S100A4 and Bone Morphogenetic Protein-2 Codependently Induce Vascular Smooth Muscle Cell Migration via Phospho–Extracellular Signal-Regulated Kinase and Chloride Intracellular Channel 4

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Rationale: S100A4/Mts1 is implicated in motility of human pulmonary artery smooth muscle cells (hPASMCs), through an interaction with the RAGE (receptor for advanced glycation end products).

Objective: We hypothesized that S100A4/Mts1-mediated hPASMC motility might be enhanced by loss of function of bone morphogenetic protein (BMP) receptor (BMPR)II, observed in pulmonary arterial hypertension.

Methods and Results: Both S100A4/Mts1 (500 ng/mL) and BMP-2 (10 ng/mL) induce migration of hPASMCs in a novel codependent manner, in that the response to either ligand is lost with anti-RAGE or BMPRII short interference (si)RNA. Phosphorylation of extracellular signal-regulated kinase is induced by both ligands and is required for motility by inducing matrix metalloproteinase 2 activity, but phospho–extracellular signal-regulated kinase 1/2 is blocked by anti-RAGE and not by BMPRII short interference RNA. In contrast, BMPRII short interference RNA, but not anti-RAGE, reduces expression of intracellular chloride channel (CLIC)4, a scaffolding molecule necessary for motility in response to S100A4/Mts1 or BMP-2. Reduced CLIC4 expression does not interfere with S100A4/Mts1 internalization or its interaction with myosin heavy chain IIA, but does alter alignment of myosin heavy chain IIA and actin filaments creating the appearance of vacuoles. This abnormality is associated with reduced peripheral distribution and/or delayed activation of RhoA and Rac1, small GTPases required for retraction and extension of lamellipodia in motile cells.

Conclusions: Our studies demonstrate how a single ligand (BMP-2 or S100A4/Mts1) can recruit multiple cell surface receptors to relay signals that coordinate events culminating in a functional response, ie, cell motility. We speculate that this carefully controlled process limits signals from multiple ligands, but could be subverted in disease. (Circ Res. 2009;105:639-647.)

Key Words: bone morphogenetic protein ▪ S100 protein ▪ vascular smooth muscle cells ▪ intracellular chloride channel ▪ migration

S100A4 (also known as Mts1, metastasin, p9Ka, pEL98, CAPL, calvasculin, Fsp-1, placental calcium-binding protein) belongs to the family of EF-hand calcium-binding proteins. Increased expression of S100A4/Mts1 is observed in cancer cells and contributes to tumor cell motility and metastatic progression, through interaction with myosin heavy chain II. Our group has shown that S100A4/Mts1 also induces human pulmonary artery smooth muscle cell (hPASMC) migration and proliferation by interacting with RAGE (receptor for advanced glycation end products). Migration and proliferation of hPASMCs have been linked to the pathogenesis of pulmonary arterial hypertension (PAH). This is in keeping with our previously reported finding that a small percentage of transgenic mice overexpressing S100A4/Mts1 develop occlusive pulmonary vascular changes. We have also previously reported that expression of S100A4/Mts1 requires a cooperative interaction between the serotonin receptor and transporter. This, too, is relevant to the pathogenesis of pulmonary hypertension, because heightened serotonin activity has been linked, both clinically and experimentally, to the pathogenesis of PAH.

Mutations causing loss of function of bone morphogenetic protein (BMP) receptor (BMPR)II have been linked to >60% of cases of familial PAH and 25% of cases of sporadic PAH.
but the penetrance is low, suggesting that other modifier genes and/or environmental factors may be important. Thus, understanding the interactions between signaling via BMPRII and other cell surface receptors, such as RAGE, via S100A4/Mts1 is helpful in uncovering factors that modify propensity to vascular disease in association with a BMPRII mutation.

Our studies reveal a previously unknown codependence between signals emanating from BMPRII and RAGE in mediating cell motility. Suppression of RAGE, but not BMPRII, resulted in loss of phospho-extracellular signal-regulated kinase (pERK) and matrix metalloproteinase (MMP)2 activity, whereas loss of BMPRII but not RAGE reduced levels of the intracellular chloride channel (CLIC4), a scaffolding protein previously implicated in motility. Loss of CLIC4 resulted in impaired activation and distribution of the small GTPases RhoA and Rac1, necessary for cell retraction and extension of filopodia and lamellipodia during cell migration.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Cell Culture

hPASMCs were used between passages 5 and 9, and synchronized using a defined starvation medium. In some experiments, cells were preincubated with the phospho (p)ERK1/2-inhibitor PD 98059 at 25 and 100 μmol/L or with the mitogen-activated protein kinase kinase (MEK) inhibitor U0126 10 μmol/L, or with a rabbit polyclonal anti-RAGE antibody.

Migration Assay Using a Modified Boyden Chamber

Cells were added to fibronectin-coated microporous inserts of 24 well plates, and the migratory stimulus was added to the well in the bottom of the chamber for 6 hour. The cells that had migrated to the bottom of the insert were fixed and stained with the Diff Quick Kit. The cells in three different fields (×200 to ×400) at the center of the cells were counted under the microscope.

Transfection With Short Interference RNA

To suppress expression of BMPRII and CLIC4 in hPASMCs, we transfected short interference (si)RNA SMARTpool from Dharmacon (Lafayette, Colo) and Lipofectamine 2000 (Invitrogen, Carlsbad, Calif) as described in the Online Data Supplement. Suppression of BMPRII and CLIC4 was documented 48 hour later both by quantitative real-time polymerase chain reaction (PCR) and by western immunoblot.

Western Immunoblotting and Phosphorylation Assays

Cell lysates were prepared and run under reducing conditions before transfer to a nitrocellulose membrane. The membrane was blocked and the blots were incubated with polyclonal antibodies against human CLIC4, S100A4/Mts1 or His tag. Densitometry was carried out using the quantity one software by Bio-Rad (Hercules, Calif).

cDNA Microarray Analysis

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

Quantitative TaqMan Real-Time PCR

RNA was isolated by TRIzol (Invitrogen) and reverse-transcribed using Superscript III (Invitrogen). RT-PCR was performed on a 7900HT Sequence Detection System with TaqMan preverified Assays-on-Demand gene expression probes (Applied Biosystems, Foster City, Calif) for BMPRII (assay ID Hs 01556128_g1), CLIC4 (assay ID Hs 00983246_g1), using the absolute quantification method with β2-microglobulin (assay ID Mm 01269327_g1) as the endogenous control.

Immunocytochemistry

hPASMCs were seeded on collagen I–coated 4-chamber slides fixed in 4% paraformaldehyde. We used, a polyclonal rabbit and monoclonal goat CLIC4 antibody, a polyclonal rabbit myosin heavy chain (MHC)IIA antibody, a mouse monoclonal Rac1, and a rabbit polyclonal α smooth muscle actin antibody. Nuclei were counterstained with DAPI. Images were acquired with a Leica microscope using Openlab version 3.1.4 software (Improvement, Coventry, UK).

Luminescence Caspase 3/7 Apoptosis Assay

In PASMCs 48 hours after transfection with control RNA interference (RNAi), BMPRII RNAi and CLIC4 RNAi, caspase 3/7 activity was assessed with Luminescence Caspase-Glo 3/7 Assay (G8091; Promega, Madison, Wis) in a 96-well plate as described in the kit.

Rac1 and RhoA and Cd42 Pulldown Assays

Rac1, RhoA, and Cad42 activation were assayed after stimulation with recombinant S100A4/Mts1, using kits for Rac1 and RhoA (Upstate technology, Temecula, NY) and for Cad42 (EZ-Detect Cdc42 Activation kit, Pierce, Rockford, Ill).

Gelatin Zymography

To assess MMP activity, electrophoresis was carried out on an 8% SDS-PAGE copolymerized with gelatin and stained with 0.05% Coomassie brilliant blue and destained in 5% ethanol with 7.5% acetic acid. Areas of proteolysis were quantified 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 1-way ANOVA, followed by a Bonferroni post hoc test or Dunnett’s when comparisons involved ≥3 groups. A probability value of <0.05 was considered significant.
Results

**S100A4/Mts1- or BMP-2–Induced hPASMC Migration Requires RAGE and BMPRII**

Stimulation of hPASMCs with recombinant S100A4/Mts1 (500 ng/mL) or with BMP-2 (10 ng/mL) resulted in a 2-fold increase in migration compared to unstimulated (control) cells, as assessed in Boyden chambers at 6 hours. Although we expected that the S100A4/Mts1 migratory response of hPASMCs would be blocked by anti-RAGE, the same was true for BMP-2 mediated hPAMSC migration (Figure 1A). Similarly, when we reduced the levels of BMPRII mRNA and protein12 with siRNA to 20% of those in hPASMCs transfected with control siRNA (Figure 1B), the migratory response to both BMP-2 and to S100A4/Mts1 was repressed (Figure 1C). We previously published that in cells from the same harvests, the reduction in BMPRII protein by siRNA was associated with reduced phosphorylation of Smad 1/5.12 The migratory response to the motogen platelet-derived growth factor (PDGF)-BB was not influenced by knocking down BMPRII (Figure 1D) or by anti-RAGE.3

**pERK1/2 Is Necessary but Not Sufficient for S100A4/Mts1- and BMP-2–Induced hPASMC Migration**

In a search for common or complementary signaling mechanisms activated via RAGE or BMPRII in response to either S100A4/Mts1 or BMP-2, we identified pERK1/2 (Figure 2A). Other signaling molecules involved in migration (c-Jun N-terminal kinase, p38, Smads) were not induced by both ligands (data not shown). Blockade of pERK1/2 with the specific mitogen-activated protein kinase inhibitor PD98059 at 25 and 100 μmol/L or with the MEK inhibitor U0126 10 μmol/L abrogated the migratory response to both BMP-2 and S100A4/Mts1 (Figure 2B). Interestingly, pERK1/2 was blocked by anti-RAGE but not by BMPRII siRNA, suggesting that both BMP-2 and S100A4/Mts1 activate pERK through RAGE (Figure 2C). In addition, cells that were treated with BMPRII siRNA and stimulated with BMP-2 showed ERK phosphorylation that could be blocked with anti-RAGE, indicating that BMP-2 can induce pERK in a BMPRII independent manner via RAGE (Figure 2D). However, it also implies that, with loss of BMPRII, neither BMP-2– nor S100A4/Mts1-mediated pERK is sufficient to induce the migratory response of hPASMCs. We therefore set out to determine downstream targets of BMPRII important in migration that were influenced by stimulation with both S100A4/Mts1 and BMP-2.

**CLIC4 Expression Depends on BMPRII and Is Necessary for S100A4/Mts1- and BMP-2–Mediated Migration of hPASMCs**

To identify BMPRII-dependent gene transcripts, we used Stanford cDNA microarrays11 to mine for mRNAs in hPASMCs that were altered by BMPRII siRNA but not by anti-RAGE in response to BMP-2 and S100A4/Mts1 stimulation for 6 hours.
Of all 14,500 annotated genes on the array, 23 were BMPRII-dependent genes meaning that they were similarly expressed after S100A4/Mts1 and BMP-2 stimulation and had a similar alteration in expression pattern with BMPRII siRNA but not the RAGE antibody. Of those, 6 genes have

Figure 3. CLIC4 lies downstream of BMPRII, not of RAGE and is necessary in S100A4/Mts1- and BMP-2–induced migration of hPASMCs. A, Quantitative RT-PCR of CLIC4 mRNA normalized to β2 microglobulin (β2M) mRNA in hPASMCs without stimulation and following S100A4 and BMP-2 for 6 hours with or without 30 minutes of pretreatment with anti-RAGE (1:1000) or 48 hours after transfection with BMPRII RNAi (80% knockdown) or CLIC4 RNAi (B). C, Western immunoblot and densitometry show a reduction in protein to less than 10% after CLIC4 RNAi. D, Relative migration with or without stimulation with S100A4 or BMP-2 of hPASMCs transfected with control (Con) or CLIC4 RNAi. E, Immunolocalization of CLIC4 in hPASMCs under control conditions and following 2 hours of stimulation with S100A4 and BMP-2. CLIC4, localized with a primary antibody and a secondary antibody conjugated to fluorescein (fluorescein isothiocyanate [FITC]), exhibits some nuclear staining under control conditions. In response to stimulation with BMP-2 and S100A4, CLIC4 staining becomes increasingly perinuclear and diffusely cytoplasmic and localizes to the cell membrane, particularly in filopodia (arrow). Nuclei counterstained with DAPI. Bars represent 10 μm.

Figure 2. Phosphorylation of ERK, induced by S100A4/Mts1 and BMP-2, is necessary for migration of PASMCs downstream of RAGE but is not BMPRII-dependent. A, Western immunoblot showing pERK1/2 (kDa 42/44) relative to total ERK1/2 in response to S100A4 and BMP-2. B, Migration assays with or without 30-minute preincubation with mitogen-activated protein kinase inhibitor PD 98059 (25 μmol/L, 100 μmol/L) and MEK inhibitor U0126 (10 μmol/L). DMSO as vehicle control for PD 98059. C and D, Western immunoblot and densitometry of pERK1/2 over total ERK1/2 of anti-RAGE–BMPRII RNAi, unstimulated (Con) or following stimulation with BMP-2 and S100A4 for 10 minutes. Each value is normalized to total ERK on the same blot, but lanes are nonconsecutive, indicated by the lines between them (C). Bars represent means±SEM for n=6 in B and n=3 in C. *P<0.001 vs control unstimulated. E, Immunolocalization of CLIC4 in hPASMCs under control conditions and following 2 hours of stimulation with S100A4 and BMP-2. CLIC4, localized with a primary antibody and a secondary antibody conjugated to fluorescein (fluorescein isothiocyanate [FITC]), exhibits some nuclear staining under control conditions. In response to stimulation with BMP-2 and S100A4, CLIC4 staining becomes increasingly perinuclear and diffusely cytoplasmic and localizes to the cell membrane, particularly in filopodia (arrow). Nuclei counterstained with DAPI. Bars represent 10 μm.

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been previously associated with motility (tenasin C, fibrillin 1, myosin light chain polypeptide kinase, α-tubulin, Ras- GTPase activating protein, and CLIC4). Of those 6 genes, CLIC4 showed the greatest alteration in expression with loss of BMPRII (4-fold reduction). Confirmation of this reduction in gene expression was verified by quantitative RT-PCR (Figure 3A). Knockdown of CLIC4 following pretreatment with BMPRII siRNA was dependent on a ligand-receptor interaction, because knocking down of BMPRII in the absence of ligand stimulation did not reduce CLIC4 levels (Figure 3A). Migration assays were carried out to test the functional importance of these findings, comparing the response of hPASMCs following knockdown of BMPRII or CLIC4 after transfection with siRNA. We documented a reduction in CLIC4 mRNA to 20% of the values obtained with control siRNA (Figure 3B), as well as a comparable decrease in CLIC4 protein (Figure 3C). We show in Figure 3D that migration of hPASMCs in response to S100A4/Mts1 or BMP-2 was abrogated by CLIC4 siRNA. As with BMPRII siRNA, the migratory response to PDGF-BB was not influenced by CLIC4 siRNA (Online Figure I).

Previous reports described the localization of CLIC4 in multiple subcellular compartments. We observed by immunocytochemistry that there is some nuclear staining of CLIC4 under conditions of serum starvation, but as early as 10 minutes after stimulation of hPASMCs with S100A4/Mts1 or BMP-2, CLIC4 moves to a perinuclear and cytoplasmic location and concentrates at or near cell membranes as well as in filopodia (Figure 3E). We therefore set out to determine why maintaining normal CLIC4 levels and pERK1/2 are necessary for hPASMC migration.

**CLIC4 and pERK Do Not Influence S100A4/Mts1 Uptake or Binding to MHCIIA**

We investigated whether CLIC4 and/or pERK might be required for internalization of S100A4/Mts1 or for binding of S100A4/Mts1 to MHCIIA, processes implicated in motility. His-tagged S100A4/Mts1 uptake into the cell did not depend on CLIC4 (Online Figure II, A). In addition, blockade of pERK1/2 had no effect on rS100A4/Mts1 uptake (Online Figure II, B). Binding of S100A4/Mts1 to MCHIIA was also neither dependent on pERK 1/2 (Online Figure II, C) nor on CLIC4 (data not shown).

**MMP2 Activity Depends on pERK1/2, Not on CLIC4**

Previous studies have shown that pERK is necessary for MMP2-mediated motility in aortic smooth muscle cells. We observed in hPASMCs that S100A4/Mts1 mediates pro-MMP2 activity, in a pERK-dependent manner in that the response is lost in cells pretreated with the inhibitor PD98059 (Figure 4). We observed primarily an increase in pro-MMP2, because both the amount and any alteration in the active form of MMP2 was difficult to appreciate by zymography. This observation is consistent with that of other investigators using cytokines to stimulate MMP2 activity in smooth muscle cells (SMCs). In contrast, reducing CLIC4 by siRNA did not alter pro-MMP2 activity in response to S100A4/Mts1 (Figure 4).

**Loss of CLIC4 Causes Accumulation of MHCIIA Around Vacuolar Structures**

Following transfection of cells with either BMPRII siRNA or with CLIC4 siRNA but not control siRNA, changes in the cytoskeleton were observed related to the alignment of MHCIIA (Figure 5A through 5D). Loss of CLIC4 resulted in disruption of the linear pattern of myosin staining and caused accumulation of MHCIIA around vacuolar structures. The vacuolar structures are also apparent by staining with an α-smooth muscle actin antibody (Online Figure III). Quantification shows that 50% to 60% of cells with knockdown in BMPRII or CLIC4 show vacuole formation, in contrast to 2% to 3% of cells after transfection with control siRNA (Online Figure V). Cell survival assays measuring caspase 3/7 activity do not show differences between transfected and nontransfected cells, suggesting that the vacuole appearing structures do not reflect cell damage (Figure 5E). We confirmed CLIC4 localization not only in the cell body but also extending to cell processes such as lamellipodia and filopodia (arrows). In addition, we demonstrated by immunocytochemistry that CLIC4 staining is markedly reduced in BMPRII RNAi and CLIC4 RNAi treated cells and that the homogeneous distribution of CLIC4 is interrupted by the vacuolar structures (Figure 5F).

**CLIC4 Delays and Alters S100A4/Mts1 Induced RhoA and Rac1 Activation**

Stimulation of hPASMC migration with both BMP-2 (not shown) and S100A4/Mts1 resulted in rapid activation of RhoA and Rac1 (Figure 6A and 6B). In contrast, suppression of CLIC4 with siRNA resulted in delayed Rac1 (Figure 6A) and RhoA activities (Figure 6B). We stained cells for Rac1 to assess changes in localization and, interestingly, have found that the distribution of Rac1 is altered after BMPRII and CLIC4 knockdown. In nontransfected or control RNAi cells, Rac1 is located both at the periphery and in the perinuclear...
region in response to S100A4, whereas with BMPRII or CLIC4 RNAi, the perinuclear localization persists but peripheral staining for Rac1 is markedly reduced (see arrows) (Figure 6C). These data support altered small GTPase signaling in response to downregulation of CLIC4.

In assessing the role of Cdc42, we found that total Cdc42 is increased after CLIC4 siRNA versus control RNAi independent of S100A4/Mts1 stimulation. Active to total Cdc42 in response to S100A4/Mts1 stimulation is not significantly affected by CLIC4 versus control siRNA (Online Figure IV).

Discussion

We propose a novel codependence between RAGE and BMPRII and indicate how a single ligand (BMP-2 or S100A4/Mts1) can recruit additional cell surface receptors to relay signals necessary to orchestrate a functional response, in this case to coordinate cytoskeletal changes with MMP activity and Rho/Rac activation required for cell motility (Figure 7). We speculate that this carefully controlled process limits signals from multiple ligands but could be subverted in disease.

S100A4/Mts1 was described as a protein differentially regulated in metastatic breast cancer and subsequent experimental animal and clinical studies established this protein as a “metastasis” factor in a variety of cancers.19 Our previous studies indicated that RAGE is the receptor for S100A4/Mts1,3 and work by others showed that S100A4/Mts1 internalization and binding to MHCIIA mediates cell motility.2 That BMP-2 is also a motogen is consistent with studies previously reported in human aortic smooth muscle cells.20 However, in rat aortic smooth muscle cells BMP-2 and BMP-4 were shown to suppress motility but in those studies, the BMPs were only used in conjunction with serum or PDGF-mediated motility and in very high doses of 100 ng/mL.21 BMP-2 promotes mesenchymal cell migration in association with atrioventricular valvulogenesis22 and also induces migration of neural crest cells.23

That S100A4/Mts1-RAGE signals depended on crosstalk with BMPRII is a novel finding, but crosstalk between RAGE and Smad2/3 signals both in cultured vascular smooth muscle cells,24 as well as in mesangial cells have been related to diabetic vascular disease and nephropathy.24 In work from our laboratory,24a we have shown that BMP-2 mediates motility of endothelial cells in a pSmad 1/5 dependent manner.

It has been suggested that the S100 proteins may have interactions with proteins other than RAGE through the hinge

Figure 5 Continued. S100A4/Mts1 for 10 minutes using a primary antibody against MHCIIA and a secondary antibody linked to fluorescein (FITC) under nontransfected conditions (A), following transfection with control siRNA (B), BMPRII siRNA (C), and CLIC4 siRNA (D). Reducing CLIC4 either by BMPRII RNAi or CLIC4 RNAi interrupts linear alignment of MHCIIA (FITC) around what appear to be vacuoles (arrows in C and D). E, Luminescent assay for caspase-3/7 activities in control, BMPRII, and CLIC4 RNAi-treated cells. No significant differences between siRNA-treated and control nontransfected cells are seen (bar represents mean±SEM of n=6). F, Immunofluorescence of MHCIIA (FITC) and CLIC4 (rhodamine) in hPASMCs under nontransfected control conditions (A) and following CLIC4 RNAi (B). Nuclear counterstain DAPI. Bars=50 μm.
domain, so it is possible that S100A4/Mts1 could interact with BMPRII or with some other receptor that recruits BMPRII, such as BMPRIA or BMPRIB. This is because blocking RAGE does not abrogate S100A4/Mts1-mediated processes that depend on BMPRII such as maintaining CLIC4 levels. Because S100A proteins can interact with lipid rafts and because BMPRII exists in lipid rafts, it is possible that there is a direct association at the level of the cell membrane. Conversely, it is possible that BMP-2 interacts with RAGE or recruits RAGE in a non–BMPRII-dependent manner, ie, through BMPRIA or BMPRIB and an activin type II receptor because the absence of BMPRII does not repress BMP-2–mediated pERK1/2 in contrast to pretreatment with anti-RAGE. Because pERK is known to bind RAGE, it is perhaps not surprising that S100A4/Mts1- and BMP-2–mediated motility is pERK-dependent. To further substantiate

Figure 6. Suppression of CLIC4 Alters S100A4/Mts1 activation of RhoA and Rac1. Western immunoblotting and densitometry of active Rac1/total Rac1 (A) and active RhoA/total RhoA (B) from hPASMC lysates 10 minutes to 6 hours after stimulation with S100A4/Mts1. C, Immunofluorescence of MHCIIA and Rac1 in hPASMCs stimulated with S100A4/Mts1 for 10 minutes using a primary antibody against MHCIIA and Rac1 and a secondary antibody linked to fluorescein (FITC) and rhodamine (red) under nontransfected control conditions and following transfection with nontargeting siRNA, BMPRII siRNA, and CLIC4 RNAi. Reducing CLIC4 either by BMPRII siRNA or CLIC4 siRNA reduces peripheral distribution of Rac1. Bars represent means±SEM from n=3 experiments. *P<0.01, **P<0.001 vs unstimulated control.
the role of RAGE in mediating the pERK signal, we have made multiple attempts to reduce RAGE expression using a variety of RAGE siRNAs and transfection strategies but could not successfully knockdown RAGE in PASMCs.

In addition to regulating MHCIIA-mediated motility, S100A4/Mts1 also induces MMP2 activity, which is essential for vascular SMC migration. RAGE and MMP2 activity have been implicated in restenosis and in atherosclerosis, S100A4/Mts1 is thought to be a marker of intimal SMCs and mediates cancer cell invasiveness through MMP2. 

Our observation that BMPRII is necessary to maintain levels of CLIC4 is novel and unexpected as was the further observation that reduced levels of CLIC4 are sufficient to suppress motility. These findings are consistent with the association of CLIC4 with a signaling complex required for motility of spermatozoa. On the other hand, upregulation of CLIC4 during conversion of fibroblasts to myofibroblasts correlates with reduced motility. Thus, the exact role of CLIC4 in cell motility may depend on a particular signaling pathway, cell type, or biological context. CLIC4 is found in lipid rafts and acts as a scaffolding protein for protein kinases and phosphatases and may play a key role in microtubule dynamics, particularly because it localizes with the centrosome of the cell during division and at the apical junctions of polarized epithelial cells. Most recently, CLIC4 has been shown to associate with G-protein coupled receptors such as the histamine H3 receptor, suggesting that CLIC4 could play a role in expression/presentation of receptors at the cell surface. We have shown that peroxisome proliferator-activated receptor (PPAR)γ is downstream of BMP-2 signaling in SMCs, and loss of PPARγ/β also decreases CLIC4.

We observed malalignment of myosin with loss of BMPRII or CLIC4 giving the appearance of "tenestractions" or vacuoles in the cytoskeleton. Although nuclear CLIC4 is associated with apoptosis, it is possible that loss of cytoplasmic CLIC4 leads to autophagy and that these vacuolar structures represent autophagosomes induced as a cell survival mechanism. Alternatively, the vacuoles could represent some stage of intracellular lumen formation, because reduction of CLIC4 and a Caenorhabditis elegans CLIC, EXC-4, have been linked to defects in lumen/tube formation in endothelial cells and intracellular secretory canals, respectively. Based on these findings, our data indicate that the cytoskeletal changes and intracellular vacuoles are indeed induced by loss of CLIC4. We suggest that this leads to delayed activity of Rac1 and RhoA and alterations in the localization of Rac1, and this impairs migration, because these GTPases are critical regulators of cytoskeletal dynamics and play key roles in cell retraction and extension of filopodia. Alternatively, reduced activity of RhoA and Rac1 might reflect loss of the function of CLIC4 act as a scaffolding molecule, suggesting that CLIC4 may permit timely activation and translocation of these GTPases. CLICs can interact directly or indirectly with members of the ERM (ezrin–radixin–moesin) family of membrane–cytoskeletal linkers, proteins reported to be involved in regulation of signaling by Rho family GTPases. Interestingly, we could not show a significant change in Cdc42 activation yet total Cdc42 seems to be increased with CLIC4 RNAi.

Recent studies in cultured hPASMCs, by our group indicate that loss of BMPRII function increases proliferation in response to agonists such as PDGF-BB. Because loss of BMPRII function reduces motility, it is possible that migration is less important than proliferation in the pathogenesis of occlusive lesions in PAH. Alternatively, additional modifications are necessary for loss of function of BMPRII to result in both heightened proliferation and migration, for example, upregulation of RAGE or heightened activity of other mito/motogens such as PDGF-BB that are unimpeded by reduced BMPRII. This would be in keeping with the low penetrance of disease in patients with loss of function of BMPRII and with the relatively mild phenotype in mice with haploinsufficiency of BMPRII.

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Disclosures
None.

References


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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 x10³ 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% CO2 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 SMARTpool® 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 NA2, 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 20 sec. After washing with ice-cold buffer, beads were re-suspended in Laemmli buffer and boiled for 5 min followed by SDS-PAGE and western immunoblot analysis as described above.

Cdc42 activation after stimulation with rS100A4/Mts1 was assessed using the EZ-Detect™ Cdc42 Activation kit (Pierce, Rockford, IL) at 0, 10, 30 min as well as 4 and 6 h 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 un-fractionated 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 (1 mg/ml, Sigma-Aldrich, Saint Louis, MO). The gel was washed for 1 h at RT in a 2.5% (v/v) Triton X-100 solution, transferred to an enzyme assay buffer (0.1 M Tris, pH pH=7.4, 10 mM CaCl₂) and incubated for 24 h 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:


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 (Histag). 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, BMPRII siRNA, and CLIC4 siRNA (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.