 24 h incubation, transfected cells were sub-cultured for an additional 72h. To silence both MyoIIA and MyoIIB, the incubations contained 100nM MYH9 and 100nM MYH10. Preliminary experiments were performed using the 4 siRNA open reading frames provided by the manufacturer. Specific knockdown was first confirmed for all 4 oligonucleotides. A mixture of the two most effective siRNA's was used for subsequent experiments. For mock transfection, cells were treated with the transfection reagent with no siRNA.

Floating and stressed collagen matrix contraction

Methods for preparing cell-containing collagen matrices and measuring floating and stressed collagen matrix contraction have been described previously [26, 27]. Briefly, collagen matrices (200 µl, 1.5mg/ml bovine type I collagen, 2×10⁵ cells/matrix) were polymerized within 12 mm circular scores made on the bottom of 24-well plates in a humidified incubator at 37° C. For floating matrix contraction, polymerized matrices were released from the culture surface after 1 hr and incubated 4 h. For stressed matrix contraction, polymerized matrices were cultured overnight DMEM/FBS, rinsed with DMEM and then released from the culture surface and incubated 1 h. Incubation medium contained FBS and PDGF as indicated.

At the end of the incubations, samples were fixed with 3% paraformaldehyde in phosphate buffered saline. Overall appearance of collagen matrices was recorded using an Epson 4870 photo scanner, and matrix diameter measured. Contraction was quantified as starting (12mm) – final matrix diameter.

Cell clustering and collagen matrix flattening

Methods for preparing collagen matrices with surface-seeded cells and measuring cell clustering and matrix flattening have been described previously [25]. Briefly, collagen

matrices (200 μ l, 1 mg/ml rat tail type I collagen) were polymerized 1 hr on 12 mm circular scores made on the bottom of 24-well plates. 2×10^4 cells in 1ml of DMEM/FBS were cultured on collagen matrices for 18 h at 37°C with 5% CO₂. At the end of the incubations, samples were fixed 10 min at room temperature with 3% paraformaldehyde in phosphate buffered saline and subjected to immunofluorescence analysis as indicated in the figure legends. The extent of cell clustering was determined using Hoechst-stained samples, defining a cell cluster as 10 nucleus within a 100 μ m square. Matrix flattening was measured microscopically by focusing from top to bottom in the center of the matrix. Flattening was quantified as height of control matrices (no cells, ~1.8 mm) minus height of experimental samples.

Statistical Analyses

Quantitative data are presented as averages \pm standard deviations. All experiments were carried out at least three times. Statistical analyses of the results (Student's T-Test, one-tailed distribution, two-sample unequal variance) was performed with Excel, and the results are shown in Supplemental Table 1.

Immunoblotting

Immunoblotting was performed as previously described [25, 26]. Protein concentration was determined using the Bradford protein assay kit (Expedeon Ltd., Harston, Cambridgeshire, UK). Equal protein samples were subjected to SDS-PAGE and electroblotted onto PVDF membrane (Millipore, Bedford, MA). After blocking with 3% BSA (fraction V)/0.1%TTBS (TBS, 20 mM Tris-Base and 150 mM NaCl, pH 7.5, plus Tween 20) for 1 h at room temperature (RT), the upper part of the blot was incubated with primary antibodies to MyoIIA/IIB and the lower part of the blot was incubated with primary antibodies to actin for 2 h at RT. HRP-conjugated goat-anti-mouse (1:5000) or anti-rabbit (1:5000) was used as secondary antibodies. Proteins were detected by Pierce ECL Western blotting substrate (Thermo Scientific, Rockford, IL).

Fluorescence microscopy

Cell preparations were processed for immunofluorescence staining as described previously [25, 26, 28]. Samples were fixed for 10 min at room temperature with 3% paraformaldehyde in PBS, blocked with 2% BSA (fraction V) /1% glycine in PBS for 30 min, and permeabilized for 10 min using 0.5 % Triton X-100 in PBS. Staining for actin was accomplished using Alexa fluor 488 phalloidin (1:500). Staining for nuclei was accomplished using propidium iodide (PI) (1:500) or Hoechst 33342 (2 μ g/ml). Staining for extracellular FN was accomplished by incubating samples with anti-FN (1:200) before permeabilization. At the end of the incubations, samples were mounted on glass slides with Fluoromount-G. Microscopic images were collected with a Nikon Elipse 400 fluorescent microscope using 10X/0.45 and 20X/0.75 Plan Apo and 40X/0.75 Plan Fluor infinity corrected objectives, Photometrics SenSys camera and MetaView acquisition software (Universal Imaging Corp.). Image processing was carried out using Photoshop (Adobe).

RESULTS

Fibroblast spreading on collagen matrices

Fibroblasts cultured in or on 3D collagen matrices become well spread with bipolar morphology and prominent actin stress fibers [17]. Figure 1 shows a representative immunoblot confirming substantial knockdown of MyoIIA and MyoIIB by isoform-specific siRNA gene silencing (Figure 1A) and the effects of silencing MyoIIA and MyoIIB on fibroblast spreading on collagen matrices (Fig 1B). Mock-treated cells cultured overnight on the surfaces of collagen matrices developed bipolar shape and actin stress fibers. Cell-cell interactions were observed. MyoIIA silencing by siRNA, although not complete, was sufficient to inhibit cells from undergoing these morphological changes. As a result, fibroblasts exhibited dendritic morphology without stress fibers (Fig 1B). The effect was specific for MyoIIA since cells treated with siRNA to silence MyoIIB showed morphological changes identical to mock-treated cells.

When fibroblasts interact with 3D collagen matrices or with soft 2D collagen-coated polyacrylamide substrates, the trajectory of initial cell spreading depends on the starting growth factor environment. Cells cultured in medium with serum exhibit Rho kinase-dependent global inward contractile force that attenuates cell extensions and causes a delay in cell spreading compared to Rac-activating conditions such as platelet-derived growth factor (PDGF) [27-30]. Figure 2 shows the difference in morphology of control cells (Mock) after spreading 4 hr on 3D collagen matrices. Fibroblasts in fetal bovine serum (FBS)-containing medium tended to be rounded with short extensions; fibroblasts in PDGF-containing medium were well spread with much longer, dendritic extensions. Silencing MyoIIA but not MyoIIB blocked the FBS effect and permitted cells to spread with dendritic morphology as rapidly as fibroblasts in PDGF-containing medium. Blebbistatin treatment had a similar effect as silencing MyoIIA.

Fibroblast contraction of collagen matrices

The results in Figures 1 and 2 suggested that for fibroblasts interacting with collagen matrices, serum-stimulated global inward contractile force and formation of actin stress fibers required MyoIIA but not MyoIIB. We then carried out experiments to determine the role of myosin II in the different iterations of the collagen matrix contraction model: stressed matrix contraction (SMC) stimulated by FBS and floating matrix contraction stimulated by PDGF and FBS. Figure 3 shows the appearance of collagen matrices after contraction and quantification of contraction as a function of blebbistatin concentration. Blebbistatin inhibited SMC-FBS and FMC-PDGF at 2.5 μM , but 10-20 μM blebbistatin was required to inhibit FBS-FMC [c.f. 22].

Figures 4A and 4B show experiments analyzing the role of myosin isoforms in stressed matrix contraction. As expected, serum but not PDGF stimulated FMC above basal conditions (BSA, absence of added growth factors) [27]. Silencing MyoIIA inhibited SMC as well as addition of 20 μM blebbistatin (Figure 4A). Parallel experiments demonstrated that silencing MyoIIB did not have a detectable effect on SMC (Figure 4B). Figures 4C and 4D show experiments to analyze the role of myosin isoforms in floating matrix contraction.

With FMC, both serum and PDGF stimulated contraction above basal levels. Silencing either MyoIIA or MyoIIB separately had no effect on FMC (Figure 4C). However, silencing both MyoIIA and MyoIIB together inhibited PDGF-FMC but not FBS-FMC (Figure 4D).

Given the close relationship between myosin II and mechanical tension, the observation that either MyoIIA or MyoIIB was sufficient for PDGF-FMC but that neither was required for FBS-FMC led us to test if there was a growth factor-dependent difference in the role of mechanosensing in FMC. Consistent with this possibility, Figure 5 shows that the focal adhesion kinase inhibitor PF-228 blocked PDGF-FMC but had no effect on FBS-FMC.

Collagen matrices with surface-seeded fibroblasts -- cell clustering and matrix flattening

The observation that FBS-FMC required neither MyoIIA or MyoIIB or both together was an unusual finding. We studied this observation further in a second, independent experimental model -- morphogenic clustering of cells seeded on the surfaces on collagen matrices [25, 31]. Clustering requires contraction-dependent fibronectin matrix assembly and can be blocked by Rho kinase inhibitor and blebbistatin. In addition to but independent of cell clustering, collagen matrices with surface-seeded fibroblasts undergo matrix flattening as the cells spread, a process that resembles collagen matrix contraction [32, 33]. Therefore, we also tested the effects of silencing MyoIIA and MyoIIB on FBS-stimulated matrix flattening.

Figures 6A and 6B show the appearance and quantification of FBS-stimulated cell clustering detected by distribution of cell nuclei and the effect of increasing concentrations of blebbistatin on clustering. Figure 6C shows matrix flattening in the same incubations, which was measured as decrease in matrix height. The results demonstrated that 2.5 μ M blebbistatin inhibited cell clustering, whereas 10 μ M blebbistatin was required to block matrix flattening. This difference in sensitivity to inhibition by blebbistatin was similar to the findings for FBS-FMC compared to FBS-SMC (Figure 3).

Figures 7 and 8 show parallel experiments testing the effects of siRNA silencing MyoIIA and MyoIIB on cell clustering, FN matrix assembly, and matrix flattening. Silencing MyoIIA but not MyoIIB inhibited cell clustering. However, silencing MyoIIA or MyoIIB or both together had no effect on matrix flattening (Figure 7). Silencing MyoIIA but not MyoIIB also caused a marked change in FN matrix assembly (Figure 8). Therefore, under conditions in which cell contraction-dependent FN matrix assembly was modified and cell clustering was inhibited by silencing MyoIIA, flattening of collagen matrices was unaffected by silencing MyoIIA or MyoIIB or both together.

DISCUSSION

The myosin II inhibitor blebbistatin was reported to inhibit fibroblast contraction of 3D collagen matrices depending on mechanical tension and growth factor environment [22-24]. In the current studies, we tested the effects of siRNA silencing of myosin IIA and myosin IIB to understand better the motor functions of myosin II isoforms in matrix contraction. As summarized in Table I and discussed below, the results suggested three different motor mechanisms: MyoIIA but not MyoIIB was required for stressed matrix contraction; either

MyoIIA or MyoIIB was sufficient for PDGF-stimulated floating matrix contraction; neither MyoIIA or MyoIIB appeared to play a role in FBS-stimulated floating matrix contraction.

Human fibroblasts cultured in collagen matrices initially exhibit diffuse organization of the actin cytoskeleton. Overall cell morphology tends to be rounded (Rho-dependent) in serum-containing medium and dendritic (Rac-dependent) in PDGF-containing medium [27-29]. If the matrices are attached, then as matrix reorganization occurs in serum or PDGF-containing medium, cells develop tension indicated by formation of actin stress fibers and reorganization of the initially punctuate distribution of activated $\beta 1$ integrins into focal adhesions that also contain vinculin [28].

In the current studies with siRNA-silenced human fibroblasts, we found that MyoIIA but not MyoIIB was required for formation of actin stress fibers and for global inward contractile force that resulted in rounded cell morphology in FBS-containing medium. We cannot exclude the possibility that absence of a MyoIIB effect occurred because of residual MyoIIB remaining after siRNA knockdown. However, others have reported a similar differential role of MyoIIA vs. MyoIIB in stress fiber formation and Rho-dependent rounding of cells interacting with 2D surfaces [23, 34-36].

Previous work established that stressed matrix contraction was a cell contraction process. Upon release of stressed matrices, cell shortening and stress fiber disruption occur [17] dependent on Rho kinase and myosin II [22, 37]. We found that MyoIIA was necessary for stressed matrix contraction. MyoIIA also was shown to be required for contraction of stressed fibrin matrices [35]. MyoIIA and MyoIIB both can contribute to cell tractional force [e.g., 38, 39]. However, differences in localization such as the enrichment of MyoIIA compared to MyoIIB in stress fibers [40] is consistent with the idea that cells can utilize these isoforms to generate force as part of different cellular functions. Examples include the differential role of MyoIIA vs. MyoIIB in stress fiber formation already discussed and the requirement for MyoIIA but not MyoIIB in focal adhesion formation by cells interacting with 2D surfaces [23, 35, 36]. Our findings suggest that collagen matrix contraction provides an additional case in which tractional force generated through different myosin isoforms is utilized differently.

Unlike stressed matrix contraction, floating collagen matrix contraction occurs with cells before tension develops (e.g., absence of stress fibers and focal adhesions). During matrix contraction, increased cell spreading rather than shortening occurs [17], contrary to what one would expect if cell contraction was the mechanism of matrix contraction. We found that either MyoIIA or MyoIIB was sufficient for PDGF-FMC since siRNA silencing of both myosin II isoforms was required to inhibit matrix contraction.

Differences in collagen matrix stiffness may explain the selective requirement for MyoIIA in stressed matrix contraction vs. the ability of MyoIIA or MyoIIB to function in PDGF-FMC. The stiffness of collagen matrices has been measured by many laboratories, typically using shear rheometry or uniaxial testing. Typical stiffness values reported for newly polymerized collagen matrices are 0.5-50 Pa (storage modulus) and 0.5-33 kPa (tensile modulus) [41]. Collagen density is the major determinant of stiffness, and the 1.5 mg/ml

matrices that we use measure ~5 Pa (storage modulus) initially but increase to ~700 Pa when the matrices are fully contracted [42]. Therefore, the matrix stiffness encountered by fibroblasts is much higher for stressed matrix contraction (matrices pre-contracted overnight before release from the culture surface) compared to floating matrix contraction (matrices newly polymerized). An attractive hypothesis to explain our findings is that tractional force exerted by cells through myosin IIA is required to remodel stiff matrices, whereas myosin IIA or myosin IIB provide sufficient force to remodel matrices that are soft, at least under PDGF-containing medium conditions. Possible mechanisms of collagen translocation would include rearward force exerted during cell protrusion [43, 44] and/or hand-over-hand cycles of cell protrusion and retraction [45, 46].

Other evidence supports the idea that matrix stiffness affects MyoIIA and MyoIIB differently. In 3D collagen matrices, cells respond to increased stiffness by increasing expression of MyoIIA but not MyoIIB [24]. On soft, polyacrylamide 2D surfaces, MyoIIA but not MyoIIB becomes organized with cellular stress fibers as substrate stiffness increases [47].

In contrast to PDGF-FMC, FBS-FMC appeared to be independent of MyoIIA and MyoIIB since blocking either myosin isoform alone or both together did not inhibit matrix contraction. Other data also shows that PDGF-FMC and FBS-FMC depend on different mechanisms. Rho kinase was reported to be the Rho effector required for PDGF-FMC, whereas mDia1 is the Rho effector required for FBS-FMC [22, 26]. Also, in the current studies we found that the focal adhesion kinase inhibitor blocked PDGF-FMC but not FBS-FMC.

The possibility that fibroblasts might utilize a MyoIIA and MyoIIB-independent motor mechanism to translocate collagen during FBS-FMC was surprising so we tested a second, experimental model -- morphogenic cell clustering, which occurs when fibroblasts are cultured on the surfaces of collagen matrices. Morphogenic cell clustering requires fibronectin matrix assembly [25], which takes place at the cell surface through a cell-contraction dependent mechanism [48-50]. We found that blocking MyoIIA but not MyoIIB prevented fibronectin matrix assembly that was observed during cell clustering but, similar to the findings for FBS-FMC, silencing either MyoIIA or MyoIIB or both together did not inhibit flattening of collagen matrices, a contraction-like process that occurs as fibroblasts surface-seeded on collagen matrices undergo cell spreading [32, 33]. Although others have reported that MyoIIB but not MyoIIA played a role in FBS-FMC by mouse fibroblasts [46], those studies were carried out with lower cell concentrations than we use, conditions that would permit cell spreading to precede matrix contraction.

One might argue theoretically that even less force is required for FBS-FMC compared to PDGF-FMC so that residual MyoIIA and/or MyoIIB remaining after siRNA knockdown would be sufficient to mediate contraction. This interpretation seems unlikely since the concentration of blebbistatin required to inhibit FBS-FMC was much higher than the concentration of blebbistatin required to inhibit PDGF-FMC. A higher blebbistatin concentration also was required to inhibit contraction of attached collagen matrices during cell clustering. Although blebbistatin is specific for myosin II amongst the different types of

myosin [51], this drug also has been shown to inhibit some myosin-independent cell motile processes [52]. Therefore, a different motor mechanism besides myosin II might be involved in FBS-FMC. Possibilities worth investigating in the future include a role for kinesin family members that could generate force through microtubule depolymerization [53, 54] or a role for a dynein-dependent reward transport mechanism [55, 56]. That microtubules are required for FBS-FMC was shown before [57, 58], but the role of microtubules in matrix contraction has never been clear. Once cell tension develops, disrupting microtubules stimulates rather than inhibits matrix contraction [59].

Ironically, although the motor mechanism remains to be determined, serum-dependent floating matrix contraction is the longest studied and original model introduced to mimic wound contraction in vitro [18]. As described in the Introduction, two different mechanisms of wound closure have been described that occur by different mechanisms depending on mechanical tension within the wound. We suggest that FBS-stressed matrix contraction resembles the myofibroblast/granulation tissue mechanism, which depends on mechanosensing and cell contraction; and that FBS-floating matrix contraction resembles the fibroblast/wound margin mechanism, which occurs independently of mechanosensing and cell contraction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. We tested the role of myosin II isoforms in fibroblast interactions with 3D collagen matrices.
2. MyoIIA was required for stressed matrix contraction and morphogenic cell clustering.
3. Either MyoIIA or MyoIIB was sufficient for PDGF-floating matrix contraction.
4. Neither MyoIIA or MyoIIB was necessary for serum-floatin matrix contraction.
5. Motor mechanisms for collagen contraction depend on cell tension and growth factor stimulation.

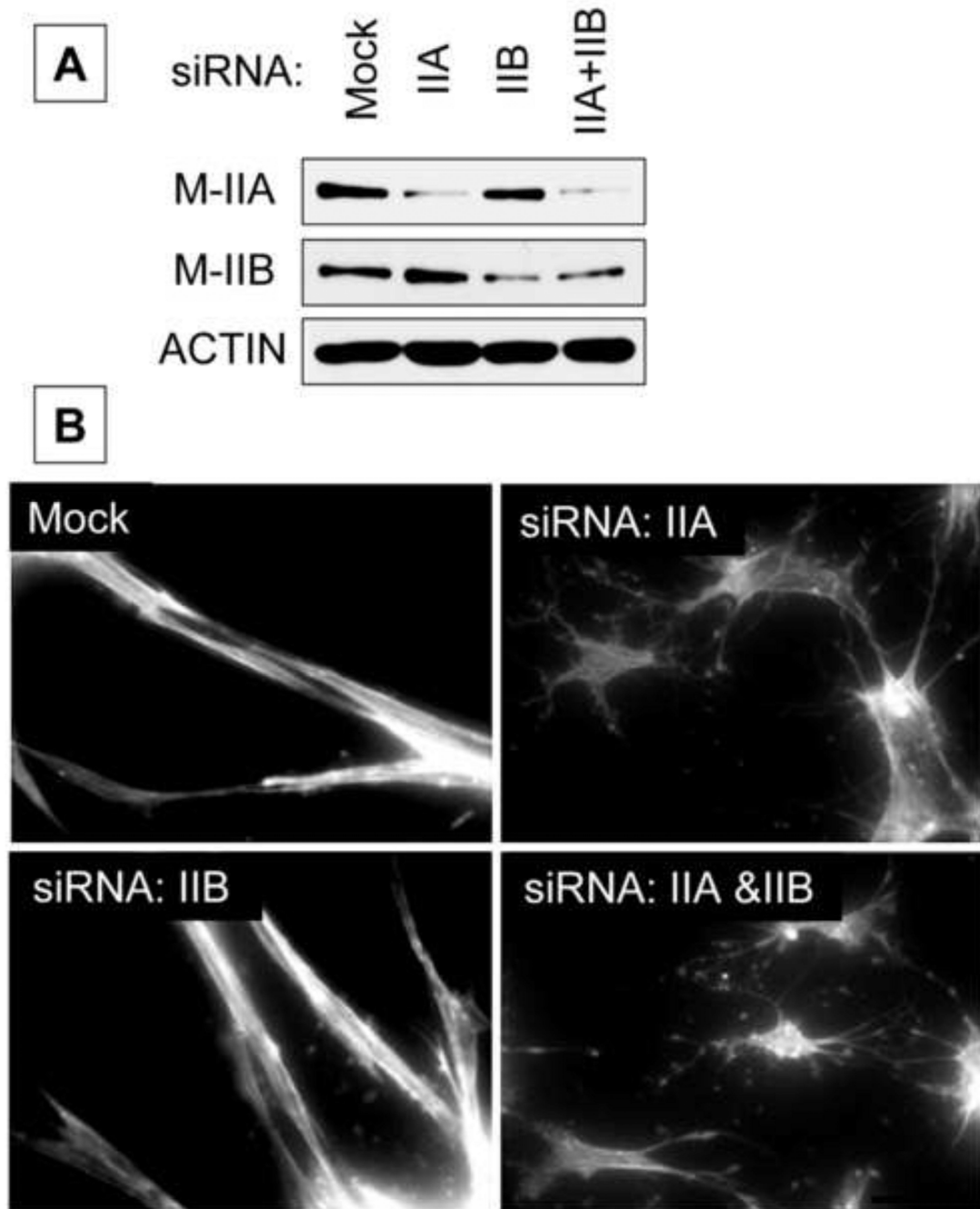


Figure 1. Silencing MyoIIA prevents fibroblasts from spreading in bipolar shape with actin stress fibers during overnight culture

A) Representative immunoblotting results showing MyoIIA and MyoIIB expression after treatment of cells by isoform-specific siRNA gene silencing. B) Mock and myosin II isoform-silenced cells as indicated were cultured 18 h on collagen matrices in DMEM/FBS. At the end of the incubations, samples were fixed and stained for actin. Silencing MyoIIA but not MyoIIB inhibited cells from developing actin stress fibers. The effect was specific for MyoIIA since cells treated with siRNA to silence MyoIIB showed morphological changes identical to mock-treated cells. Bar = 50 μ m.

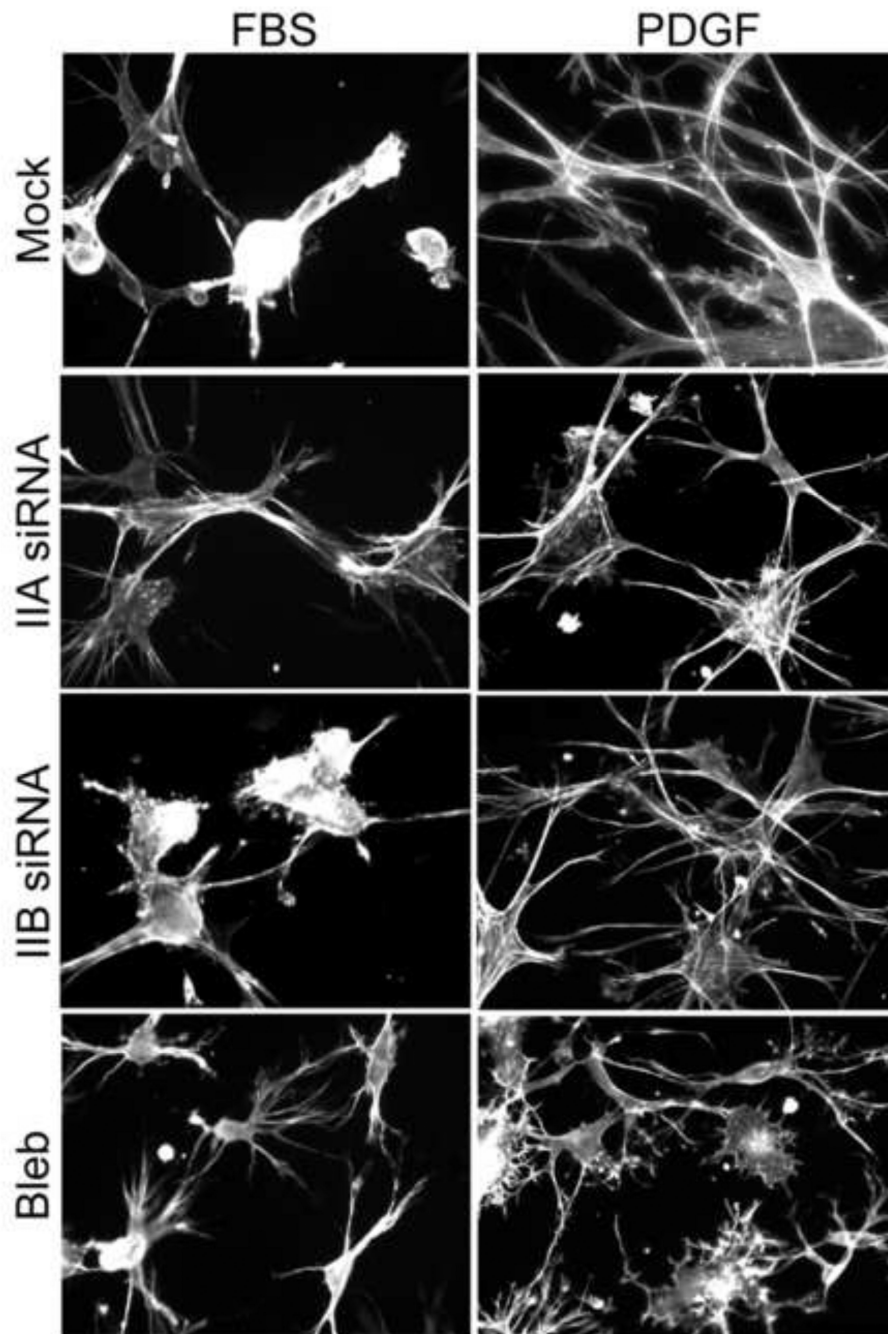


Figure 2. Silencing MyoIIA blocks serum-stimulated global inward cell contraction that attenuates initial fibroblast spreading
 Mock and myosin II isoform-silenced cells as indicated and untreated cells plus 20 μ M blebbistatin were cultured on polymerized collagen matrices 4 h in DMEM/FBS and DMEM/PDGF as shown. At the end of the incubations, samples were fixed and stained for actin. Blebbistatin treatment or silencing MyoIIA but not MyoIIB blocked FBS-stimulated round cell morphology and permitted cells to spread with dendritic morphology as rapidly as in PDGF-containing medium. Bar = 50 μ m.

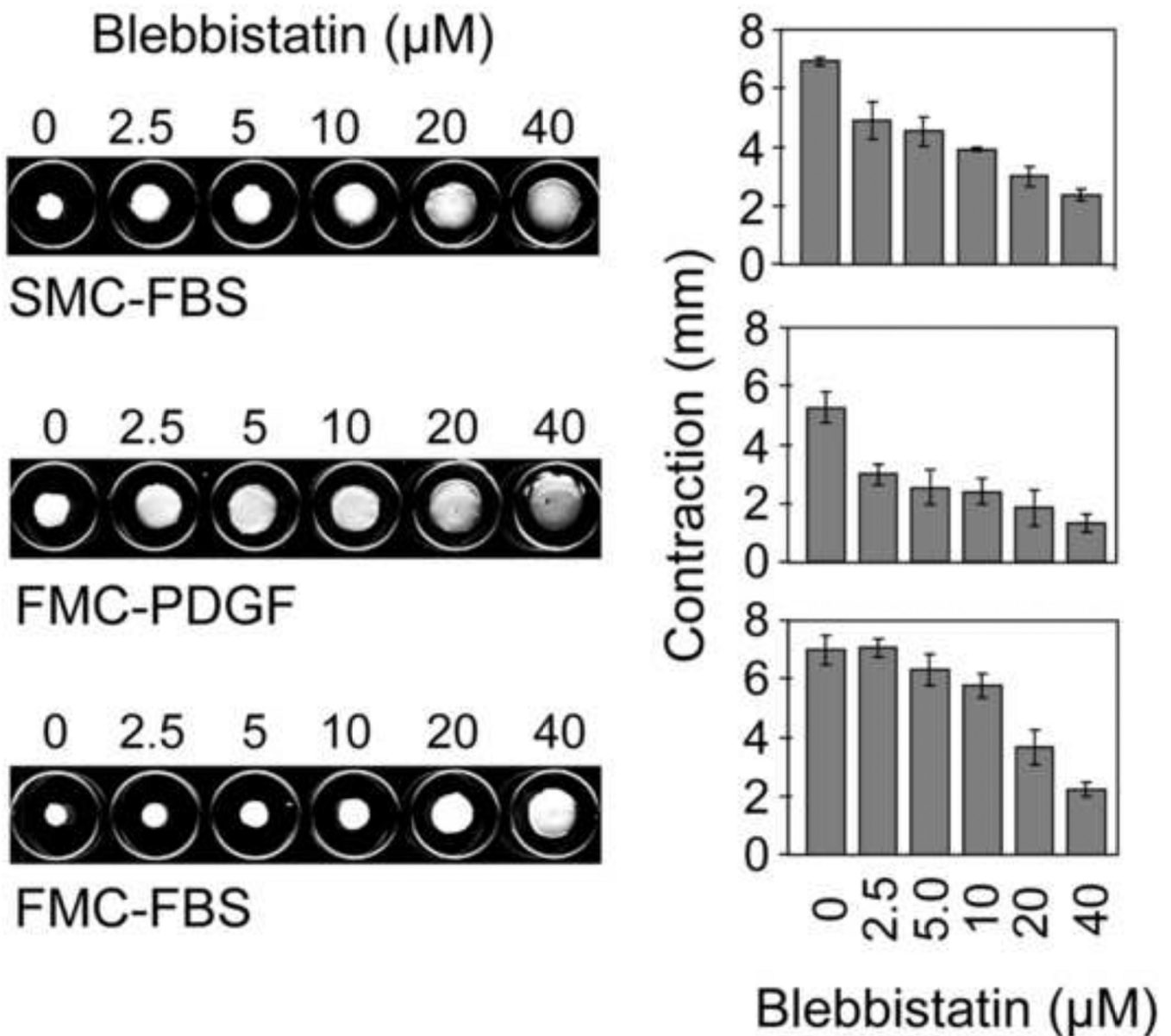


Figure 3. Differential effects of blebbistatin on fibroblast contraction of stressed and floating collagen matrices

Stressed collagen matrix contraction was carried out in DMEM/FBS. Floating collagen matrix contraction was carried out in DMEM/PDGF and DMEM/FBS. Blebbistatin as indicated was added to the incubations at the times that the matrices were released from the underlying culture dishes to initiate contraction -- 18 hr for SMC and 1 hr for FMC. Data shown are the appearance of collagen matrices from a representative experiment and the averages \pm SD of quantitative contraction measurements (starting – final matrix diameter) for triplicate samples. Blebbistatin inhibited SMC-FBS and FMC-PDGF at 2.5 μM , but 10-20 μM blebbistatin was required to inhibit FBS-FMC.

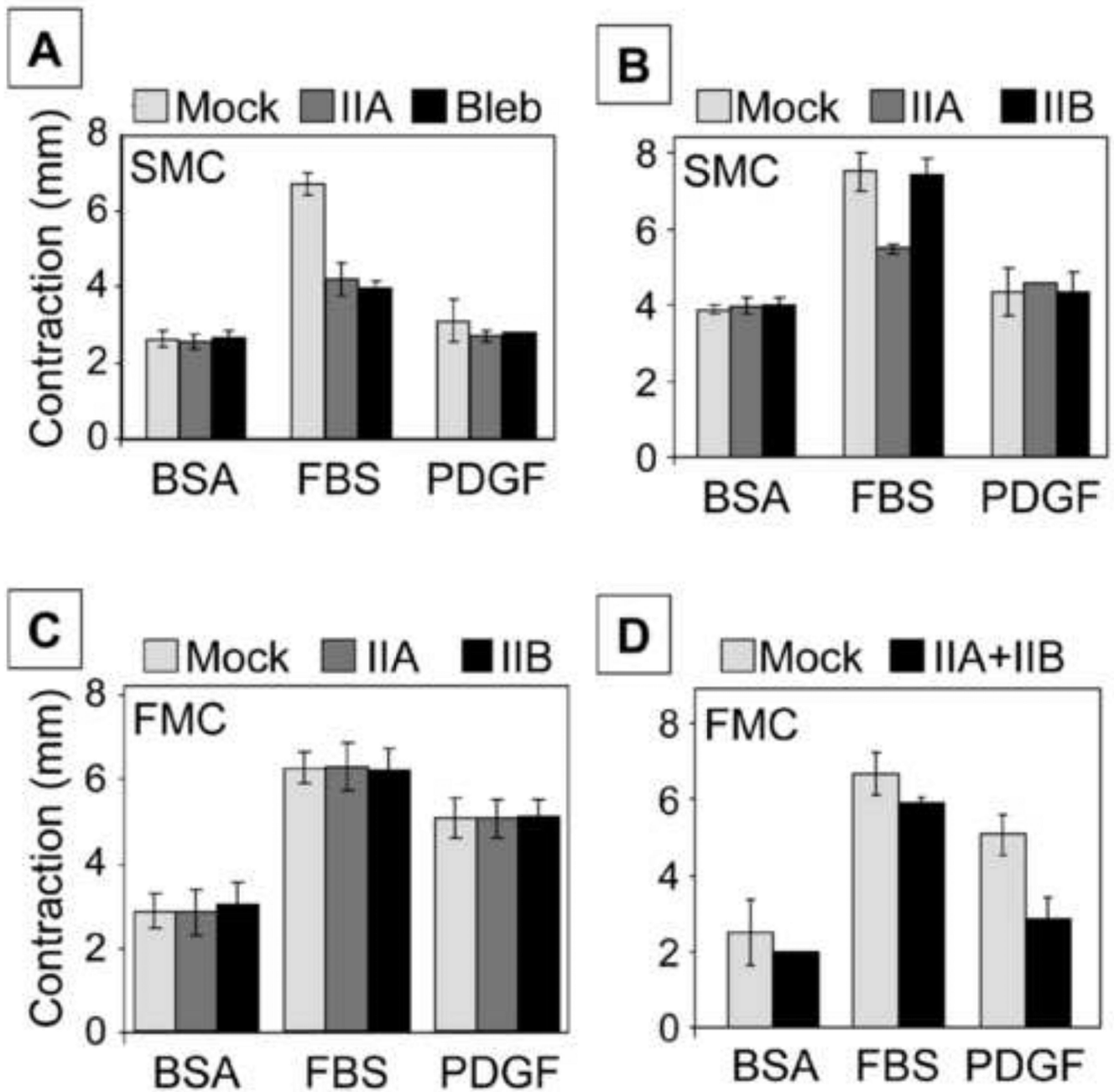


Figure 4. Differential effects of silencing MyoIIA and IIB on floating and stressed collagen matrix contraction

A,B) Stressed matrix contraction was carried out with mock and myosin II isoform-silenced cells as indicated and untreated cells plus 20 μ M blebbistatin (bleb) in DMEM/BSA, DMEM/FBS, and DMEM/PDGF as shown. C,D). Floating collagen matrix contraction was carried out with mock and myosin II isoform-silenced cells as indicated in DMEM/BSA, DMEM/FBS, and DMEM/PDGF as shown. Data presented are the averages \pm SD of quantitative contraction measurements (starting – final matrix diameter) for triplicate samples. Complete set of p values are shown in supplemental Table 1. Silencing MyoIIA

inhibited SMC as well as addition of 20 μ M blebbistatin, whereas silencing MyoIIB did not have a detectable effect. Silencing either MyoIIA or MyoIIB separately had no effect on FMC; silencing both together inhibited PDGF-FMC but not FBS-FMC.

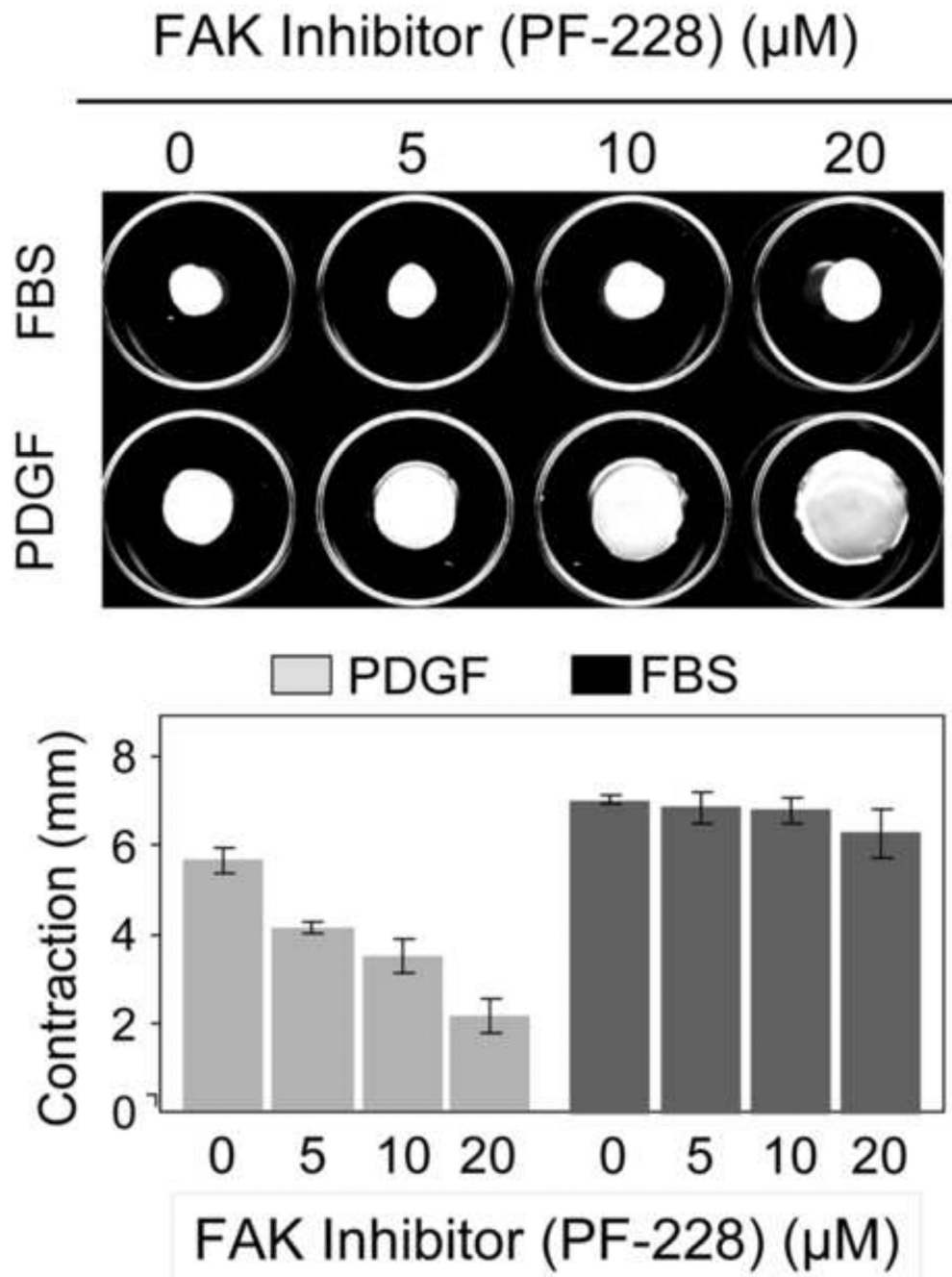


Figure 5. Differential effects of FAK inhibitor on fibroblast contraction of floating collagen matrices in PDGF and FBS-containing medium

Floating collagen matrix contraction was carried out in DMEM/PDGF and DMEM/FBS. FAK inhibitor PF-22 as indicated was added to the incubations at the time the matrices were released from the underlying culture dish to initiate contraction. Data shown are the appearance of collagen matrices from a representative experiment and the averages \pm SD of quantitative contraction measurements (starting – final matrix diameter) for triplicate samples. FAK inhibitor PF-228 blocked PDGF-FMC but had no effect on FBS-FMC.

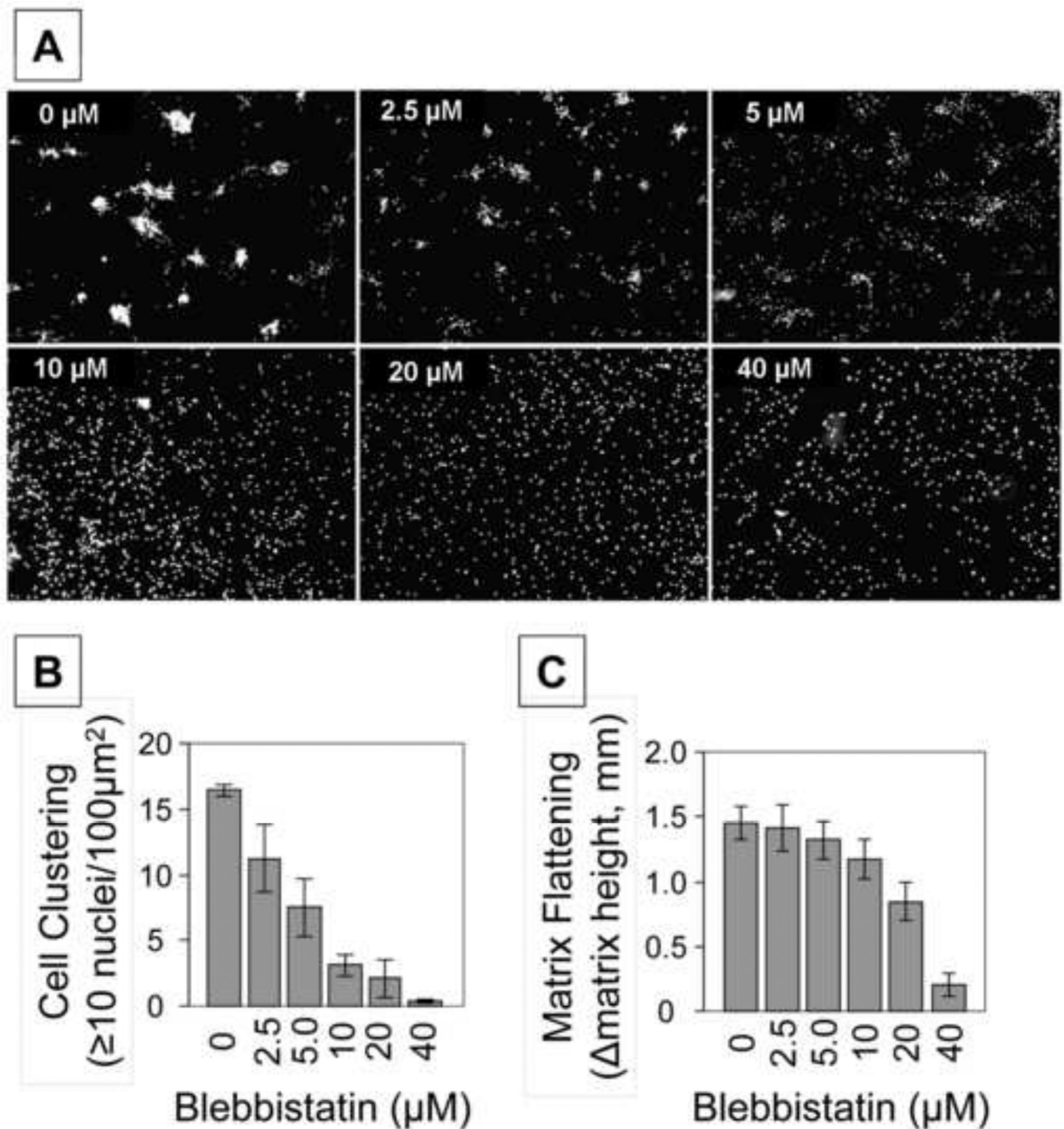


Figure 6. Differential effects of blebbistatin on fibroblast clustering and contraction of attached collagen matrices

A,B) Fibroblast clustering on collagen matrices was carried out in DMEM/FBS with blebbistatin added to the incubations as indicated. At the end of the incubations, samples were fixed and stained for cell nuclei (Hoechst). Data shown are the appearance of cell clusters from a representative experiment (Bar = 200 μm) and the averages \pm SD of quantitative clustering measurements (cell cluster = 10 nucleus in $100 \mu\text{m}^2$) for triplicate samples. C) Using the same samples as in A,B, contraction of attached collagen matrices was determined as the difference in height between control matrices (no cells) and

experimental matrices. Data shown are the averages \pm SD for triplicate samples. Blebbistatin inhibited cell clustering at 2.5-5 μ M, whereas 10-20 μ M blebbistatin was required to block matrix flattening.

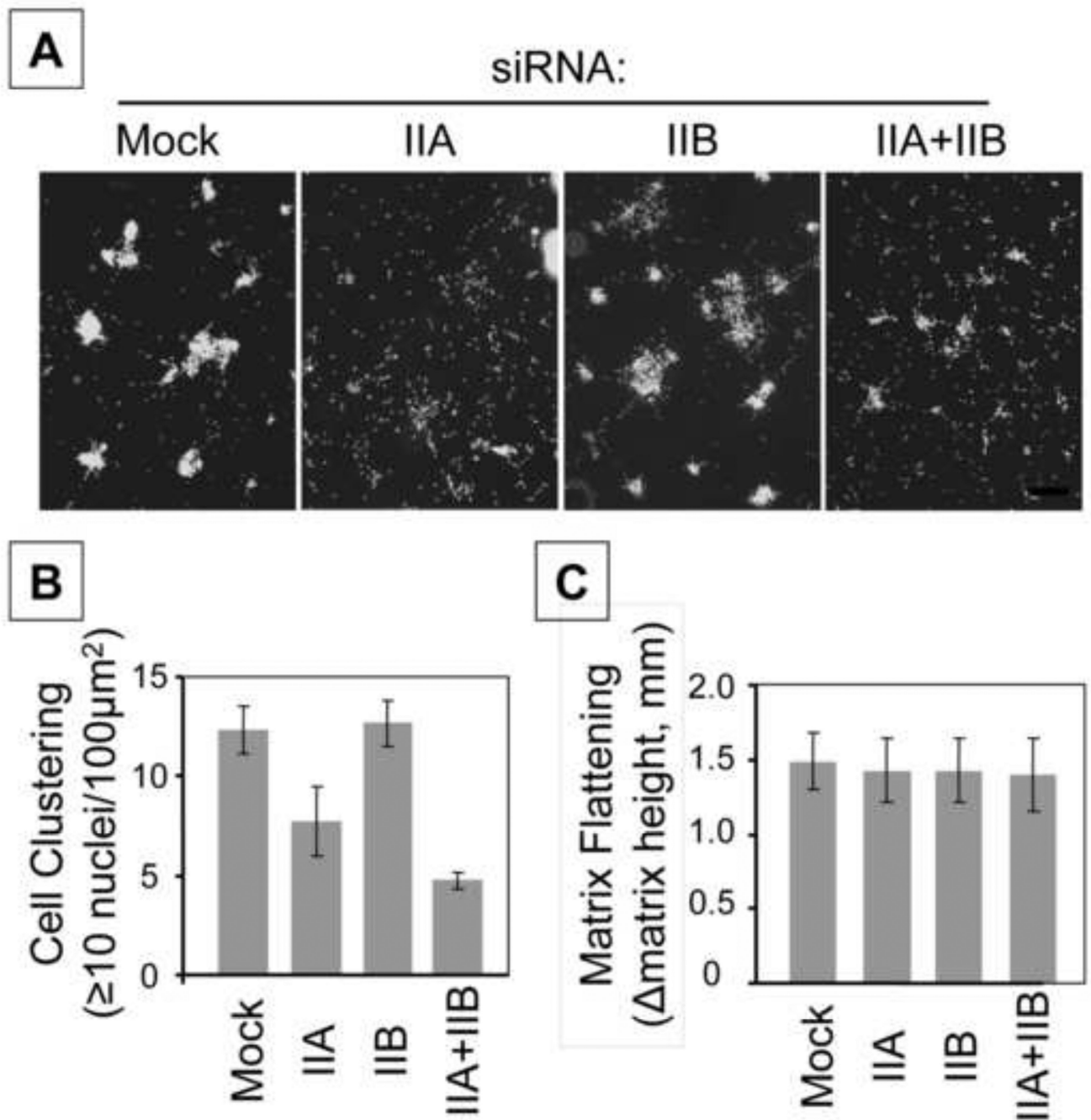


Figure 7. Silencing MyoIIA blocks cell clustering on collagen matrices not attached gel contraction

A,B) Cell clustering was carried out with mock and myosin II isoform-silenced cells as indicated. At the end of the incubations, samples were fixed and stained for cell nuclei (Hoechst). Data shown are the appearance of cell clusters from a representative experiment (Bar = $200\mu\text{m}$) and the averages \pm SD of quantitative clustering measurements (cell cluster = 10 nucleus in $100\mu\text{m}^2$) for triplicate samples. C) Using the same samples as in A,B, contraction of attached collagen matrices was determined as the difference in height between control matrices (no cells) and experimental matrices. Data shown are the averages

± SD for triplicate samples. Silencing MyoIIA inhibited cell clustering, whereas silencing MyoIIA or MyoIIB or both together had no effect on matrix flattening.

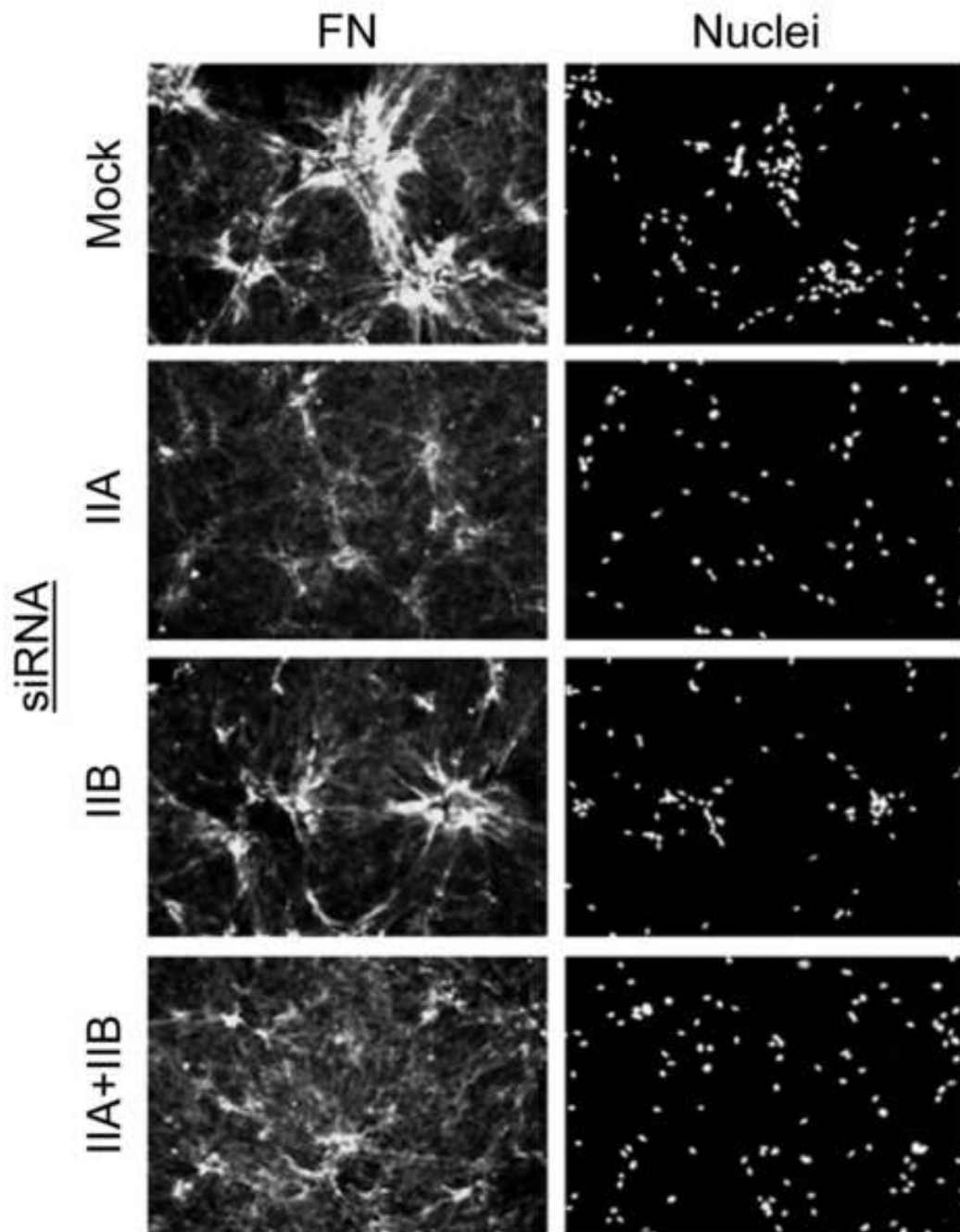


Figure 8. Silencing MyoIIA modifies fibronectin fibrillar matrix organization

Samples are the same as in Figure 7 except stained for extracellular fibronectin (FN) and cell nuclei (Hoechst). Silencing MyoIIA but not MyoIIB caused a marked change in FN matrix assembly. Bar = 100 μ m.

Table 1Summary of myosin II isoform functions in collagen matrix contraction¹

| 3D Collagen Matrix Model | Myosin II Isoform | Inhibition by Blebbistatin | Rho Effector |
|--|--------------------|----------------------------|--------------------|
| FBS -- Stressed Matrix Contraction | IIA | 2.5 – 5.0 μ M | Rho Kinase [22,37] |
| PDGF -- Floating Matrix Contraction | IIA or IIB | 2.5 – 5.0 μ M | Rho Kinase [22,26] |
| FBS -- Floating Matrix Contraction | Neither IIA or IIB | 10 – 20 μ M | mDial [26] |
| FBS -- Cell Clustering with Cells on Top of Matrix | IIA | 2.5 – 5.0 μ M | Rho Kinase [25] |
| FBS -- Matrix Flattening with Cells on Top of Matrix | Neither IIA or IIB | 20 – 40 μ M | not tested |

¹See text for details