Homotypic and Endothelial Cell Adhesions via N-Cadherin Determine Polarity and Regulate Migration of Vascular Smooth Muscle Cells

Peter J.B. Sabatini, Ming Zhang, Rosalind Silverman-Gavrila, Michelle P. Bendek, B. Lowell Langille

Abstract—Migration of smooth muscle cells from the arterial media to the intima is central to several vascular pathologies including restenosis. This study demonstrates that, like directional migration of other cells, smooth muscle migration is accompanied by a dramatic, polarized reorganization of the cell cytoskeleton that is accompanied by activation of the Rho GTPase Cdc42 and inactivation of glycogen synthase kinase-3β. We also show, for the first time, that signals generated at the posterior–lateral aspects of wound edge cells by the cell–cell adhesion molecule N-cadherin are required for polarization and rapid migration of vascular smooth muscle. Importantly, when a cohort of migrating smooth muscle cells encounter CHO cells or the A10 smooth muscle cell line, neither of which expresses N-cadherin, polarity is only slightly suppressed. However, when smooth muscle cells encounter stably transfected, N-cadherin–expressing A10 cells or (N-cadherin–expressing) vascular endothelium, they rapidly lose their polarized phenotype. The latter finding indicates that endothelial signaling to innermost smooth muscle cells via N-cadherin may be critical to normal vessel wall stability. We infer that asymmetrical distribution of N-cadherin is necessary for the establishment of cell polarity during migration and that N-cadherin ligation is highly effective in abrogating polarized migration. Finally, we showed that endothelial cell polarity does not depend on N-cadherin; therefore, this molecule may be an attractive target for therapies to prevent restenosis without suppressing endothelial repair and risking late thrombosis.

Key Words: smooth muscle cell ■ migration ■ N-cadherin ■ polarity ■ restenosis
depends on the establishment of an intracellular, posterior–
anterogradient in signaling that involves activation of the
Rho GTPase Cdc42 and then inhibition of glycogen synthase
kinase (GSK)-3β by noncanonical Wnt signaling.15,16

Elegant work with astrocytes and fibroblasts has proven that
integrin activation at the cell anterior can initiate the
signaling gradient that drives cell polarity and directional
migration.17 However, cell–cell contact localizes to the pos-
terior–lateral aspects of wound edge cells; therefore, the
intracellular signals that result from this contact are also
potential initiators of cell polarity. In this context, we were
intrigued by observations that N-cadherin, an important
mediator of smooth muscle cell–cell adhesion, suppresses
Cdc42 activity.18 Therefore, disinhibition of this signaling at
the leading edge of wound edge cells, attributable to loss of
cell–cell contact, could elicit the intracellular gradients that
drive cell polarity and directional migration. Importantly,
N-cadherin mediates adhesion between smooth muscle and
endothelial cells;19,20 therefore, loss of endothelium in vivo
might elicit such polarized signaling in innermost smooth
muscle cells within the media.

In the present study, in vitro wound models were used to
demonstrate that N-cadherin is an obligatory source of
signaling that drives polarity and accelerates migration of
vascular smooth muscle cells and that the downstream sig-
naling acts via regulation of Cdc42 and GSK-3β. Furthermore,
we used a model of heterotypic cell contact to demonstrate
that when smooth muscle cells contact other cells
expressing N-cadherin (endothelial cells or A10 cells trans-
fected with N-cadherin), polarization is dramatically sup-
pressed. By contrast, contact of smooth muscle cells with
cells that do not express N-cadherin (CHO or A10 cells) only
modestly suppresses polarization. The inhibitory effects of
endothelium on smooth muscle polarity and migration may
indicate an important role for endothelial signaling to inner-
most smooth muscle cells via N-cadherin in stabilizing the
structure of mature, quiescent arteries. Finally, polarity of
migrating endothelium did not depend on N-cadherin–related
signals; therefore, N-cadherin provides a useful target for
restenosis therapy that might avoid the risk of persistent
endothelial denudation and late thrombosis that characterizes
antimitogenic therapies.

**Materials and Methods**

Detailed methods are provided in the online data supplement at
http://circres.ahajournals.org.

**Cell Culture**

Porcine arterial smooth muscle cells were used at passages 4 to 9 and
porcine endothelial cells at passages 4 to 7. Additional experiments
used the MOVAS smooth muscle cell line.20 CHO cells and A10
cells were obtained from the American Type Culture Collection.

**Wound-Healing Assay**

Postconfluent cultures were wounded by dragging a 200-μL pipette
tip across the bottom of the plate. When indicated, taxol, nocodazole,
EGTA, LiCl, or SB415286 (GSK-3β inhibitor) were added to the
media 1 hour before wounding.

In some experiments, a monoclonal anti–N-cadherin antibody
(GC-4, which binds to the extracellular domain of N-cadherin), or a
nonspecific mouse IgG1, antibody was added to the media 16 hours
before wounding to prevent N-cadherin–mediated adhesion. Alter-
natively, various concentrations of a linear N-cadherin–specific
blocking peptide21 (N-Ac-LRAHAVDING-NH2) or a scrambled
control peptide (N-Ac-HLNARAGAIVD-NH2) was added 1 hour
before wounding.

**Image Processing**

In d and f of Figure 6, the green channel was suppressed within
Adobe Photoshop to improve visibility of MTOC (red channel). No
other image processing was used.

**Quantification of Cell Polarity**

Wound edge cells were described as “polarized” if the MTOC
(identified using γ-tubulin antibody) was anterior to the midpoint
of the nucleus, or “highly polarized” if their MTOC was anterior to the
nucleus (Figure 1c). With this scheme, 50% of cells are labeled
polarized in randomly oriented cultures. ANOVA followed by Tukey
or Bonferroni comparisons were used to establish significance
among different time points and treatments, a Student t test was used
to compare a single treated group with control cultures, and a
Dunnett’s test was used when multiple experimental groups were
compared with a single control.

**Transfections**

Subconfluent smooth muscle cells were transfected with 4 μg/mL of
the Cdc42-GFP plasmid (gift from G. Downey, University of
Toronto, Toronto, Canada) with Effectene (Qiagen) transfection kits.
To generate stably expressing N-cadherin–GFP or α-tubulin–GFP
A10 cell lines, the N-cadherin–GFP (gift from Cecile Gauthier-
Rouvire, Centre de Recherches de Biochimie Macromoléculaire,
Centre National de la Recherche Scientifique, IFR 122, 34293

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**Figure 1.** The microtubule system of vascular smooth muscle cells polarizes during migration. a, Confocal micrograph of postconfluent
portions of porcine aortic smooth muscle cells immunostained for γ-tubulin to localize MTOCs (arrows), with nuclear counterstain (pro-
piudium iodide) at 12 hours after wounding. MTOCs are consistently anterior to the nuclear midpoint of cells at the wound edge. (Scale
bar=50 μm.) The dashed box outlines the region enlarged to the right. b, Same as for a but immunostained for acetylated tubulin (sta-
bilized microtubules) at 6 hours after wounding. c, Method for quantifying position of the MTOCs of wound edge cells. Cells are “highly
polarized” if the MTOC is wound-proximal to the nucleus and “polarized” if the MTOC is wound-proximal to the center of the nucleus
(includes highly polarized cells). d, Graph showing percentage of cells with polarized or highly polarized MTOCs (see text) at 4, 6, and
12 hours after wounding (*P<0.05 compared with 4 hours; #P<0.05 compared with 6 hours). e, Percentage of cells at edges of 6-hour
wounds that are highly polarized in cultures that are untreated (Control), treated with nocodazole (Noc), or treated with taxol (*P<0.05
compared to 6-hour control). All means represent 3 independent experiments plotted with SEs.
Montpellier, France) or α-tubulin-GFP (Clontech) plasmids were transfected into A10 cells with Effectene (Qiagen), and clones resistant to G418 were isolated.

Rho GTPase Activity Assay
Smooth muscle cells were grown to confluence on 90-mm² Petri dishes and then wounded twice in orthogonal directions with a comb containing 13 protrusions for a total of 26 wounds in a grid-like pattern. Cdc42/Rac1 and RhoA activation assay kits were purchased from Cytoskeleton (Denver, CO) and used to detect active Cdc42, Rac1, and RhoA according to the instructions of the manufacturer.

Western Blot
Cells were grid-wounded as described above and were probed using antibodies against GSK-3, phospho(Ser9)-GSK-3β, Cdc42, Rac1, RhoA, N-cadherin, and horseradish peroxidase–conjugated secondary antibodies. An ECL detection kit (Amersham Bioscience) was used to detect protein, which was quantified by densitometry.

Heterotypic Adhesion and Smooth Muscle Cell Polarity
Cocultures of porcine smooth muscle cells with CHO cells, porcine aortic endothelial cells, α-tubulin–expressing A10 cells (no N-cadherin expression), or N-cadherin–GFP expressing A10 cells were used to assess the effect of differential N-cadherin expression on cell polarity on wound closure. A cloning ring was placed on a glass cover slip, and porcine smooth muscle cells were seeded on the inside at the same time as either endothelial cells, CHO cells, or A10 cells were seeded on the outside of the cloning ring. One day later, the cloning ring was removed to allow heterotypic cell wound repair. At 6 hours after first heterotypic cell contact, the cells were fixed and immunostained for γ-tubulin to identify the MTOC and with cell type–specific antibodies. Nuclei were counterstained with TOTO-3 and the GFP signal was used to differentiate A10 cells from porcine arterial smooth muscle cells.

In Vivo Studies
Animal experiments were performed according to the guidelines of the Canada Council on Animal Care. Adult male Sprague–Dawley rats (Charles River, Constant, Quebec, Canada) weighing 375 to 415 g were anesthetized by IP injection of 4.6 mg/kg xylazine (Ketaset; Ayerst Veterinarian Laboratories, Guelph, Ontario, Canada). Endothelium was removed from the left carotid artery, en face preparations were stained with propidium iodide and visualized by confocal microscopy. Micrographs established that smooth muscle cells could migrate individually through fenestrae in the internal elastic lamina but that they formed clusters of intimal cells that spread outward (Figure 1 in the online data supplement). Therefore, smooth muscle cells at the periphery of these intimal colonies are subjected to the same polarized signaling from cell–cell adhesions as wound edge cells in vitro.

N-Cadherin–Mediated Adhesion Is Required for Cell Polarity and Efficient Directional Migration of Smooth Muscle Cells
To test whether N-cadherin signaling is required for cell polarity, we treated smooth muscle cell cultures with antibody against the extracellular domain of the protein to prevent homophilic binding.25,26 Immunofluorescence staining confirmed posterior–lateral localization of N-cadherin on wound edge cells, and it revealed loss of most of this junctional N-cadherin in the presence of N-cadherin antibody (Figure 2a and 2b). As previously reported,27 a slowing of wound repair (supplemental Figure II) was observed in the presence of the N-cadherin antibody, and time lapse phase contrast movies demonstrated that wound edge cells rapidly oriented in the direction of migration in untreated cultures, whereas this orientation was lost in the presence of the antibody (Figure 2c and 2d and supplemental Movies 1 and 2).

Impairment of cell alignment and migration was accompanied by a complete loss of cell polarity, as indicated by MTOC position. MTOCs became randomly positioned (Figure 2e), with the percentage of cells that were highly polarized at 6 hours decreasing from >40% in control cultures to <10% in the presence of antibody (Figure 2f). Control IgG was without effect. N-Cadherin dependence of polarity was confirmed when a N-cadherin–blocking peptide was used instead of antibody, whereas the control peptide had no effect (Figure 2g). Finally, cadherin-mediated adhesion is calcium-dependent; therefore, calcium chelation is often used to disrupt/reestablish adherens junctions (calcium switch experiments).28 Accordingly, EGTA treatment of cultures also prevented cell polarity at wound edges, whereas adding
back calcium restored cell polarity within 6 hours (Figure 2f). Dependence of polarity on N-cadherin was not restricted to the establishment of polarity during initiation of movement because late addition of the N-cadherin antibody to already polarized cells (6-hour wound) abolished cell polarity within 6 hours (data not shown).

These interventions unequivocally establish that N-cadherin–mediated adhesion is required for the initiation and maintenance of smooth muscle cell polarity. Similar experiments showed that polarity of an immortalized mouse aortic smooth muscle cell line (MOVAS) also depend on N-cadherin–mediated adhesion (data not shown).

**N-Cadherin Regulates Cell Polarity by Controlling Cdc42 Activation**

Rho GTPases are important regulators of cell migration and polarity; therefore, we used standard pull-down assays to detect active (GTP-bound) RhoA, Rac1, and Cdc42 after comb-wounding porcine smooth muscle cell cultures (Figure 3). RhoA activity was slightly and transiently suppressed after wounding, whereas Rac activity increased gradually, ultimately displaying a persistent >2-fold elevation over control levels. Notably, Cdc42 activity increased rapidly after wounding and was highest during the first 6 hours. Cdc42 activity returned to resting levels between 6 to 12 hours after wounding.

Cdc42 is a proven regulator of cell polarity, and it is sensitive to N-cadherin signaling, at least in C2C12 cells. Therefore, we tested whether N-cadherin regulates wound-induced Cdc42 activation. Maximal activation at 3 hours after wounding was completely suppressed when cultures were wounded in the presence of N-cadherin antibody (Figure 4a and 4b); furthermore, a high degree of localization of Cdc42-GFP to the cell anterior was suppressed (Figure 4d and 4e). In contrast, modest inactivation of RhoA at 1 hour postwounding was unaffected by the antibody (Figure 4c).
Cdc42 regulation of cell polarity of astrocytes and endothelium is effected via inhibition of GSK-3. Accordingly, GSK-3 underwent rapid inhibitory phosphorylation after wounding of smooth muscle cultures that slowly decreased at later times (Figure 5a and 5b). In shear-stressed endothelium and in migrating astrocytes, GSK-3 inhibition must be spatially regulated to control cell polarity because wholesale inhibition of the kinase prevents MTOC reorientation. Accordingly, we found that 2 GSK-3 inhibitors, LiCl and SB415286, prevented the establishment of cell polarity in wound edge smooth muscle cells (Figure 5c).

These results implicate N-cadherin–mediated inhibition of Cdc42 at the leading edge of the cell exposed to blocking antibody.

Heterotypic Cell–Cell Contact Elicits N-Cadherin–Dependent Suppression of Smooth Muscle Cell Polarity

Arterial smooth muscle cells exhibit rapid migration to the intima and proliferation after loss of endothelium in vivo that is suppressed on reendothelialization. To test whether N-cadherin–specific adhesion inhibits directional migration of porcine arterial smooth muscle in already polarized cells, we exploited the A10 smooth muscle cell line, which does not express N-cadherin (Figure 6a) in heterotypic cell contact experiments. A10 cells stably expressing α-tubulin–GFP, to differentiate the cells from porcine arterial smooth muscle, were grown outside of cloning rings that enclosed porcine smooth muscle cell colonies, and then the barrier was removed allowing the 2 cell populations to meet. We then repeated the experiment using A10 cells that stably expressed N-cadherin–GFP. Interestingly, 6 hours after contact with N-cadherin–expressing A10 cells, a robust loss in the percentage of highly polarized cells was observed, whereas only a modest decrease occurred when primary smooth muscle cell contacted A10 cells that do not express N-cadherin (Figure 6b through 6f).

In vivo, smooth muscle cells in the innermost layer of the media contact endothelium, which expresses N-cadherin; therefore, we reasoned that N-cadherin signaling from endothelium may suppress polarity of these smooth muscle cells.
under resting conditions. To test this possibility in vitro, we grew endothelial cells or CHO cells (which do not express N-cadherin) outside of cloning rings that enclosed smooth muscle cell colonies and repeated the heterotypic contact experiments with these cell types. N-Cadherin antibody was used to distinguish CHO cells from smooth muscle cells, whereas a platelet endothelial cell adhesion molecule-1 antibody discriminated endothelial cells from smooth muscle cells. Forty percent of control cells at wound edges (no contact after same outward migration time, \(=36\) hours) were highly polarized (quantitatively matched data from 6-hour wounds), whereas heterotypic contact with endothelium abolished polarity of smooth muscle cells (Figure 6b). When endothelium was replaced with CHO cells, which do not express N-cadherin, only a modest suppression of smooth muscle cell polarity occurred (Figure 6b). These results reveal that engagement of N-cadherin is a potent signal that abrogates cell polarity on wound closure.

**N-Cadherin Does Not Regulate the Polarity of Migrating Endothelium**

Wound-healing experiments were performed using postconfluent porcine arterial endothelial cells. As previously reported, these cells displayed a high degree of cell polarity at wound edges, but this polarity was not affected by treatment with N-cadherin antibody. Accordingly, at 6 hours after wounding, 81\(\%\) of cells were highly polarized in the absence of N-cadherin–blocking antibody, and 69\(\%\) were highly polarized when the antibody was present (\(P>0.05\)) (supplementary Figure III).

**Discussion**

Angioplasty and stent implantation are attractive therapies for arterial occlusive disease because they are much less invasive than bypass procedures. The development of drug-eluting stents has been particularly encouraging because these devices suppress restenosis caused by intimal accumulation of vascular smooth muscle; however, recent concern has arisen over increased risk of late thrombosis with these devices. The likely source of late thrombosis is use of drugs that globally target cell proliferation and therefore suppress endothelial repair, as well as intimal smooth muscle accumulation. The present study focused on the early migration of smooth muscle cells to the luminal surface of the vessel as a possible target for restenosis therapy, because this process may be amenable to smooth muscle–specific therapies. We explored polarized signaling that innermost smooth muscle cells receive from underlying medial cells after loss of endothelium as potential stimuli for migration to the intima.

We examined the role of N-cadherin because it exhibits a polarized distribution to the posterior–lateral aspects of cells migrating at the leading edge of a cohort of cells and because its ligation can suppress activation of a key regulator of directional migration, Cdc42, in other cells. Such suppression has the potential to introduce the posterior-to-anterior gradient of Cdc42 activity in wound edge cells that drives polarity. We found that migrating smooth muscle cells also display polarity and that N-cadherin signaling can drive this critical cell behavior. The capacity of an N-cadherin antibody, an N-cadherin–targeted peptide, and calcium switch experiments to disrupt cadherin-dependent cell–cell contact leaves no doubt that the process requires N-cadherin–mediated signaling in smooth muscle cells.

The mechanisms that drive this polarity resemble those occurring in astrocytes and fibroblasts, including a dependence on intact microtubules (nocodazole experiments) and...
their dynamic instability (taxol experiments) and a dependence on the regulation of GSK-3β. The latter probably reflects tight spatial control of inhibition of the kinase that is initiated after injury because we and others have found that its wholesale inhibition with exogenous agents blocked polarity.15,29

In other cell types, integrin signaling at the leading edge of the cell, presumably as new cell substrate contacts are made, initiates polarity,17 but this anterior signaling is inadequate in smooth muscle cells because disruption of N-cadherin ligation fully ablated the process. It remains possible that signaling between N-cadherin and integrins could cooperate in driving cell polarity. For example, the nonreceptor tyrosine kinase Fer can shuttle between the cytoplasmic domains of N-cadherin and β1 integrins to modulate adhesion mediated by both complexes.30 The effects of this communication on cell polarity merit further investigation.

Endothelial cells engage in heterotypic cell contacts with smooth muscle cells through fenestrae of the internal elastic lamellae (our unpublished observations and see Spagnoli et al21); therefore, an attractive hypothesis was that endothelium could signal to innermost smooth muscle cells. Resulting signals would balance those from deeper smooth muscle and thereby suppress inward migration. Loss of endothelium, eg, during angioplasty, could then liberate a stimulus for inward migration. In fact, Paik et al have shown that N-cadherin–mediated interactions between endothelial cells and smooth muscle cells promotes vascular maturation and stability during development.19 In this study, we found that polarity of migrating smooth muscle cells is abolished specifically on contact with N-cadherin–expressing cells, including other smooth muscle cells and endothelium. These findings, combined with the effects of blocking N-cadherin adhesion, indicate that N-cadherin signaling is necessary for establishment of cell polarity following injury and a strong signal for loss of polarity on wound closure.

Although endothelial cells express appreciable levels of N-cadherin, there has been a consensus that homotypic endothelial adherens junctions are populated exclusively by VE-cadherin, which can displace N-cadherin from junctional complexes.32 Because of this displacement, polarized N-cadherin distribution in wound edge endothelial cells would not be anticipated. More recent in vivo work has challenged the universality of this concept33; nonetheless, we found that cell polarity of migrating endothelium was independent of N-cadherin ligation. The finding is important because it emphasizes the potential for disruption of N-cadherin ligation to selectively inhibit smooth muscle migration without affecting endothelial repair in a therapeutic setting.

All of the experiments in this study were performed using cultured cells grown on a 2D substrate, whereas arterial medial smooth muscle cells are normally embedded in a 3D extracellular matrix. Nonetheless, our findings are relevant to in vivo smooth muscle cell migration. This is because endothelial cell loss induces smooth muscle cells to crawl through fenestrae in the internal elastic lamellae, and they subsequently migrate laterally to cover the internal elastic lamellae. Consequently, the cells that lead this outgrowing cohort exhibit N-cadherin–mediated adhesion at the cell posterior, whereas the anterior is free of adherens junctions, because they migrate over a 2D surface.

Finally, we have emphasized the importance of directional migration of smooth muscle cells during restenosis, but the same concepts may apply to bypass graft failure and possibly to the migration of smooth muscle cells to atherosclerotic lesions. Furthermore, targeted migration of these cells or their precursors, sometimes over large distances, is central to developmental morphogenesis of the artery wall. Examples include the early investment of the proximal aorta, pulmonary trunk, and carotid arteries by cells originating in the cardiac neural crest, population of the distal aorta by cells derived from somites, and invasion of the heart by coronary precursor cells from the proepicardial organ (for review, see Majesky26). N-Cadherin is of proven importance in vessel wall assembly, and it will be of interest to explore its capacity to direct migration during vascular morphogenesis.

In summary, we have shown that smooth muscle cells display cell polarity during directional migration and that this polarity requires signals derived from N-cadherin–mediated adhesion and its regulation of a classical polarity pathway that involves Cdc42 and GSK-3β. The process is ablated by endothelial–smooth muscle cell contact and depends on N-cadherin expression in both cell types, because suppression of polarity is more efficient in the presence of cadherins. Failure of N-cadherin to suppress polarity of endothelial cells indicates that this molecule is a candidate target as a smooth muscle–specific mediator of intimal hyperplasia.

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We are grateful to Dr Gregory Downey for providing the Cdc42-GFP construct and Dr Cecile Gauthier-Rouviere for providing the N-cadherin–GFP construct. Dr Mansoor Husain kindly provided the MOVAS cell line.

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Disclosures

None.

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**Movie S1:** Smooth muscle cells align in the direction of migration at wound edges. Time-lapse video (0.003 frames/sec) of untreated MOVAS cells for 8h following wounding (scale bar=50 μm).

**Movie S2:** N-cadherin antibody prevents wound edge smooth muscle cells from aligning in the direction of migration. Time-lapse video (0.003 frames/sec) of MOVAS cells pre-treated with ACAM (50 μg/ml) for 8h following wounding (scale bar=50 μm).
Figure S1. *En face* fluorescence micrographs of luminal surface of rat carotid arteries at 4 days after removal of endothelium. **A.** Optical sections captured by confocal microscopy at the abluminal surface (left), midpoint (middle) and luminal surface of the IEL. Upper frames show propidium iodide staining of smooth muscle cell nuclei and lower frames show autofluorescence of elastin in IEL. Images display a single smooth muscle captured while migrating through a fenestration in the IEL (arrow). **B.** Confocal micrograph at site where a cohort of smooth muscle cells has migrated to the luminal surface and has initiated an outwardly expanding population of intimal cells.
**Figure S2.** Graph shows distance migrated into wound as a function of time for untreated (solid line) and N-cadherin antibody-treated (dashed line) cultures.
Figure S3. Graph representing percent of endothelial cells at the wound edge that are highly polarized 6 hours after injury when left untreated (control) or treated with the N-cadherin antibody.
Materials and Methods

Cell Culture: Porcine aortic or carotid arterial smooth muscle cells were derived from vessels freshly harvested at a local abattoir by explant outgrowth and used at passages 4-9. Endothelial cells were derived by enzymatic digestion of porcine aortas and used at passages 4-7. The MOVAS smooth muscle cell line\(^1\) was kindly provided by Dr. M. Husain (Toronto, Canada). CHO cells and A10 cells were obtained from the ATCC. The porcine cells were cultured in medium 199 (GIBCO (Invitrogen), Burlington, ON) with 10% calf serum. For CHO cells medium was supplemented with 1% non-essential amino acids (GIBCO). MOVAS and A10 cells were grown in DMEM with 10% calf serum.

Wound Healing Assay: Smooth muscle or endothelial cells were plated on glass cover slips and post-confluent cultures were wounded by dragging a 200μl pipette tip across the bottom of the plate. When indicated, taxol (Sigma, Oakville, ON; 15nM), nocodazole (Sigma; 100nM), EGTA (Sigma; 4mM), LiCl (Sigma; 30mM) or SB415286 (Biomol, Plymouth Meeting, PA; 30μM) were added to the media 1h prior to wounding.

In experiments to disrupt N-cadherin-mediated cell adhesion and signaling, a monoclonal anti-N-cadherin antibody (GC-4, Sigma, 10μg/ml for porcine and 50μg/ml MOVAS cells), which binds to the extracellular domain of N-cadherin, was added to the media 16 hours prior to wounding to prevent N-cadherin-mediated cell-cell adhesion. Antibody was refreshed at the time of wounding. A non-specific mouse IgG\(\lambda\) antibody (Sigma) was used for control experiments. Alternatively, various concentrations of a linear N-cadherin specific blocking peptide\(^2\) \((N-Ac\text{-LRAHADVING-NH}_2)\) or 5mg/ml of a scrambled control peptide \((N-Ac-\ldots)\)
HLNARGAIVD-NH₂) was added to post-confluent porcine smooth muscle cells 1h prior to wounding.

**Immunofluorescence Staining:** Cells were fixed with 99.9% methanol for 2 min at room temperature or they were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.2% Triton X-100 for 5 minutes. Anti-γ-tubulin (Sigma), α-tubulin (Sigma), N-cadherin (BD Transduction, Mississauga, ON or Santa Cruz Biotechnology, CA), acetylated-tubulin (Sigma) and CD31 (Santa Cruz) antibodies were used with either FITC/Alexa488 or CY3/Alexa568 conjugated secondary antibodies (Molecular Probes, Eugene, OR). Nuclei were counterstained with propidium iodide (Calbiochem, Gibbstown, NJ; 20μg/ml) or TOTO-3 (Molecular Probes) for 15 min. All samples were visualized using a BioRad 1024 or Olympus FV1000 laser scanning confocal microscope with 60X oil-immersion (NA=1.4) objectives.

**Image Processing:** In panels d and f of Figure 6, the green channel was suppressed within Adobe Photoshop to improve visibility of MTOC (red channel). No other image processing was employed.

**Quantification of Cell Polarity:** Wound edge cells were described as polarized if the MTOC (identified using γ-tubulin antibody) was anterior to the midpoint of the nucleus, or highly polarized if their MTOC was anterior to the nucleus (Figure 1c). With this scheme, 50% of cells are labeled polarized in randomly oriented cultures. ANOVA followed by Tukey or Bonferroni comparisons were used to establish significance among different time points and treatments, a
Student’s t-test was used to compare a single treated group with control cultures, and a Dunnett’s test was employed when multiple experimental groups were compared with a single control.

**Plasmids and Transfections:** Wild-type Cdc42-GFP encoded plasmids were a gift from G. Downey (Toronto, Canada), N-cadherin-GFP encoded plasmids were a gift from Cecile Gauthier-Rouviere (Paris, France) and α-tubulin-GFP encoded plasmids were purchased from Clontech (Clontech #6117-1, Palo Alto, CA). Subconfluent smooth muscle cells were transfected with 4μg/ml of the Cdc42-GFP plasmid with Effectene (Qiagen) transfection kits. After 48h, cells were wounded and 6h later fixed in 4% paraformaldehyde and imaged with a laser scanning confocal microscope.

To generate stably expressing N-cadherin-GFP or α-tubulin-GFP A10 cell lines, the N-cadherin-GFP or α-tubulin-GFP plasmids were transfected into A10 cells with Effectene (Qiagen) and treated with G418. Clones resistant to G418 were isolated and passaged to grow clones then frozen at -80°C.

**Rho GTPase Activity Assay:** Cdc42/Rac1 and RhoA Activation Assay Kits were purchased from Cytoskeleton™ (Denver, CO) and used to detect active Cdc42, Rac1 and RhoA according to the manufacturer’s instructions. Briefly, smooth muscle cells were grown to confluence on 90 mm² Petri dishes then wounded twice in orthogonal directions with a comb containing 13 protrusions for a total of 26 wounds in a grid-like pattern. The cultures were incubated either with or without anti-N-cadherin antibody. Cell lysates were incubated with 25μg/ml of PAK-coated beads or 60μg/ml Rhotekin-coated beads then loaded onto a 15% SDS-PAGE gel, and a
western blot was performed for Cdc42, Rac1 or RhoA as described below. In addition, 10μg of protein was collected and loaded onto a separate 15% gel to determine total protein content.

**Western Blot:** Cells were grid-wounded in 60 mm² dishes as described above and lysed in RIPA buffer (1mM Sodium Orthovanadate, 50mM NaF, and a protease inhibitor cocktail (Roche, Laval, QC). Protein (10μg) was loaded onto a 12% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane (PVDF). Antibodies used included GSK-3 (Sigma), phospho(Ser9)-GSK-3β (Cell Signaling, Pickering, ON), Cdc42 (Cytoskeleton), Rac1 (Cytoskeleton), RhoA (Cytoskeleton), N-cadherin (BD Transduction) and horseradish peroxidase conjugated anti-mouse, anti-rabbit (Amersham Biosciences, Piscataway, NJ) or anti-sheep antibodies (Jackson ImmunoResearch Laboratories). An ECL detection kit (Amersham Bioscience) was used to detect protein.

Densitometric analysis was performed using Image J software to quantify the relative expression of the proteins (e.g. phospho(ser9)GSK-3β was normalized to total GSK-3β levels and represented as a fold change compared to 0h after wounding) and a Dunnett’s test was used to determine statistical significance (p<0.05) relative to values at 0h.

**Heterotypic Adhesion and Smooth Muscle Cell Polarity:** Co-cultures of porcine smooth muscle cells with CHO cells, porcine aortic endothelial cells, α-tubulin expressing A10 cells (no N-cadherin expression), or N-cadherin-GFP expressing A10 cells were used to assess the effect of differential N-cadherin expression on cell polarity upon wound closure. A cloning ring (Bellco, 10 mm diameter, 1 mm wall thickness) was placed on a glass cover slip and porcine smooth muscle cells were seeded on the inside at the same time as either endothelial cells, CHO
cells, or A10 cells were seeded on the outside of the cloning ring. One day later the cloning ring was removed to allow heterotypic cell wound repair. At 6h after first heterotypic cell contact, the cells were fixed and immunostained for γ-tubulin to identify the MTOC, and for either N-cadherin (Santa Cruz) to discriminate smooth muscle from CHO cells, or PECAM-1 (Santa Cruz) to discriminate endothelial from smooth muscle cells. Nuclei were counterstained with TOTO-3 (Molecular Probes) and the GFP signal was used to differentiate A10 cells from porcine arterial smooth muscle after cell contact.

**Live Cell Imaging:** Phase contrast movies (0.003 frames/sec) of migrating MOVAS cells were captured using a TE300 Nikon microscope with a 10X (NA=0.25) objective at 37°C with a CCD Elmo B/W TV Camera (SE360S) controlled by Simple PCI software (C·Imaging).

**In Vivo Studies:** Animal experiments were performed according to the guidelines of the Canada Council on Animal Care. Adult male Sprague-Dawley rats (Charles River, Constant, Quebec, Canada) weighing 375 to 415 g were anesthetized by intraperitoneal injection of 4.6 mg/kg xylazine (Rompum; Bayer Inc., Etobicoke, Ontario, Canada) and 70 mg/kg ketamine (Ketaset; Ayerst Veterinarian Laboratories, Guelph, Ontario, Canada). Endothelium was then removed by passing a loop of 5-0 monofilament nylon into the left carotid artery via the external carotid and withdrawing the nylon while rotating, exactly as previously described, and then incisions were closed. Four days later, arteries were perfusion fixed at 100 mmHg. Arteries were excised then opened longitudinally, stained with the DNA label propidium iodide, and viewed en face at 60X magnification using a Bio-Rad 1024 laser scanning confocal microscope mounted on a Nikon Optiphot stand and objectives.
Reference List

