Cdc42 regulates extracellular matrix remodeling in three dimensions

Running title: Cdc42 regulates ECM in 3 dimensions

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Extracellular matrix (ECM) actively participates in normal cell regulation and in the process of tumor progression. The Rho GTPase Cdc42 has been shown to regulate cell-ECM interaction in conventional two-dimensional culture conditions by using dominant mutants of Cdc42 in immortalized cell lines that may introduce non-specific effects. Here we employ three-dimensional culture systems for conditional gene targeted primary mouse embryonic fibroblasts (MEFs) that better simulate the reciprocal and adaptive interactions between cells and surrounding matrix, to define the role of Cdc42 signaling pathways in ECM organization. Cdc42 deficiency leads to a defect in global cell-matrix interactions reflected by a decrease in collagen gel contraction. The defect is associated with an altered cell-matrix interaction that is evident by morphologic changes and reduced focal adhesion complex formation. The matrix defect is also associated with a reduction in synthesis and activation of MMP9 and altered fibronectin deposition patterning. Cdc42 mutant rescue experiment found that downstream of Cdc42, PAK, but not Par6 or WASP, may be involved in regulating collagen gel contraction and fibronectin organization. Thus, in addition to the previously implicated roles in intracellular regulation of actin organization, proliferation and vesicle trafficking, Cdc42 is essential in ECM remodeling in 3-dimensions.

Extracellular matrix (ECM) actively participates in normal cell regulation and in the process of tumor progression. The Rho GTPases Rac1, RhoA, and Cdc42 are known to regulate intracellular activities such as focal complex formation, integrin localization, adhesion, cell morphology and cytoskeletal...
organization, as well as MMP expression and activation, activities that are important for ECM contraction (15-19). Most previous studies have been performed under traditional 2D culture conditions, although the roles of Rac1 and RhoA have been examined in regulating 3D ECM remodeling to some extent (17,20,21). Further, most studies employed broad ranged activators, inhibitors, and/or dominant mutants of Rho GTPases, which are inherently limited by their non-specific nature toward individual Rho GTPases involved in the cellular process (22,23). In particular, the potential role of Cdc42 in ECM remodeling has not been reported in 3D systems.

The goal of our studies is to define the signaling role of Cdc42 in 3D ECM remodeling. We have utilized a collagen 3D culture system and Cdc42 conditional gene deletion in primary mouse embryonic fibroblasts (MEFs) that simulates the reciprocal and adaptive interactions between cells and surrounding matrix. We report that Cdc42 is a key regulator of cell-ECM interactions, specifically in maintaining normal cell morphology, focal complex formation, MMP9 expression and activation, and extracellular fibronectin organization. The inside-out effect by Cdc42 on collagen contraction and fibronectin matrix organization is associated with the effector, PAK. Our findings implicate Cdc42 as an important regulator of ECM organization in 3D.

Experimental Procedures

Generation of Primary Cdc42loxP/loxP MEFs

The generation and genotyping of Cdc42 conditional knockout mice was described previously (24). MEFs were generated from mouse embryos at gestational ages E12-13. MEFs were cultured in DMEM (Invitrogen) and supplemented with 10% heat-inactivated fetal bovine serum (FBS) as described previously (25). These cells were used at low passage numbers (<5) to avoid accumulation of genetic abnormalities.

Adenoviral Deletion

Deletion of Cdc42 from Cdc42loxP/loxP MEFs was achieved by infecting twice with 25MOI adenoviral Cre recombinase a day apart. The experiments were initiated at 4 days after infection to ensure efficient Cdc42 protein deletion. Wild-type MEFs were also infected as positive controls.

Collagen Gel Contraction Assay

For collagen gel contraction, MEFs were trypsinized, suspended in cold normal media, counted, and suspended at 50k, 100k, or 150k cells/60µl of collagen I solution. Collagen I solution consisted of 46.5% normal media, 2% NaHCO3, 1.5% 1M Hepes, 50% PurCol (3.0 mg/mL, Inamed Biomaterials) The 60µl collagen I solution mixed with cells was placed as a droplet in the center of a 6-well sterile non-tissue culture treated dish and incubated at 37°C for 20 minutes to solidify the gel. 2mls of normal MEF media was added to each well and the collagen I gel/cell solution was lifted off the bottom with a cell lifter. The initial reading (time point 0) was taken at this step and the dish was put back into the incubator until further time point readings. Images were taken on an optical microscope (Leica S8AP0; Leica DFC320; LAS; βLeica Microsystems Inc.) and ImageJ software (NIH) was used to calculate the surface area. Percent contraction was calculated as the change in surface area from the initial surface area divided by the initial surface area. Cell extensions were measured from the cell surface to the tips of the extensions by using confocal images and ImageJ for all cells within a given field (20x objective) in 3 representative images.

3D Fibronectin Assembly Assay

 Coverslips were pretreated and cells were plated as previously described (26). Briefly, coverslips were coated with 0.2% gelatin solution and MEFs were plated at 120,000 cells/15mm circular coverslip. Ascorbic acid (50µg/ml) was added to the medium every other day to encourage matrix deposition. Cells were fixed at 2, 4, 6, 8 and/or 9 days post plating. Fibronectin, f-actin, and nuclei were stained and the cells and matrix were imaged, as described above. Matrix thickness was assessed using the z-stack feature from the confocal by measuring the maximum matrix thickness across a stack of images from five independent sets of cells.

Western Blotting and Immunofluorescence
For western blotting, MEFs in 3D collagen1 after 6 hours of plating were lysed in lysis buffer containing 20mM Tris-HCl (pH 7.6), 100mM NaCl, 10mM MgCl, 1% Triton X-100, 0.2% sodium deoxycholate, 2mM PMSF, 0.5mM DTT, 50mM NaF, 1mM Na3VO4, 0.1µM okadaic acid, 0.1% SDS, and protease inhibitors (Sigma) and protein extracts of 50µg were used for analysis. For immunoprecipitation, cells were incubated in a buffer containing 50mM Hapes, pH 7.3, 150mM NaCl, 1% Triton X-100, 5mM MgCl2, 1mM EDTA, 1mM Na3VO4, 10mM NaF, 10% glycerol, and protease inhibitors (2mM phenylmethylsulfonyl fluoride, 20µg/ml leupeptin, 100µg/ml aprotinin) for 15 min at 4ºC. Extracts were clarified by centrifugation at 14,000rpm for 15 mins. 1µl of the PAK antibody was incubated with each lysate for 1hr at 4ºC, then 30µl of suspended protein A/G agarose beads (Santa Cruz Biotechnology) were incubated with each lysate for 2hrs at 4ºC, all performed while rotating. Beads were pelleted, washed, lysed. Proteins were resolved by electrophoresis in a 4-15% gradient gel and electrotransferred to a PVDF membrane. Primary antibodies used were anti-cofilin, -pcofilin(ser3), -PAK, -pPAK1(thr423)/PAK2(thr402), -ERK, -pERK1/2(thr202/tyr204), -MLC, -pMLC19, -ppaxillin(tyr118) (Cell Signaling Technology), anti-Cdc42, -FAK, -pFAK(pY397), -paxillin (BD Transduction Laboratories), anti-β-actin, -fibronectin (Sigma), anti-pWASP83/484 (BETHYL Laboratories), anti-WASP (Santa Cruz Biotechnology), and GAPDH (Fitzgerald Industries). Secondary antibodies used were horseradish peroxidase-linked anti-mouse or anti-rabbit IgG (Amersham Biosciences). Representative images are shown.

For immunofluorescence experiments, MEFs were blocked with 2% BSA and stained with rhodamine-phalloidin (Molecular Probes), phospho-paxillin (Cell Signaling Technology), TOPRO3 (Invitrogen), DAPI (Invitrogen), α tubulin, β tubulin, vinculin, and fibronectin (Sigma). Alexa Fluor 488 (Invitrogen) was used as secondary antibodies for α and β tubulin, and Cy5 (Jackson ImmunoResearch Laboratories, Inc.) for vinculin and phospho-paxillin. A laser confocal microscope (Zeiss LSM 510, Zeiss) was used to image MEFs and capture optical slices from 3D samples, and the LSM Image Browser (Carl Zeiss MicroImaging GmbH) was used to view the images.

**Flow Cytometry**

MEFs were prepared in cell dissociation buffer for 10 mins at room temperature, washed with PBS and suspended in 1% fetal bovine serum/PBS. Antibodies (0.5µl) were added to 250k MEFs at room temperature for 20 mins and subjected to FACS analysis using Canto III (BD Biosciences). Data were analyzed with FACS Diva software. Antibodies PE-CD49d (integrin α4), PE-CD49e (α5), PE-CD61 (β3), APC-CD11b (αM) and FITC-CD29 (αL) came from BD Biosciences and PE-CD11a (αL) came from eBiosciences.

**Reflection Microscopy**

MEFs in 3D were prepared as per the immunofluorescence protocol and imaged using the Zeiss LSM 510 (Zeiss). 3D samples were stained for f-actin (rhodamine-phalloidin) only and imaged for fluorescence (f-actin) and reflected light (collagen fibrils). The HeNe laser (633nm) was used for reflected light.

**Gelatinase Activity Assay**

Cells (20,000) were plated in 3D with the addition of 0.33µl of DQ™ gelatin (Molecular Probes) per 60µl gel. After 6 hrs, cells were fixed, stained with rhodamine-phalloidin, and imaged. Quantification of protease activity was evaluated on ImageJ (NIH). Values were obtained by converting the fluorescent images to 8-bit and summing the intensity values over 90-255 pixel intensity values (any value below 90 pixel intensity value was considered to be background) and dividing by the number of nuclei. On average, 90 nuclei were in each sample. Values
were averaged over 3 experiments and the standard deviations were derived.

Zymography

Culture media (30µl) after 6 hrs of cell plating from the contraction experiment was applied to a 10% Tris-HCl Bio-Rad Ready Gel containing 10% gelatin. Gels were incubated in 2.5% Triton X-100 for 1 hour, then incubated for additional 18 hours at 37°C in 50mM Hepes (pH 7.5), 200mM NaCl, 5mM CaCl₂, and 20µM ZnCl₂. The gels were then stained with Coomassie Blue.

Quantitative Real-Time RT-PCR

3D cells (250,000 cells/ 60 µl gel) were pelleted for protease expression profiling, and cells from the fibronectin matrix assembly were gathered for fibronectin and collagen I expression profiling. mRNA was harvested using an RNeasy Micro Kit (Qiagen Inc.). Synthesis of cDNA was carried out using 1µg of RNA using the High-Capacity cDNA Reverse Transcription (Applied Biosystems Inc.). The cDNA for MMP9, collagen 1, and fibronectin was amplified using SYBR Green PCR Master Mix reagent (Applied Biosystems) for MMP9, collagen 1, fibronectin, and GAPDH. Quantitative real-time RT-PCR was performed and analyzed on an ABI Prism 7500 sequence detection system (Applied Biosystems). Relative expression was calculated using the standard curve. The following primer sets were used: MMP9 forward: 5′-GCC TCA AGT GGG ACC ATC AT-3′; reverse: 5′-CTC GCG GCA AGT CTT CAG A-3′, MMP2 forward: 5′-GCA GGG TGG TGG TCA TAG CTA -3′; reverse: 5′-CAC GCT CTT GAG ACT TTG GTT CT -3′, collagen 1 forward: 5′-GCC TTG GAG GAA ACT TTG CTT -3′; reverse: 5′-GCA CGG AAA CTC CAG CTG AT -3′, fibronectin forward: 5′-TAC CAA GGT CAA TCC ACA CCC C -3′; reverse: 5′-CAG ATG GCA AAA GAA AGC AGA GG -3′, and GAPDH forward: 5′-GAC GGC CGC ATC TTC TTG T G -3′; reverse: 5′-TCT CCA CTT TGC CAC TGC AA -3′.

Retroviral Transduction

Cdc42-/- MEFs were reconstituted with retrovirus expressing HA-tagged Cdc42 with EGFP (MIEG3 vector). Wild type Cdc42 (WT) or effector binding mutant of Cdc42, Cdc42D38A or Cdc42I173A/L174A, was expressed at a similar level in cells as the endogenous Cdc42. These effector binding mutants contain a single point mutation in Cdc42 which inhibits binding to PAK or Par6/WASP (27). 48 hour after viral transduction, cells were sorted by the GFP marker and used in subsequent functional assays.

Results

Cdc42 deficiency in MEFs impairs global ECM remodeling activity

To understand the role of Cdc42 in primary cell regulation, we used MEFs from Cdc42loxP/loxP homozygous mice generated previously (24) since direct Cdc42 knockout mice (i.e. Cdc42−/−) are embryonic lethal. Using an adenovirus expressing the Cre recombinase, we were able to efficiently delete the Cdc42 gene (Supplemental Figure 1A) and completely remove Cdc42 protein (Supplemental Figure 1B) without affecting cell viability within the time frame of the subsequent assays. This method allowed us to investigate the specific involvement of Cdc42 in primary cells without possible limitations imposed by using the traditional non-specific bacterial toxins or dominant mutants to interfere with Cdc42 activity.

To assess the requirement of Cdc42 in global extracellular matrix remodeling, we employed the collagen contraction assay that manifests the ability of fibroblasts to contract the surrounding matrix. Cdc42−/− MEFs showed significantly decreased activity in collagen contraction over WT MEFs (Figure 1A). While increasing cell numbers caused an increased contractility for both WT and Cdc42−/− cells, Cdc42−/− MEFs displayed reduced contraction activity versus WT MEFs proportional to the cell numbers (Supplemental Figure 2). These results indicate that Cdc42 is essential in the fibroblast-mediated collagen contraction and organization.

Cdc42 deletion causes a defective cell morphology in 3D

Since Cdc42 has been shown to regulate cell morphology in 2D, we hypothesized that it also affects cellular morphology in 3D which may contribute to the ability of the cell to reorganize ECM. At the initial time of plating in collagen
matrix, both WT and Cdc42-/ cells were spherical in appearance; however, over time WT MEFs were able to extend protrusions whereas the Cdc42-/ MEFs remained mostly spherical (Figure 1B). At 6 hours after plating (latest time point assessed from Figure 1A), it is seen that WT MEFs have significantly more of these extensions than Cdc42-/ MEFs (Figure 1C). Cytoskeleton staining revealed that in WT MEFs, microtubules were well aligned along these extensions and f-actin was accumulated at the tips of the protrusions, whereas in Cdc42-/ MEFs there were no analogous microtubule or actin structures (Figure 2A). In 2D, Cdc42-/ MEFs exhibited irregular, elongated protrusions as opposed to WT MEFs that showed a spread-out morphology (Supplemental Figure 3A) (24). These data indicate that while Cdc42 is required for normal cell morphologies in both 2D and 3D conditions, it distinctly regulates cell protrusion in 3D.

Cdc42 deletion causes defective focal complex formation in 3D

The drastic defective morphology of Cdc42-/ MEFs prompted us to further examine the effect of Cdc42 loss on adhesion to the matrix in 3D, because Cdc42 has previously been shown to play a role in cell adhesion to the underlying ECM in 2D (24). In 3D, WT MEFs had characteristic focal complexes at the end of cellular protrusions and along the length of the extended cell body, whereas Cdc42-/ MEFs showed no detectable focal complexes, even at small extensions as revealed by phosphorylated paxillin immunostaining (Figure 2B). In 2D, WT MEFs showed prominent focal complexes as seen by phosphorylated paxillin and vinculin immunostaining, whereas Cdc42-/ MEFs lacked these punctuated patches (Supplemental Figure 3B). These data indicate that Cdc42 is involved in cell adhesion in both 3D and 2D to the ECM.

Cdc42 deletion causes defective local ECM remodeling by MEFs

The observations that loss of Cdc42 leads to a decrease in global ECM remodeling, a loss of normal MEF morphological maintenance and a loss of focal complex components, led us to hypothesize that local ECM remodeling surrounding Cdc42-/ MEFs in 3D is also altered. To assess the effect of Cdc42 deficiency on the immediately surrounding matrix, we plated Cdc42-/ MEFs cells in 3D collagen, and visualized the cell bodies by rhodamine-phalloidin staining of f-actin and the collagen fibers around the cells by reflection microscopy. The ECM surrounding WT MEFs consisted of collagen fibrils that appear to extend from and align with the cellular protrusions (Figure 3A). These aligned fibrils were not seen surrounding the Cdc42-/ MEFs, in which case the collagen alignment appeared random (Figure 3B). A dark spherical region surrounding the WT MEF cell body suggests that these MEFs are able to degrade the immediate surrounding matrix, while Cdc42-/ MEFs lack this region. These data suggest that Cdc42 is involved in regulating the surrounding matrix and in matrix degradation.

Effect of loss of Cdc42 on extracellular fibronectin assembly

Fibronectin is a major constituent of the ECM produced by fibroblasts. In vitro, MEFs can make their own 3D extracellular matrix composed of mainly fibronectin, which mimics in vivo mesenchymal matrices (26). To determine whether fibronectin matrix organization was altered by Cdc42 deficiency, we analyzed the MEF secreted ECM for fibronectin fiber structure and matrix thickness from WT and Cdc42-/ MEFs. WT MEFs produced an ECM with short, random fibronectin fiber alignment, whereas Cdc42-/ MEFs generated fibronectin fibers that were long and well aligned (Figure 3C). The WT MEFs were also more proficient at producing fibronectin, as indicated by a thicker matrix compared to the Cdc42-/ MEFs (Figure 3D and Supplemental Figure 4). Fibronectin and collagen synthesis and cellular fibronectin protein levels remained unchanged upon Cdc42 deletion (Supplemental Figure 5). These data indicate a role for Cdc42 in fibronectin ECM assembly surrounding the cells.

Effect of loss of Cdc42 on protease processing

Proteases as well as constituents of the ECM secreted from cells play a role in the ECM organization. To examine if Cdc42 may regulate protease processing, we plated the cells in 3D collagen embedded with gelatin-fluorescence, that
when cleaved by proteases becomes fluorescently activated. WT MEFs exhibited significantly higher protease activity than Cdc42-/- MEFs at 6, 24, and 48 hours after plating (Figure 4A,B). Specific protease activities were detected around the periphery of WT MEFs, while mainly fluorescent signals inside cells were visible in Cdc42-/- MEFs (Figure 4C). These data suggest that Cdc42 is involved in protease production and/or activation in ECM. To determine which proteases might be affected by the loss of Cdc42, we performed the collagen contraction assay and analyzed the surrounding media on a gel zymogram. The zymogram analysis revealed that WT MEF media contained both active and inactive forms of matrix metalloproteinase 9 (MMP9), whereas the media from Cdc42-/- MEFs contain only the inactive form of MMP9 (Figure 4D). In contrast, MMP2 activity was not affected (Supplemental Figure 6A). In addition, transcription of MMP9 (Figure 4E), but not MMP2 (Supplemental Figure 6B), was found decreased in Cdc42-/- MEFs. These data strongly indicate that cellular Cdc42 is required for the expression and activation of MMP9.

To examine if MMPs play a role in cellular matrix contractility, WT MEFs were treated with varying concentrations of a general MMP inhibitor, GM6001, and the collagen contractility assay was performed. This MMP inhibitor suppressed the gel contraction in a dose dependent manner, from 80% to 75-62% at 5-20µM of the inhibitor at the 5 hour time point (Supplemental Figure 7). These results suggest that MMPs are partially involved in the contractility of matrix. Thus, Cdc42 regulates MMP9 expression and activation, which may contribute to the Cdc42-mediated ECM remodeling.

Cdc42 deficiency alters focal complex and actin organization signal in 3D

Cdc42 controls a variety of downstream pathways to affect cell morphology, actin organization, and focal adhesion patterning that may be important in ECM remodeling. Signaling components important in these pathways include FAK, paxillin, WASP, PAK, coflin, MLC, and integrins. To determine which downstream signaling molecules were affected by Cdc42 deletion in MEFs cultured in 3D, we analyzed phosphorylation states of these proteins by Western blotting. The phosphorylation activities of cytoskeletal organizing proteins PAK, WASP, and coflin and focal adhesion proteins FAK and paxillin were decreased in the Cdc42-/- MEFs (Figure 5A,B). Integrin β3, a major subunit of intergins expressed in WT MEFs, was significantly decreased in Cdc42-/- MEFs as measured by flow cytometry (Figure 5C). On the other hand, integrins β1 and α5 remained unchanged, while α4, αL, and αM subunits that were expressed at low levels in MEFs were also significantly altered upon Cdc42 deletion (Figure 5C). These data indicate that loss of Cdc42 causes defects in downstream signaling pathways regulating actin organization and focal adhesion, which may contribute to the observed defect in ECM remodeling.

PAK, but Not PAR6 or WASP, interaction is involved in ECM thickness and contractility regulated by Cdc42

To determine which downstream signaling components of Cdc42 might be involved in ECM organization, we expressed the Cdc42 effector binding mutants, Cdc42 D38A and Cdc42I173A/L174A, that lack binding activity to the downstream effector PAK or PAR6/WASP (27), as well as WT Cdc42, in Cdc42-/- MEFs by retroviral transduction. PAK most notably regulates actin and microtubule dynamics, PAR6 is known to regulate microtubule polarity, while WASP is important for F-actin nucleation reaction. At a similar expression level to that of endogenous Cdc42 (Figure 6A), the WT or PAR6/WASP deficient, but not PAK deficient, mutant of Cdc42 was able to rescue collagen contraction defect by Cdc42 knockout (Figure 6B), suggesting that PAK, but not PAR6/WASP, may be involved in collagen contraction. The PAK binding defective mutant, but not the PAR6/WASP binding mutant, was able to rescue fibronectin matrix thickness (Figure 6C). These data suggest that the PAK pathway is involved in Cdc42 mediated collagen contraction and native fibronectin organization by MEFs.
Discussion
Cdc42 has previously been shown to regulate cell morphology, actin organization, and focal adhesion complexes in vitro, and basement membrane organization and forebrain development in vivo (24,28-30). There are several inherent limitations with these studies. Firstly, they are performed in 2D culture conditions, which hamper the ability to analyze cell-ECM interactions that occur in 3D. Secondly, in vivo studies show phenotypes which may have arisen due to inter-cellular interactions of neighboring cells and the downstream signaling pathways, and specific cellular functions involved are difficult to dissect. In the current work, we have genetically defined the role of Cdc42 in ECM remodeling in 3D, and provide evidence that collected cell functions, including ECM adhesion, cytoskeleton organization, protease (MMP9) activation, and extracellular matrix component production and organization, may be involved in generating the ECM environment (Fig. 5D). Further, we implicate the immediate downstream signaling effector PAK as a candidate pathway through which Cdc42 exerts these functions.

The fibroblast contraction of a collagen gel is a commonly used model system to evaluate cell-ECM interactions in 3D environments. The simplicity of the contraction assay allows for the analysis of cell-ECM interactions without compounding factors seen in vivo. In collagen matrices, normal fibroblasts extend processes over time to contract the matrix. Addition of LPA or PDGF was shown to stimulate RhoA and Rac1, respectively, and was found to increase matrix contraction, which was inhibited by pertussis toxin (21,31). In 2D, Cdc42 has been shown to be downstream of LPA stimulation and inhibited by pertussis toxin (32). Our studies using Cdc42/- MEFs show that matrix contraction is significantly reduced, demonstrating that Cdc42 is essential for efficient matrix contraction (Figure 1 and Supplemental Figure 2). This functional outcome in 3D is closely related to the observed cytoskeleton and morphological changes, and to the signaling decreases of phospho-PAK, -WASP, and -cofilin, as previous studies have found that PAK and cofilin inhibition decreases matrix contraction (20).

The general paradigm of matrix contraction is thought to be through cell-matrix interactions, which generate cellular tractional force through the cell migratory machinery and cytoskeletal dynamics, and mechanical loading (2,33). Cdc42 is known to regulate cell migration in 2D by modulating cell morphology and focal adhesion. We have previously shown that conditional Cdc42/- MEFs have irregular shaped morphologies with contracting cell bodies and reduced focal adhesion complexes (24), while others found Cdc42 knockout ES cells to be smaller and exhibit round morphologies (28). WT MEFs in 3D exhibit extended morphologies with actin rich extensions and phospho-paxillin containing focal adhesion complexes, whereas loss of Cdc42 results in a round morphology with a disorganized cytoskeleton (Figure 1B,C and Figure 2) and a lack of detectable focal adhesion complexes (Figure 2B). Furthermore, the phosphorylation status of focal adhesion proteins, FAK and paxillin, were decreased in the Cdc42/- MEFs (Figure 5B), as the integrin β3 expression which is known to bind to fibronectin among other substrates (Figure 5C). Consistent with previous findings in 2D, these data suggest that Cdc42 is necessary for regulating cell morphology and focal complex formation in 3D. Because efficient cell-ECM binding is necessary for effective matrix contraction (34,35), the lack of focal complexes in Cdc42/- MEFs likely contributes to the decreased ability to contract the matrix. Although these cells have altered surface integrin expressions, they still retain partial activity in contracting the matrix, which is likely due to an amoeboid-like migration reminiscent of cancer cell migration when MMPs are removed (36). The reduction in MMPs may affect proper processing of the fibronectin fibrils which results in decreased matrix contraction and a differential cellular phenotype (i.e. round) in 3D than in 2D. This effect on the inside-out cell-matrix interaction may be related to the activities of actin organization proteins such as PAK, cofilin, and WASP, which were decreased in the Cdc42/- MEFs (Figure 5A).

ECM contraction can also be a result of the ability of the cell to locally remodel the ECM. In 3D collagen gels, ECM fiber alignment surrounding the
fibroblasts is rearranged (5,37). WT MEFs show local collagen fibril alignment organized along the long axis of the cells' extension, while Cdc42−/− MEFs lack collagen fibril reorganization capability (Figure 3A). This finding fits well with the observed defects in focal adhesion complexes and cytoskeletal organization of the knockout cells, since both are required for the cellular traction and mechanical force which are in turn required for the fibril alignment force. Other major regulators and linkers of cellular force and ECM remodeling include integrins. With the observation that focal complex is disrupted in the Cdc42 deficient fibroblasts, we expect that integrin activities are also defective downstream of Cdc42 and contribute to the altered ECM organization. In addition to the defective collagen fibrils, we found that MEFs deficient in Cdc42 are defective in 3D fibronectin deposition, but not synthesis, particularly in altering fibronectin thickness and alignment structure (Figure 3C-E). Whether such altered ECM can promote or suppress tumor cell migration, invasion or proliferation will be interesting to test. A recent study evaluating native fibronectin matrix production shows that tumor associated fibroblasts generate fibronectin with similar morphology and aligned fibronectin pattern (38) as the Cdc42−/− MEFs, raising the possibility that fibronectin organization around the tumors may be modulated by a reduced Cdc42 activity in the tumor associated fibroblasts.

MMPs were previously thought to be involved in regulating fibroblast-mediated ECM contraction through ECM cleavage and degradation, and may do so by interfering with tractional forces and migration and competing with cell-matrix proteins (8,13,14). Since Cdc42 has previously been shown to regulate 2D cell adhesion and migration, and as the activation and secretion of MMPs (15,24,39), we wanted to further evaluated the role of Cdc42 on MMP activity in 3D. In our system, MMP9 activity is decreased upon Cdc42 deletion and mostly remains inside the Cdc42−/− cell (Figure 4A-C). The transcription and activity levels of MMP9 are also decreased in Cdc42−/− media, whereas MMP2 transcription and activity levels are unchanged (Figure 4D,E & Supplemental Figure 6). Consistently, ERK activity, which may affect MMP2 transcription, is not affected in Cdc42−/− MEFs (Supplemental Figure 8). Because MMPs are necessary for efficient matrix remodeling and contractivity, it is likely that the altered MMP9 activity due to Cdc42 deletion is contributing to the phenotype of inefficient 3D ECM remodeling. Future studies are warranted to determine the role of Cdc42 in 3D ECM remodeling, particularly in the context of recent finding from Friedl’s group that shows elegantly that the nature of the collagen gel and pore size determines whether ameboid movement is MMP dependent or independent (40, 41), and work from Yamada’s group that implicates cell adhesion structures and ECM fibrils in the formation of 3D matrices (42, 43).

We have made an attempt to delineate the effector signaling events involved in this Cdc42 function. Among the known effector targets, PAK specifically has been shown to play a role in LPA and PDGF stimulated ECM contraction. In agreement with these, we show that PAK activity is decreased in Cdc42−/− cells and mutant of Cdc42 deficient in binding to PAK could not rescue the contractility activity, suggesting that in our system PAK plays a role in mediating Cdc42 signaling to ECM remodeling. Additionally, microtubule dynamics have been shown to play a role in fibroblast morphology in 3D (44). Since Cdc42 is known to regulate microtubule dynamics and polarity (22), the rounded morphology in Cdc42−/− MEFs may also be due to altered microtubule organization. However, addition of PAR6/WASP binding deficient Cdc42 mutant to Cdc42−/− MEFs was able to rescue collagen contraction and fibronectin remodeling, indicating that Cdc42 regulated microtubule polarity may not be involved in ECM remodeling. It remains to be seen how other signaling pathways controlled by Cdc42, e.g. IQGAP, N-WASP, and MCRK, may contribute to different aspects of the global ECM remodeling.

In conclusion, our study identifies a novel function for Cdc42 in regulating ECM structure in 3D. How Cdc42 would exert such a role in physiologically and pathologically relevant conditions involving dynamic interactions of cells with ECM such as wound healing, embryonic development, or tumor...
progression is an important topic that requires future experimentations. Altogether, Cdc42 and its signaling pathways play critical roles in cell morphogenesis and matrix remodeling, manipulation and interference of which may benefit diseases including cancer and regenerative medicine such as tissue engineering.

Footnotes

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Contributions of the work

NSS performed the experiments, analyzed data, and wrote the paper, YF performed the experiments for Figure 5C, 6A and B, SM and HOL contributed to the work of Figure 3C, FSC made virus from the REW13 mutant DNA, FG initially made the REW13 mutants, JC oversaw experiments performed by HOL, JM oversaw experiments performed by FSC, and YZ oversaw all experimental design, data analysis and edited the work.

References


Figure Legends

Figure 1. Cdc42 deletion in MEFs decreases collagen contraction and prevents cell extensions in 3D. WT or Cdc42−/− MEFs were plated in collagen1 at 150,000 cells/60 ul collagen gel. (A) Gel contraction increases over time, however with a significant reduction using Cdc42−/− MEFs. n=3, mean±SD, p<0.01. (B) Cells were fixed and immunostained with phalloidin to assess cellular morphology at 6hr, 24hr, and 48hr. WT MEF cells continue to elongate and spread while Cdc42−/− MEFs remain compact. (C) Cdc42−/− MEFs show a significant decrease in filopodia-like protrusions compared with WT MEFs in 3D at 6hrs after plating. Images are representative of 3 independent experiments; mean±SD, p<0.005.

Figure 2. Cytoskeletal organizations are disorganized and focal adhesions are reduced in Cdc42−/− MEFs in 3D. WT or Cdc42−/− MEFs were plated in collagen1 at 20k cells/collagen gel. (A) Cells were fixed and immunostained with anti-α/β tubulin (green) and phalloidin (red) at 6 hrs after plating. WT MEFs exhibit spread morphologies with aligned microtubules and actin rich tips of extensions, while Cdc42−/− MEFs lack this organization. (B) At 24hrs after plating in 3D, WT MEFs reveal distinct punctate focal adhesion patterns, and Cdc42−/− MEFs have little to no focal adhesion complexes as shown with phospho-paxillin (light blue) immunostaining. Arrows in WT inset pictures (iii, iv) reveal focal adhesion complexes, whereas in the Cdc42−/− MEFs the patterning was scarce. (B(i)) depicts a compressed confocal z-stack, whereas (B(ii)) depicts a confocal z-stack across one XY plane. Colors in (B) represent actin in red, α/β tubulin in green, and paxillin in light blue.

Figure 3. Cdc42 is necessary for local exogenous collagen remodeling and native fibronectin organization and alignment in 3D. WT or Cdc42−/− MEFs were plated in collagen1 at 20,000 cells/60 ul 50% collagen gel (A,B) or allowed to produce their own matrix (C). At 6 hrs after plating in 3D, WT MEFs appear to grab the matrix (A,i,ii,iii) and pull on the collagen fibrils (arrows). Arrowheads indicate a hole in the matrix near WT cell. Cdc42−/− MEFs remain compact (B,i,ii,iii). (i,ii,iii) depicts representative z-stacks (at ~4μm intervals) and shows collagen matrix fibril alignment measured by reflection microscopy. The images on the far left are the compressed z-stacks with phalloidin immunostaining of F-actin. (C,D) Cdc42−/− MEFs were encouraged to produce a 3D FN matrix with the addition of ascorbic acid (50μg/ml) every other day, resulting in a thinner and more aligned fibronectin matrix than WT MEFs at 8 days post plating. n=5, mean±SD, p<0.001.

Figure 4. Cdc42 is necessary for MMP9 activation and expression in 3D. To detect extracellular matrix changes due to gelatinases, WT or Cdc42−/− MEFs were plated in collagen1 at 20k cells/collagen gel with
fluorescent gelatin (green) mixed in the collagen gel and cleavage of the gelatin was detected by fluorescence. (A,B) Gelatinase activity is significantly higher in the WT than in the Cdc42-/- MEFs, n=3, mean±SD, p<0.01. (A) is imaged at 24 hrs after plating. (C) Active gelatinase activity is shown along the outside periphery of WT MEFs, but remains inside Cdc42-/- MEFs at 6hrs after plating. Z-stacks along the green and red line planes are also shown. Cdc42-/- MEFs show a reduction of MMP9 activation (D) as shown by zymography from WT and Cdc42-/- MEF media at 6hrs post plating (D) and MMP9 transcripts as shown by RT-PCR (E) at 24hrs post plating, n=3, mean±SD.

Figure 5. Cdc42 regulates the activation states of WASP, cofilin, PAK, paxillin, and FAK and expression of integrins in 3D. WT or Cdc42-/- MEFs plated in 3D collagen1 after 6hrs of plating were analyzed using western blotting. The phosphorylation status of (A) actin cytoskeletal modulators WASP, cofilin, and PAK and (B) focal adhesion components paxillin and FAK are decreased upon Cdc42 deletion in MEFs. Phosphorylation status of MLC appears to be level, however total protein levels are decreased upon Cdc42 deletion. (B) Actin and gapdh were run in total cell lysates. (C) Flow cytometry analysis of integrin subunits, β1, β3, α4, αL, αM and α5 in WT and Cdc42-/- MEFs. n=3, mean±SD.

Figure 6. Rescue of ECM remodeling by Cdc42 mutant reconstitution. (A) Genomic DNA genotyping and Western blot confirm endogenous Cdc42 deletion and WT or mutant Cdc42 protein reconstitution in Cdc42loxP/loxP MEFs with or without cre treatment. Lane 1, Cdc42loxP/loxP cells; lane 2, Cdc42loxP/loxP+cre; lane 3, Cdc42loxP/loxP+cre+WT HA-Cdc42; lane 4, Cdc42loxP/loxP+cre+HA-D38A; lane 5, Cdc42loxP/loxP+cre+HA-I173A/L174A. (B) The collagen matrix contraction assays were carried out with Cdc42 effector binding mutant MEFs). n=3, mean±SD, and p<0.004 for Cdc42-/- + PAK mutant compared to WT for the 2-6 hr time points and for Cdc42-/- + PAR6 mutant compared to WT for the 2 hr time point. (C) The thickness of fibronectin matrix produced by Cdc42 WT or mutant reconstituted MEFs was measured by confocal z-stack at 9 days after plating of the cells. n=7, mean±SD, p<0.001.
Figure 1

(A) Graph showing the percentage of contraction over time for WT and Cdc42-/- cells. The line for WT shows a significant increase in contraction with time, while the line for Cdc42-/- shows a smaller increase. The p-value for the difference between the two groups is less than 0.008. The data is represented as mean ± SD, with n = 3.

(B) Images showing cell morphology at 6, 24, and 48 hours for WT and Cdc42-/- cells. The images show a comparison of cell extensions and overall cell morphology over time.

(C) Bar graph showing the percentage of cells with extensions ≥5μm in 3D. The WT group shows a significantly higher percentage compared to the Cdc42-/- group. The p-value for the difference is less than 0.003. The data is represented as mean ± SD, with n = 3.
Figure 3
Figure 4
Figure 5
Figure 6
Cdc42 regulates extracellular matrix remodeling in three dimensions
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