RESEARCH ARTICLE

Rho guanine nucleotide exchange factors involved in cyclic-stretch-induced reorientation of vascular endothelial cells

Hiyori Abiko1,‡, Sachiko Fujiwara1,‡, Kazumasa Ohashi1,§, Ryuichi Hiatori1, Toshiya Mashiko1, Naoya Sakamoto2,*, Masaaki Sato2 and Kensaku Mizuno1,§

ABSTRACT
Cyclic stretch is an artificial model of mechanical force loading, which induces the reorientation of vascular endothelial cells and their stress fibers in a direction perpendicular to the stretch axis. Rho family GTPases are crucial for cyclic-stretch-induced endothelial cell reorientation; however, the mechanism underlying stretch-induced activation of Rho family GTPases is unknown. A screen of short hairpin RNAs targeting 63 Rho guanine nucleotide exchange factors (Rho-GEFs) revealed that at least 11 Rho-GEFs – Abr, alsin, ARHGEF10, Bcr, GEF-H1 (also known as ARHGEF2), LARG (also known as ARHGEF12), p190RhoGEF (also known as ARHGEF28), PLEKHG1, P-REX2, Solo (also known as ARHGEF40) and α-PiX (also known as ARHGEF6) – which specifically or broadly target RhoA, Rac1 and/or Cdc42, are involved in cyclic-stretch-induced perpendicularly reorientation of endothelial cells. Overexpression of Solo induced RhoA activation and F-actin accumulation at cell–cell and cell–substrate adhesion sites. Knockdown of Solo suppressed cyclic-stretch- or tensile-force-induced RhoA activation. Moreover, knockdown of Solo significantly reduced cyclic-stretch-induced perpendicularly reorientation of endothelial cells when cells were cultured at high density, but not when they were cultured at low density or pretreated with EGTA or VE-cadherin-targeting small interfering RNAs. These results suggest that Solo is involved in cell–cell-adhesion-mediated mechanical signal transduction during cyclic-stretch-induced endothelial cell reorientation.

KEY WORDS: Cyclic stretch, Endothelial cells, Mechanotransduction, Rho-GEF, Solo/ARHGEF40, VE-cadherin

INTRODUCTION
Mechanical forces play fundamental roles in various cell functions, including morphogenesis, migration, proliferation, apoptosis and differentiation, and thereby make important contributions to the regulation of many pathophysiological processes such as embryogenesis, organogenesis, angiogenesis, tumorigenesis and tissue remodeling and homeostasis (Wozniak and Chen, 2009; Lecuit et al., 2011). In blood vessels, vascular endothelial cells are constantly exposed to mechanical forces, including periodic stretching/relaxation and shear stress caused by pulsatile blood pressure and flow, respectively (Hahn and Schwartz, 2009). Endothelial cells in the vessel exhibit an elongated spindle-like morphology, orient their long axis parallel to the blood flow, and form a monolayer sheet that covers the inner layer of the vessel. When endothelial cells are cultured in vitro under static conditions, they have a polygonal shape and are orientated randomly. However, when they are artificially subjected to uniaxial cyclic stretch, they become elongated and align perpendicular to the stretch axis (Dartsch and Betz, 1989; Yano et al., 1996). Concomitantly, actin stress fibers are aligned perpendicular to the stretch direction. These results suggest that the mechanical forces of periodical stretching and relaxation contribute to the morphology and orientation of endothelial cells within vessels and to the rearrangement of their stress fibers. Cells sense mechanical forces through cell–substrate and cell–cell adhesion sites. Integrins and cadherins are involved in mechanical-stretch-induced cell responses (Geiger et al., 2009; Huveneers and de Rooij, 2013). Several signaling pathways involving RhoA, focal adhesion kinase (FAK, also known as PTK2), Src and Ca2+ are involved in cyclic-stretch-induced reorientation of endothelial cells and their stress fibers (Yano et al., 1996; Naruse et al., 1998; Kaunas et al., 2005). However, it is not well understood how mechanical forces are sensed and transduced to biochemical signals that lead to actin cytoskeletal rearrangement and cell reorientation.

Rho family small GTPases play key roles in actin cytoskeletal reorganization (Etienne-Manneville and Hall, 2002). RhoA is activated by various mechanical stimuli. Inhibition of RhoA or its downstream effectors, Rho-associated protein kinase (ROCK) or mammalian Diaphanous (mDia), changes cyclic-stretch-induced cell and stress fiber reorientation, indicating that RhoA plays a crucial role in mechanical signal transduction (Kaunas et al., 2005; Goldyn et al., 2009; Lee et al., 2010; Lessey et al., 2012). Rac1 is also activated by mechanical stresses and is involved in the mechanical-stretch-induced responses of endothelial cells and other cell types (Tzima, 2006; Liu et al., 2007; DiPaolo et al., 2013). However, the mechanisms by which mechanical force induces activation of RhoA and/or Rac1 are unknown. Rho guanine nucleotide exchange factors (Rho-GEFs) are responsible for the conversion of Rho GTPases from inactive GDP-bound forms to active GTP-bound forms, and these activated forms associate with downstream effector proteins to induce diverse cellular responses (Schmidt and Hall, 2002; Rossman et al., 2005; Cook et al., 2014). In the human genome, there are ~70 distinct members of the Dbl-related Rho-GEF gene family, and they are thought to stimulate the GDP-GTP exchange of Rho family GTPases (Cook et al., 2014). They possess a Dbl homology (DH)
domain that is responsible for Rho-GEF activity and a pleckstrin homology (PH) domain that mediates membrane association or modulates the Rho-GEF activity and the specificity of the DH domain (Rossman et al., 2005). Given the crucial roles of Rho family GTPases in mechanotransduction, it is plausible that Rho-GEFs make important contributions to mechanical-force-induced cell responses upstream of Rho family GTPases. Two RhoA-targeting GEFs, LARG (also known as ARHGEF12) and GEF-H1 (also known as ARHGEF2), have been shown to be involved in the integrin-mediated response to mechanical force (Guilluy et al., 2011). However, little is known about the role of each Rho-GEF in mechanical signal transduction.

This study aimed to identify Rho-GEFs that are involved in cyclic-stretch-induced reorientation of human umbilical vein endothelial cells (HUVECs). A screen of a short hairpin RNA (shRNA) library targeting 63 human Rho-GEFs revealed that at least 11 Rho-GEFs – Abr, alsin, ARHGEF10, Bcr, GEF-H1, LARG, p190RhoGEF (also known as ARHGEF28), PLEKHG1, P-REX2, Solo (also known as ARHGEF40) and α-PiX (also known as ARHGEF6) – are involved in the cyclic-stretch-induced reorientation of HUVECs and their stress fibers to the direction perpendicular to the stretch axis. We also provide evidence that Solo, a RhoA-targeting GEF, contributes to cyclic-stretch-induced cell and stress fiber reorientation by transducing mechanical force signals at cell–cell adhesion sites.

RESULTS

Cyclic stretch induces the reorientation of HUVECs and their stress fibers

Cyclic uniaxial stretch causes endothelial cells and their stress fibers to reorientate in a direction perpendicular to the stretch axis (Yano et al., 1996; Kaunas et al., 2005). Prior to the screening of Rho-GEFs involved in the cyclic-stretch-induced cell and stress fiber reorientation responses, we analyzed the effects of cyclic stretch stimuli on the orientation of HUVECs and their stress fibers. Cells were cultured to almost 100% confluency on a fibronectin-coated silicone chamber and subjected to uniaxial cyclic stretch (20% stretch) at frequency of 1 Hz for 1 hour. Then, cells were fixed and stained with Alexa-Fluor-546–tagged phalloidin to detect cell morphology and actin cytoskeletal organization. Whereas untreated HUVECs had a polygonal shape and were almost randomly oriented prior to cyclic stretch, whereas they aligned with uniaxial cyclic stretch adopted an elongated shape and aligned their long axis and stress fibers in a direction almost perpendicular to the stretch axis (Fig. 1B). Cell orientation was quantified by fitting each cell outline to an ellipse (Fig. 1A). Histogram analysis showed that HUVECs were almost randomly oriented prior to cyclic stretch, whereas they aligned almost perpendicular to the stretch axis after cyclic stretch treatment (Fig. 1C). The stress fiber orientation before and after cyclic stretching was also analyzed by measuring the angle (θ) between the stress fiber and the stretch direction (Fig. 1D). The percentages of the cells and stress fibers oriented perpendicular to the stretch axis (70°<θ<110°) were 34% and 20% before stretching and 67% and 74% after stretching, respectively. The value should be 22% (40° divided by 180°) if cells are oriented randomly. Thus, as previously reported (Yano et al., 1996; Kaunas et al., 2005), cyclic stretch stimuli induced the reorientation of HUVECs and their stress fibers to the direction perpendicular to the stretch axis.

Fig. 1. Cyclic stretch induces cell and stress fiber reorientation. (A) Analysis of cell and stress fiber orientation. Cell shape was analyzed by fitting each cell outline to an ellipse. Cell and stress fiber orientation was analyzed by measuring the orientation angle (θ) of the long axis of the ellipse or stress fiber relative to the stretch axis. (B) Effects of cyclic stretch on cell and stress fiber orientation. HUVECs were co-transfected with CFP and control shRNA plasmids and cultured for 24 hours. Cells were replated onto a fibronectin-coated silicone stretch chamber, cultured for 24 hours and subjected to 20% cyclic uniaxial stretch for 1 hour at 1 Hz. Cells were fixed and stained with Alexa-Fluor-546–phalloidin. Scale bar: 50 μm. (C,D) Histograms of the percentage of cells (C) and stress fibers (SF, D) with the indicated orientation angle θ, measured as in (A).

Construction of a human Rho-GEF shRNA library

To identify Rho-GEFs that are involved in the cyclic-stretch-induced reorientation of HUVECs, we constructed a shRNA library targeting human Dbl-like Rho-GEFs and searched for the Rho-GEF shRNAs that perturb the cyclic-stretch-induced cell reorientation response. To obtain effective shRNA constructs against each human Rho-GEF, candidate shRNA sequences were designed according to a previously reported method (Ui-Tei et al., 2004) and inserted into the pSUPER vector (Brummelkamp et al., 2002). To assess the silencing effect, each shRNA plasmid was introduced into Jurkat cells together with the reporter plasmid, which contained a chimeric cDNA composed of luciferase cDNA and a cDNA fragment (360–540 base pairs) of the target gene (supplementary material Fig. S1A). The primer sequences used...
to amplify the Rho-GEF cDNA fragments are listed in supplementary material Table S1. If any shRNA construct effectively suppressed expression of its target gene, luciferase expression was expected to be reduced. Thus, the knockdown effect of each shRNA on expression of its target gene could be easily assessed by measuring luciferase activity. Using this reporter assay, one or two effective shRNA constructs targeting each of the 63 human DbI-like Rho-GEFs were obtained (supplementary material Fig. S1B). The shRNA sequences targeting each Rho-GEF are listed in supplementary material Table S2.

Identification of Rho-GEFs involved in cyclic-stretch-induced reorientation of HUVECs and their stress fibers

We examined the effect of knockdown of each Rho-GEF on cyclic-stretch-induced alignment of HUVECs perpendicular to the stretch axis. Cells were transfected with each Rho-GEF shRNA plasmid, transferred to a fibronectin-coated silicone chamber and then subjected to cyclic stretch (20% stretch) at 1 Hz for 1 hour. Cell orientation was analyzed by measuring the orientation angle (θ) of the long axis of the cell relative to the stretch direction (Fig. 1A), and the percentage of cells that were oriented perpendicular to the stretch axis (70°<θ<110°) was calculated (Fig. 2A). Of the shRNAs targeting the 63 Rho-GEFs, the shRNAs targeting 11 Rho-GEFs (Abr, alsin, ARHGEF10, Bcr, GEF-H1, LARG, p190RhoGEF, PLEKHG1, P-REX2, Solo and α-PIX) suppressed the cyclic-stretch-induced realignment of HUVECs perpendicular to the stretch axis (Fig. 2A). Fig. 2B and supplementary material Fig. S2A show the fluorescence images of phalloidin staining of HUVECs transfected with each Rho-GEF shRNA after cyclic stretching and the histograms of the percentage of cells oriented at the indicated angles. We also analyzed the effect of knockdown of each of the 11 Rho-GEFs on cyclic-stretch-induced stress fiber reorientation. Knockdown of each Rho-GEF suppressed the cyclic-stretch-induced perpendicular reorientation of stress fibers in HUVECs (supplementary material Fig. S2B,C). These results suggest that at least 11 Rho-GEFs are involved in the cyclic-stretch-induced realignment of HUVECs and their stress fibers to the direction perpendicular to the stretch axis.

To examine whether the 11 Rho-GEFs identified in this study are expressed in HUVECs, we performed reverse transcription (RT)-PCR analysis using the primers listed in supplementary material Table S1. Transcripts of all these Rho-GEFs were detected in HUVECs (supplementary material Fig. S3A).

Characteristics of Rho-GEFs involved in cyclic-stretch-induced reorientation of HUVECs

Schematic structures of the 11 Rho-GEFs identified as being involved in the cyclic-stretch-induced reorientation of HUVECs are shown in supplementary material Fig. S3B. As a whole, they are structurally diverse with distinct domain structures (except for the DH and PH domains) and are distributed widely in the phylogenetic tree of Rho-GEFs; however, two pairs of Rho-GEFs are classified into the same subgroups (Abr and Bcr; GEF-H1 and p190RhoGEF) (Cook et al., 2014). As summarized in supplementary material Table S3, the reported targets of these Rho-GEFs are also diverse, as follows: Abr targets RhoA, Rac1 and Cdc42; alsin targets Rac1; ARHGEF10 targets RhoA, RhoB and RhoC; Bcr targets RhoA, Rac1 and Cdc42; GEF-H1 targets RhoA; LARG targets RhoA; p190RhoGEF targets RhoA; P-REX2 targets Rac1, Rac2, Rac3, Cdc42, RhoG and TC10 (also known as RHQ); Solo targets RhoA and RhoC; and α-PIX targets Rac1 and Cdc42 (Cook et al., 2014; see also references in supplementary material Table S3). The target of PLEKHG1 is unknown; therefore, we examined which Rho family GTPases are the targets of PLEKHG1. 293T cells were co-transfected with YFP-tagged wild-type PLEKHG1 [PLEKHG1(WT)] or its inactive L258E mutant, in which Leu-258 was replaced with Glu, and FLAG-tagged RhoA, Rac1 or Cdc42. Thereafter, cell lysates were subjected to GST pull-down assays using the GST-tagged RhoA-binding domain (RBD) of rhoetkin and the p21 (Rac1/Cdc42)-binding domain (PBD) of PAK1, to which active GTP-bound forms of RhoA and Rac or Cdc42 specifically bind, respectively. This assay revealed that PLEKHG1(WT) activated Rac1 and Cdc42, but not RhoA, whereas PLEKHG1(L258E) did not activate any Rho family GTPases (supplementary material Fig. S3). In addition, overexpression of CFP-tagged PLEKHG1(WT) in HeLa cells induced membrane ruffling and dorsal stress fibers, in place of marginal stress fibers observed in the surrounding PLEKHG1-non-expressing cells (supplementary material Fig. S3D). These phenotypes are frequently observed in Rac-activated cells (Vallenius, 2013), further suggesting that PLEKHG1 activates Rac. These results suggest that a wide spectrum of Rho-GEFs with distinct domain structures and target specificities are involved in cyclic-stretch-induced cell reorientation.

Solo induces F-actin accumulation at cell–cell and cell–substrate adhesion sites

Solo (also named ARHGEF40 and Scambio) is a GEF that targets RhoA and RhoC (Curtis et al., 2004; Tse et al., 2005). Its zebrafish ortholog, called Quattro (Quo), is involved in convergent extension cell movements in gastrula, a process related to mechanotransduction (Daggett et al., 2004). However, the cellular function of Solo is unknown. Here, we focused on the role of Solo in cyclic-stretch-induced cell and stress fiber orientation. To examine the role of Solo in actin cytoskeletal remodeling, we analyzed the effect of Solo overexpression on actin organization. HUVECs were transfected with YFP-tagged Solo(WT) or its GEF-inactive L1217E mutant, in which Leu-1217 was replaced with Glu, and stained with Alexa-Fluor-568–phalloidin to detect F-actin. Overexpression of YFP–Solo(WT) induced the formation of aberrantly accumulated F-actin and thick stress fibers near to cell–cell contact sites in both confluent and subconfluent cells, whereas this was not observed in surrounding cells that did not express YFP–Solo(WT) (Fig. 3A,B). In some instances, expression of YFP–Solo(WT) induced aberrant accumulation of actin filaments at focal adhesions, the sites of stress fiber termination and cell attachment to the extracellular matrix (ECM) (Fig. 3C). In both cases, YFP–Solo(WT) accumulated at regions where F-actin accumulated. By contrast, expression of YFP–Solo(L1217E) had no apparent effect on actin organization and it localized diffusely in the cytoplasm (Fig. 3D). These results suggest that Solo has the potential to promote the formation of actin stress fibers and focal adhesions by activating RhoA. Time-lapse fluorescence imaging of HUVECs co-expressing CFP–Solo(WT) and YFP–Lifeact revealed that Solo colocalized and moved together with F-actin-assembled structures (supplementary material Fig. S4A and Movie 1), suggesting that Solo functions in the F-actin-assembled regions.

Solo is involved in cyclic-stretch-induced RhoA activation

To confirm that Solo activates RhoA, YFP-tagged Solo(WT) or its C-terminal fragment (amino acids 1058–1519), which contains
Fig. 2. See next page for legend.
Fig. 2. Screening of Rho-GEFs that are involved in cyclic-stretch-induced reorientation of HUVECs. (A) Effects of Rho-GEF-targeting shRNAs on cyclic-stretch-induced cell reorientation perpendicular to the stretch axis. HUVECs were co-transfected with plasmids encoding CFP and each Rho-GEF-targeting shRNA and cultured for 24 hours. Cells were replated onto a fibronectin-coated silicone stretch chamber, cultured for 24 hours and subjected to 20% cyclic uniaxial stretch for 1 hour at 1 Hz. Cells were fixed and stained with Alexa-Fluor-546–phalloidin. The orientation angle of the cell was measured as in Fig. 1 and the percentages of cells with an orientation angle of $70^\circ < \theta < 110^\circ$ were calculated. Data represent the means of two independent experiments (at least 50 cells per experiment). (B) Fluorescence images of HUVECs that were transfected with control or Rho-GEF-targeting shRNAs, subjected to cyclic stretch and stained with Alexa-Fluor-546–phalloidin. The lower panels show the histogram of the percentage of cells with the indicated orientation angle ($\theta$). Scale bar: 50 $\mu$m.

To examine the role of Solo in cyclic-stretch-induced RhoA activation, we analyzed the effect of Solo knockdown on cyclic-stretch-induced RhoA activation. We used Madin-Darby canine kidney II (MDCKII) cells in this experiment, because MDCKII cells reoriented perpendicular to the stretch direction (Fig. 4C) and consistently exhibited RhoA activation in response to cyclic stretching. The small interfering RNAs (siRNAs) targeting dog Solo effectively suppressed the expression of endogenous Solo in MDCKII cells (Fig. 4D). Cells were treated with control or dog Solo-targeting siRNAs and subjected to cyclic stretch for 1 minute at 1 Hz. Measurement of activated RhoA by GST–RBD pull-down assays revealed that RhoA is activated by cyclic stretch and that knockdown of Solo significantly decreases the level of cyclic-stretch-induced RhoA activation (Fig. 4E). This indicates that Solo is involved in the cyclic-stretch-induced RhoA activation.

Effect of double knockdown of Solo and LARG on cyclic-stretch-induced cell and stress fiber orientation

To examine the relationships between the roles of Solo and other RhoA-targeting GEFs, we analyzed the effects of double knockdown of Solo and LARG on cyclic-stretch-induced cell...
Fig. 4. See next page for legend.
Fig. 4. Solo is involved in cyclic-stretch-induced RhoA activation. (A) Structures of Solo(WT) and its C-terminal fragment (amino acids 1058–1519). (B) Expression of Solo(WT), but not Solo(1058–1519), induces RhoA activation. 293T cells were transfected with YFP, YFP–Solo(WT) or YFP–Solo(1058–1519) and cultured for 48 hours. RhoA activity was measured by using GST–phokin(RBD) pull-down assays. Relative RhoA activity is shown as means ± s.d. of three independent experiments. **P<0.01 (one-way ANOVA followed by Dunnett’s test). (C) Cyclic stretch induces perpendicular orientation of MDCKII cells and their stress fibers. MDCKII cells were subjected to cyclic stretch, as in Fig. 1. Cells were fixed and stained with Alexa-Fluor-546–phalloidin. Scale bar: 20 μm. (D) Effect of Solo-targeting siRNAs on expression of Solo in MDCKII cells. Cells were transfected with control or dog Solo-targeting siRNAs and cultured for 48 hours. Cell lysates were analyzed by immunoblotting with an anti-Solo antibody. (E) Effect of Solo knockdown on cyclic-stretch-induced RhoA activation. MDCKII cells were transfected with Solo-targeting siRNAs and cultured for 24 hours. Cells were replated onto a fibronectin-coated silicone stretch chamber, cultured for 24 hours and subjected to cyclic stretch for 1 minute at 1 Hz. Cell lysates were subjected to GST–phokin(RBD) pull-down assays. Relative RhoA activity is shown as means ± s.d. of 12 independent experiments. *P<0.05; **P<0.01 (one-way ANOVA followed by Dunnett’s test). (F) Suppression of Solo and LARG expression by siRNAs. HUVECs were transfected with Solo- or LARG-targeting siRNAs and cultured for 48 hours. Cell lysates were analyzed by immunoblotting with the indicated antibodies. (G) Effects of double knockdown of Solo and LARG on cyclic-stretch-induced cell and stress fiber reorientation. HUVECs were transfected with Solo- or LARG-targeting siRNAs, or both, and subjected to cyclic stretch, as in Fig. 1. Cells were fixed and stained with Alexa-Fluor-546–phalloidin, and the orientation parameters (cos2) of the cell body and stress fibers were measured. Data represent means ± s.d. of three independent experiments (at least 100 cells or 500 stress fibers per experiment). ***P<0.001; n.s., not significant (one-way ANOVA followed by Tukey’s test).

Effects of EGTA or VE-cadherin siRNA treatment on Solo-knockdown-induced suppression of perpendicular orientation of cells and stress fibers

To further examine the role of Solo in cell-cell-contact-mediated reorientation of HUVECs, we analyzed the effects of Solo knockdown on cyclic-stretch-induced cell and stress fiber reorientation in the presence or absence of EGTA, which is expected to inhibit cell-cell adhesion through inhibition of cell adhesion molecules, including vascular endothelial (VE)-cadherin. Treatment with EGTA reduced VE-cadherin localization in the cell–cell contact sites in HUVECs (Fig. 6A). When control siRNA-transfected cells were cultured in the presence of EGTA and subjected to cyclic stretch, the values of the orientation parameters of cell bodies (−0.31) and their stress fibers (−0.45) were increased, compared with those in control EGTA-non-treated cells (−0.71 and −0.73 for cell bodies and stress fibers, respectively) (Fig. 6B,C), indicating that EGTA treatment suppresses cyclic-stretch-induced perpendicular orientation of cells and stress fibers. The orientation parameters of cells and stress fibers after cyclic stretch were significantly increased by Solo knockdown in EGTA-non-treated cells, but they did not significantly differ between control cells and Solo-knockdown cells after EGTA treatment (Fig. 6B,C), which suggests that Solo is involved in cyclic-stretch-induced cell and stress fiber reorientation primarily through cell–cell-contact-mediated mechanical force transduction.

VE-cadherin mediates cell–cell adhesions and mechanical-stress-induced endothelial cell responses (Tzima et al., 2005; Liu et al., 2007). To further examine the role of Solo in cell-cell-adhesion-mediated endothelial cell reorientation, we analyzed the effects of Solo knockdown on cyclic-stretch-induced reorientation under the VE-cadherin-depleted conditions. Treatment with VE-
cadherin-targeting siRNAs reduced the expression of endogenous VE-cadherin in HUVECs (Fig. 7A). When HUVECs were treated with VE-cadherin siRNAs and subjected to cyclic stretch, the values of the orientation parameters of cell bodies (−0.50) and their stress fibers (−0.59) were significantly increased, compared with those in control siRNA-treated cells (−0.75 and −0.86 for cell bodies and stress fibers, respectively) (Fig. 7B,C), indicating that depletion of VE-cadherin suppresses cyclic-stretch-induced perpendicular orientation of cells and stress fibers. The orientation parameters of cells and stress fibers after cyclic stretch were significantly increased by Solo knockdown in control HUVECs (Fig. 5A; Fig. 6C, −EGTA), but they were not significantly changed by Solo knockdown in VE-cadherin-depleted cells (Fig. 7B,C). Taken together with the findings in Figs 5 and 6, these results suggest that Solo is primarily involved in cell–cell-contact-mediated mechanical force transduction during the cyclic-stretch-induced cell and stress fiber reorientation responses.

Knockdown of Solo suppresses tensile-force-induced RhoA activation

To further examine the role of Solo in mechanical force transduction, we analyzed whether knockdown of Solo affects the tensile-force-induced RhoA activation. E-cadherin-coated magnetic beads were put on cultured MDCKII cells stably expressing CFP–RhoA and tensile force was applied by placing a permanent magnet on the top of the culture dish (Fig. 8A). To measure activated RhoA, cell lysates were subjected to GST–
rhotein pull-down assays. This assay revealed that RhoA is significantly activated 10 minutes after tensile force application and that knockdown of Solo suppresses tensile-force-induced RhoA activation (Fig. 8B). This result suggests that Solo is involved in the cadherin-mediated mechanical-force-induced RhoA activation.

DISCUSSION

Mechanical forces play essential roles in many cell activities and functions. Rho family GTPases, including RhoA, Rac1 and Cdc42, are crucially involved in mechanical-force-induced cell responses, such as cell shape change, migration and proliferation, mostly by regulating actin cytoskeletal reorganization (Tzima, 2006; Birukov, 2009; Lessey et al., 2012). However, little is known about the mechanisms by which mechanical forces induce the activation of Rho family GTPases. Using a shRNA library targeting 63 Rho-GEFs, we identified 11 Rho-GEFs that are involved in cyclic-stretch-induced alignment of HUVECs and their stress fibers perpendicular to the stretch axis. Consistent with the important roles of RhoA and Rac1 in mechanical force responses, the 11 Rho-GEFs identified in this study have distinct target specificities towards RhoA, Rac1 and Cdc42.
Among these 11 Rho-GEFs, GEF-H1, LARG and p190RhoGEF are RhoA-targeting GEFs that are involved in integrin-mediated mechanotransduction at focal adhesions (Lim et al., 2008; Tomar and Schlaepfer, 2009; Guilluy et al., 2011). In addition, single-nucleotide polymorphism analysis revealed that the genes encoding ARHGEF10 and PLEKHG1 are associated with atherothrombotic stroke and high blood pressure traits, respectively (Matsushita et al., 2010; Franceschini et al., 2013), suggesting that they have roles in blood vessel homeostasis and functions. However, little is known about the regulation mechanisms and functional roles of these Rho-GEFs in mechanotransduction in vascular endothelial cells. Our results indicate that multiple Rho-GEFs with distinct target specificities are involved in the transmission of mechanical signals to activate distinct Rho family GTPases.

Cyclic-stretch-induced cell and stress fiber reorientation is a multistep process that involves disruption and/or movement of focal adhesions and stress fibers along the stretch direction, lamellipodium formation and cell elongation at the cell ends perpendicular to the stretch direction, and reassembly and alignment of focal adhesions and stress fibers perpendicular to the stretch direction. The 11 Rho-GEFs identified in this study appear to play crucial roles in the cyclic-stretch-induced cell and stress fiber reorientation responses by coordinately regulating the activities of their specific target Rho family GTPases. Thus, it is plausible that RhoA-targeting GEFs, such as ARHGEF10, GEF-H1, LARG, p190RhoGEF and Solo, are involved in the reassembly of focal adhesions and stress fibers perpendicular to the stretch axis, whereas Rac1/Cdc42-targeting GEFs, such as alsin, PLEKHG1, P-REX2 and α-PIX, are involved in lamellipodium formation and cell elongation at the cell ends perpendicular to the stretch axis. Inhibition of RhoA or its effectors, ROCK or mDia, causes cyclic-stretch-induced stress fiber orientation in the direction parallel to the stretch axis (Kaunas et al., 2005). Knockdown of each of the 11 Rho-GEFs or double knockdown of Solo and LARG was insufficient to completely block perpendicular alignment of cells or induce parallel orientation of stress fibers, indicating that multiple Rho-GEFs are required for the cyclic-stretch-induced RhoA activation and reorientation of cells and their stress fibers perpendicular to the stretch axis. Further studies on the mechanisms that regulate the activation and localization of each Rho-GEF will provide better understanding of the functional roles of individual Rho-GEFs and Rho family GTPases and the mechanism underlying their spatiotemporal coordination during cyclic-stretch-induced actin cytoskeletal remodeling and cell reorientation.

Cells sense external mechanical forces primarily through cell attachment sites, including cell–substrate and cell–cell adhesions. Integrin-mediated linkages between the actin cytoskeleton and the ECM at focal adhesions are involved in the sensing of mechanical signals, such as the spatial distribution of adhesive ligands and matrix rigidity (Roca-Cusachs et al., 2012; Schwarz and Gardel, 2012). Cell–cell adhesions mediated by cadherins or other transmembrane proteins are also involved in mechanosensing, such as the shear-stress-induced response and stretch-induced...

(supplementary material Table S3). Among these 11 Rho-GEFs, GEF-H1, LARG and p190RhoGEF are RhoA-targeting GEFs that are involved in integrin-mediated mechanotransduction at focal adhesions (Lim et al., 2008; Tomar and Schlaepfer, 2009; Guilluy et al., 2011). In addition, single-nucleotide polymorphism analysis revealed that the genes encoding ARHGEF10 and PLEKHG1 are associated with atherothrombotic stroke and high blood pressure traits, respectively (Matsushita et al., 2010; Franceschini et al., 2013), suggesting that they have roles in...
Cadherin-coated magnetic beads were put on cultured MDCKII cells stably et al., 2004). Cell-cell-contact-mediated mechanotransduction movement during gastrulation of the zebrafish embryo (Daggett zebrafish ortholog of Solo, is involved in convergent extension cell induced cell and stress fiber reorientation of HUVECs. Quo, a VE-cadherin-mediated mechanotransduction during cyclic-stretch appears to predominantly contribute to cell–cell contact- and effect on cyclic-stretch-induced cell reorientation when cells were low density. Furthermore, knockdown of Solo had no apparent density, but it had no apparent effect when cells were cultured at 37˚C under a 5% CO₂ atmosphere in Medium 199

These results suggest that Solo is involved in mechanotransduction at cell–cell and/or cell–ECM adhesion sites. Knockdown of Solo suppressed cyclic-stretch-induced RhoA activation, but its C-terminal DH-containing fragment does not, indicating that the N-terminal region of Solo is required for its activation in cells. One possible explanation for this result is that the Rho-GEF activity ofSolo is enhanced by the tensional-force-induced conformational change in the DH domain and that the N-terminal region functions as one of the supporting points for this process. Thus, Solo might function by itself as a mechanosensor that is activated by mechanical-force-induced conformational changes. Identification of the Rho-GEFs that are involved in cyclic-stretch-induced cell reorientation in this study will help future studies to elucidate the mechanisms by which mechanical forces activate Rho-GEFs and their downstream Rho family GTPases.

Fig. 8. Effect of Solo knockdown on tensile-force-induced RhoA activation. (A) Schematic of the process of tensile force application. The E-cadherin-coated magnetic beads were put on cultured MDCKII cells stably expressing CFP–RhoA. Tensile force (F) was applied by placing a permanent magnet on the top of the culture dish. (B) Effect of Solo knockdown on tensile-force-induced RhoA activation. Tensile force was applied to MDCKII cells as in A. Cell lysates were subjected to GST–rhotein(RBD) pull-down assays. Relative RhoA activity is shown as means ± s.d. of six independent experiments. *P<0.05; n.s., not significant (one-way ANOVA followed by Dunnett’s test).

MATERIALS AND METHODS
Antibodies
A rabbit polyclonal antibody recognizing human and dog Solo was raised against the C-terminal peptide (LSRQSHARALSDPTTPL) of human Solo. Other antibodies were purchased as follows: rabbit polyclonal antibodies against GFP (A-6455, Life Technologies, Grand Island, NY), LARG (sc-25638, Santa Cruz Biotechnology, Dallas, TX) and VE-cadherin (ALX-210-232-C100, Enzo Life Sciences, Farmingdale, NY), mouse monoclonal antibodies against FLAG (M2, Sigma-Aldrich, St Louis, MO), Rho (sc-418, Santa Cruz Biotechnology) and β-actin (AC-15, Sigma-Aldrich), and Alexa-Fluor-488-labeled goat anti-rabbit-IgG antibody (A11034, Life Technologies).

Cell culture and transfection
HUVECs were prepared from human umbilical cord veins as described previously (Ueki et al., 2010) or purchased from Life Technologies. Cells were cultured at 37°C under a 5% CO₂ atmosphere in Medium 199 (Life Technologies) supplemented with 20% fetal calf serum (Life Technologies) and 10 ng/ml human basic fibroblast growth factor (Austral Biologicals, San Ramon, CA). The cells were used within seven passages. Jurkat T cells were obtained from the Cell Resource Center, Tohoku University (Sendai, Japan) and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. HeLa and MDCKII cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Cells were transfected with proliferation of endothelial cells (Tzima et al., 2005; Liu et al., 2007; Huveneers and de Rooij, 2013). Overexpression of Solo caused marked effects on the actin cytoskeletal organization in HUVECs, including the formation of thick stress fibers and aberrant F-actin accumulation at cell–cell contact sites and focal adhesions, and Solo localized at sites of F-actin accumulation. These results suggest that Solo is involved in mechanotransduction at cell–cell and/or cell–ECM adhesion sites. Knockdown of Solo suppressed cyclic-stretch-induced reorientation of HUVECs perpendicular to the stretch axis when cells were cultured at high density, but it had no apparent effect when cells were cultured at low density. Furthermore, knockdown of Solo had no apparent effect on cyclic-stretch-induced cell reorientation when cells were treated with EGTA or VE-cadherin siRNA. Therefore, Solo appears to predominantly contribute to cell–cell contact- and VE-cadherin-mediated mechanotransduction during cyclic-stretch-induced cell and stress fiber reorientation of HUVECs. Quo, a zebrafish ortholog of Solo, is involved in convergent extension cell movement during gastrulation of the zebrafish embryo (Daggett et al., 2004). Cell–cell-contact-mediated mechanotransduction plays an essential role in convergent extension (Lecuit et al., 2011). Therefore, Quo likely mediates mechanical force signal transduction in zebrafish, in a manner similar to how Solo functions in the stretch-induced reorientation of HUVECs.

It is unknown how mechanical stimuli activate the 11 Rho-GEFs identified in this study. Recent studies identified several candidate mechanosensor molecules, such as p130Cas (also known as BCAR1), talin, vinculin, α-catenin and filamin, which directly sense and transduce mechanical force signals into chemical signals (Sawada et al., 2006; del Rio et al., 2009; Grashoff et al., 2010; Yonemura et al., 2010; Ehrlicher et al., 2011). Rho-GEFs are activated by their association with other proteins or by modifications, such as phosphorylation. For example, GEF-H1 is activated by its release from microtubules, whereas p190RhoGEF is activated by FAK association and FAK-mediated tyrosine phosphorylation (Lim et al., 2008; Tomar and Schlaepfer, 2009). To examine the possibility that Solo is phosphorylated after cyclic stretch, we analyzed the gel mobility shift and tyrosine phosphorylation of Solo before and after cyclic stretch of HUVECs and MDCKII cells by immunoblot analysis with anti-Solo and anti-phospho-tyrosine antibodies; however, neither the gel mobility shift nor the phospho-tyrosine signal of Solo was detected (supplementary material Fig. S4B). Although Dbl-like Rho-GEFs have a common catalytic DH domain, many Rho-GEFs have additional sequences. We showed that expression of full-length Solo induces RhoA activation in cells, but its C-terminal DH-containing fragment does not, indicating that the N-terminal region of Solo is required for its activation in cells. One possible explanation for this result is that the Rho-GEF activity of Solo is enhanced by the tensional-force-induced conformational change in the DH domain and that the N-terminal region functions as one of the supporting points for this process. Thus, Solo might function by itself as a mechanosensor that is activated by mechanical-force-induced conformational changes. Identification of the Rho-GEFs that are involved in cyclic-stretch-induced cell reorientation in this study will help future studies to elucidate the mechanisms by which mechanical forces activate Rho-GEFs and their downstream Rho family GTPases.
plasmids and siRNAs using Lipofectamine LTX and RNAiMax (Life Technologies), respectively.

**Plasmid construction and siRNAs**
The cDNAs of human Solo and PLEKHG1 were amplified by RT-PCR from total mRNA isolated from MCF10A cells. Expression plasmids encoding YFP-tagged Solo and PLEKHG1 were constructed by inserting the PCR-amplified cDNAs into the pEYFP-C1 vector (Clontech, Mountain View, CA). The plasmids for GEF-inactive Solo(L1217E) and PLEKHG1(L258E) were constructed using a site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). Expression plasmids encoding FLAG-tagged RhoA, Rac1 and Cdc42 were constructed by inserting the cDNAs for RhoA, Rac1 and Cdc42 into the pFLAG-C1 vector, which was constructed by replacing the YFP cDNA in the pEYFP-C1 vector with the FLAG epitope sequence. The siRNAs targeting human Solo, LARG and VE-cadherin were purchased from Sigma-Aldrich. The sequences of siRNAs targeting dog Solo are listed in supplementary material Table S4.

**Construction of a shRNA library targeting Rho-GEFs**
To construct a shRNA library targeting each of the human Rho-GEFs, candidate 19-nucleotide shRNA sequences were designed according to the method of Ui-Tei et al. (Ui-Tei et al., 2004) and subcloned into the pSUPER vector (OligoEngine, Madison, WI) (Brummelkamp et al., 2002). To assess the knockdown efficiency, each shRNA plasmid was transfected into Jurkat cells together with the pNUL reporter plasmid, which encodes a chimeric cDNA composed of luciferase cDNA and the cDNA fragment (360–540 bp) of the target Rho-GEF gene, as reported previously (Tsuiji et al., 2010; Hayashi et al., 2013). The cDNA fragments (360–540 bp) of Rho-GEFs were obtained by RT-PCR amplification from total poly(A) RNA of human placenta (Clontech) using the primers listed in supplementary material Table S1. Two days after transfection, Jurkat cells were lysed with 90 μl of Bright-Glo Luciferase Assay System reagent (Promega, Madison, WI). The levels of luciferase expression were measured by determining the luminescence intensity of lysates of Rho-GEF-targeting shRNA- and control shRNA-transfected cells using an LMax II luminometer ( Molecular Devices, Sunnyvale, CA). The intensity of luminescence in Rho-GEF shRNA-transfected cells relative to that in control shRNA-transfected cells was used as a measure of silencing efficiency. Using this reporter assay, one or two effective shRNA constructs targeting each of the 63 human Rho-GEFs were obtained. The target sequences are listed in supplementary material Table S2. GL2 (5′-CCGTACCGGGAATACTTTCGA-3′) was used as the control shRNA.

**Cyclic stretch experiments**
HUVECs were removed from the dish using 0.05% trypsin and 0.05% EDTA and transferred to a silicone chamber coated with 1 μg/ml fibronectin. For MDCKII cells, 0.25% trypsin and 30 μg/ml fibronectin were used. Cells were subjected to cyclic stretch (20% uniaxial stretch) for 1 hour at a frequency of 1 Hz with an automated stretch system (STB-140, Strex, Osaka, Japan). Cells were fixed and stained with Alexa-Fluor-568- or Rhodamine-labeled phalloidin (Life Technologies) to detect F-actin. Cell orientation was analyzed by fitting each cell outline to an ellipse and measuring the angle (θ) of the long axis of the ellipse with respect to the stretch axis. Stress fiber orientation was analyzed by measuring the angle (θ) between stress fibers and the stretch direction. The angles of five brighter stress fibers per cell (more than 60 cells) were measured. The levels of cell and stress fiber reorientating were estimated by constructing a histogram in which cell frequency was plotted against cell orientation angle or calculating the orientation parameter (cos2θ) of cell bodies and stress fibers, as reported previously (Goldyn et al., 2009).

**Immunostaining and time-lapse analysis**
Cells were fixed with phosphate-buffered saline (PBS) containing 4% formaldehyde for 30 minutes and permeabilized with PBS containing 0.05% Triton X-100 for 5 minutes. After washing with PBS, cells were stained with Alexa-Fluor-568-labeled phalloidin or an anti-VE-cadherin antibody. Alexa-Fluor-488-labeled anti-rabbit IgG antibody was used as the secondary antibody. Fluorescence images were obtained using a fluorescence microscope (DMI 6000B, Leica Microsystems, Wetzlar, Germany) equipped with a PL Apo 63× oil-immersion objective lens (NA 1.4) and ImageJ image analysis software. Time-lapse fluorescence images were obtained using a LSM 710 confocal microscope (Carl Zeiss, Jena, Germany) equipped with a PL Apo 63× oil objective lens (NA 1.4) and ImageJ software.

**Immunoblotting**
Cells were lysed with ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM MgCl2, 1 mM Na3VO4, 1 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin). To detect the silencing effect of siRNAs, cells were lysed with 1× SDS sample buffer containing 1% SDS. Cell lysates were subjected to immunoblot analysis as described previously (Ohashi et al., 2000).

**Pull-down assays of GEF activity**
Active GTP-bound forms of RhoA, Rac1 and Cdc42 were analyzed by pull-down assays using GST fusion proteins of the RBD of rhoetokin and the PBD of PAK1, as described previously (Nishita et al., 2002). 293T cells were transiently transfected with YFP–Solo (WT or 1058–1519) or co-transfected with FLAG-tagged RhoA, Rac1 or Cdc42 and YFP–PLEKHG1 (WT or L258E) and cultured for 24 hours. Cell lysates were then incubated with GST–rhoetokin(RBD) or GST–PAK1(PBD) bound to glutathione–Sepharose. The bead pellets were washed and analyzed by immunoblotting using anti-RhoA or anti-FLAG antibodies. To analyze the effect of Solo knockdown on stretch-induced RhoA activation, MDCKII cells were transfected with siRNAs targeting dog Solo, cultured for 24 hours and transferred to a silicone chamber coated with 30 μg/ml fibronectin. After incubation for 24 hours, cells were subjected to cyclic stretch for 1 minute at 1 Hz, and cell lysates were subjected to pull-down assays using GST–rhoetokin(RBD) for measuring the RhoA-GEF activity.

**Force application**
Recombinant human E-cadherin–Fc chimera protein (R&D systems, Minneapolis, MN) was bound to the Protein-G coated magnetic beads (280 nm, Dynabeads Protein G, Life Technologies) by incubating at a ratio of 1 μg of protein per 1 mg of beads in 10 mM HEPES (pH 7.2), 50 mM NaCl, 1 mM CaCl2, 0.1% BSA for 4 hours at 4˚C, then washed four times to remove unbound proteins and resuspended in 10 mM HEPES (pH 7.2), 50 mM NaCl, 0.1% BSA. MDCKII cells stably expressing CFP–RhoA were cultured subconfluent in 35-mm dishes and the E-cadherin-coated magnetic beads were put on the cells and incubated for 40 minutes. Force application was conducted as reported previously (Guilluy et al., 2011). A ceramic permanent magnet (DynaMag-2, Vertis, Tokyo, Japan) was used to generate perpendicular tensile forces on beads attached to the dorsal surface of cells. The manufacturer’s value for the magnetization is an average of 4000 gauss. For all experiments, the pole face was parallel with and 1 cm from the culture dish surface.

**Statistical analysis**
Statistical data are expressed as means±s.d. of more than three independent experiments. Statistical analyses were carried out by using Prism 4 (GraphPad Software, La Jolla, CA). The P-values were calculated using one-way ANOVA followed by Tukey’s test or Dunnett’s test for multiple data set comparisons. In all cases, P<0.05 was considered statistically significant.

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**Competing interests**
The authors declare no competing or financial interests.

**Author contributions**
H.A., S.F., R.H. and T.M. performed the experiments and analyzed the data. N.S. and M.S. provided expertise. K.O. designed the study, performed the experiments
and analyzed the data. K.M. designed the study, discussed the data and wrote the manuscript.

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Supplementary material
Supplementary material available online at http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.157503/-/DC1

References


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Fig. S1. Construction of a shRNA library targeting each Rho-GEF. (A) Scheme of the protocol used to monitor the silencing efficiency of shRNA constructs. The cDNA fragment (360-540 bp) of a target gene was fused downstream of the luciferase cDNA and transfected into Jurkat cells together with each individual shRNA construct. Effective shRNA constructs target the sequences of the target gene on the luciferase-target chimeric mRNA. Thus, the reduction in luciferase activity reflects the silencing efficiency of the shRNA construct. (B) Luciferase reporter analysis to monitor the silencing effect of shRNA constructs targeting each of 63 Rho-GEFs. The relative luminescence intensity in Rho-GEF-targeting shRNA-transfected cells was compared with that in control shRNA-transfected cells. Data represent means ± s.d. of triplicate experiments. The target sequences for Rho-GEFs are listed in Supplementary Table S2.
Fig. S2. Effects of Rho-GEF knockdown on cyclic stretch-induced cell and SF reorientation. (A) Effects on cell reorientation. HUVECs were transfected with Rho-GEF shRNAs, subjected to cyclic stretch, and stained with Alexa546-phalloidin, as in Fig. 2. The upper panels show the fluorescence images of HUVECs. The lower panels show the histogram of the percentage of cells with the indicated orientation angle ($\theta$). (B) Effects on SF reorientation. HUVECs were transfected with control or Rho-GEF shRNAs, subjected to cyclic stretch, and stained with Alexa546-phalloidin, as in Fig. 2. The orientation angle ($\theta$) between SF and the stretch axis was measured for 5 brighter SFs per cell (at least 50 cells per experiment) and the percentages of SFs with an orientation angle of $70^\circ < \theta < 110^\circ$ were counted. Data represent the means of two independent experiments. (C) The histogram of the percentage of SFs with the indicated orientation angle ($\theta$).
Fig. S3. Expression and schematic structures of 11 Rho-GEFs and the Rac1/Cdc42-GEF activity of PLEKHG1. (A) Expression of 11 Rho-GEF mRNAs in HUVECs. Total RNA was isolated from HUVECs using an Isogen II Kit (Nippon Gene, Tokyo, Japan) and reverse-transcribed to yield single-stranded cDNAs using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, Basel, Switzerland). The cDNAs were subjected to PCR amplification for 30 cycles consisting of denaturation at 98°C for 10 seconds, primer annealing at 55°C for 30 seconds, and extension at 68°C for 50 seconds, using specific primers listed in Supplementary Table S1. (B) Schematic structures of 11 Rho-GEFs identified in this study to be involved in cyclic stretch-induced cell reorientation. C1, protein kinase C conserved region 1; C2, protein kinase C conserved region 2; CC, coiled-coil; CH, calponin homology; DEP, domain found in disheveled, Egl-10, and pleckstrin; DH, Dbl homology; MORN, membrane occupation and recognition nexus; PDZ, domain found in PSD-95, Dlg, and ZO-1; PH, pleckstrin homology; RCC1, regulator of chromosome condensation 1; RGS, regulator of G protein signaling; RhoGAP, Rho GTPase-activating protein; SH3, Src homology 3; S/T-Kinase, serine/threonine kinase; VPS9, vascular sorting protein 9; WD40, Trp-Asp 40. (C) Pull-down assays of the GEF activity of PLEKHG1. FLAG-tagged RhoA, Rac1, or Cdc42 was co-transfected with control YFP or YFP-tagged PLEKHG1 (WT or L258E) into 293T cells. Cell lysates were subjected to GST pull-down assays using GST-rhotekin(RBD) or GST-PAK1(PBD). Bound proteins were analyzed by immunoblotting with an anti-FLAG antibody. (D) Effect of PLEKHG1 expression on cell shape and actin organization. HeLa cells were transfected with CFP-PLEKHG1(WT), cultured for 24 hours, fixed, and stained with Alexa546-phalloidin. Scale bar, 20 μm.
Fig. S4. Time-lapse fluorescence images of CFP-Solo and YFP-Lifeact and effects of cyclic stretch on gel mobility and tyrosine phosphorylation of Solo. (A) Time-lapse fluorescence images of CFP-Solo and YFP-Lifeact. HUVECs were co-transfected with CFP-Solo(WT) and YFP-Lifeact and cultured for 24 hours. Fluorescence images of CFP-Solo (green) and YFP-Lifeact (red) were acquired every 30 seconds for 30 min (see Supplementary Movie 1). Arrows indicate aberrant accumulation of F-actin that colocalized with CFP-Solo. Scale bar, 20 μm. (B) Effects of cyclic stretch on gel mobility and tyrosine phosphorylation of Solo. HUVECs and MDCKII cells were subjected to cyclic stretch, as in Figure 1. At indicated times, cell lysates were analyzed by immunoblotting with anti-Solo and anti-phospho-tyrosine antibodies.
Movie 1. Time-lapse fluorescence imaging of CFP-Solo and YFP-Lifeact. HUVECs were co-transfected with CFP-Solo(WT) and YFP-Lifeact and cultured for 24 hours. Fluorescence images of CFP-Solo (green) and YFP-Lifeact (red) were acquired every 30 seconds for 30 min.

Table S1. The primer sequences used to amplify the cDNA fragments of human Rho-GEFs and to analyze the expression of Rho-GEF mRNAs.

Download Table S1

Table S2. The shRNA sequences that target 63 human Rho-GEFs.

Download Table S2
Table S3. Targets and functions of the 11 Rho-GEFs involved in cyclic stretch-induced cell orientation response.

<table>
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Table S4. The siRNAs used in this study.

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