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Matrix rigidity regulates spatiotemporal dynamics of Cdc42 activity and vacuole formation kinetics of endothelial colony forming cells

Seung Joon Kim [a], Qiaoqiao Wan [a,c], Eunhye Cho [a], Bumsoo Han [b,c], Mervin C. Yoder [d,e,f], Sherry L. Voytik-Harbin [c,g], Sungsoo Na [a,*]

[a] Department of Biomedical Engineering, Indiana University-Purdue University Indianapolis, Indianapolis, IN 46202, USA
[b] School of Mechanical Engineering, Purdue University, West Lafayette, IN 47907, USA
[c] Weldon School of Biomedical Engineering, Purdue University, West Lafayette, IN 47907, USA
[d] Department of Pediatrics, Indiana University School of Medicine, Indianapolis, IN 46202, USA
[e] Herman B. Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN 46202, USA
[f] Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN 46202, USA
[g] Department of Basic Medical Sciences, Purdue University, West Lafayette, IN 47907, USA

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ABSTRACT

Recent evidence has shown that endothelial colony forming cells (ECFCs) may serve as a cell therapy for improving blood vessel formation in subjects with vascular injury, largely due to their robust vasculogenic potential. The Rho family GTPase Cdc42 is known to play a primary role in this vasculogenesis process, but little is known about how extracellular matrix (ECM) rigidity affects Cdc42 activity during the process. In this study, we addressed two questions: Does matrix rigidity affect Cdc42 activity in ECFC undergoing early vacuole formation? How is the spatiotemporal activation of Cdc42 related to ECFC vacuole formation? A fluorescence resonance energy transfer (FRET)-based Cdc42 biosensor was used to examine the effects of the rigidity of three-dimensional (3D) collagen matrices on spatiotemporal activity of Cdc42 in ECFCs. Collagen matrix stiffness was modulated by varying the collagen concentration and therefore fibril density. The results showed that soft (150 Pa) matrices induced an increased level of Cdc42 activity compared to stiff (1 kPa) matrices. Time-course imaging and colocalization analysis of Cdc42 activity and vacuole formation revealed that Cdc42 activity was colocalized to the periphery of cytoplasmic vacuoles. Moreover, soft matrices generated faster and larger vacuoles than stiff matrices. The matrix-driven vacuole formation was enhanced by a constitutively active Cdc42 mutant, but significantly inhibited by a dominant-negative Cdc42 mutant. Collectively, the results suggest that matrix rigidity is a strong regulator of Cdc42 activity and vacuole formation kinetics, and that enhanced activity of Cdc42 is an important step in early vacuole formation in ECFCs.

1. Introduction

Endothelial colony forming cells (ECFCs) are circulating cells with *in vivo* vessel forming properties that can be isolated from human umbilical cord blood [1]. A growing body of evidence suggests that ECFCs have a therapeutic potential as a cell-based strategy in repair of damaged tissues due to their high telomerase activity and proliferative potential [2–4]. Thus, understanding the molecular mechanisms that govern ECFC-driven blood vessel formation will provide important insights into therapeutic vascularization [5–7]. Vessel formation or morphogenesis is often described as a program of cellular events including vacuole formation within individual cells, coalescence of vacuoles to form multicellular lumens, and sprouting and branching events that yield multi-cellular networks of interconnected tubes. Significant advancements have been made in identification of major cellular processes, soluble growth factors, and associated molecular players underlying vasculogenesis and angiogenesis [8,9].

In addition to these findings, it is now well recognized that the fibril microstructure and mechanical properties (e.g., stiffness) of the extracellular matrix component of the cell microenvironment modulate vessel formation *in vitro* and *in vivo* [5–7,10]. Two-dimensional (2D) culture studies using bovine aortic endothelial cells and human umbilical vein endothelial cells demonstrated that substrates with compliant or intermediate rigidity promote the vascular cord structure and capillary network, while stiffer substrates or higher matrix density decrease the cell's capillary network formation ability [11,12]. In addition to these traditional,

* Corresponding author. Address: Department of Biomedical Engineering, Indiana University-Purdue University Indianapolis, 723 West Michigan Street, SL220, Indianapolis, IN 46202, USA. Fax: +1 317 278 2455.
E-mail address: sungna@iupui.edu (S. Na).

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monolayer culture studies, more physiologically relevant studies within 3D matrices have recently demonstrated a regulatory role of the ECM stiffness in the vascular lumen formation [13–16]. These works suggest that a matrix-integrin-cytoskeleton signaling axis drives vessel formation and represents convergence of biochemical and biophysical signaling pathways.

Major signaling downstream of integrin-mediated binding involves small Rho GTPases, including Rac1 and Cdc42 [17]. These bi-molecular switches cycle between an active (GTP-bound) and inactive (GDP-bound) states, which are regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) [18]. Selective inhibition and gene transfer studies indicate that Cdc42 and Rac1 drive vacuole formation and coalescence while RhoA appears to stabilize capillary tube networks [17]. Although the role of Cdc42 in vessel formation is well documented [19,20], little is known about how ECM rigidity modulates Cdc42 activity during vacuole formation.

Here we report, for the first time, the spatiotemporal activity of Cdc42 at the subcellular level during ECFC vacuole formation. Since vacuole formation and the associated signaling activity are known to be highly dynamic [21], we employed live cell imaging in conjunction with FRET technique to capture the complexity of the interactions. In order to examine the role of ECM stiffness in Cdc42 activities and vacuole formation, we used 3D collagen matrices with different rigidities. The spatiotemporal correlation was performed between Cdc42 activation and vacuole formation. To further examine the role of Cdc42 in early vacuole formation in ECFCs, we used constitutively active and dominant negative Cdc42 mutants.

2. Materials and methods

2.1. Cdc42 biosensor

A FRET-based Cdc42 biosensor was used to monitor Cdc42 activity in ECFCs at the subcellular resolution [22]. The biosensor consists of truncated Cdc42, its substrate PAK1 (p21 protein-activated kinase 1; a serine-threonine kinase activated by Cdc42 GTPase), and a pair of cyan florescent protein (CFP) and yellow fluorescent protein (YFP) (Fig. 1A). Upon activation, Cdc42 proceeds with conformational changes so that it binds specifically to its substrate PAK1 within the biosensor. The binding of Cdc42 with PAK1 results in the close association of CFP with YFP, which leads to an increase of FRET from CFP to YFP upon excitation of CFP at 433 nm. Thus Cdc42 activity can be represented as the emission intensity ratio of YFP/CFP. This ratiometric imaging approach has been well characterized and extensively used to measure intracellular signaling [22–24].

2.2. Cell culture and transfection

Human umbilical cord blood ECFCs were obtained from EndGenitor Technologies (Indianapolis, IN) and cultured as described previously [2]. The cells were cultured in EGM-2 media (Lonza, Walkersville, MD) supplemented with 10% FBS (Invitrogen, Grand Island, NY) and 1.5% of antibiotic-antimycotic (Invitrogen). Prior to experiments, the cells were maintained at 37 °C and 5% CO_2 in a humidified incubator. The DNA plasmids were transfected into the cells using a Neon transfection system (Invitrogen). A GFP-actin was used to confirm the transfection. To examine the role of Cdc42 in vacuole formation, constitutively active (Cdc42-L61) and dominant negative (Cdc-N17) Cdc42 mutants were used [25].

2.3. Preparation of collagen solutions

Oligomeric type I collagen were extracted and purified from the dermis of market weight pigs as described previously [26]. Lyophilized collagen was dissolved in 0.01 N hydrochloric acid (HCl) and collagen concentration was determined using a Sirius Red assay [27]. Collagen solutions were neutralized with 10X PBS (1X PBS had 0.17 M total ionic strength and pH 7.4) and 0.1 N sodium hydroxide to achieve neutral pH (7.4). Collagen solutions were polymerized under identical reaction conditions to produce 3D matrices as described previously [5]. Positive displacement pipettes (Microman; Gilson, Middleton, WI) were used to accurately deliver all collagen solutions.

2.4. Stiffness measurement of 3D collagen matrices

Viscoelastic properties of polymerized collagen matrices as a function of collagen concentration were measured in oscillatory shear on a stress-controlled AR2000 rheometer (TA Instruments, New Castle, DE) using a stainless steel 40-mm diameter parallel

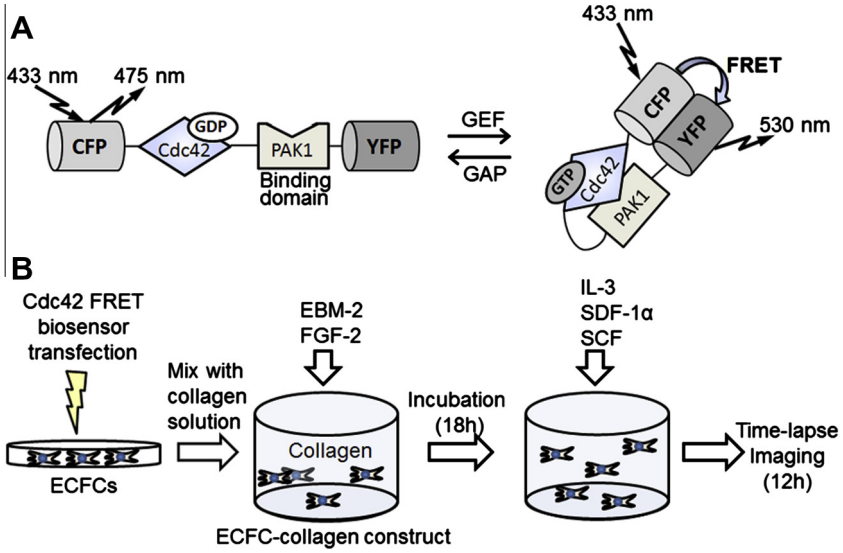

Fig. 1. (A) Molecular structure of a FRET-based Cdc42 biosensor. Adapted from Ref. [22]. (B) Schematic diagram showing timeline of ECFC transfection, collagen-based tissue construct formation, and spatiotemporal analysis of ECFC vacuole formation and Cdc42 activity.

plate geometry as previously described [26]. Briefly, a shear strain sweep from 0.01% to 5% strain at 1 Hz was used to measure shear storage modulus (G′) and shear loss modulus (G″). Reported values are at 1% strain. Shear testing was performed on three independent matrices per matrix formulation. Collagen concentrations yielding matrix stiffness (G′) values of 150 Pa and 1 kPa were selected for this study.

2.5. Preparation of 3D vascularized tissue constructs

Transfected ECFCs (2×10^6 cells/ml) were suspended in neutralized oligomer solutions, pipetted (150 μl) into small glass cylinders (Fisher Scientific, Pittsburgh, PA) positioned on a glass-bottom dish (MatTek, Ashland, MA), and allowed to polymerize for 15 min at 37 °C. ECFC-collagen constructs were cultured overnight in endothelial cell basal media (EBM-2; Lonza, Walkersville, MD) containing 40 ng/ml of fibroblast growth factor (FGF-2; R&D Systems, Minneapolis, MN) and reduced serum II (RS II) to maintain the cell viability and prime the vacuole formation [28]. After 18 h of incubation, media was replaced with fresh EBM-2 media supplemented with 200 ng/ml of stem cell factor (SCF; EMD Millipore, Billerica, MA), stromal cell-derived factor-1α (SDF-1α; R&D Systems), and interleukin-3 (IL-3; R&D Systems) to induce vacuole formation [28]. A schematic of the process and timeline for collagen-ECFC construction preparation as well as vacuole induction and analysis is shown in Fig. 1B.

2.6. Fluorescence microscopy

Images were collected using an automated fluorescence microscope (Nikon, Melville, NY) equipped with a charge-coupled device camera (Evolve 512; Photometrics, Tucson, AZ), a filter wheel controller (Sutter Instruments, Novato, CA) and a Perfect Focus System (Nikon) that maintains the microscope focus during time-lapse imaging. The following filter sets were used (Semrock, Rochester, NY): CFP excitation: 438/24 (center wavelength/bandwidth in nm); CFP emission: 483/32; YFP (FRET) emission: 542/27. Cells were illuminated with a 100 W Hg lamp through an ND64 (~1.5% transmittance) neutral density filter to alleviate photobleaching. Time-lapse images were acquired at 1 h intervals with a 40X (0.75 numerical aperture) objective. Deconvolution was conducted to reduce out-of focus fluorescence using NIS-Elements software (Nikon). During imaging, the cells were maintained at 37 °C using an air stream incubator (Nevtek, Williamsville, VA).

2.7. Image analysis

Acquired images (512 × 512 pixels, 0.4 μm/pixel) were analyzed by NIS-Elements software and ImageJ software (NIH, Bethesda, MD) [29]. The images bisecting the center of an individual cell were used for this study. Both CFP and YFP images were background subtracted and aligned pixel to pixel. Corresponding pixels in the aligned CFP and YFP images were computed to generate YFP/CFP ratio images. The emission ratios were averaged over the whole cell and were normalized to time point 0 h. The spatial analysis of Cdc42 activity was conducted by drawing the line on the body of a cell including vacuoles. The ECFC vacuole area was measured by manually tracing the vacuoles in the DIC images and computing the area using NIS-Elements.

2.8. Statistical analysis

All statistical data were analyzed using Prism 5 software (GraphPad Software, La Jolla, CA) and presented as the mean ± standard error of the mean (SEM). One-way ANOVA followed by Tukey's test was used for multiple comparisons. Student's *t*-test was used to compare the difference between two groups. A *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Cdc42 activation pattern is spatiotemporally correlated with ECFC vacuole formation sites within 3D collagen matrices

Since Cdc42 plays a key role in cell–matrix interactions and vessel formation [17], we reasoned that spatiotemporal activity of Cdc42 within an individual ECFC would be closely associated with vacuole formation dynamics. To test this hypothesis, we conducted live-cell, time-lapse imaging of Cdc42 activity and vacuole formation. ECFCs were transfected with a FRET-based Cdc42 biosensor and cultured in either soft (150 Pa) or relatively stiff (1 kPa) collagen matrices. Vacuole formation was induced within tissue constructs 18 h after seeding. Time-lapse imaging then was conducted on individual cells over a 12 h period. In both soft and rigid matrices, the Cdc42 activity was increased when vacuole formation was initiated and the strong Cdc42 activation sites colocalized with the sites of vacuole formation (Fig. 2A and B). The line profile data of Cdc42 activation around the vacuoles further demonstrates the colocalization of Cdc42 activation and vacuole formation sites (Fig. 2C and D). These results suggest that locally elevated Cdc42 activation within the ECFCs may be required to initiate vacuole formation.

3.2. Matrix rigidity affects dynamics of Cdc42 activation

Published reports show that Cdc42 is mechanoresponsive and plays an important role in integrin-mediated mechanotransduction [30] and cell–ECM interaction [31]. To test whether the level of Cdc42 activation would be regulated by ECM rigidity, ECFCs were transfected with a Cdc42 biosensor and cultured within 3D collagen matrices prepared at 150 Pa and 1 kPa stiffness. Here, the collagen concentration of the polymerization reaction was increased to increase matrix stiffness which occurs as a result of increased fibril density [26]. Time-lapse imaging was conducted for 9 h after the induction of vacuole formation and Cdc42 activity was quantified as the average YFP/CFP emission ratio of the biosensor measured across an individual ECFC. Distinct dynamics of Cdc42 activation were observed in response to different matrix rigidities (Fig. 3A). In soft matrices, Cdc42 activity gradually increased until it was maximal (~13%) at 3 h, and returned to the basal level at 7 h. In stiff matrices, Cdc42 activity also gradually increased until 3 h, but the maximal activation level was not substantial as compared to that in soft matrices. In addition, the basal level of Cdc42 activity (before the induction of vacuole formation at time zero) was higher in soft matrices than that in stiff matrices (Fig. 3B).

3.3. Matrix rigidity affects vacuole formation kinetics

To examine the role of matrix rigidity on the kinetics of vacuole formation, the timecourse of initial vacuole formation was monitored and quantified for individual ECFC for 6 h after induction of vacuole formation. Soft matrices induced earlier vacuole formation (62.5% of cells within 3 h), whereas vacuoles appeared later in stiff matrices (Fig. 3C). Interestingly, dynamics of Cdc42 shows similar trend as that of vacuole formation of ECFCs in soft matrices, but not in stiff matrices (compare Fig. 3A and C). Taken together, the data suggest that higher basal level and enhanced activity of Cdc42 in soft matrices may contribute to the synchronized Cdc42 activity and vacuole formation.

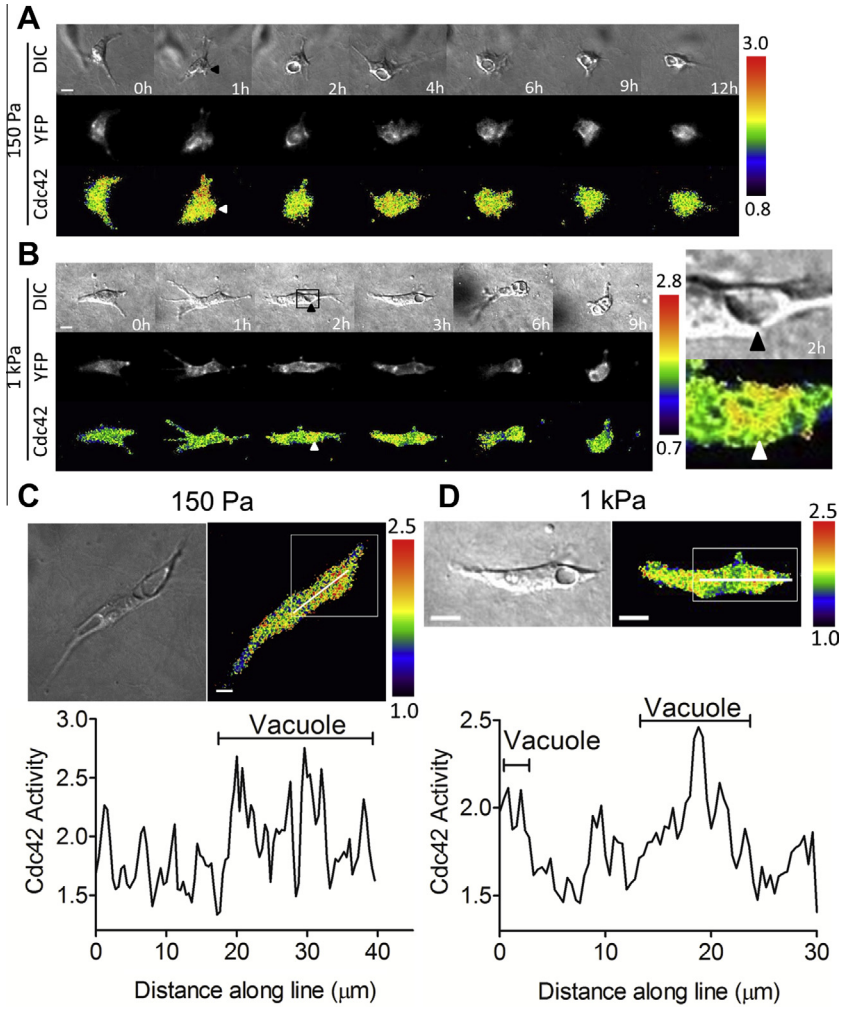

Fig. 2. Colocalization of Cdc42 activation and vacuole formation. (A) Time-lapse images of early vacuole formation and Cdc42 activity (18–30 h following seeding) for an individual ECFC within a (A) soft (150 Pa) and (B) stiff (1 kPa) collagen matrix. Color bars represent emission ratio of YFP/CFP of the biosensor, an index of Cdc42 activation. Close-up view of boxed area in (B) shows colocalization of regional Cdc42 activation with the vacuole periphery. Arrowheads indicate strong Cdc42 activation and vacuoles. (C, D) Cdc42 activity profile along the line from *the left* to *the right* on the cell body in a (C) soft and (D) stiff matrix. Scale bars, 10 μm.

3.4. Matrix rigidity affects vacuole size

We postulated that if matrix rigidity affects the dynamics of Cdc42 activity and vacuole formation, it would alter the morphology of the vacuoles. To test this hypothesis and further examine the effect of matrix rigidity on early vacuole formation in ECFCs, we compared the vacuole area in soft and stiff matrices at 6 h after vacuole formation induction. The vacuole area measurements based on the DIC images revealed that soft matrices induced the formation of larger (124 ± 24 μm^2) intracellular vacuoles than stiff matrices (63 ± 12 μm^2) (Fig. 4A and B).

3.5. Cdc42 mediates early vacuole formation

To further examine the role of Cdc42 activity in early vacuole formation, we co-transfected ECFCs with a GFP-actin and either one of Cdc42 mutants (a constitutively active Cdc42-L61 or a dominant negative Cdc42-N17). As a control, ECFCs were only transfected with a GFP-actin. Only transfected cells (confirmed by GFP) were considered for analysis after 6 h of vacuole formation induction. The data revealed that Cdc42-L61 significantly enhanced early vacuole formation (33 ± 1.9% as compared to control, 23 ± 2.3%). Although fewer vacuoles (9 ± 2.5%) were formed in Cdc42-N17-transfected ECFCs, Cdc42-N17 failed to completely block the vacuole formation (Fig. 4C), suggesting that Cdc42 mediates, at least in part, matrix rigidity-induced vacuole formation and other Rho GTPases, such as Rac1, may participate in this process [17].

4. Discussion

Vascularization, driven by ECFCs, is essential for tissue survival and development of a functional bioengineering graft. It is known that ECFCs, a subset of EPCs, have a great therapeutic potential in repair of damaged tissues [2,4,32]. In ECFCs, the formation of vacuoles is an important step that subsequently leads to lumen formation and vascular morphogenesis. This process is known to be dependent on the matrix-integrin-cytoskeleton signaling axis through the involvement of Rho family GTPases and MT1-MMP [20]. Inhibition of Cdc42 and Rac1, for example, decreases capillary lumen formation, suggesting a critical role of Rho family GTPases in capillary morphogenesis [17,20,21,33,34], and this process is in part regulated by the rigidity of ECM [5–7,13,14,35]. While substantial progress has been made to uncover the mechanism of ECM-driven vascularization, it remains elusive how individual ECFCs respond to the rigidity of the matrix spatially and temporally to regulate Cdc42 activity and vacuole formation. Furthermore, the spatiotemporal correlation between Cdc42 activation and vacuole formation has not been studied at the subcellular level.

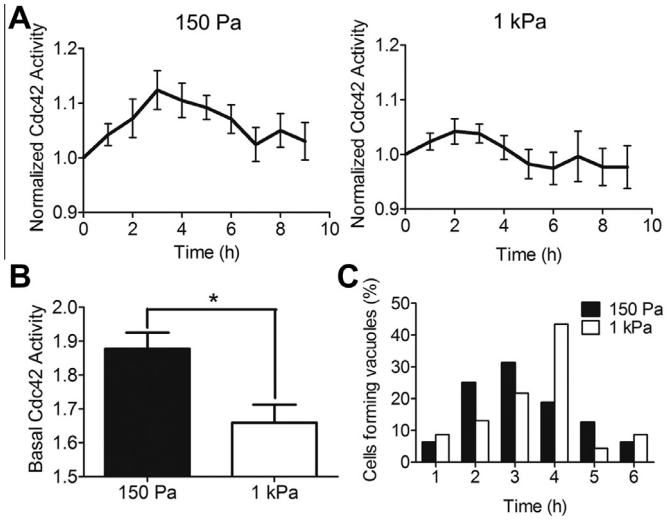

Fig. 3. Effect of matrix rigidity on the dynamics of Cdc42 activity and vacuole formation kinetics. (A) Cdc42 activation time course during early vacuole formation in soft and stiff matrices. 150 Pa: $n = 11$ cells; 1 kPa; $n = 13$ cells. (B) Basal levels of Cdc42 activity of ECFCs (150 Pa: $n = 7$ cells; 1 kPa: $n = 8$ cells) grown in two different matrices before induction of vacuole formation. $^{*}p < 0.05$. (C) Percentage of vacuole-forming cells as a function of time following vacuole induction in soft and stiff matrices. Data collected from >150 cells for 3 different experiments per group.

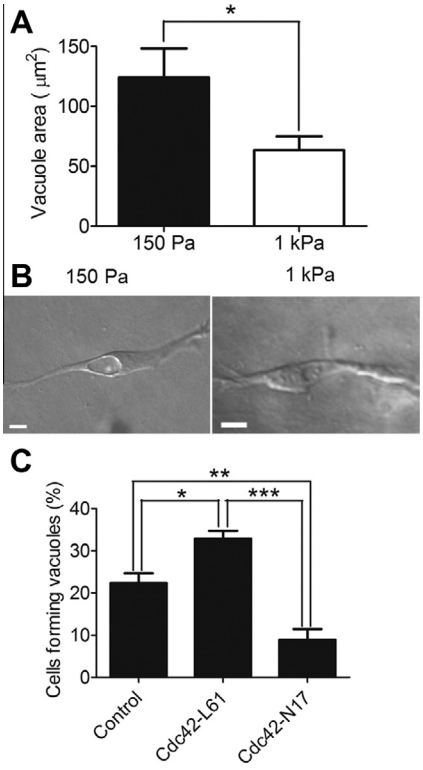

Fig. 4. Role of matrix rigidity and Cdc42 in vacuole formation. (A) ECFCs form larger vacuoles in soft matrices than in stiff matrices (150 Pa: $n = 14$ cells; 1 kPa: $n = 16$ cells). (B) Representative DIC images of ECFCs forming vacuoles in two different matrices. Scale bars, 10 μm. (C) The percentage of vacuole forming cells increases significantly with expression of Cdc42-L61 compared to untreated control. In contrast, expression of Cdc42-N17 yields a significant decrease in the percentage of vacuole-forming cells. Data collected from >200 cells for 5 different experiments per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

The present study shows, for the first time, the spatiotemporal dynamics of Cdc42 activity and its correlation with vacuole formation kinetics at the subcellular level. By employing live cell imaging in conjunction with a FRET-based Cdc42 biosensor, we were able to simultaneously monitor the Cdc42 activity and vacuole formation in the same cell. We have shown that Cdc42 is highly activated near the vacuole sites of ECFCs in both soft and stiff 3D collagen matrices. This result is consistent with a previous report showing that Cdc42 is involved in the initiation of vacuole formation [17]. We also observed that the rigidity of collagen matrices affects the kinetics of vacuole formation, which is temporally coincided with a marked increase in Cdc42 activity in soft, but not in stiff matrices. We do not know the exact mechanism on this distinct dynamic between Cdc42 activity and vacuole formation, but based on our data using constitutively activated Cdc42, a high basal level of Cdc42 may be required for the synchronized behavior observed in soft matrices. Given that the Cdc42 activity and vacuole formation are known to influence vascular morphogenesis, the dynamic activity patterns of Cdc42 and vacuole formation and the role of ECM rigidity in regulating them, demonstrated by our live cell imaging data, will be of significance in regulation of the ECM-induced signaling and vascular morphogenesis.

Accumulating evidence has shown the effect of matrix rigidity on vacuole and lumen size. In addition to the present study, we have previously shown that soft matrices increases vacuole size, whereas stiffer matrices decrease it [6], which is likely due to changes in spatial constraints imposed by collagen concentration [5] or collagen matrix porosity [36]. This result is consistent with another group's report using ECFCs and hyaluronic acid-gelatin hydrogels as a substrate [35]. Other reports using bovine endothelial cells [14] or human endothelial cells [13] have shown that soft collagen gels form a dense and thin network of small lumens and stiff gels form larger lumens. Based on the present study as well as previous reports including ours [5–7], it is becoming increasingly clear that matrix rigidity may play a significant role in regulating vessel formation process.

In summary, our findings about the distinct activation patterns of Cdc42 and vacuole formation kinetics suggest they play critical roles in the response of ECFCs to ECM rigidity. By modulating the spatiotemporally dynamic Cdc42 activity, the rigidity of ECM can regulate vacuole formation kinetics and vacuole size, which play important roles in vascularization process. Further studies are needed to address how Cdc42 interacts with other Rho GTPases, including Rac1, to mediate the activity of matrix metalloproteinases such as MT1-MMP in response to matrix rigidity.

Acknowledgments

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