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Cell-Based Assays to Study ERK Pathway/Caveolin1 Interactions

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Abstract

Caveolin1, the main component of caveolae, plays a major role in regulating cell motility, gene expression, and cytoskeleton remodeling downstream of many membrane receptors. Here, we summarize different techniques set up to study changes in cell morphology and cell motility regulated by ERK/caveolin1 interactions during induction of epithelial mesenchymal transition (EMT) in mesothelial cells (MCs).

Introduction

The plasma membrane represents a barrier that protects the cell from the outside environment and also contains the receptors that sense the signals emanated from other cells or from the extracellular milieu. The plasma membrane contains differentiated domains that ensure proper sorting of the huge number of signaling molecules arriving at the plasma membrane. One of these domains is named caveolae. Caveolae are membrane invaginations with a 60–80 nm diameter with a particular protein and lipid composition [1]. Caveolins (Cav1, 2 and 3) are the major proteins responsible for the formation of caveolae and together with the Cavin family (Cavin1 to 4) shape caveolae. Caveolae recruit many signaling proteins, including receptors, adaptors, and nonreceptor kinases, that do not necessarily play a role in caveolae biology and may use this membrane domain as a signaling platform [2]. Many studies have shown a cross-talk between ERK signaling and caveolar components, especially Cav1. In many tissues and organs including heart, lung, endothelium, and peritoneum, Cav1 reduction is sufficient to induce a constitutive hyperactivation of the ERK pathway [3– 6]. It has been suggested that Cav1 through its scaffolding domain (CSD) may directly bind and maintain in an inactive state several kinases, including Src, AKT, and MEK [7, 8]. The lack of direct evidence for interaction between the CSD and caveolin-binding motifs (CBM) in Cav1-interacting proteins has challenged the CSD/CBM hypothesis [9]; thus, understanding the effect of Cav1 or CSD on signaling pathways is far from complete [1]. The recent observation that Cav1 may modulate the MEK-ERK1/2 signaling pathway through organization of Ras microclusters emphasize the role of Cav1 in the regulation of MEK-ERK1/2-driven events, such as epithelial–mesenchymal transition (EMT) [10–13].

EMT is a complex and stepwise process that occurs during embryonic development and tumor progression, and that has more recently been described in chronic inflammatory and fibrogenic diseases [14]. EMT is characterized by the disruption of intercellular junctions, replacement of apical–basolateral with front-to- back polarity, and acquisition of migratory and invasive phenotypes. ERK activation may control the EMT program through induction of Snail, an EMT master gene [11, 15].

The EMT of peritoneal mesothelial cells (MCs), or mesothelial-to-mesenchymal transition (MMT), may occur as a response to recurrent pro-inflammatory or pro-fibrogenic insults affecting the peritoneal membrane, including peritoneal dialysis (PD) [16]. In the peritoneum, an excess of ERK signaling induced by the absence of Cav1 is key to the EMT process and pathology [5]. In all cases, transdifferentiated MCs may invade the submesothelial space where they exert an angiogenic and fibrogenic activity [17]. Interestingly, MCs that have undergone EMT in vivo may be isolated from effluent of patients undergoing PD. These cells constitute a useful experimental system to study MCs plasticity and recovery of an epithelial-like phenotype (mesenchymal-to-epithelial transition, MET) [11, 18, 19].

Different readouts may be used to analyze EMT/MET dynamics. The elliptical factor (EF) or aspect ratio (AR) measures the change from a cobblestone shape, which is characteristic of epithelial cells, towards a spindle-like shape, characteristic of cells with mesenchymal features. The analysis of cell height is another readout of EMT induction. This indirectly measures the change from apico-basal polarity, which is characteristic of epithelia, towards a flattened morphology with front-back polarity, characteristic of fibroblasts. A first step of EMT induction is cell scattering, followed by the acquisition of migratory and invasive abilities. The acquisition of an invasive phenotype is a final step of EMT induction and is acquired through a profound

reprogramming of cell proteome, including the induced expression of metalloproteases such as matrix metalloproteinase (MMP) 2–9, able to degrade the extracellular matrix.

The role of the MEK-ERK pathway in these processes may easily be monitored treating cells with pharmacological inhibitors. Old-generation compounds such as PD98052 or U0126 are still used but more potent inhibitors, such as CI-1040 or PD0325901, are the preferred choice. Alternatively, specific genetic silencing or dominant-negative constructs may be used. In this chapter, we describe how morphological changes induced by ERK-Cav1 signaling during EMT of MCs may be studied. We describe functional assays such as wound healing, migration/invasion through polycarbonate filters, as well as a 3D invasion assay. Moreover, cell shape and cell height measurements are described.

Materials

MCs Isolation and Cell Culture

1. Trypsin-EDTA solution: 0.125 % Trypsin, 0.01 % EDTA.

2. Fine-pointed forceps (Dumont 4), scalpel blades, and scissors.

3. Phosphate buffered saline (PBS).

4. 12- to 24-well tissue culture plates, 10-cm petri dishes, 25-cm2 tissue culture flasks, and 50-mL tubes.

5. Murine MCs cell culture medium: Dulbecco's Modified Eagle's Medium (DMEM) F12 supplemented with 20 % fetal calf serum (FCS), 50 U/mL penicillin, 50 μg/mL streptomycin, 2.50 μg/ mL Amphotericin B (Sigma) and 2 % BIOGRO-2 Supplement (containing insulin, transferrin, ethanolamine, and putrescine) (Biological Industries). Store at 4 °C for up to 1 month.

6. PureCol, Bovine Collagen Solution, Type I (Advanced Biomatrix): prepare in cold PBS at a concentration of 60 μg/ mL and coat culture plates for 1 h at 37 °C. Use a 300 μg/mL solution for coating polycarbonate inserts.

7. Human MCs cell culture medium: Earle's 199 medium (M199) supplemented with 10 % FCS, 50 U/mL penicillin, 50 μg/mL streptomycin, 2.50 μg/mL Amphotericin B and 2 % BIOGRO-2.

Functional Assays

1. Glass slides for microscopy (precleaned) and Neubauer chamber.

2. μ-Slide 8 well ibiTreat (ibidi).

3. Culture-Insert in μ-Dish 35 mm, low, ibiTreat (ibidi).

4. μ-Slide Angiogenesis, ibiTreat (ibidi).

5. Polycarbonate inserts with 8 μm pore size (Corning Costar).

6. Matrigel-Collagen solution: 40 % Matrigel Matrix Growth Factor Reduced (GFR) (BD), 30 μg/mL Collagen, Type I in serum-free medium.

7. Recombinant human TGF-β1 (R&D Systems).

8. MEK inhibitors CI-1040 and PD0325901 (Selleckchem), resuspended in DMSO at a concentration of 10 and 1 mM, respectively.

9. Fixation solution: 4 % paraformaldehyde (from 16 % stock, Electron Microscopy Sciences), 5 % sucrose in PBS. Prepare before use.

10. Bovine Serum Albumin (BSA) solution: 2 % BSA in PBS, prepare fresh.

11. Permeabilization solution: 0.5 % Triton X-100 in Dulbecco's PBS. Filter solution through a 0.22 μm filter membrane to remove particulate impurities present in Triton X-100.

12. Phalloidin-Alexa Fluor 488 solution: add 1.5 mL of methanol to 300 units of lyophilized phalloidin-Alexa Fluor 488 (Molecular Probes) in the vial supplied by the manufacturer.

13. Hoechst 33342 (Thermofisher): Add 1 μL of Hoechst 33342 in 500 μL of PBS before use.

14. ProLong Gold, aqueous mounting medium with anti-fading agents (Thermofisher). Thaw right before use.

15. MetaMorph image analysis software.

16. Image J software.

Methods

Isolation and Culture of Murine MCs from Peritoneum

1. Euthanize the mouse and spray it with 70 % ethanol.

2. Using scissors and forceps cut the outer skin of the peritoneum and gently pull it back to expose the inner skin lining the peritoneal cavity.

3. Cut parietal peritoneum (try to obtain as much peritoneal tissue as possible). To further increase cell number, MCs covering the surface of internal organs such as liver may also be isolated (see Note 1).

4. Place peritoneum samples in a 50-mL Falcon and wash them with 10 mL of cold PBS (see Note 2).

5. Remove PBS and add 5 mL of trypsin-EDTA solution at 37 °C with occasional agitation for 10–15 min.

6. Add 5 mL of culture medium with 20 % serum (to inactivate trypsin-EDTA).

7. Centrifuge peritoneum samples (5 min at 500–700 × g) and remove the supernatant.

8. Resuspend the cells in DMEM F12 with 20 % FCS plus 2 % BIOGRO-2 and plate in culture dishes already coated with Type I Collagen solution (60 μg/mL) (see Note 3).

9. The next day, carefully remove unattached cells (see Note 4).

10. When cells reach confluence they can be split (1:2) no more than two times. Cell culture medium should be changed every other day. After 7–10 days cells start to lose their cobblestone appearance.

Isolation and Culture of MCs from Human Omentum

1. Cut omentum samples in thin slices (2–3 cm in diameter).

2. Wash with cold PBS.

3. Digest samples by placing two slices in a 50-mL Falcon with 15 mL of Trypsin-EDTA solution. Incubate for 15 min in a water bath at 37 °C with occasional agitation.

4. Add 15 mL of medium containing 20 % FCS to stop trypsin- EDTA activity.

5. Centrifuge omentum samples (5 min at 500–700 × g) and remove the supernatant.

6. Count the cells with a Neubauer chamber.

7. Plate 2.5–5 × 104 cells in a 25-cm2 tissue culture flask in culture medium for human MCs (see Note 5).

Isolation and Culture of MCs from Peritoneal Effluent of PD Patients

1. Hang bags containing nocturnal peritoneal dialysates from clinically stable patients for 3–4 h at 4 °C to facilitate deposition of floating cells at the bottom of the bag.

2. Remove the upper supernatant, leaving 200–300 mL of fluid per bag.

3. Fill 6 Falcon tubes (50 mL each) and centrifuge at 500–700 × g for 10 min.

4. Wash twice the cells with PBS.

5. Count the cells with Neubauer chamber.

6. Plate the cells in a 25-cm2 tissue culture flask (see Note 6).

Acquisition of Spindle-Like Morphology

1. Gently place glass coverslips for microscopy on the wells of 24-well tissue-culture plate.

2. Plate 1 × 105 MCs and culture them until they reach 100 % confluence (see Note 7).

3. Treat MCs for 1 h with MEK inhibitor CI-1040 (2 μM final concentration) or PD0325901 (100 nM final concentration), or treat with DMSO (same volume as drugs) as a control.

4. Incubate the cells (both treated and untreated with CI-1040) with recombinant human TGF-β1 (0.5–2 nM) for 24–48 h (see Note 8).

5. Remove culture medium.

6. Fix the cells with Fixation solution for 20 min at room temperature.

7. Permeabilize the cells with Permeabilization solution for 5 min on ice.

8. Block the unspecific binding with 2 % BSA for 20 min at room temperature.

9. Wash cells with 1 mL of PBS three times.

10. To visualize F actin in cells, add 2 µL of phalloidin-Alexa Fluor 488 solution to 500 µL of Dulbecco's PBS in the well. Incubate for 30 min at room temperature.

11. Wash cells with 1 mL of PBS three times.

12. Mount coverslips on slides with mounting solution (ProLong Gold). Gently lift glass coverslips using needle and forceps and drain all the liquid onto a Kimwipe. Place a drop (20–50 µL) of mounting solution on the coverslip side that contains labeled cells. Invert the coverslip on a microscope slide.

13. Leave the mounting solution to polymerize for at least 1 h at room temperature. Examine slides on a confocal laser-scanning microscope. The samples can be stored light-protected at 4 °C for 1 week.

Measurement of Cell Shape

EF Measured with MetaMorph

We describe two methods to measure cell elongation: the elliptical factor (EF) measured with MetaMorph (proprietary software) and the Aspect Ratio (AR) calculated with ImageJ (free software).

1. Wash the cells and stain for F actin as described in Subheading 3.4.

2. Acquire the images of cells in a confocal laser-scanning microscope.

3. In order to analyze the images using MetaMorph, they must be first separated into the individual channels if more than one channel was acquired.

4. Segment the cells as follows. Select "threshold" in the opened window and then select "inclusive threshold," scroll the bar on the left until whole cells are segmented. The selected areas are the segmented regions.

5. In the main menu, go to "measure" and then to "integrated morphometry analysis."

6. In the window go to "select measurements" and click "area" box and in "shape/position/orientation" box mark "ell. form factor." In the same window go to "preferences" and click the boxes: "measure all regions," "fill holes in objects," and "exclude objects touching edge."

7. Click "measure" in the "integrated morphometry analysis" window and the table with all the values is in the "object data" tab in the same window. Select the values and copy to an excel document. The EF is the ratio between the length and the breadth of each object. The higher the ratio, the more elongated the cell is (Fig. 1a).

AR Measured with ImageJ

1. Split the channels in case various channels were acquired.

2. Take the channel with the actin staining and threshold the image (go to Image → adjust → threshold) until the cell is selected; the cell should be labeled in red. Select "apply" in the same window to create a mask on the cell, it should change to black.

3. Go to the “hand (tracing) tool” in the ImageJ main panel and click on the cell to select it.

4. Go to the main menu and select “analyze” → “set measurements.” Select “shape descriptors” and “fit ellipse”; in case other parameters are needed, they can be selected here.

5. Go to “analyze” → “measure.” In the measurements window, the major and minor axis of the best fitting ellipse are shown (labeled as “major” and “minor”) together with the AR, which is the major-axis/minor-axis. The biggest the AR value, the more elongated the cell is. A perfect circle would have an AR of 1.

Measurement of Cell Height

1. In order to measure cell height use an inverted confocal micro- scope that can register the position of the objective. The pinhole must be set at the lowest possible. Use a 63× objective.

2. Focus on a single cell using the zoom (see Note 9). Focus on the basal surface of the cell (objective upwards) until no signal is observed. Record the basal z-position at which there is no signal.

3. Move the focus in the opposite direction, towards the apical region of the cell until the signal is lost. Record the apical z-position. Subtract the apical position from the basal position to obtain the cell height (Fig. 1b).

Scratch Assay

1. Plate 1 × 105 MCs in a 12-well tissue-culture plate and culture them until they reach 100 % confluence.

2. Create a scratch wound on the cell surface using a micropipette tip (see Note 10).

3. Image the wound area by bright-field microscopy every 30 min for 24 h.

4. Measure the width of the wound and calculate the wound closure rate.

Induction of Cell Invasion Through Collagen Type I Gels Using Polycarbonate Inserts

1. Precoat polycarbonate inserts with 40 µL of Collagen solution, Type I (300 µg/mL) and incubate 1 h at 37 °C to allow gel formation.

2. Pretreat MCs with CI-1040 (2 µM), PD0325901 (100 nM) or DMSO (see Subheading 3.4, step 3) in M199 medium containing 10 % FBS for 12 h.

3. Resuspend 5 × 104 MCs in 100 µL of assay medium (M199 without FBS) and place them in the upper chamber. Add the invasion stimulus (600 µL of M199 with 10 % FBS) with MEK inhibitor or DMSO to the lower chamber.

4. Allow MCs to invade for 24 h.

5. Fix the inserts by adding 200 µL of Fixation solution to the upper chamber for 20 min.

6. Remove noninvading cells on the upper face of the membrane with a cotton swab.

7. Cut the filters with a scalpel blade.

8. Stain nuclei of invading cells with Hoechst 33342 solution for 1 h.

9. Wash the filters three times with 1 mL of PBS.

10. Count invading cells (four fields per sample) using a fluorescence microscope (40× magnification).

3D Invasion Assay

1. Treat MCs (2 × 104) with CI-1040 (2 μM), PD0325901

(100 nM) or DMSO (see Subheading 3.4, step 3) for 12 h.

2. Seed MCs in ibidi 15-well Angiogenesis chambers (2 × 104 cells per well, each point in triplicate) and allow them to attach for 3 h.

3. Lay 15 μL of Matrigel-Collagen solution over the cells.

4. After 1 h, add 50 μL of full medium containing 20 % FCS with or without MEK inhibitor (see Subheading 3.4, step 3) and incubate cells for 24–48 h.

5. After 24 h, gently remove 25 μL of full medium from the top of the well and replace with the same volume of full medium containing MEK inhibitor or DMSO at double concentration relative to that used at step 1.

6. Fix the cells with 50 μL of Fixation solution for 20 min (see

Note 11).

7. Wash by removing carefully 50 μL from the wells and filling them with 50 μL of PBS three times. Alternatively, place the plate in a bigger container and gently fill it with PBS until plate is submerged. Take care of not letting PBS enter rapidly into the wells.

8. Permeabilize by adding 50 μL of Permeabilization solution for 5 min.

9. Wash by removing carefully 50 μL from the wells and filling them with 50 μL of PBS three times as in step 7.

10. To stain actin and nuclei, add 2 μL of phalloidin-Alexa Fluor 488 and 1 μL of Hoechst 33342 to 500 μL of Dulbecco's PBS. Gently add 50 μL per well and incubate for 1 h.

11. Wash by removing carefully 50 μL from the wells and filling them with 50 μL of PBS three times as in, step 7.

12. Image samples with a confocal microscope fitted with a 40× oil objective. Set first the basal surface of the cell. Record the basal z-position.

13. Move the focus towards the edge of the invasion front until no signal is observed. Record the apical z-position. Maximum projection images may consist of 24 individual images (5 μM distance from one image to another) with a z distance of 120 μm (Fig. 1c).

Notes

1. In order to increase the number of cells, the liver may also be extracted. Remove the gallbladder, cut off the ligaments connected to the liver lobes and diaphragm carefully, take out the liver lobes gently without disturbing the liver surface, and put the liver in a 10-cm petri dish with sterile cold PBS. Wash repeatedly with PBS to remove contaminating blood cells. After this, transfer the livers to a 50-mL tube, and add 10 mL of trypsin-EDTA solution at 37 °C with occasional agitation for 10–15 min [20]. Follow the next steps of purification from Subheading 3.1, step 7.

2. Washing with PBS is necessary to remove contaminating blood cells derived from isolation procedures, as well as recirculating leukocytes. Adherent macrophages are not removed during PBS wash and may be maintained during MC culture. MCs secrete factors favoring macrophage trophism [21].

3. Coating with Collagen Type 1 and BIOGRO-2 are strictly needed for murine MCs proliferation. For human MCs Collagen is not needed, whereas BIOGRO-2 enhances proliferation. Generally, cells from two peritoneums may be plated in a single p12 plate well.

4. In order to increase cell number, unattached cells may be replated in another Collagen Type 1-coated well from a p12 plate. Cells are similar to cells attached to the first well.

5. Cells may be split 1:2 no more than 2–3 times. Generally the cells are used within 15 days from extraction. After this period cells start to acquire senescent features, with increased size and vacuoles in the cytoplasm.

6. Typically 2.5–5 × 104 cells are obtained per bag. For growing these cells, see Note 5.

7. Confluence is necessary for MCs to acquire a cobblestone epithelial-like phenotype. Murine MCs need Collagen Type 1-coating to grow properly in these experimental conditions, and the use of ibidi chambers is recommended for these cells.

8. After 24 h cells start to acquire a spindle-like conformation, and the effect is complete after 48 h. This change in cell conformation, along with several biochemical and functional changes, is a landmark of EMT induction. Interestingly, TGFβ1 treatment causes a strong reduction in Cav1 levels; thus, lack of Cav1 is associated to EMT induction in these cells [5]. In cells treated with MEK inhibitors, the induction of spindle- like shape is blocked as well as changes in cell height and induction of migratory/invasive abilities [5]. In the case of treatments lasting more than 24 h, culture medium should be changed and replaced with new medium containing MEK inhibitors +/− TGF-β1 every 24 h.

9. Alternatively, several cells can be measured at the same time using low zoom, which will give rise to an average height for all the cells in the same field of view. In cases with low cell heterogeneity, this approach may be more practical.

10. Alternatively, cell plates with culture inserts may be used. They have the advantage of facilitating the comparison and reproducibility of the experiments, since the removal of the inserts results in formation of empty spaces to be filled by migrating cells which have similar area in every well.

11. Due to the very small volume of liquid contained in Angiogenesis slides, some additional measures are required. In order to limit evaporation of culture medium, the lateral borders of the slides should be filled with 50–100 μL of distilled water or PBS. Moreover, great care should

be taken during the washing procedure. Gently remove and add no more than 50 µL of PBS per time. Rapid aspiration of culture medium/ PBS will alter the Matrigel-Collagen layer.

Figure legends

Fig. 1: ERK1/2 signaling mediates cellular responses induced by loss of Cav1. (a) Left: Confocal IF of phalloidin and cytokeratin expression and localization in MCs from WT and Cav1-/- mice. Cells were treated with 2 µM CI-1040 (CI) or DMSO for 48 h. Fixed and permeabilized cells were stained with phalloidin (F-actin). Cell nuclei were stained with Hoechst 33342. (a) Right: Quantification of the elliptical factor in experiments shown in (a). Bars represent means + s.e.m. of three independent experiments. ERK inhibition leads to reacquisition of cobblestone/epithelial-like shape in Cav1-/- MCs. *P < 0.05. (b) Left: Representative X–Z images are shown from the experiment described in (a). Arrows indicate the maximal apical dimensions used to calculate cell height. Right: quantification of cell height in the experiment described above. ERK inhibition in Cav1-/- MCs leads to an increase in cell height to levels comparable to WT MCs. *P < 0.05. (c) Effect of MEK inhibition on three-dimensional invasion by WT and Cav1-/- MCs. MCs were pretreated (24 h) with DMSO or CI-1040 (CI) (2 µM) and then overlaid with a Matrigel-Collagen matrix. Invasion was monitored over 24 h. Three-dimensional invasion was enhanced by adding 10 % FCS to the well. Cells were fixed and stained with phalloidin (blue), and Hoechst 33342 (cell nuclei; light blue). ERK inhibition blocks the increased invasion observed in Cav1-/- MCs. *P < 0.05. Adapted from ref. 5 with permission from Wiley

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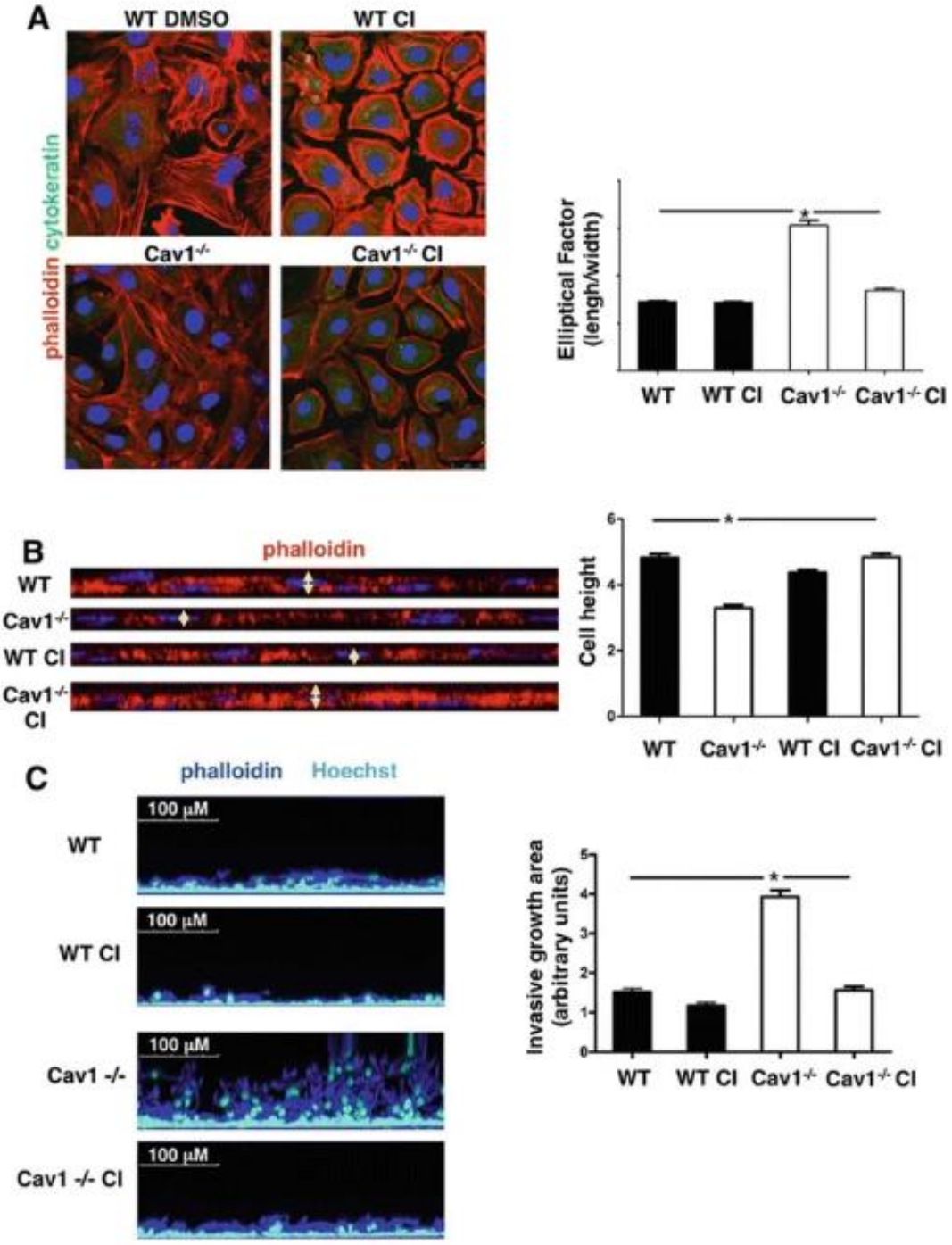
Figure 1


A
WT DMSO
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Cav1-/-
Cav1-/- Cl
phalloidin cytokeratin
Elliptical Factor (lengh/width)
WT
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Cell height
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100 µM
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WT Cl
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Cav1 -/- Cl
Invasive growth area (arbitrary units)