# Substrate Stiffness Regulates PDGF-Induced Circular Dorsal Ruffle Formation Through MLCK

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**Abstract**—As atherosclerosis progresses, vascular smooth muscle cells (VSMCs) invade from the medial layer into the intimal layer and proliferate, contributing to atherosclerotic plaque formation. This migration is stimulated in part by platelet-derived growth factor (PDGF), which is released by endothelial cells and inflammatory cells, and vessel stiffening, which occurs with age and atherosclerosis progression. PDGF induces the formation of circular dorsal ruffles (CDRs), actin-based structures associated with increased cell motility. Here we show that mechanical changes in matrix stiffness enhance the formation of CDRs in VSMCs in response to PDGF stimulation. Our data indicate that matrix stiffness increases cellular contractility, and that intracellular pre-stress is necessary for robust CDR formation. When treated with agonists that promote contractility, cells increase CDR formation, whereas agonists that inhibit contractility lead to decreased CDR formation. Substrate stiffness promotes CDR formation in response to PDGF by upregulating Src activity through myosin light chain kinase. Together, these data indicate that vessel stiffening accompanying atherogenesis may exacerbate VSMC response to PDGF leading to CDR formation.

**Keywords**—Cell migration, Traction force, Actin, Vascular smooth muscle cells, Cell contractility.

## INTRODUCTION

During atherosclerosis, the vessel wall stiffens due to excess extracellular matrix (ECM) deposition, matrix crosslinking, and elastin fragmentation.[16,56] Vascular smooth muscle cells (VSMC), which populate the arterial media, convert from a quiescent phenotype and migrate into the vascular intima,[7,12] leading to plaque formation and blood vessel occlusion.[28,43] This migration is thought to be stimulated by the release of growth factors, particularly platelet-derived growth factor (PDGF), from endothelial cells[14] or macrophages once they infiltrate into the vessel wall.[40,44] Vascular stiffening accompanies atherosclerosis and is measured by clinicians to determine cardiovascular risk.[30,48] Matrix stiffness has been shown to modulate VSMC migration[22,37] and proliferation,[9,31] cell behaviors that occur with atherosclerotic plaque generation. Research from our lab and others showed that vascular stiffening may promote endothelial permeability[21,24] and leukocyte transmigration,[46,47] hallmarks of atherogenesis. Thus, arterial stiffening may be a contributing factor for the progression of atherosclerosis.

Circular dorsal ruffles (CDRs) are transient actin-based structures that have been shown to form in several different cell types in response to growth factor signaling.[4,32] CDRs are thought to play a role in directed VSMC migration in response to PDGF signaling.[17,19] It is believed that wide-scale actin cytoskeletal rearrangements induced by CDRs allows cells to become polarized and motile.[5] Quiescent contractile VSMCs typically exhibit robust stress fibers and cell–matrix attachments that inhibit cell migration. When stimulated with growth factors, actin stress fibers disassemble and are reorganized into CDRs, leaving a meshwork of disassembled cortical actin to be used for lamellipodia extension and cell migration.[25]

Upon PDGF binding to its receptor, Src is quickly phosphorylated and activated. Phosphorylated Src activates and modulates a number of downstream effector proteins required to reorganize actin into CDRs.[4,32] PDGF receptor activation by PDGF was recently shown to be modulated by substrate stiffness.[9] Whether this substrate stiffness receptor sensitization to PDGF translates into changes in CDR formation remain unknown.

Since CDRs are actin-based structures and because actin is known to form more robust stress fibers in

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response to matrix stiffness,[3,13] we hypothesized that CDR formation in VSMCs is modulated by the mechanical properties of the extracellular microenvironment. Our data indicate that substrate stiffness enhances F-actin organization and cellular contractility priming cells for robust PDGF-stimulated CDR formation mediated by myosin light chain (MLC) kinase activity.

## MATERIALS AND METHODS

### Cell Culture and Gel Synthesis

A7R5 rat aortic VSMCs obtained from American Type Culture Collection (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptavidin (Invitrogen). Polyacrylamide (PA) gels were prepared as described previously[21] and coated with 0.002 to 0.2 $\mu$g/mL rat tail collagen type I (BD Biosciences, San Jose, CA). Briefly, 22 × 22 mm or 48 × 65 coverslips (VWR International, Radnor, PA) were activated by subsequent washing in 0.1 N sodium hydroxide (VWR), 3-aminopropyltrimethoxysilane (VWR), and 0.5% glutaraldehyde (Sigma-Aldrich, St. Louis, MO). 1, 2.5, 5, 10, and 30 kiloPascal (kPa) gels were polymerized onto activated coverslips according to the ratios 3:0.1, 5:0.1, 7.5:0.175, 7.5:0.35, and 12:0.28% acrylamide:bisacrylamide (Bio-Rad, Hercules, CA), respectively. Cells were seeded onto PA gels and incubated overnight prior to further experimentation.

### Drug Treatments

To induce CDR formation, VSMCs were treated with 10 ng/mL platelet-derived growth factor (PDGF-BB) (R&D Systems, Minneapolis, MN) for 5 min. In some studies, cells were pre-treated with 10 nM calyculin A for 5 min (Sigma-Aldrich) or 10 $\mu$M ML-7 for 30 min (Sigma-Aldrich).

### Immunofluorescent Staining

A7R5s on PA gels were fixed in 3.7% formaldehyde and subsequently washed in 1% Triton X-100 (Mallinckrodt Baker, Phillipsburg, NJ), 0.2% Tween (Mallinckrodt Baker), and blocked with 3% bovine serum albumin (Sigma-Aldrich). Cells were then immunostained with a 1:200 dilution of rabbit polyclonal MLC kinase or cortactin primary antibody (sc-22223 and sc-11408 respectively, Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were Alexa Fluor 488 or 568 donkey anti-mouse or anti-rabbit (Invitrogen). Actin was stained with Alexa Fluor 488 or 568 phalloidin (Invitrogen) and nuclei with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). Images were captured on a Zeiss Axio Observer.Z1m microscope with a Hamamatsu ORCA-ER camera. Confocal imaging of actin staining was used to perform measurements of cell area after 24 h in culture using ImageJ software (v. 1.46, National Institutes of Health, Bethesda, MD).

### Western Blotting

A7R5 cells cultured on 48 × 65 mm PA gels were lysed with a modified radio-immunoprecipitation assay (RIPA) buffer [150 mM sodium chloride, 50 mM Tris-hydrochloride, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Nonidet P40, 25 mM sodium fluoride, 1 mM sodium orthovanadate, 1:500 dilution of protease inhibitor cocktail (Sigma-Aldrich)]. Cell lysate was cleared by centrifugation at 14,000×$g$ and the supernatant was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After protein transfer onto nitrocellulose, blots were probed using antibodies against phosphorylated MLC at threonine-18 and serine-19 (pMLC) (#3674, Cell Signaling Technology, Beverly, MA), total MLC (#3672, Cell Signaling Technology), phosphorylated Src at Tyr-416 (pSrc) (#2101, Cell Signaling Technology), total Src (#2108, Cell Signaling Technology), phosphorylated Erk at thr 202/204 (pErk) (#9106, Cell Signaling Technology), and total Erk 1/2 (#9102, Cell Signaling Technology). Anti-rabbit horseradish peroxidase (HRP) conjugated secondary antibodies were obtained from Cell Signaling Technology or Santa Cruz Biotechnology. After incubation in SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL), blots were exposed and imaged using a FujiFilm Image-Quant LAS-4000. Protein densitometry was performed using ImageJ software (v. 1.46, National Institutes of Health, Bethesda, MD).

### Traction Force Microscopy

A7R5 cells were cultured overnight on PA gels embedded with 0.5 $\mu$m diameter fluorescent beads (Invitrogen). Individual cells and the stressed fluorescent bead field beneath the cell were imaged. After cell removal with 0.25% trypsin–EDTA (Invitrogen), a second fluorescent image of the unstressed bead field was imaged. Bead displacements were calculated from the stressed and unstressed images and used to compute cellular traction vectors and total magnitudes of force using the LIBTRC analysis library developed by Dr. Micah Dembo (Department of Biomedical Engineering, Boston University).

### Statistical Analysis

Statistical analyses were performed using JMP 8 (SAS Institute, Cary, NC), GraphPad Prism 5 (GraphPad Software, Inc.), or Excel 2010 (Microsoft, Corporation, Redmond, WA). Parametric one-way or two-way ANOVA with *post hoc* Tukey's honest significance test were performed where appropriate. *P* values <0.05 were considered statistically significant.

Data in Fig. 2 were fit to a dose–response curve[38] described by Eq. (1):

$$Y = E_{min} + \frac{E_{max} - E_{min}}{1 + \left(\frac{x}{ED_{50}}\right)^h}, \qquad (1)$$

where $Y$ is the response (in this case, percentage of cells exhibiting CDRs), $E_{min}$ is the minimum response, $E_{max}$ is the maximum response, $x$ is the dose (in this case, stiffness), $h$ is the Hill slope, and $ED_{50}$ is the effective dose that elicits a half-maximal response. The values for $E_{min}$, $E_{max}$, and $h$ were constrained to stay constant between collagen densities.

## RESULTS

### Matrix Stiffening and Increased Collagen Density Promotes CDR Formation

Using PA gels varying in stiffness from 1 to 30 kPa, we first investigated A7R5 VSMCs actin organization with respect to substrate stiffness. This particular stiffness range was chosen according to measurements reported previously of healthy and atherosclerotic vessels.[29,36] With increasing stiffness, the actin cytoskeleton of VSMCs becomes increasingly organized into F-actin-rich stress fibers (Fig. 1a). On more compliant substrates (1 and 2.5 kPa gels), cells do not form many stress fibers; however, on stiff substrates (10 kPa and above), they exhibit robust stress fibers that extend the length of the cell. In the same way, cell spreading area increased gradually from 1 to 5 kPa, and remained constant for higher stiffnesses (Fig. 1b). After inducing CDR formation in VSMCs with PDGF, cells were fixed and stained for actin and the CDR marker cortactin. The colocalization of these proteins was used to determine the formation of CDRs with respect to matrix stiffness (Fig. 1c). Quantification of the percentage of cells that exhibit CDRs reveals that a larger percentage of cells cultured on stiff substrates formed CDRs compared to those cultured on more compliant substrates (Fig. 1d).

Because matrix protein density has been shown to alter VSMC spreading and migration speeds,[37] we tested whether the collagen density conjugated to the PA gels affects the formation of CDRs. We modeled the percentage of cells that exhibit CDRs vs. substrate stiffness and collagen density as a pharmacokinetic dose–response curve, where stiffness was modeled as the dose. Compared to 0.002 or 0.02 mg/mL collagen, 0.2 mg/mL collagen shifted the stiffness–response curve to the left, indicating that on substrates conjugated with 0.2 mg/mL collagen, a higher percentage of cells formed CDRs on lower stiffness (Fig. 2). Additionally, the effective dose that elicits a half-maximal response, or $ED_{50}$, decreased from ~12 kPa on 0.002 and 0.02 mg/mL collagen-conjugated gels to ~7 kPa on 0.2 mg/mL collagen-conjugated gels. Together, our data suggest that stiffer substrates and higher collagen densities promote the formation of CDRs.

### Cell Force is Required for CDR Formation

Recent data indicates that CDR formation may be related to cell contractility,[55] and because our data shows that increased substrate stiffness promotes robust stress fiber formation, we asked whether stress fiber organization resulted in higher cell contraction and whether these forces are required for the formation of CDRs. Cell contractility as assessed through MLC phosphorylation state increased with substrate stiffness (Fig. 3). Additionally, traction force microscopy was performed to quantify cell traction forces of VSMCs cultured on 2.5, 5, 10, and 30 kPa substrates. In accordance with our MLC phosphorylation data, cells cultured on stiffer substrates exhibited higher traction forces (Figs. 4a and 4b).

Our data indicate that cells that generate higher traction forces also exhibit more CDRs. As such, we sought to determine whether force is a prerequisite for CDR formation. To upregulate or downregulate cell force, cells were treated with either calyculin A, an inhibitor of MLC phosphatase, or ML-7, an inhibitor of MLC kinase, respectively. Regardless of stiffness, cells treated with calyculin A increased their traction force generation significantly (Fig. 5a, green bars). Cells cultured on stiffer substrates that were treated with ML-7 exhibited lower traction forces compared to their respective untreated controls (Fig. 5a, blue bars). Notably, a higher percentage of cells pretreated with calyculin A formed CDRs in response to PDGF treatment compared to cells not treated with contractility-altering drugs. Conversely, significantly fewer cells formed CDRs when pretreated with ML-7. Thus, increased substrate stiffness positively affects MLC regulated cell contractility which in turn enables robust CDR formation.
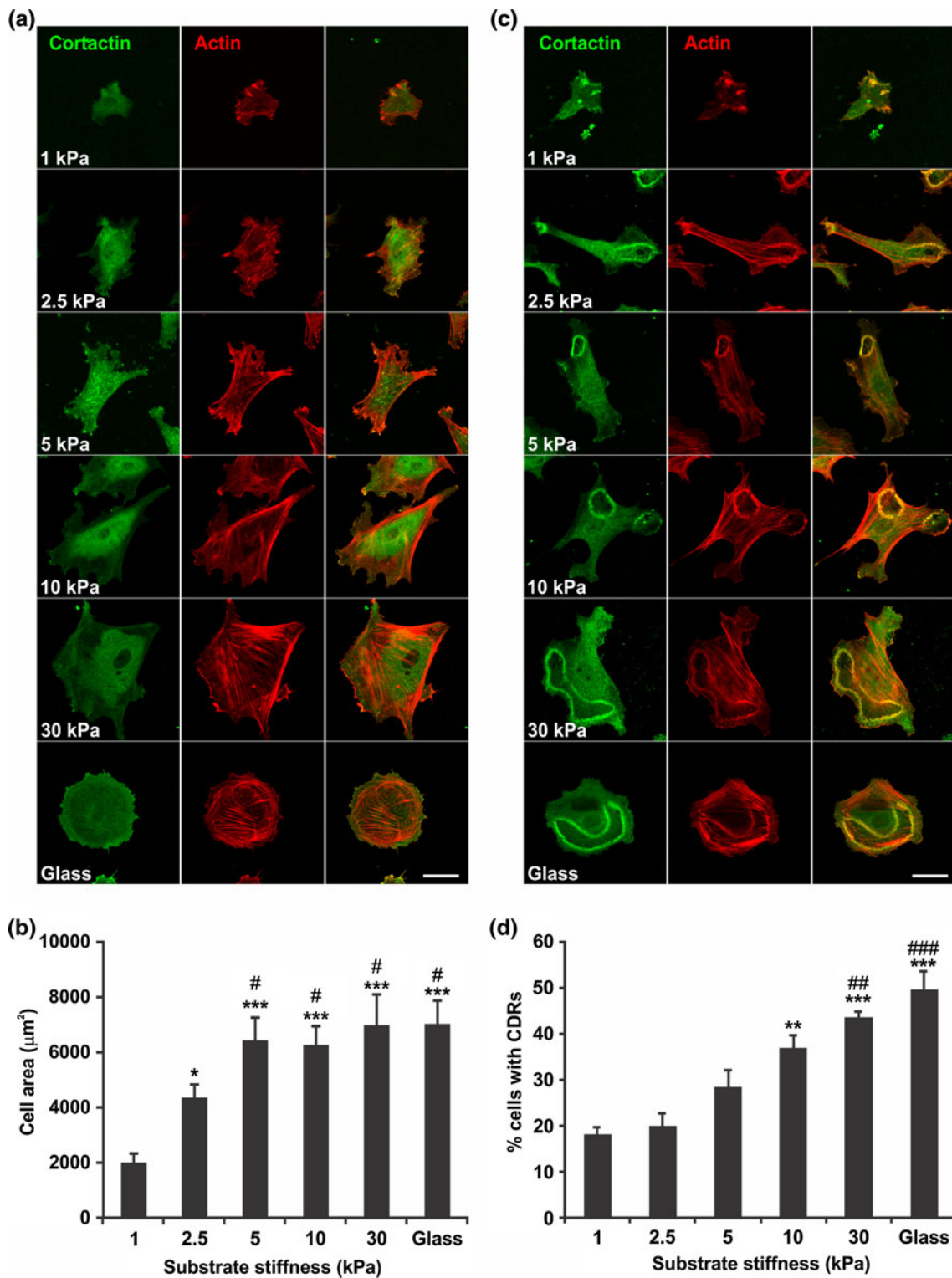
FIGURE 1. Increased substrate stiffness promotes circular dorsal ruffle formation. (a) Representative images of cortactin (green) and actin organization (red) of A7R5 vascular smooth muscle cells cultured on collagen-coated polyacrylamide gels varying in stiffness from 1 to 30 kPa. (b) Corresponding cell area with respect to substrate stiffness. Data are mean ± SEM. *$p < 0.05$ and ***$p < 0.001$ vs. 1 kPa, #$p < 0.05$ vs. 2.5 kPa. (c) Cortactin (green) and actin organization (red) after 5 min PDGF stimulation induced circular dorsal ruffles. (d) The percentage of cells exhibiting PDGF-induced CDRs on polyacrylamide gels derivatized with 0.2 mg/mL rat tail type I collagen ($n = 6$ independent experiments). Data are mean ± SEM. **$p < 0.01$ vs. 1 or 2.5 kPa, ***$p < 0.001$ vs. 1 or 2.5 kPa, ##$p < 0.01$ vs. 5 kPa, ###$p < 0.001$ vs. 5 kPa (Tukey's test). Scale bar, 40 $\mu$m.
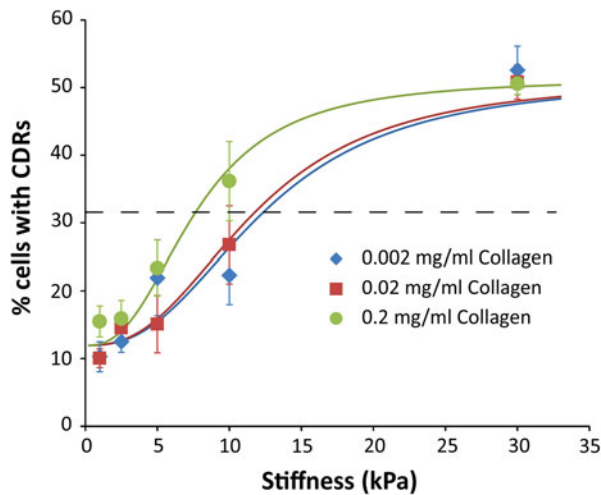
**FIGURE 2. Collagen density promotes circular dorsal ruffle formation.** The percentage of cells forming CDRs on gels derivatized with 0.2, 0.02, and 0.002 mg/mL collagen ($n = 3$ independent experiments). The dashed black line represents the half-maximal response. Data are mean ± SEM.
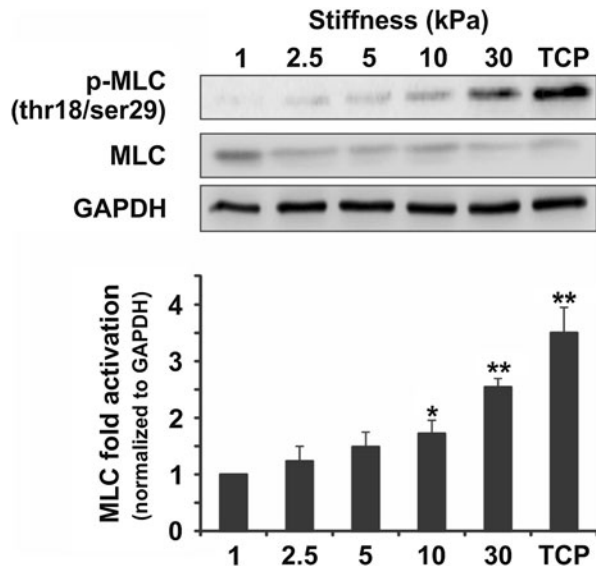


**FIGURE 3. Substrate stiffness upregulates MLCK activity to prime cells for robust CDR formation.** Representative immunoblots of phosphorylated MLC (p-MLC) at residues threonine-18 and serine-19 and total MLC with respect to cells cultured on different substrate stiffnesses or on tissue culture plastic (TCP). GAPDH was used as loading control. The mean fold activation values ± SEM are provided ($n = 3$ independent experiments). $*p < 0.05$ and $**p < 0.01$ vs. 1 or 2.5 kPa (Tukey's test).

### PDGF-Induced Src Activity Depends on Substrate Stiffness Modulation

It has been shown previously that the formation of CDRs following PDGF-receptor activation by PDGF stimulation involves a Src-dependent signaling cascade.[32] Additionally, growing evidence indicate that substrate stiffness affects cell surface receptor dependent signal transduction,[1,35] including PDGF receptors.[9] Accordingly, when Src was inhibited with pharmacological inhibitor PP1, treated VSMCs were unable to form CDRs in response to PDGF (Fig. 6a), highlighting the essential role of Src in CDR formation. As such, we sought to determine whether substrate stiffness regulates PDGF-induced Src activity in A7R5 cells. Lysates from PDGF-treated cells cultured on gels of varying stiffness were probed for Src phosphorylation at residue tyrosine 416 (Y416), a marker for activated Src.[4] Indeed, Src phosphorylation was found to increase with substrate stiffness (Fig. 6b), suggesting that PDGF-induced Src activation is mediated by substrate stiffness. Additionally, inhibition of Src activation by PP1 did not result in any changes in MLC phosphorylation for cells plated on 10 kPa gels (Fig. 6c). Together, these results suggest that contractility mediated CDR formation occurs upstream of Src activation in response to PDGF receptor activation.

### MLC Kinase Modulation of Cell Contractility is Required for PDGF-Induced Src Activation

Substrate stiffness regulates cellular contractility in part through the action of the MLC kinase (MLCK).[10] Of note, Src was recently shown to complex with MLCK and affect its function.[6] Interestingly, upon PDGF stimulation, immunofluorescent staining indicates that MLCK colocalizes with CDRs (Fig. 7a). Given our above results indicating CDR formation decreases after MLCK inhibition, we assessed if Src activation was dependent on MLCK-mediated cell contractility. As shown in Fig. 7b, PDGF stimulation increases Src phosphorylation at Y416 compared to the controls while the inhibition of MLCK-induced cell contractility by pretreatment with ML-7 is sufficient to block this activation. Investigation of Erk phosphorylation downstream of the PDGF receptor reveals that Erk activation by PDGF is greatly diminished by the use of ML-7 (Fig. 7b). Together, these results suggest that MLCK regulation of cell contractility is required for activation of different PDGF pathways. In combination with the above TFM and CDR data, these results show that substrate stiffness regulates PDGF-induced CDR formation through the modulation of cell contractility.

### DISCUSSION

Here, we present data that implicate vascular stiffening as a factor that promotes the formation of VSMC CDRs, structures believed to play a large role
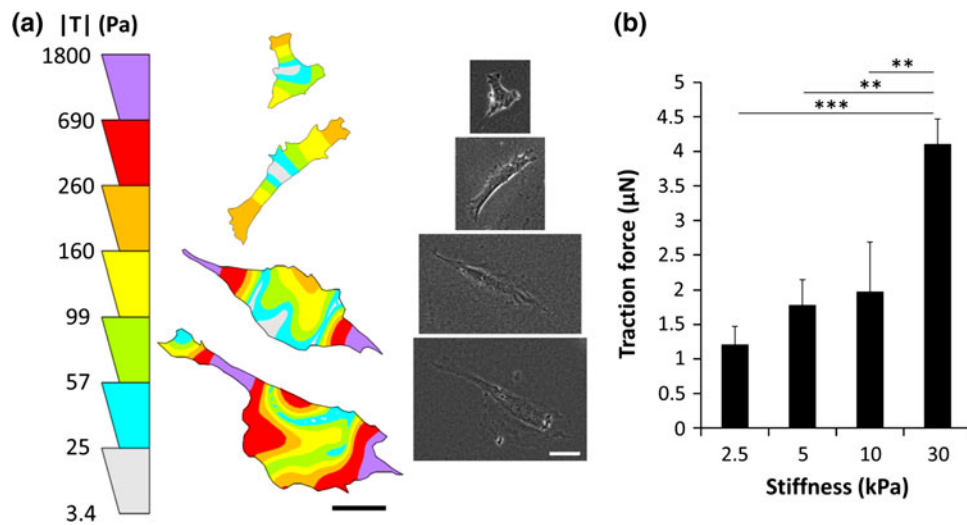
FIGURE 4. Substrate stiffness regulates intracellular pre-stress. (a) Representative traction force maps and phase images of A7R5 VSMCs on polyacrylamide gels. Scale bars, 50 $\mu$m. (b) The total magnitudes of cell traction force measurements with respect to substrate stiffness ($n$ = 12–26 cells, three independent experiments). Data are mean ± SEM. **$p < 0.01$, ***$p < 0.001$ (Tukey's test).
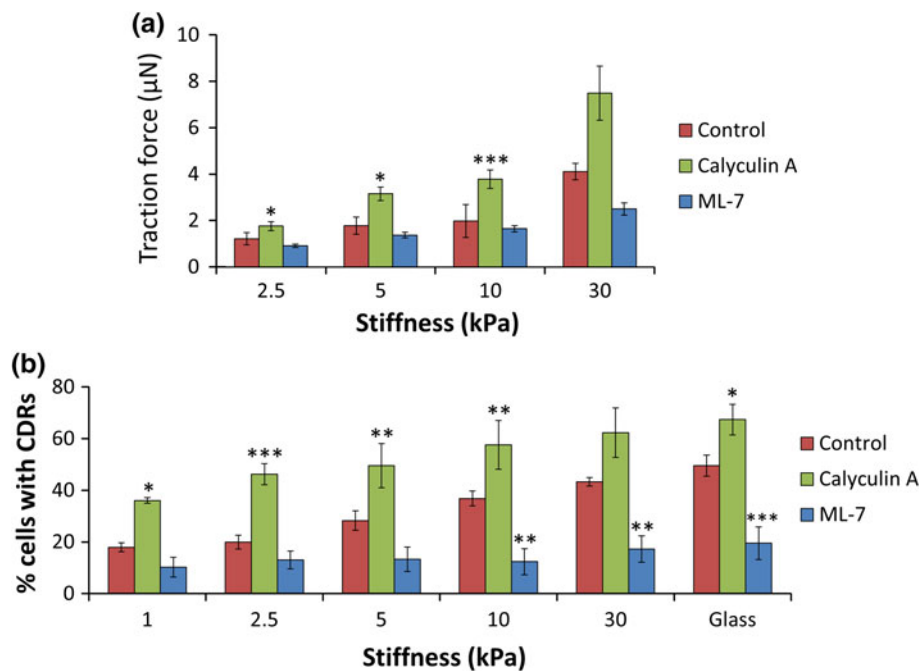


FIGURE 5. Pharmacological control of cell force modulates CDR formation. (a) Traction force measurements of untreated (red bars, $n$ = 12–26), calyculin A treated (green bars, $n$ = 46–60), and ML-7 treated (blue bars, $n$ = 37–51) cells. Data are mean ± SEM. *$p < 0.05$ compared to respective untreated controls (Tukey's test). (b) The percentage of cells cultured on gels that exhibit CDRs ($n$ = 3 independent experiments). Data are mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to respective untreated controls (Tukey's test).

in initiating cell motility. Our results indicated that CDR formation in VSMCs is dependent on substrate stiffness. On more compliant substrates, cells displayed fewer CDRs compared to cells cultured on stiffer substrates. Moreover, our data indicate that cell contractility is a prerequisite for CDR formation, and upregulation of cell force increases PDGF-induced Src

activity that leads to greater CDR formation. Although the total magnitude of cell force increases with substrate stiffness, it remains to be seen whether local forces, particularly at sites of CDR formation, regulate actin dynamics to form CDRs. Overall, substrate stiffness promotes the formation of CDRs through upregulation of MLCK-mediated cell contractility.
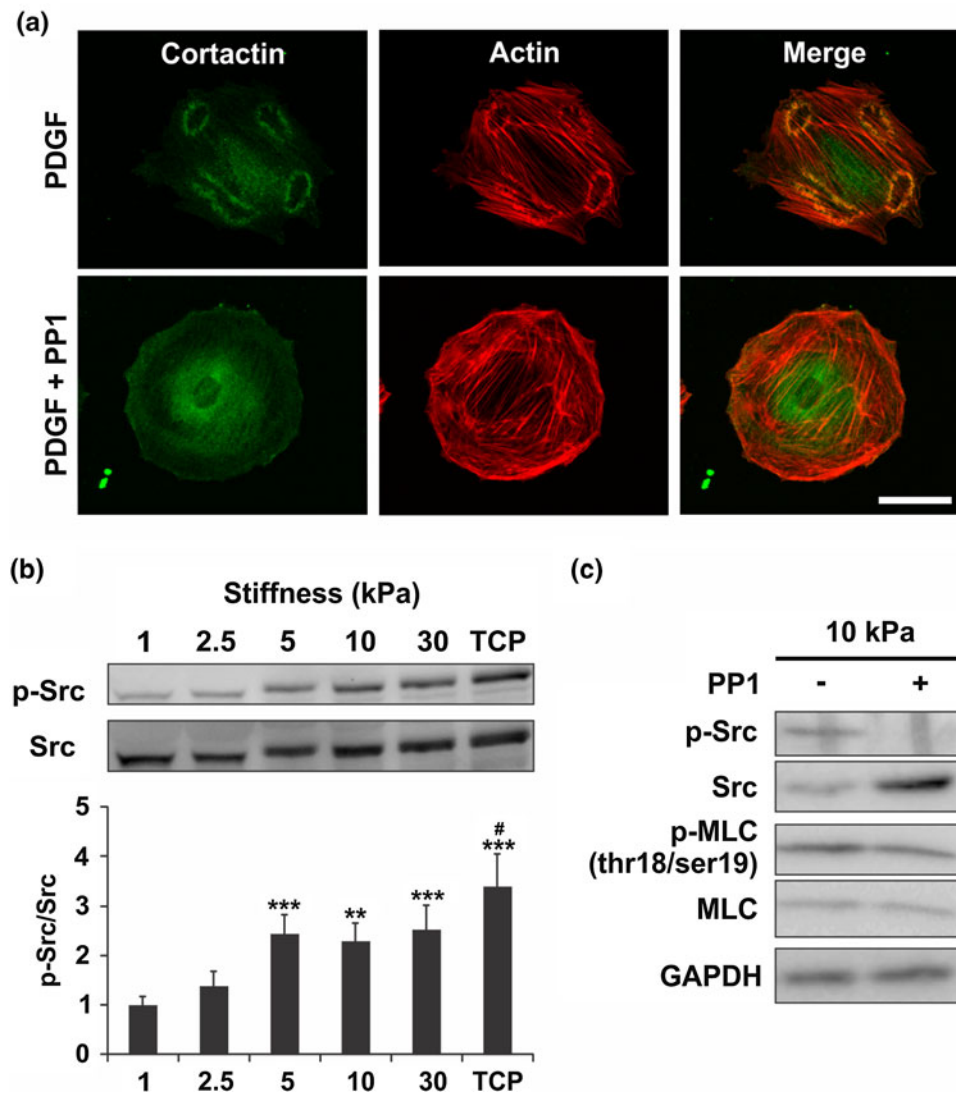
FIGURE 6. Src activity increases with substrate stiffness and is required for CDR formation. (a) Representative images of cortactin (green) and actin organization (red) of cells plated on collagen coated glass after 5 min PDGF showing the absence of CDR in response to PP1 pretreatment. Scale bar, 40 $\mu$m. (b) Representative immunoblots of phosphorylated Src (p-Src) and total Src and the ratio of pSrc to total Src in cells cultured on gels of varying stiffness or TCP ($n$ = 6 independent experiments). GAPDH was used as loading control. Data are mean ± SEM. **$p < 0.01$ vs. 1 or 2.5 kPa, ***$p < 0.001$ vs. 1 or 2.5 kPa, #$p < 0.05$ vs. 5, 10, or 30 kPa (Tukey's test). (c) Western blotting on total protein extracts of cells plated on 10 kPa gels after 5 min PDGF treatment with or without PP1 pretreatment showing decreased levels of p-Src but equal levels of p-MLC in response to Src inhibition.

It is widely accepted that, during atherosclerosis progression, VSMCs switch from a quiescent "contractile" phenotype to a migratory "synthetic" phenotype, where they are able to degrade their matrix and invade into the intima.[34] Previous data suggests that PDGF signaling, which is both a potent migratory signal and initiator of CDRs, is a major contributing factor to the VSMC phenotypic switch.[41] Our data suggests that the matrix stiffening that occurs concomitant with atherosclerosis progression may exacerbate phenotypic switching by increasing the percentage of cells that exhibit CDR formation in response to PDGF signaling. This is likely due to heightened MLCK activity of cells in stiffer microenvironments, as evidenced by our data showing increased threonine-18/serine-19 MLC phosphorylation and CDRs inhibition by ML-7. Several upstream activators may also directly contribute to CDR formation, particularly RhoGTPase and Rho-associated kinase (ROCK). RhoGTPase activity, which has been shown to increase with substrate stiffness,[18,21] activates ROCK, which in turn regulates MLC at serine-19 to regulate stress fiber formation[49] and cell contractility.[8] ROCK also functions to prevent MLC phosphatase from deactivating MLC.[51] Hence, we cannot exclude that RhoGTPase and ROCK contribution to
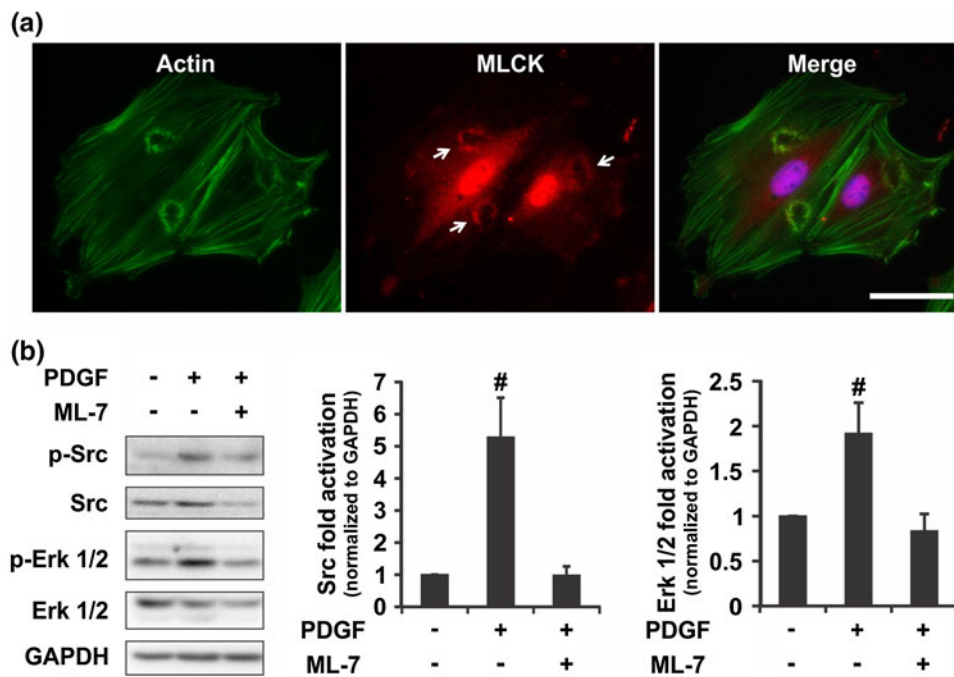
FIGURE 7. Involvement of MLCK in PDGF stimulation. (a) Representative immunofluorescent images of F-actin (green) and MLCK (red). White arrows indicate the localization of MLCK at CDRs. Scale bar, 50 $\mu$m. (b) Western blotting on total protein extracts of cells plated on 10 kPa gels after 5 min PDGF treatment with or without ML-7 pretreatment showing increased phosphorylation levels of Src and Erk after PDGF treatment. ML-7 prevents PDGF-mediated activation of both Src and Erk. The mean fold activation values $\pm$ SEM are provided ($n = 3$ independent experiments). $*p < 0.05$ vs. untreated control. GAPDH was used as loading control.

intracellular pre-stress may affect CDR formation. Indeed, a recent model by Zeng et al.[55] propose a direct Rho involvement in regulating CDR lifetime. Therefore, there is significant evidence that cell contractility is an important regulator of CDR formation.

Previous data suggests that substrate rigidity enhances PDGF receptor phosphorylation intensity and duration.[9] Here we show that Src activation also increases with substrate stiffness downstream of the PDGF receptor, resulting in increased CDR formation in a process that requires cell contractility. However, regulation of cell contractility involves processes that take place at focal adhesions. In fact, key focal adhesion signaling proteins, including focal adhesion kinase (FAK) and Src, are known to be regulated by matrix stiffness,[35,53] and mechanical activation of Src requires an intact actin cytoskeleton and functional contractility.[33,50] In turn, increased cell contractility is required for proper targeting of signaling and scaffolding proteins to focal adhesions, actin-based structures, and membrane microdomains.[2,6,11,15] Conversely, recent studies have linked growth factor induced CDR formation with pre-existing signals emanating from focal adhesions. Notably, Azimifar et al.[4] show that epithelial growth factor induced CDR formation requires proper Src localization to focal adhesions by associating with integrin-linked kinase (ILK). Additionally, integrins $\beta1$ and $\beta3$ have been reported to be involved in CDR formation.[4,17,23] Alternatively, expression of different paxillin constructs could result in either up- or down-regulation of PDGF induced CDRs formation in fibroblast.[45] Interestingly, Src, paxillin, and integrins are known regulators of the cell contractility pathway[15,20,35,45,52] and were all shown to be present within CDRs after PDGF stimulation.[4,17,23,45] As such, integrin-mediated cell contractility regulation may be required for targeting proteins upstream of PDGF to regulate CDR formation.

In addition to VSMC migration, our data may also have implications in cancer, where stiffening of the tumor microenvironment has been shown to promote cancer cell migration and metastasis.[26,35,54] Invasive cancer cells form actin-based structures in response to growth factors stimulation that are similar to CDRs in their protein makeup called invadopodia.[4] Like CDRs, invadopodia are hypothesized to enhance cell migration[42]; however, they play a more significant role in cell invasion by releasing MMPs to degrade their surrounding extracellular matrix.[27,39] Because invadopodia formation is also dependent on Src activity,[32] our data suggests that substrate stiffness may also regulate invadopodia formation. Indeed, recent findings by Alexander et al.[1] showed that breast carcinoma cells formed more invadopodia and degraded more of their matrix when cultured on stiffer substrates. However, a direct involvement of cell contractility remains an open question.

Here, we provide evidence that substrate stiffness regulates the formation of CDRs in VSMCs in a contractility-dependent process. Specifically, intracellular pre-stress, which is modulated by matrix stiffness, primes cells for robust CDR formation. Increased CDR formation on stiffer substrates likely contributes to the migration of VSMCs, possibly by phenotypically switching quiescent "contractile" VSMCs into more migratory "synthetic" cells through large-scale actin rearrangement. Collectively, our data suggests that the changes in vessel stiffness that accompany atherosclerosis enhance CDR formation and therefore likely contribute to the advancement of disease.

## ACKNOWLEDGMENTS

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