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Cadherins, RhoA, and Rac1 Are Differentially Required for Stretch-Mediated Proliferation in Endothelial Versus Smooth Muscle Cells

Wendy F. Liu, Celeste M. Nelson, John L. Tan, Christopher S. Chen

Abstract—Abnormal mechanical forces can trigger aberrant proliferation of endothelial and smooth muscle cells, as observed in the progression of vascular diseases such as atherosclerosis. It has been previously shown that cells can sense physical forces such as stretch through adhesions to the extracellular matrix. Here, we set out to examine whether cell–cell adhesions are also involved in transducing mechanical stretch into a proliferative response. We found that both endothelial and smooth muscle cells exhibited an increase in proliferation in response to stretch. Using micropatterning to isolate the role of cell–cell adhesion from cell–extracellular matrix adhesion, we demonstrate that endothelial cells required cell–cell contact and vascular endothelial cadherin engagement to transduce stretch into proliferative signals. In contrast, smooth muscle cells responded to stretch without contact to neighboring cells. We further show that stretch stimulated Rac1 activity in endothelial cells, whereas RhoA was activated by stretch in smooth muscle cells. Blocking Rac1 signaling by pharmacological or adenoviral reagents abrogated the proliferative response to stretch in endothelial cells but not in smooth muscle cells. Conversely, blocking RhoA completely inhibited the proliferative response in smooth muscle cells but not in endothelial cells. Together, these data suggest that vascular endothelial cadherin has an important role in mechanotransduction and that endothelial and smooth muscle cells use different mechanisms to respond to stretch. (*Circ Res.* 2007;101:e44–e52.)

Key Words: atherosclerosis ■ cadherin ■ cell cycle progression ■ mechanical stretch
■ mechanotransduction ■ Rho

Mechanical forces regulate the function of many tissue types. In the vascular system, hemodynamic forces such as stretch and shear stress are required to maintain the structural organization and functional activity of endothelial and smooth muscle cells that form the vessel wall.¹ In the context of tissue engineering, applied forces are important for creating functional vessels that can withstand the mechanical stresses present in vivo.² Unlike shear stress, which appears to predominantly act on endothelial cells, mechanical stretch associated with sustained hypertension is experienced by both the endothelium and underlying smooth muscle layers. Aberrant levels of stretch induce inflammation and cell proliferation, leading to thickening of the vessel wall, which further heightens vascular resistance, blood pressure, and the vicious cycle of vascular disease progression.^{3,4} Stretch has been reported to induce proliferation in both endothelial and smooth muscle cells, but the molecular mechanisms of this response remain poorly understood.

Numerous surface receptors including stretch-activated ion channels and receptor tyrosine kinases have been implicated in transducing stretch into proliferative responses.^{5,6} The

integrin receptors that mediate cell adhesion to the extracellular matrix (ECM) are thought to be the most significant players because these receptors mechanically link cells to their surrounding environment and also play a central role in proliferative signaling.⁷ Stretch induces proliferation in cells attached to the ECM proteins fibronectin, collagen, or vitronectin, but not elastin or laminin.⁸ Furthermore, stretch activates integrins,⁹ and stretch-mediated proliferative signaling requires the phosphorylation of the integrin-associated signaling protein focal adhesion kinase.¹⁰

Although several investigators have demonstrated that cell–ECM adhesions are critical in the transduction of mechanical stretch into biochemical signals, cell–cell adhesion may also play an important role. Cell–cell junctions, and in particular the adherens junctions, can withstand substantial forces between neighboring cells and transmit force to the cytoskeleton.¹¹ Adherens junctions are composed of cadherin molecules that bind homophilically to cadherins on neighboring cells and, like integrin-mediated adhesions, are linked intracellularly to the actin cytoskeleton through a number of scaffolding proteins. It has recently been demonstrated that the endothelial-specific

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From the Department of Biomedical Engineering (W.F.L., C.M.N., J.L.T.), Johns Hopkins University School of Medicine, Baltimore, Md; Department of Bioengineering (W.F.L., C.S.C.), University of Pennsylvania, Philadelphia.

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Correspondence to Christopher S. Chen, Skirkanich Hall, Suite 510, 210 South 33rd St, Philadelphia, PA 19104. E-mail chrischen@seas.upenn.edu
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cadherin, vascular endothelial (VE)-cadherin, as well as the cell–cell adhesion receptor, platelet endothelial cell adhesion molecule-1, are required for endothelial alignment and gene expression in response to shear stress.^{12,13} However, it remains unclear whether this requirement is restricted to the endothelial response to shear or whether cell–cell junctions are also involved in the transduction of mechanical stimuli such as stretch. Moreover, the role of cell–cell junctions in mechanotransduction in other vascular cell types such as smooth muscle cells is not well described.

Intracellularly, the small GTPases RhoA and Rac1, which were originally described as regulators of stress fibers and lamellipodia, respectively, have been shown to have important roles in the proliferative response to applied mechanical stress. Stretch induces the translocation of RhoA to the cell membrane, and inhibition of RhoA signaling impairs stretch-induced extracellular signal-regulated kinase activity and proliferation.^{14,15} Schwartz and colleagues demonstrated that Rac1 activity is downregulated within 5 minutes of the onset of stretch and that constitutive activation of Rac1 blocked stretch-induced stress fiber formation.¹⁶ In other studies, blocking Rac1 activity prevented phosphorylation of p38 and extracellular signal-regulated kinase by stretch.¹⁷ Because both RhoA and Rac1 activity are also regulated by cadherin engagement,^{18–20} mechanical force could modulate these proliferative pathways, in part, through the involvement of cadherin receptors.

In this study, we examined whether cadherin-mediated cell–cell contact is involved in sensing stretch in vascular cells. We observed that confluent cultures of endothelial and smooth muscle cells exhibited an increase in proliferation in response to stretch. Using micropatterning to isolate the role of cell–cell from cell–matrix adhesion, we found that cell–cell adhesion is required for endothelial but not smooth muscle cell proliferation in response to stretch. Blocking the engagement of VE-cadherin abrogated the stretch-mediated response in the endothelial cells. Furthermore, endothelial cells required Rac1 activity, whereas smooth muscle cells required RhoA activity, to respond to stretch. Together, these data demonstrate a novel role for cadherins in mechanotransduction and suggest a mechanism by which endothelial and smooth muscle cells sense forces differently.

Materials and Methods

Cell Culture and Reagents

Bovine pulmonary artery endothelial cells (VEC Technologies) and bovine pulmonary artery smooth muscle cells (a gift from D. Ingber, Harvard University, Cambridge, Mass) were maintained as previously described.²¹ Reagents were obtained as follows: Y27632 (Calbiochem), NSC23766 (Calbiochem), BV9 (Cell Sciences), polyclonal anti-VE-cadherin (Alexis), phalloidin (TRITC labeled; Sigma), lucifer yellow (Invitrogen), and palmitoleic acid (Sigma). RhoA-N19 adenovirus was generated using the AdEasy XL system (Stratagene) as previously described.¹⁹ Rac1-N17 adenovirus was a generous gift from A. Ridley (University College, London, England). β 2-Chimerin adenovirus was a generous gift from M. Kazanietz (University of Pennsylvania, Philadelphia).

Preparation of Micropatterned and Unpatterned Flexible Substrates

Micropatterned substrates were prepared by adapting previously developed methods to microcontact print fibronectin.²² Briefly,

stamps were fabricated by casting poly(dimethyl siloxane) (Dow Corning) on a photolithographically generated master pattern. Stamps were coated with 50 μ g/mL fibronectin (BD Biosciences) for at least 1 hour and then thoroughly dried. Protein was transferred onto surface-oxidized BioFlex culture plates (Flexcell International). The remaining unstamped regions were blocked by submerging the substrate in 0.2% wt/vol Pluronic F127 (BASF) for at least 1 hour. Unpatterned substrates were prepared either by the identical stamping procedure using a flat stamp or by adsorbing 50 μ g/mL fibronectin to the center (\approx 5-cm² circular region) of each well, where stretch was most uniformly applied. Cellular responses with printed and adsorbed fibronectin were indistinguishable (data not shown). The remainder of the well was blocked with 0.2% wt/vol Pluronic F127 for at least 1 hour.

Stretch Procedures

Synchronized cells were seeded onto BioFlex culture plates that were either uniformly coated or micropatterned with fibronectin for at least 8 hours and then equibiaxially stretched using the Flexercell Tension Plus baseplate (Flexcell International) connected to a house vacuum source (15 pounds per square inch) to achieve up to 40% strain. Unless otherwise indicated, we stretched cells with 40% strain to ensure robust stimulation of proliferation. For cyclic stretch experiments, cells were stretched with 20% strain at 1 Hz using the Flexercell Tension Plus system (Flexcell International). Stretch was applied for the duration of the experiments.

Proliferation Assays

Synchronization in the G₀ phase of the cell cycle was achieved by holding cells at confluence for 2 days. Cells were then seeded into unpatterned or patterned flexible culture wells in full-serum media. 5-Bromo-2'-deoxyuridine (BrdUrd) (GE Healthcare) was added to the media 8 hours after seeding, and cells were then stretched for an additional 24 hours. Incorporation of BrdUrd was detected using a monoclonal antibody against BrdUrd (GE Healthcare) according to the instructions of the manufacturer. Unless otherwise indicated, at least 300 cells were examined across a minimum of 3 experiments for all conditions reported. Statistical analysis was performed by Student's *t* test, 1-way ANOVA, or 2-way ANOVA. Tukey's honestly significant difference (HSD) test was used for pairwise comparisons.

Microscopy, Immunofluorescence, and Image Acquisition

Images of fixed samples were acquired using a TE200 epifluorescence microscope (Nikon) equipped with a mercury arc lamp, a \times 10 Plan Fluor NA 0.3 dry lens, a \times 60 Plan Apo NA 1.4 oil immersion lens, Spot camera, and software (Diagnostic Instruments). For immunostaining, cells were fixed in formaldehyde and permeabilized with 0.05% triton in PBS, blocked with goat serum (Invitrogen) in PBS, and incubated in primary and AlexaFluor 594- or 488-conjugated secondary antibodies (Molecular Probes). In some cases, image levels were adjusted using Adobe Photoshop.

RhoA and Rac1 Activity Assays

Cells were stretched for 6 hours in full-serum media, lysed, and assayed for GTP-loaded RhoA and Rac1 by pull-down assay as described previously.^{23,24} Rhotekin-PBD beads were obtained from Millipore or Cytoskeleton Inc. Pak1-PBD beads were obtained from Millipore and also made using GST-tagged recombinant Pak1-PBD produced in BL21 cells containing the pGEX-PBD vector (a gift from L. Romer, Johns Hopkins University, Baltimore, Md). Proteins were separated by denaturing SDS-PAGE, electroblotted onto poly(vinylidene difluoride), immunoblotted with specific primary antibodies, and detected with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) and SuperSignal West Dura chemiluminescent substrate (Pierce Chemical). Densitometric analysis was performed using a Versadoc imaging system equipped with Quantity One software (Bio-Rad). Quantitative data were

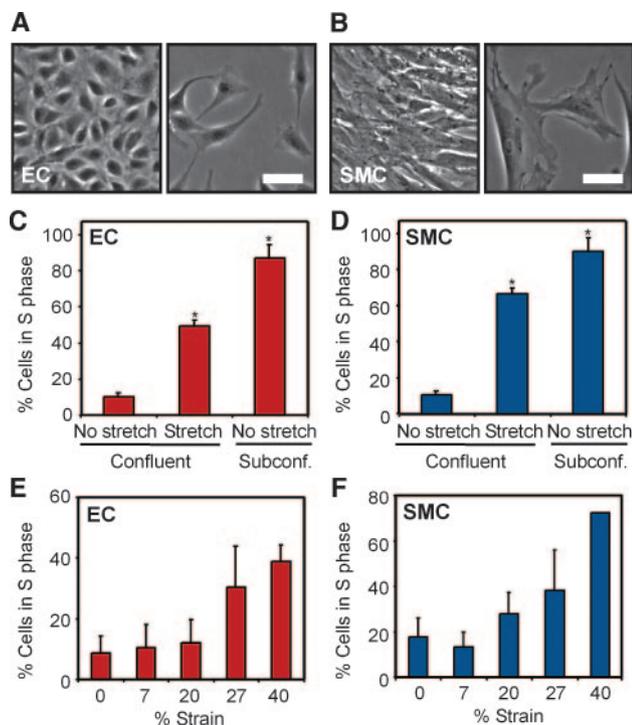


Figure 1. Stretch-mediated proliferation depends on cell seeding density for endothelial (EC) and smooth muscle (SMC) cells. A and B, Phase-contrast images of endothelial (A) and smooth muscle (B) cells seeded at confluence (10^5 cells/cm², left) and subconfluence (10^4 cells/cm², right). C and D, Graphs of percentages of stretched and unstretched endothelial (C) and smooth muscle (D) cells entering the S phase. E and F, Graph of percentages of endothelial (E) and smooth muscle (F) cells entering the S phase when stretched to the indicated magnitudes. Scale bars=50 μ m. Error bars indicate SE of 3 independent experiments. * $P < 0.05$ when compared with no stretch, confluent cells as calculated by Student's *t* test.

averaged across 3 separate experiments, and statistical differences were analyzed by Student's *t* test.

Results

Stretch Stimulates Proliferation in Confluent Endothelial and Smooth Muscle Cells

To first explore whether mechanical stretch induced proliferation in vascular cells, we examined entry into the S phase of bovine pulmonary artery endothelial and smooth muscle cells in response to stretch. Cells were synchronized in G₀ by culture to confluence and reseeded at 10^5 cells/cm², a density comparable to a confluent monolayer (Figure 1A and 1B). Cells were incubated for at least 8 hours to allow cells to form stable adhesions and then stretched for 24 hours. We found that stretch significantly increased levels of proliferation in both endothelial cells and smooth muscle cells, as measured by incorporation of BrdUrd (Figure 1C and 1D). Similarly, cyclic stretch increased proliferation when compared with unstretched controls (Figure 1A and 1B in the online data supplement). We also found that in both cell types, stretch increased proliferation in a magnitude-dependent manner, with the highest levels of proliferation observed in cells that were stretched with the highest magnitudes of strain (Figure 1E and 1F).

These data confirmed that stretch stimulates proliferation in vascular cells, as others have observed.^{25,26} However, it

was not clear based on this experiment whether cells required cell–cell contact to transduce mechanical forces or whether cell–ECM adhesion was sufficient. To address this question, we examined proliferation of cells seeded at subconfluence (10^4 cells/cm²), where cells have fewer contacts with neighboring cells. However, endothelial and smooth muscle cells seeded at low densities proliferated maximally even without stretch (Figure 1C and 1D), and, as such, stretch did not further stimulate proliferation. Phase-contrast images of endothelial and smooth muscle cells illustrate that decreasing cell density not only reduced cell–cell contact but also increased the area of cell–ECM adhesion (Figure 1A and 1B), which is known to directly stimulate cell proliferation.^{27,28}

Cell–Cell Contact Is Required for Endothelial Cells, but Not Smooth Muscle Cells, to Transduce Stretch

To specifically examine whether cell–cell contact was required for stretch-mediated proliferation, we used a micropatterning approach to vary cell–cell adhesion while holding the degree of cell spreading constant. Flexible substrates were patterned with fibronectin-coated islands surrounded by Pluronic F127, a nonadhesive polymer that prevents the adhesion of proteins and cells. On these substrates, cells adhere to fibronectin-coated regions and spread to fill the island without spreading onto Pluronic-coated regions. To mimic the spread area of confluent cells, but remove the presence of cell–cell contact, endothelial and smooth muscle cells were seeded onto 625- μ m² squares, such that only a single cell typically attached per island, and were compared with cells with cell–cell contact (Figure 2A and 2B). To directly compare cells with and without cell–cell contact using this system, both cell types were also seeded on large micropatterned islands (10 000- μ m² squares), such that many cells occupied a single island and formed a discrete monolayer. We also compared cells in these 2 micropatterned conditions, with cells forming monolayers on unpatterned substrates uniformly coated with ECM (Figure 2A and 2B). We confirmed that in the absence of stretch, cells in all conditions spread to similar areas (600- to 700- μ m²), as measured by outlining the cells in phase-contrast images (Figure 2C and 2D). Cells were seeded for 8 hours in these different conditions, stretched for 24 hours, fixed, and analyzed for proliferation. In endothelial cells, single and groups of cells exhibited low proliferation in the unstretched condition. With stretch, groups of endothelial cells patterned onto large islands or seeded as monolayers both increased proliferation with stretch. In contrast, the levels of proliferation of single endothelial cells patterned on small islands were equally low with and without stretch (Figure 2E). In smooth muscle cells, single cells, groups of cells, and confluent cells also exhibited low proliferation in the unstretched condition. However, in contrast to our observations in endothelial cells, smooth muscle cells in all seeding conditions, including single cells, increased proliferation on stretch (Figure 2F). This differential response in the 2 cell types to stretch as single cells versus groups of cells was also observed in patterned cells exposed to cyclic stretch (supplemental Figure 1C and 1D). The ability for single smooth muscle cells seeded on small islands to

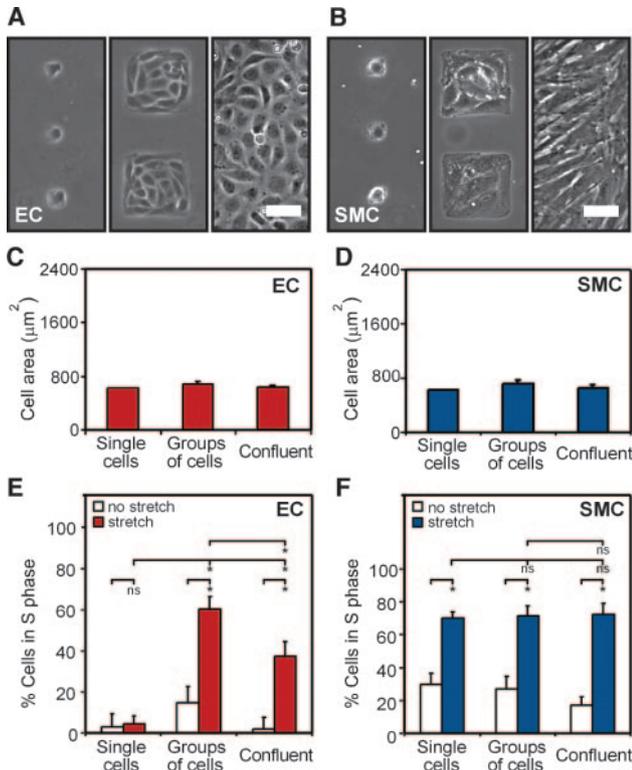


Figure 2. Cell-cell contact is required for endothelial cells, but not smooth muscle cells, to proliferate in response to stretch. A and B, Phase-contrast images of endothelial (A) and smooth muscle (B) cells seeded on micropatterned 625- μm^2 islands (left), on micropatterned 10 000- μm^2 islands (middle), and at 10^5 cells/cm 2 on unpatterned substrates (right). C and D, Graphs of average cell area of single, groups of, and confluent endothelial (C) and smooth muscle (D) cells. Differences were not statistically significant by 1-way ANOVA. E and F, Graphs of percentage of unstretched (white bars) and stretched (colored bars) single, groups of, and confluent endothelial (E) and smooth muscle (F) cells entering the S phase. Scale bars=50 μm . Error bars indicate SE of 3 independent experiments. * $P < 0.05$ and not significant (ns), as calculated by 2-way ANOVA and Tukey's HSD test. Complete results of this statistical analysis can be found in supplemental Figure III.

proliferate with stretch suggests that these cells do not require cell-cell contacts to respond to stretch stimuli, whereas endothelial cells do.

VE-Cadherin Is Required for Stretch-Induced Proliferation in Endothelial Cells

We next examined potential mediators of stretch-stimulated proliferation through cell-cell junctions. Upregulation of gap junction expression and increased gap junction communication have been previously linked to vascular disease processes associated with mechanical stretch.^{29,30} We therefore investigated the possibility that gap junctions might be involved in contact-mediated proliferation in response to stretch using a pharmacological inhibitor against gap junctions, palmitoleic acid. We first confirmed the inhibition of lucifer yellow dye transfer through gap junctions in a wounded endothelial monolayer treated with palmitoleic acid (supplemental Figure IIA). We pretreated endothelial cells seeded at confluent densities with the inhibitor for 1 hour and then stretched for 24 hours in the presence of BrdUrd, fixed,

and assayed for BrdUrd incorporation. We found that endothelial cells still exhibited a robust increase in proliferation in response to stretch even in the presence of 50 $\mu\text{mol/L}$ palmitoleic acid (supplemental Figure IIB), suggesting that gap junctions are not involved in contact-dependent proliferation in response to stretch in endothelial cells.

Because VE-cadherin is important for the mechanical integrity of cell-cell adhesions, and has also been demonstrated to be involved in the endothelial response to shear stress,^{12,31} we explored the possibility that VE-cadherin was involved in the proliferative response to stretch. Endothelial cells were seeded on flexible substrates at high densities and treated with blocking antibody against VE-cadherin 1 hour after seeding, a time point when cells have adhered but have not yet formed cell-cell contact (data not shown). Cells were fixed and stained for VE-cadherin to confirm that normal localization of cell-cell junctions was abrogated in cells treated with blocking antibody versus mouse IgG controls (Figure 3A). Eight hours after seeding, cells were stretched for 24 hours in the presence of BrdUrd, fixed, and analyzed for entry into the S phase. The cadherin blocking antibody abrogated the stretch-mediated increase in proliferation, whereas mouse IgG-treated control cells proliferated with stretch to similar levels as untreated confluent monolayers (Figure 3B). These data demonstrate that VE-cadherin is required for the contact-mediated increase in proliferation with stretch in endothelial cells.

Stretch-Mediated Endothelial Proliferation Is Associated With Increased Cortical Actin

Because tension transmitted through the actin cytoskeleton has been implicated in endothelial proliferation,³² and stretch has been shown to induce stress fiber formation,³³ we examined whether actin microfilaments change their structure or distribution over the course of the experiment. We seeded endothelial cells at high density, stretched them for 24 hours, fixed them, and labeled them with F-actin and phalloidin. Stretch increased the formation of stress fibers and cortical actin structures at the cell boundary (Figure 3C). We performed similar experiments with smooth muscle cells but could not distinguish any changes in cortical actin versus stress fiber in these cells (data not shown). Interestingly, the increased cortical actin in response to stretch was abolished with the VE-cadherin blocking antibody (Figure 3C). These observations suggested that cortical actin might be important for cadherin-mediated proliferation in stretched endothelial cells.

RhoA and Rac1 Activity Is Differentially Activated in Endothelial and Smooth Muscle Responses to Stretch

To explore the intracellular signaling pathways involved in stretch-mediated proliferation, we examined RhoA and Rac1, both of which are known regulators of actin cytoskeletal organization and proliferation.³⁴⁻³⁶ Confluent cells were stretched for 6 hours, lysed, and assayed for the degree of RhoA and Rac1 activation using pull-down assays to measure GTP-bound RhoA and Rac1. We found in endothelial cells that levels of activated Rac1 increased with stretch, whereas levels of activated RhoA decreased (Figure 4A and 4C). In

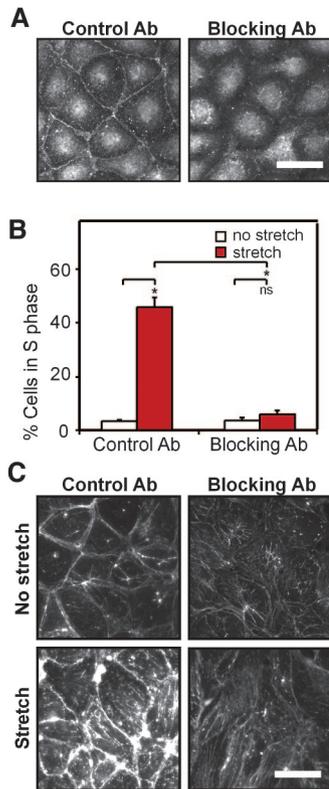


Figure 3. VE-cadherin is required for stretch-mediated proliferation of endothelial cells. A, Immunofluorescent images of VE-cadherin in endothelial cells treated with control mouse IgG antibody (left) or blocking antibody against VE-cadherin (right). B, Graph of percentages of unstretched (white bars) and stretched (red bars) cells treated with control or blocking antibody entering the S phase (bottom). C, Images of F-actin in endothelial cells treated with control mouse IgG antibody (left) or blocking antibody against VE-cadherin (right) without (top) or with (bottom) stretch. Scale bars=20 μ m. Error bars indicate SE of 3 independent experiments. * P <0.05 and not significant (ns), as calculated by 2-way ANOVA and Tukey's HSD test. Complete results of this statistical analysis can be found in supplemental Figure III.

contrast, RhoA activity increased and Rac1 activity decreased in smooth muscle cells subjected to stretch (Figure 4B and 4D). The differential activation of RhoA and Rac1 in endothelial versus smooth muscle cells suggested the possibility that these 2 GTPases have different roles in proliferative stimulation by stretch in the two cell types.

RhoA Activity Is Required for Smooth Muscle Response to Stretch, and Rac1 Activity Is Required for Endothelial Response to Stretch

We next examined whether RhoA and Rac1 activity are involved in the stimulation of proliferation in response to stretch in either vascular cell type. We first used the pharmacological inhibitor Y27632 to inhibit the RhoA effector ROCK. Cells were pretreated with Y27632 for 1 hour, stretched for 24 hours, fixed, and assayed for proliferation. Treatment with Y27632 did not have a striking effect on cell morphology in either endothelial or smooth muscle cells, although stretch-induced stress fiber formation was reduced in endothelial cells (Figure 5A and 5B and data not shown). Endothelial cells treated with Y27632 still significantly in-

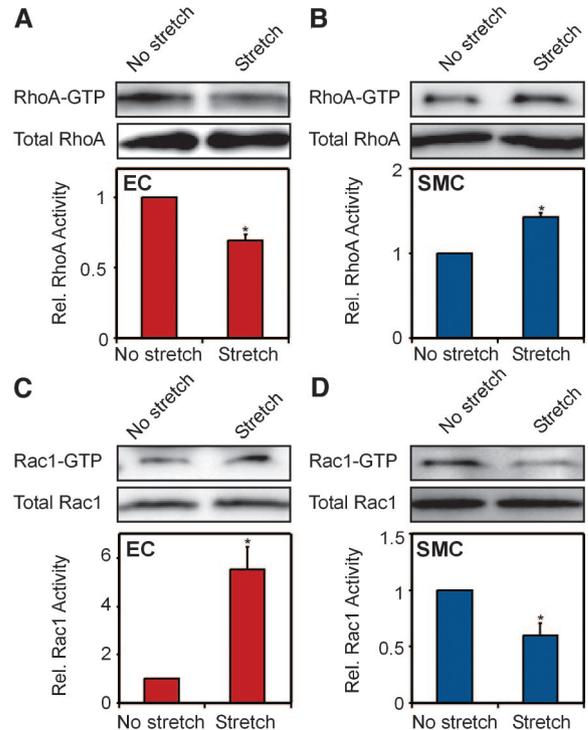


Figure 4. Stretch increases Rac1 activity in endothelial cells and RhoA activity in smooth muscle cells. A and B, Representative Western blots of GTP-bound and total RhoA levels (top) in stretched and unstretched endothelial cells (A) and smooth muscle cells (B), and graphs of the averages of 3 independent experiments (bottom). C, and D, Representative Western blots of GTP-bound and total Rac1 levels (top) in stretched and unstretched endothelial cells (C) and smooth muscle cells (D) and graphs of the averages of 3 independent experiments (bottom). Error bars indicate SE of 3 independent experiments. * P <0.05 between cells with and without stretch, as calculated by Student's *t* test.

creased proliferation in response to stretch but to a lower extent than control cells (Figure 5A). In contrast, ROCK inhibition completely abrogated the proliferative response in smooth muscle cells (Figure 5B).

To more specifically block RhoA signaling, we infected cells with an adenovirus containing a dominant-negative mutant of RhoA (Ad-RhoA-N19). Cells were infected at least 3 hours before the application of stretch and assayed for BrdUrd incorporation after 24 hours of stretch. Expression of RhoA-N19 did not alter the morphology of cells at confluence (Figure 5C and 5D). RhoA-N19-expressing endothelial cells still increased proliferation with stretch, although the level of stimulation appeared to be lower than in Ad-GFP-infected control cells (Figure 5C). As with Y27632 treatment, infection with Ad-RhoA-N19 in smooth muscle cells abolished stretch-induced proliferation completely (Figure 5D). Together, these data show that RhoA is necessary for stretch-stimulated proliferation in smooth muscle cells but that endothelial cells can still proliferate with stretch in the absence of RhoA signaling.

To examine the role of Rac1 signaling, we inhibited Rac1 activity first by using the small molecule inhibitor NSC23766.³⁷ Cells were treated with NSC23766 1 hour before stretch and analyzed for BrdUrd incorporation 24

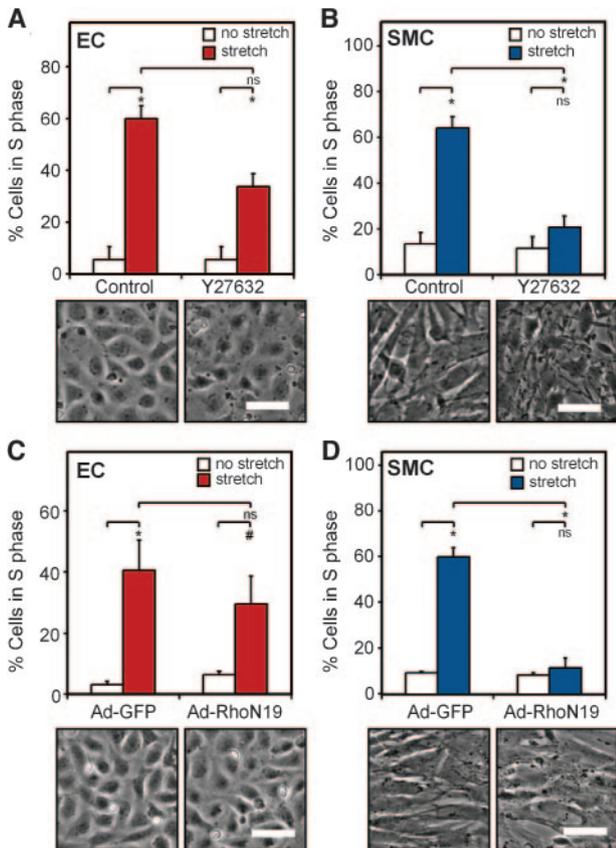


Figure 5. RhoA activity is required for smooth muscle cells to proliferate in response to stretch. A and B, Graph of percentage of stretched (colored bars) and unstretched (white bars) endothelial (A) and smooth muscle (B) cells treated with Y27632 or control untreated cells entering the S phase (top) and corresponding phase-contrast images (bottom). C and D, Graph of percentage of stretched (colored bars) and unstretched (white bars) endothelial (C) and smooth muscle (D) cells treated with Ad-RhoA-N19 or Ad-GFP entering the S phase (top) and corresponding phase-contrast images (bottom). Scale bars=50 μ m. Error bars indicate SE of 3 independent experiments. * P <0.05 and not significant (ns), as calculated by 2-way ANOVA and Tukey's HSD test. Complete results of this statistical analysis can be found in supplemental Figure III. # P <0.05, as calculated by Student's t test.

hours after stretch. We observed no morphological changes in endothelial or smooth muscle cells with this inhibitor (Figure 6A and 6B). In striking contrast to RhoA inhibition, NSC23766 completely inhibited the stimulation of endothelial cell proliferation with stretch, whereas smooth muscle cells exhibited robust stretch-mediated proliferation even in the presence of NSC23766 (Figure 6A and 6B).

We also inhibited Rac1 using an adenovirus to express a dominant-negative Rac1 (Ad-Rac1-N17). Expression of Rac1-N17 reduced the proliferation of stretched endothelial cells to levels similar to unstretched cells, but, inconsistent with NSC23766 treatment, expression of Rac1-N17 in smooth muscle cells appeared to inhibit stretch-induced proliferation (Figure 6C and 6D). Given that Rac1-N17 may affect proliferation via numerous off-target effects³⁸ and that this reagent nonspecifically blocked proliferation even in standard serum-stimulated cultures (data not shown), we overexpressed a more specific antagonist of Rac1 signaling,

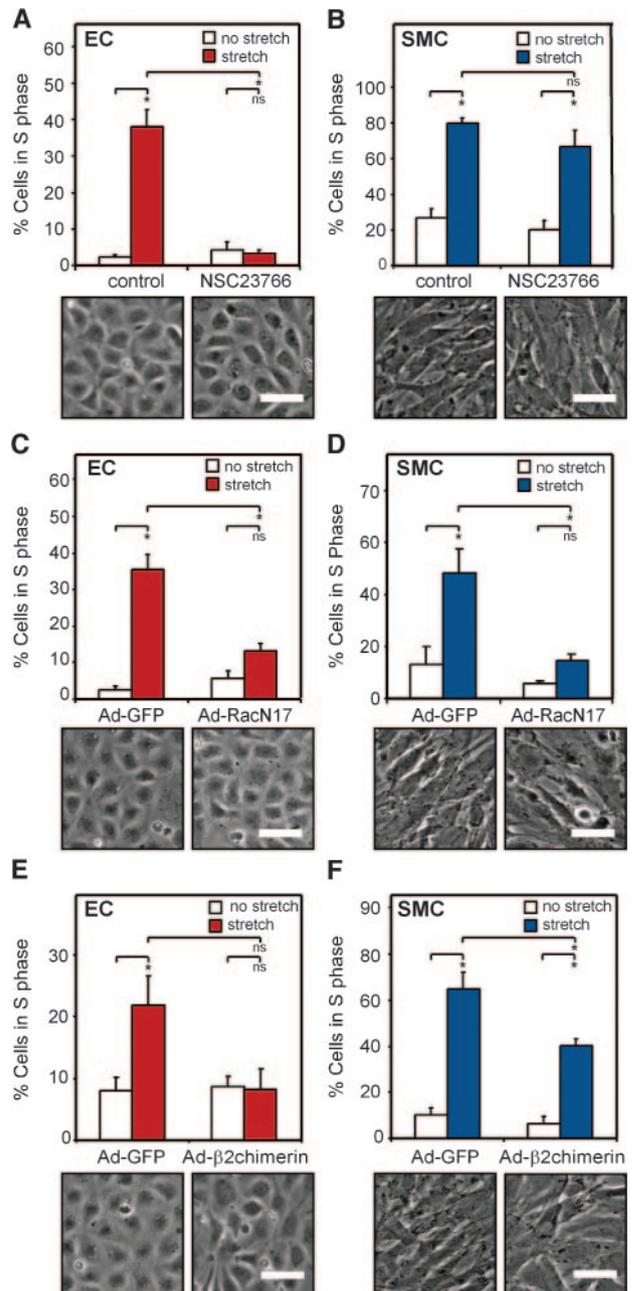


Figure 6. Rac1 activity is required for endothelial cells to proliferate in response to stretch. A and B, Graphs of percentages of stretched (colored bars) and unstretched (white bars) endothelial (A) and smooth muscle (B) cells treated with NSC23766 or control untreated cells entering the S phase (top) and corresponding phase-contrast images (bottom). C and D, Graphs of percentages of stretched (colored bars) and unstretched (white bars) endothelial (C) and smooth muscle (D) cells treated with Ad-Rac1-N17 or Ad-GFP entering the S phase (top) and corresponding phase-contrast images (bottom). E and F, Graph of percentage of stretched (colored bars) and unstretched (white bars) endothelial (E) and smooth muscle (F) cells treated with Ad- β 2-chimerin or Ad-GFP entering the S phase (top) and corresponding phase-contrast images (bottom). Scale bars=50 μ m. Error bars indicate SE of 3 independent experiments. * P <0.05 and not significant (ns), as calculated by 2-way ANOVA and Tukey's HSD test. Complete results of this statistical analysis can be found in supplemental Figure III.

the Rac GTPase-activating protein β 2-chimerin.³⁹ Similar to treatment with NSC23766, overexpression of β 2-chimerin induced no morphological changes in either cell type (Figure 6E and 6F), and Ad- β 2-chimerin expressing endothelial, but not smooth muscle, cells failed to increase proliferation in response to stretch (Figure 6E and 6F). Together, these data suggest that Rac1 may be more specifically involved in the endothelial response to stretch.

Discussion

Previous studies examining the mechanotransduction of stretch into proliferative signals in vascular endothelial and smooth muscle cells have focused on a role for cell–matrix adhesion. Here, using a micropatterning approach to hold the area of cell–matrix contact constant while varying the degree of cell–cell contact, we found that cell–cell contact was necessary for stretch-mediated proliferation in endothelial but not smooth muscle cells. We further showed that engagement of VE-cadherin is required for stretch-induced proliferation in endothelial cells. Recent studies have reported that VE-cadherin is also required for the endothelial response to shear stress because flow induced cellular alignment and changes in gene expression in confluent, wild-type cells but not sparse or VE-cadherin–null cells.^{12,31} Together, these findings suggest that VE-cadherin may play a general role in mechanotransduction.

VE-cadherin appears to transduce mechanical forces to regulate endothelial cell proliferation through the activation of Rac1. It has been shown previously that DNA synthesis is stimulated by microinjection of Rac1 and inhibited by a dominant-negative mutant of Rac1.³⁶ Mechanistically, Rac1 appears to trigger proliferative signaling through its effector p21-activated protein kinase⁴⁰ and constitutively active Rac1 stimulates c-Jun N-terminal kinase and p38,⁴¹ the nuclear factor κ B pathway,⁴² cyclin D1 expression,⁴³ Rb hyperphosphorylation, and E2F transcriptional activity.⁴⁴ It is possible that activation of Rac1 by stretch in endothelial cells drives proliferation through 1 or several of these pathways. It is interesting to note that we and others have shown previously a dependence of endothelial cell proliferation on RhoA signaling in the context of other stimuli⁴⁵ but that this pathway is not essential for stretch-induced proliferation. These data suggest that endothelial cells use different regulatory pathways to control proliferation, depending on the stimulation context, and may provide a means to target certain forms of proliferative signaling, while leaving others intact.

How VE-cadherin transduces mechanical stretch into Rac1 activity is not clear. Mechanical stretch has been shown to cause changes in cadherin expression levels and the organization of cell–cell junctions,⁴⁶ which may lead to changes in the dynamics and degree of cadherin engagement. Supporting this idea, biophysical studies using a surface force apparatus have found that force application could shift cadherin–cadherin interactions among 3 distinct binding regimes,⁴⁷ which were subsequently linked to different binding kinetics.^{48,49} Such force-mediated changes in cadherin engagement or adherens junction structure could in turn alter downstream signaling. It has previously been shown that changes in

cadherin engagement are sufficient to influence Rac1 signaling.^{18,20} Although a direct molecular link between cadherins and Rac1 is not yet known, engagement of VE-cadherin has been shown to activate Rac1 via the guanine exchange factor Tiam1.⁵⁰ Cadherins are also thought to regulate Rho GTPases through p120-catenin, a cadherin-associated scaffolding protein.^{20,51} Alternatively, because cadherins are thought to be required for the formation of other junctions,⁵² a secondary junctional structure could be responsible for sensing force. Nonetheless, these studies suggest a model whereby forces alter the engagement of cadherins, which in turn leads to Rac1-dependent proliferative signaling.

The transduction of mechanical forces through cadherins does not appear to be universal among different cell types, because in smooth muscle cells, stretch-mediated proliferation occurred in the absence of cell–cell contact and cadherin engagement. The smooth muscle cell response to mechanical stress appears to involve different integrin subtypes and changes in ECM composition, supporting the model that cell–ECM interactions are central to mechanotransduction in these cells.^{8,53} Our data suggest that cell–ECM receptors are sufficient because stretch induced a similar magnitude of proliferation in single cells when compared with cells in contact with neighbors. Furthermore, our studies show that in smooth muscle cells, RhoA activity is upregulated by stretch and stretch-induced proliferation is modulated predominantly through RhoA rather than Rac1. Integrins and integrin-associated proteins including Src and focal adhesion kinase have been linked to the regulation of RhoA activity.^{45,54,55} Notably, these adhesion-associated molecules are also regulated by mechanical force,^{9,56,57} and focal adhesion kinase in particular has been shown to be involved in stretch-mediated proliferation.¹⁰ Additionally, a role for RhoA in proliferative regulation is well established and appears to occur through its effectors, ROCK and mDia.⁵⁸ Our studies show that smooth muscle cell proliferation in response to stretch indeed requires RhoA and ROCK, important regulators of actin–myosin contractility.

The distinct mechanisms of force transduction observed in endothelial versus smooth muscle cells suggest that either endothelial cells lack the cell–ECM structures that transduce stretch into RhoA-mediated proliferation or smooth muscle cells lack the cell–cell structures that transduce stretch into Rac1-mediated proliferation. It is not clear whether the differences are simply attributable to the expression of specific adhesion molecules in each cell type. For example, perhaps the presence of VE-cadherin in endothelial cells allows these cells to sense forces through cell–cell adhesions. Likewise, $\alpha_v\beta_3$ integrin, which appears to be expressed in vascular smooth muscle but not in quiescent endothelial cells,⁵⁹ is thought to be activated by mechanical strain and also required for smooth muscle proliferation induced by stretch.^{8,9} Alternatively, FRNK, the inactive truncated splice variant of focal adhesion kinase that is expressed endogenously only in smooth muscle cells,⁶⁰ could potentially modulate force transduction in smooth muscle cells. In support of this, FRNK has been shown to regulate cell proliferation through a cytoskeletal tension-mediated pathway.⁴⁵ Finally, the way in which cell–cell and cell–ECM

adhesions are anchored and therefore mechanically linked to the actin cytoskeleton may also affect which structure is the mechanosensor. Interestingly, in our studies, stress fibers were present in smooth muscle cells, whereas cortical actin associated with cell–cell contact was enhanced by stretch in only endothelial cells, supporting the possibility that anchorage of the actin cytoskeleton to the cell–cell or cell–ECM adhesions is a prerequisite for mechanotransduction by those adhesions. Nonetheless, further studies to delineate how cell–matrix and cell–cell adhesions contribute to mechanotransduction will provide insight into how mechanical forces regulate cell function and can be used for engineering functional tissue constructs.

Hyperproliferation of endothelial and smooth muscle cells is a major component of vascular disease progression. Endothelial cells are bordered by neighboring cells within a confluent monolayer, whereas smooth muscle cells are surrounded mostly by ECM *in vivo*. Perhaps as a result of these differences in their local context, they appear to have evolved distinct mechanisms to transduce forces into biological responses. Limiting stretch-mediated endothelial cell proliferation to occur only in the presence of neighboring cells may provide a mechanism to restrict endothelial cells from invading into regions lacking a preexisting endothelium and explain the slow recovery of endothelial denudation injuries in certain settings.⁶¹ In contrast, the stretch-mediated increase in proliferation even in isolated smooth muscle cells may explain why these cells undergo more dramatic hyperproliferation when compared with endothelial cells in many vascular diseases that result from aberrant stretch such as atherosclerosis.³ These studies illustrate key differences in how endothelial and smooth muscle cells transduce forces and point to molecular strategies to differentially modulate 1 cell type versus another.

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Disclosures

None.

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