Supplement Material:

Supplementary Materials and Methods

Cell Culture: Human PASMC were purchased as proliferating cultures from Cascade Biologics (Portland, OR) and maintained in Medium 231 supplemented with Smooth Muscle Growth Supplement (SMGS), and with 100U/ml penicillin G, 100µg/ml Streptomycin sulfate, and 0.25µg/ml Amphotericin B (PSA Solution) (Cascade Biologics). Cells were received at passage 3 and used between passages 5 and 9. Before adding agonists, cells were synchronized using a defined starvation medium consisting of DMEM + 0.1%FBS+ 1%PSA. The agonists used were recombinant S100A4/Mts1, 500ng/ml (provided by NA), BMP-2, 10ng/ml, (Sigma, St Louis MO) and PDGF-BB, 20ng/ml, (Sigma). In some experiments, cells were pre-incubated with the phospho (p) ERK1/2-inhibitor PD 98059 (Calbiochem, San Diego, CA) at a concentration of 25 and100µM for 30min as well as the MEK inhibitor U0126 (10µM) or with the vehicle control, DMSO. To block the RAGE receptor, cells were preincubated with a rabbit polyclonal anti-RAGE antibody (1:1000) (provided by NA). To disrupt lipid rafts cells were pre-incubated with β-methyl-cyclodextrin at a concentration of 10µM for 1h (Sigma-Aldrich, St Louis, MO).

Migration Assay Using a Modified Boyden Chamber: Cell culture inserts for 24 well plates, 8μm pore size, polyethylene terephthalate (PET) membranes (BD Bioscience, San Jose, CA), were coated with fibronectin (Sigma) using a concentration of 13.5μg/ml in 1xPBS. hPASMCs at 70% confluence were incubated in starvation medium for 48h, washed in 1xPBS, trypsinized, centrifuged, resuspended and counted using a hemocytometer. 50 x10^3 cells were added to each insert and the migratory stimulus was added to 750μl medium in the well in the bottom of the chamber and the chambers incubated at 37°C and 5% CO_2 for 6h. The inserts were removed

and, after scraping off cells on the top of the insert, the cells that had migrated to the bottom of the insert were fixed and stained with the Diff Quick Kit (VWR, West Chester, PA). The cells in three different fields (200-400x) at the center of each well were counted under the microscope.

Transfection with siRNA: To suppress expression of BMPRII and CLIC4 in hPASMC, we used siRNA *SMART*pool® from Dharmacon (Lafayette, CO) and Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA). We complexed 100nmol/L of BMPRII siRNA (Cat. No: 005309), CLIC4siRNA (Cat. No: 013533), or non-targeting control siRNA (Cat. No: D-001206-13) with 5μl of Lipofectamine 2000 in 500μl of Opti-MEM I (Invitrogen) at 37°C for 6h, and then added the mixture to hPASMC at 70% confluence in T25 flasks in Medium 231 supplemented with SMGS + PSA, that were washed 3x with warm PBS. Transfection efficiency was close to 100%. Suppression of BMPRII and CLIC4 was documented 48h later both by quantitative Real-time polymerase chain reaction (qRT-PCR) and by western immunoblot.

Western Immunoblotting and Phosphorylation Assays: Cell lysates were prepared by adding boiling lysis buffer (10mM Tris-HCl, 1 mM sodium orthovanadate, 1% SDS (all Sigma) and 1x protease inhibitor cocktail (Roche, Indianapolis, IN) to the cells, followed by centrifugation. The supernatants were transferred to fresh microcentrifuge tubes and protein concentration was measured using the Lowry based DC Protein Assay (Bio-Rad, Hercules, CA). Twenty µg of protein from each sample were loaded on a 4-12% Bis-Tris NuPage gel and run under reducing conditions in MES running buffer (Invitrogen) prior to transfer to a nitrocellulose membrane (Invitrogen). The membrane was then blocked for 1h in 5% non-fat milk at room temperature. The blots were incubated O/N at 4°C unless otherwise stated, with the following antibodies: total ERK1/2 and pERK1/2 (Cell Signaling, Santa Cruz, CA), whole rabbit antiserum against human

CLIC4 (B134) at conc. 1:2000 (provided by MB), rabbit polyclonal S100A4/Mts1 antibody at conc. 1:100 (Cat. No Ab27957, Abcam, Cambridge, MA), rabbit Polyclonal CLIC4 at 1:400 (Cat No ARP35222, Aviva, San Diego, CA), rabbit polyclonal 6X His tag antibody 1:1000 (Cat. No Ab 9108, Abcam). Horseradish peroxidase (HRP) conjugated anti-mouse or anti-rabbit antibodies (1:5000) (GE Life Sciences/Amersham Biosciences, Piscataway, NY) were then incubated with the blots for 1h at RT prior to performing an enhanced chemiluminescence reaction (ECL), and exposure to autoradiograph film (Amersham Biosciences). Densitometry was carried out using the quantity one software by Biorad (Hercules, CA).

cDNA Microarray Analysis: Microarray analysis of cDNAs obtained from hPASMCs, data acquisition and analysis were performed as previously described[1] comparing genes that were similarly up or down regulated >2 fold by S100A4/Mts1 and BMP-2 and blocked by BMPRII but not by anti-RAGE.

Quantitative TaqMan Real-Time Polymerase Chain Reaction (q-RT-PCR): RNA was isolated by Trizol (Invitrogen) from hPASMC and reverse transcribed using Superscript III (Invitrogen). RT-PCR was performed on a 7900HT Sequence Detection System with TaqMan pre-verified Assays-on-Demand gene expression probes (system and probes from Applied Biosystems, Foster City, CA) for BMPRII (assay ID Hs01556128_m1), CLIC4 (assay ID Hs00983246_g1), and using the absolute quantification method with β2-microglobulin (assay ID Mm01269327_g1) as the endogenous control.

Immunocytochemistry: Human PASMCs ($25x10^3$) were seeded on collagen I coated 4-chamber slides. In experiments assessing the impact of loss of CLIC4, cells were seeded in chambers

24h after transfection with siRNA. Stimulation with agonist or control vehicle was performed on cells that were 60% confluent in starvation medium and the results were assessed 24h later.

The cells were fixed in 4% paraformaldehyde at RT for 10 min followed by ice-cold methanol. The fixed cells were then incubated in blocking serum (5% goat serum if secondary is produced in goat, 2% BSA and PBS) for 30min. The primary antibody was added in blocking serum to the sample and incubated overnight at 4°C. We used, a polyclonal rabbit CLIC4 antibody at 1:100 (provided by RA), a polyclonal rabbit myosin heavy chain IIA (MHCIIA) antibody at 1:500 (Cat. No PRB-440P, Covance, Princeton, NJ), and a mouse monoclonal anti-6XHis antibody at 1:800 (Ab5000, Abcam, Cambridge MA), α smooth muscle actin at 1:100 (Abcam, Cambridge, MA), rabbit CLIC4 antibody at 1:100 (Aviva, San Diego, CA) and goat CLC4 antibody 1:50 (Santa Cruz Technologies, Santa Cruz, CA). After washing, the secondary antibody (goat anti-mouse Alexa Fluor ® 594nm, goat anti-rabbit Alexa Fluor ® 480nm, donkey anti-goat Alexa Fluor ® 594nm) (Molecular Probes, Eugene, OR) was added at a concentration of 1:500 and incubated for at least 1h in the dark. The samples were washed and then mounted with Prolong Gold antifade containing the nuclear DAPI stain (Cat No P36931, Molecular Probes). Images were acquired with a Leica microscope using Openlab 3.1.4 software (Improvision, Coventry, UK).

Nickel Chromatography to Assess Uptake of Recombinant S100A4/Mts1 and Co-immunoprecipitation to determine its binding to MHCIIA: After starvation for 48h, hPASMCs were incubated with the ERK-Inhibitor PD 98059 (100μM for 30min) or with vehicle. Recombinant 6xHis-tagged S100A4/Mts1 generated by NA[2], 500ng/ml, was added for 0, 30 min and 1h. To obtain cytoplasmic extracts, cells were lysed using 200μl CER I (NE-PER Nuclear and cytoplasmic extraction reagents [Pierce #78833] following manufacturer's

instructions). For purification of His•Tag fusion proteins the His•Tag® Affinity Resins and Buffer Kit was used (Cat. #70899-3, Novagen, Gibbstown, NJ). We added 200μl binding buffer provided in the kit to 200μl cytoplasmic extract after which 100μl of cleared 50% Ni-NTA His-Bind-Slurry was added to 400μl of lysate/buffer and placed on rotary shaker overnight at 4°C. The next day, the mixture was centrifuged, the supernatant discarded, and the pellet washed twice. Then 50μl of eluate buffer, provided in the kit, was added to the final pellet, and the sample was centrifuged. The supernatant was used for western immunoblotting, performed as described above with the His-tag polyclonal antibody and (after stripping), we also blotted with the S100A4/Mts1 polyclonal antibody to determine whether rS100A4/Mts1 had been taken up by the cells and whether this had been influenced by blocking CLIC4 with siRNA or by inhibiting pERK with PD98059. Co-immunoprecipitation for MHCIIA and rS100A4/Mts1 was carried out 0, 30 and 60min after addition of the recombinant protein.

Rac1, RhoA and Cdc42 Pulldown Assays: Both Rac1 and RhoA activation were assayed using kits (Upstate technology, Temecula, NY) at times 0, 10min, 30min, 1h, 3h and 4h after stimulation with rS100A4/Mts1. Human PASMCs were washed and cell lysates were prepared by adding 500µl of ice-cold Magnesium Lysis Buffer to the cells (10mM Tris-HCl, 1.0% SDS, PMSF 0.2mM, and 100X protease and phosphatase inhibitor cocktails #1 and #2) (Upstate) followed by scraping into a 1.5ml microcentrifuge tube and storing on ice for 15min prior to centrifugation at 14,000 RPM for 10min at 4°C. The supernatants were transferred to fresh microcentrifuge tubes and stored at -80°C until use.

Active forms of RhoA or Rac1 were precipitated using glutathione beads containing Rhotekin or PAK1, respectively, following the manufacturer's protocol (Upstate). Briefly, lysates were

incubated with a slurry containing the glutathione beads for 1h at 4°C with constant rotation. At the end of this period, the beads were pelleted by centrifuging the lysates at 14,000 RPM for 20sec. After washing with ice-cold buffer, beads were re-suspended in Laemmli buffer and boiled for 5min followed by SDS-PAGE and western immunoblot analysis as described as above.

Cdc42 activation after stimulation with rS100A4/Mts1 was assessed using the EZ-Detect™ Cdc42 Activation kit (Pierce, Rockford, IL) at 0, 10, 30min as well as 4 and 6h after stimulation with S100A4/Mts1 following the protocol for cell lysis for adherent cells, affinity precipitation of activated Cdc42 with GST-PAK-1-PBD as well as the western immunoblot for active Cdc42 and unfractionated cell lysate (total Cdc42).

Gelatin Zymography: To assess matrix metalloproteinase (MMP) activity, we simulated the conditions of migration by seeding hPASMCs that had been transfected with control siRNA and CLIC4 siRNA, in the upper compartments of the Boyden chambers. Electrophoresis was carried out on an 8% SDS-PAGE co-polymerized with gelatin (1mg/ml, Sigma-Aldrich, Saint Louis, MO). The gel was washed for 1h at RT in a 2.5% (v/v) Triton X-100 solution, transferred to an enzyme assay buffer (0.1M Tris, pH pH=7.4, 10mM $CaCl_2$) and incubated for 24h at 37°C. The gel was stained with 0.05% Coomassie brilliant blue G-250 in a mixture of propanol-2: acetic acid: water (3: 1: 6 by volume) and de-stained in 5% ethanol with 7.5% acetic acid. Areas of proteolysis appeared as clear zones against a blue background. Densitometry was performed using the public JAVA image-processing program, NIH Image/ImageJ.

Statistical Analysis: The number of experiments carried out for each determination is given in the Figure legends. All quantitative results are presented as mean ± SEM. Statistical significance

was determined by one-way ANOVA followed by a Bonferroni post-hoc or Dunnett's test when comparisons involved ≥3 groups. A p value of <0.05 was considered significant.

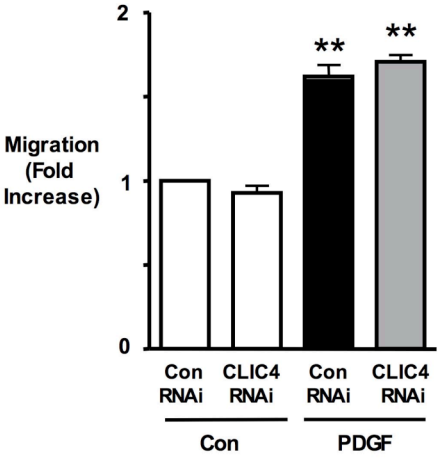
References:

1. Merklinger SL, Wagner RA, Spiekerkoetter E, Hinek A, Knutsen RH, Kabir MG, Desai K, Hacker S, Wang L, Cann GM, Ambartsumian NS, Lukanidin E, Bernstein D, Husain M, Mecham RP, Starcher B, Yanagisawa H, Rabinovitch M. Increased fibulin-5 and elastin in S100A4/Mts1 mice with pulmonary hypertension. *Circ Res*. Sep 16 2005;97(6):596-604.

2. Ambartsumian N, Klingelhofer J, Grigorian M, Christensen C, Kriajevska M, Tulchinsky E, Georgiev G, Berezin V, Bock E, Rygaard J, Cao R, Cao Y, Lukanidin E. The metastasis-associated Mts1(S100A4) protein could act as an angiogenic factor. *Oncogene*. Aug 2 2001;20(34):4685-4695.

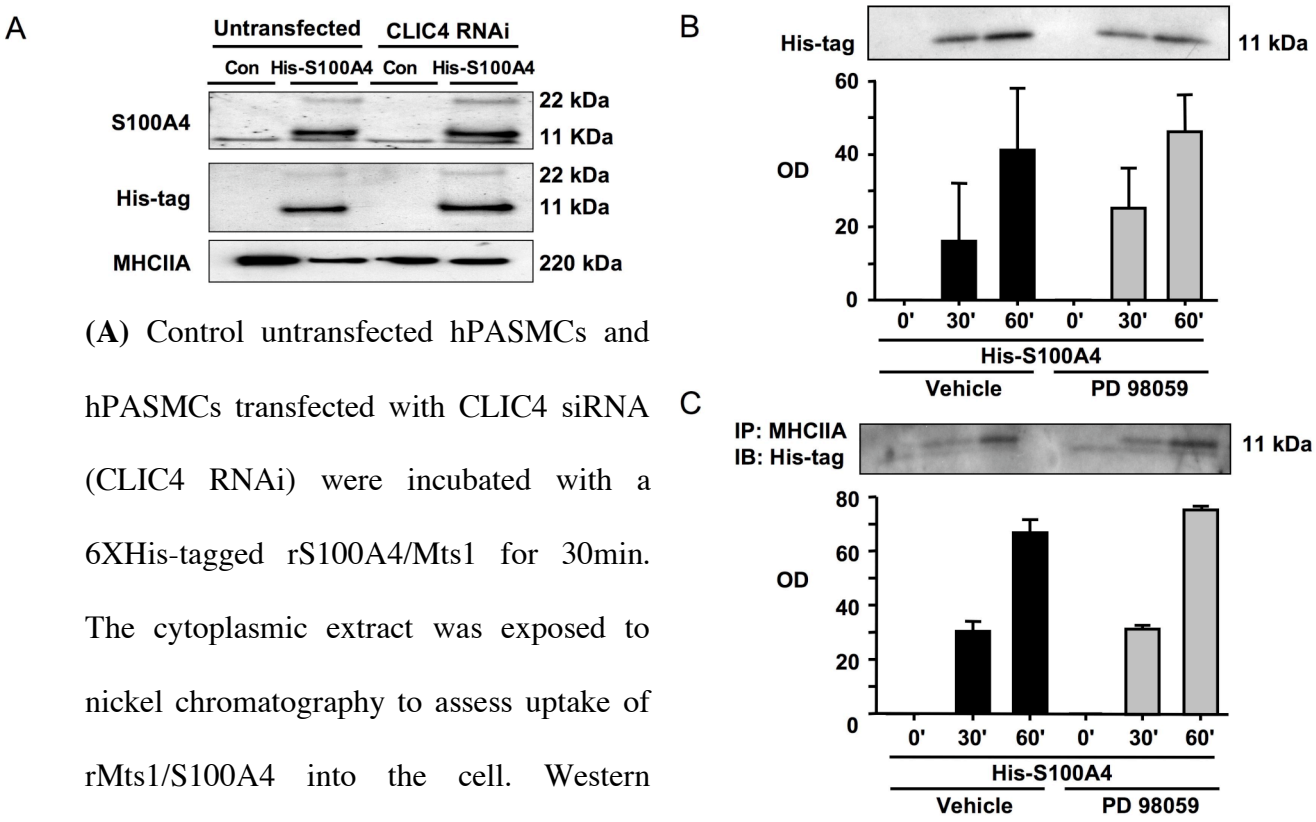
Supplementary Figures

Online Figure 1: PDGF-BB Induced Migration of hPASMC is CLIC4 Independent



Histogram shows relative migration of hPASMC transfected with control siRNA (Con RNAi), or CLIC4 siRNA (CLIC4 RNAi), under control conditions (Con) or after stimulation with PDGF-BB (20ng/ml). Bars represent mean±SEM for n=3. *p<0.001

Online Figure 2: S100A4/Mts1 Uptake is Unaffected by Reduced CLIC4 or by pERK1/2



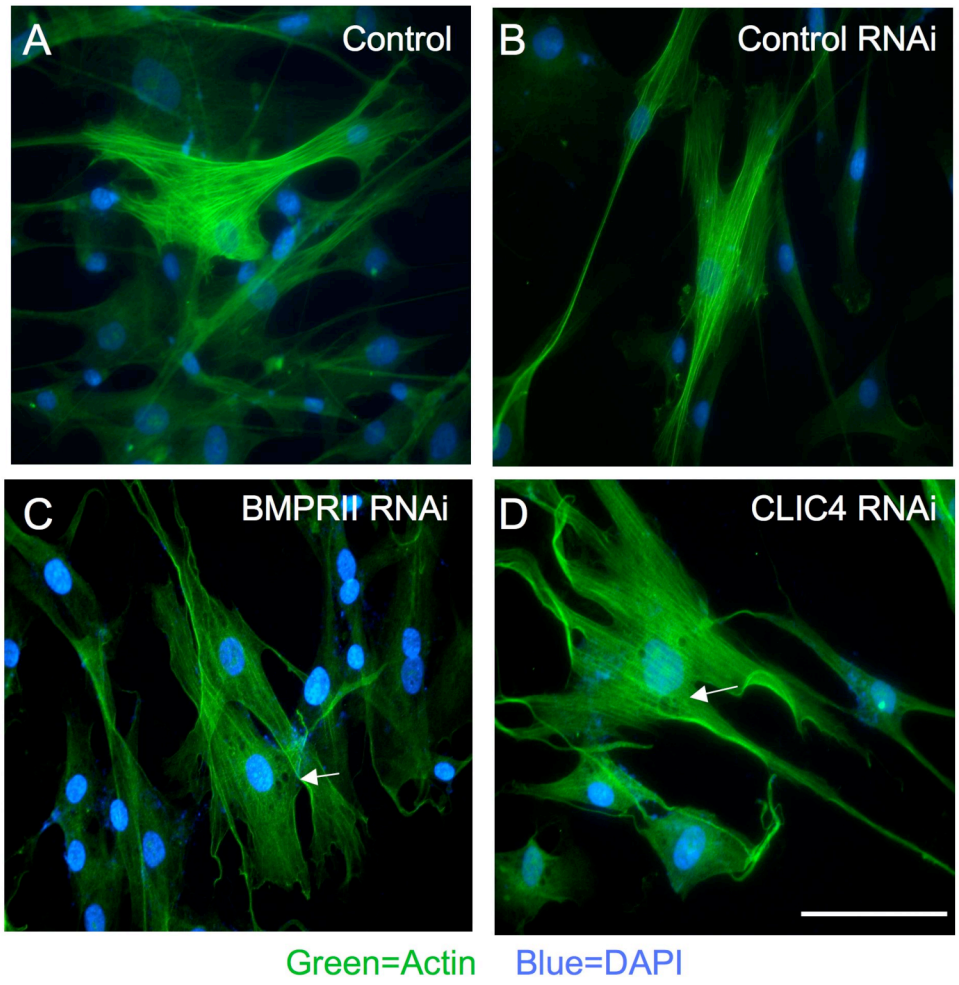
(A) Control untransfected hPASMCs and hPASMCs transfected with CLIC4 siRNA (CLIC4 RNAi) were incubated with a 6XHis-tagged rS100A4/Mts1 for 30min. The cytoplasmic extract was exposed to nickel chromatography to assess uptake of rMts1/S100A4 into the cell. Western immunoblots are representative of results from two different experiments, showing both untransfected and CLIC4 RNAi transfected cells under control conditions (Con) or following addition of His-tagged rS100A4/Mts1 (His-S100A4). Cytoplasmic endogenous and rS100A4/Mts1 (11kDa) are detected using anti-S100A4/Mts1Ab (S100A4). The endogenous S100A4 migrates slightly faster than the rS100A4/Mts1 that is detected with the His-Ab (His-tag). The band at about 22kDa reflects a dimer of rS100A4/Mts1 that does not occur with the endogenous S100A4/Mts1. There is no difference in the protein levels of MHCIIA observed at 220kDa.

(B) Representative immunoblot above and densitometric quantification below of rS100A4/Mts1 uptake under control conditions in cells pre-treated with vehicle or following pre-treatment with

the pERK inhibitor PD98059 at the doses used in Figure 2. Recombinant His-tagged S100A4/Mts1 was added and cytoplasmic levels were monitored 0, 30 and 60min later. Densitometric values are given in arbitrary units for data from three different experiments.

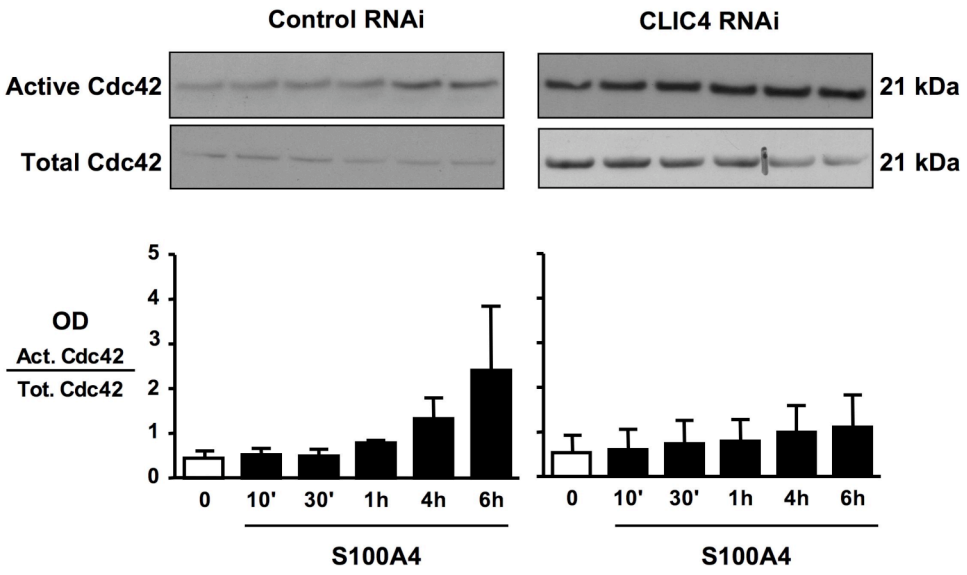
(C) Representative immunoblot above and densitometry below from three experiments, in which co-immunoprecipitation for MHCIIA and rS100A4/Mts1 was carried out 0, 30 and 60min after addition of the recombinant protein. The MHCIIA antibody for immunoprecipitation (IP) and the His-Tag antibody for immunoblotting (IB) were used in cells pretreated for 30min with the pERK1/2 inhibitor PD98059 or with vehicle.

Online Figure 3: Loss Of CLIC4 Induces Vacuoles in hPASMC, as Revealed by α Smooth Muscle Actin Immunofluorescence



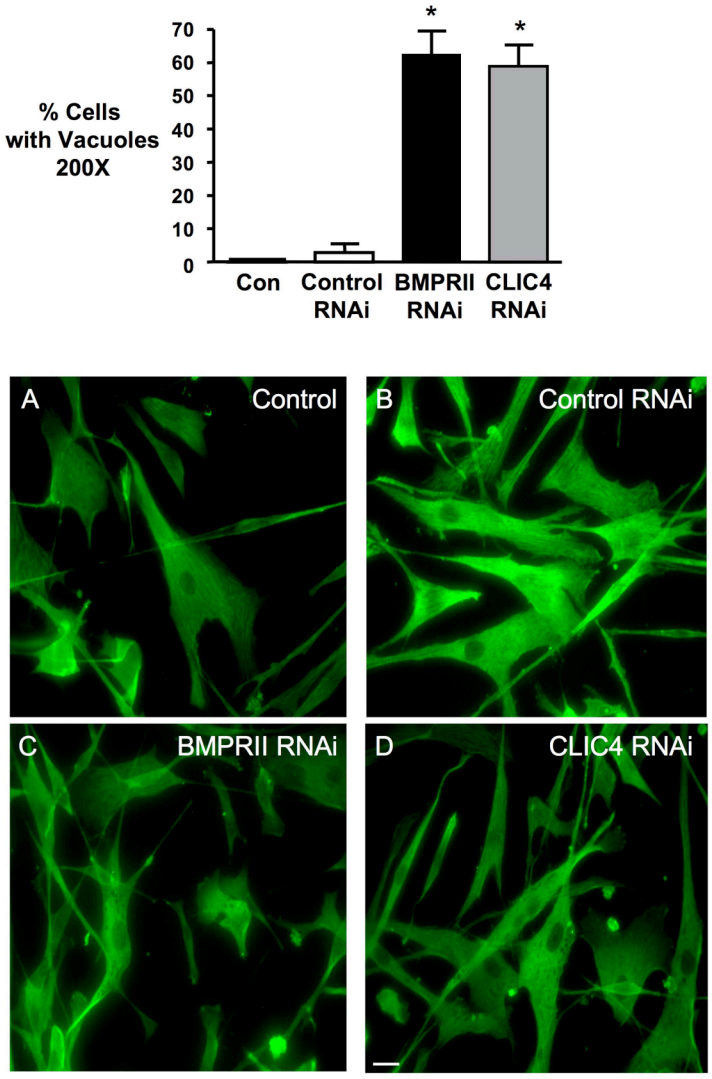
Representative immunofluorescence of α smooth muscle actin in hPASMC and a secondary antibody linked to fluorescein (FITC) under **(A)** untransfected control conditions, and following transfection with **(B)** non-targeting siRNA, **(C)** BMPRII siRNA, and **(D)** CLIC4 siRNA. Reducing CLIC4 either by BMPRII siRNA or CLIC4 siRNA interrupts linear alignment of actin fibers (FITC) around what appear to be vacuoles (arrows in **C** and **D**). Nuclear counterstain, DAPI. (Bar =50 μM)

Online Figure 4: **Cdc42 Activity Is Not Significantly Affected By Reduced CLIC4**



Representative Western immunoblotting of **(A)** active Cdc42 and total Cdc42 under control and CLIC4RNAi conditions, and **(B)** and quantitative densitometry of active Cdc42/total Cdc42 as described in the Methods from hPASMC lysates 10min to 6h after stimulation with S100A4/Mts1. Bars represent mean±SEM from n=3 experiments.

Online Figure 5: Vacuole formation is seen in about 50-60% of cells where BMPRII and CLIC4 is knocked down.



Histogram showing quantification of percentage of vacuoles in cells without transfection, control RNAi, BMPRIIsi as well as CLIC4si (N=4 each condition, magnification 200X, $p < 0.001$). Representative immunofluorescence of MHCIIA in hPASMC using a primary antibody against MHHCIIA and a secondary antibody linked to fluorescein (FITC) under **(A)** untransfected control conditions, and following transfection with **(B)** non-targeting siRNA, **(C)** BMPRII siRNA, and **(D)** CLIC4 siRNA. Vacuoles are seen in **C** and **D** yet not in **A** and **B.**