Inhibition of Diaphanous Formin Signaling In Vivo Impairs Cardiovascular Development and Alters Smooth Muscle Cell Phenotype

Laura Weise-Cross, Joan M. Taylor, Christopher P. Mack

Objective—We and others have previously shown that RhoA-dependent stimulation of myocardin-related transcription factor nuclear localization promotes smooth muscle cell (SMC) marker gene expression. The goal of this study was to provide direct in vivo evidence that actin polymerization by the diaphanous-related formins contributes to the regulation of SMC differentiation and phenotype.

Approach and Results—Conditional Cre-based genetic approaches were used to overexpress a well-characterized dominant-negative variant of mDia1 (DNmDia) in SMC. DNmDia expression in SM22-expressing cells resulted in embryonic and perinatal lethality in ≈20% of mice because of defects in myocardial development and SMC investment of peripheral vessels. Although most DNmDia/SM22Cre+ mice exhibited no overt phenotype, the re-expression of SMC differentiation marker gene expression that occurs after carotid artery ligation was delayed, and this effect was accompanied by a significant decrease in myocardin-related transcription factor-A nuclear localization. Interestingly, neointima growth was inhibited by expression of DNmDia in SMC and this was likely because of a defect in directional SMC migration and not to defects in SMC proliferation or survival. Finally, by using the tamoxifen-inducible SM MHC-CreER\(^{T2}\) line, we showed that SMC-specific induction of DNmDia in adult mice decreased SMC marker gene expression.

Conclusions—Our demonstration that diaphanous-related formin signaling plays a role in heart and vascular development and the maintenance of SMC phenotype provides important new evidence that Rho/actin/myocardin-related transcription factor signaling plays a critical role in cardiovascular function. (Arterioscler Thromb Vasc Biol. 2015;35:2374-2383. DOI: 10.1161/ATVBAHA.115.305879.)

Key Words: carotid arteries ■ mDia2 protein, mouse ■ myocardin ■ phenotype ■ smooth muscle

Smooth muscle cell (SMC) differentiation plays an important role in vascular development and maintenance. Although medial SMCs express a repertoire of contractile-associated differentiation markers, they do not terminally differentiate even in adult animals. Phenotypically modulated SMC that exhibit decreased differentiation marker gene expression and increased growth, migration, and matrix production have been shown to contribute to the progression of several important cardiovascular diseases, including atherosclerosis, hypertension, and restenosis.\(^1,2\) Therefore, a better understanding of the signaling mechanisms that control these SMC functions will be critical.

Most SMC differentiation marker genes including SM myosin heavy chain (SM MHC), SM α-actin, calponin, and SM22 are regulated by serum response factor, a ubiquitously expressed MADS (MCM1, agamous, deficiens, SRF) box transcription factor that binds to conserved CArG (CC(A/T)GG) elements within the promoters of these genes.\(^3\) The cardiac and SMC selective serum response factor cofactor, myocardin, strongly transactivates SMC-specific transcription and is required for SMC differentiation in vivo.\(^3\) Two myocardin-related transcription factors, MRTF-A and MRTF-B, have also been identified. Although they are expressed more widely, their importance in the regulation of SMC differentiation marker gene expression is supported by the phenotypes of global and tissue-specific knockouts.\(^4,5\) We and others have demonstrated that RhoA-mediated actin polymerization stimulates MRTF nuclear localization and is critical for SMC marker gene expression in at least some SMC types.\(^6,7\) However, direct in vivo evidence that this mechanism plays a significant role in the regulation of SMC differentiation or phenotypic modulation is lacking.

We have previously identified many signaling mechanisms that control RhoA activity in SMCs\(^8,9\) and have characterized the pathways downstream of RhoA that regulate MRTF-dependent SMC-specific transcription.\(^10,11\) The Rho-associated kinases 1 and 2 increase actin polymerization indirectly through a kinase cascade that inhibits cofilin ability to sever F-actin, and they also promote stress fiber formation by enhancing actin fiber bundling and cell contractility.\(^12,13\)
However, we have shown that inhibition of Rho-associated kinase signaling only partially inhibited SMC-specific promoter activity, suggesting that additional RhoA effectors were important. The diaphanous-related formins (DRFs), mDia1 and mDia2, are RhoA effectors that directly catalyze linear actin polymerization in cooperation with profilin.\textsuperscript{18–21} We have shown that both are highly expressed in SMCs, and when activated, strongly upregulate SMC marker gene expression.\textsuperscript{14} Importantly, siRNA-mediated knockdown of mDia1 and mDia2 in primary rat aortic SMCs resulted in a significant reduction in SMC-specific promoter activity, endogenous SMC marker gene expression, and MRTF nuclear localization.\textsuperscript{14}

RhoA signaling also regulates the actin and adhesion dynamics that control cell migration and division. Using fluorescent biosensors, Pertz et al\textsuperscript{22} detected RhoA activity near the leading edge of migrating cells where it is thought to promote actin-based cell protrusion. RhoA activity is also high at the rear of migrating cells where it induces the contractile forces necessary for trailing edge retraction. Several studies have shown that mDia1 and mDia2 catalyze the linear actin polymerization that is required for filopodia formation.\textsuperscript{23} RhoA signaling also controls cell adhesion by modulating focal adhesion formation and RhoA-dependent activation of mDia2 at the cleavage furrow may be important for cytokinesis.\textsuperscript{24,25} Given its pleiotropic effects on nearly all the parameters that define SMC phenotype, it will be critical to further study RhoA/mDia/actin signaling in SMCs.

The goal of this study was to define the contributions of mDia-mediated actin polymerization to SMC phenotype in vivo. We used genetic models to inhibit DRF signaling in SMCs during cardiovascular development, and we studied the effects of mDia inhibition on SMC phenotypic modulation in adult mice subjected to carotid artery ligation. Our results indicate that mDia signaling was required for normal cardiovascular development and that inhibition of mDia signaling attenuated SMC differentiation marker gene expression but reduced neointima formation by inhibiting SMC migration.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.

**Results**

**Inhibition of mDia Signaling in SM22-Expressing Cells Led to Some Embryonic/Perinatal Lethality**

To determine the contributions of actin polymerization to the regulation of SMC differentiation or phenotype in vivo, we used a genetic approach to overexpress a dominant-negative variant of mDia1 (F1FHΔ1) specifically in SMCs (Figure 1A). Because mDia1 and mDia2 are RhoA effectors with considerable functional overlap,\textsuperscript{26,27} it is important to note that this variant inhibits the function of all DRFs by dimerizing with endogenous proteins or binding nonproductively with barbed actin filaments.\textsuperscript{15,28} Another consideration for our approach was that DNmDia inhibits actin polymerization more specifically than alternative interventions that target Rho and its many effectors or Rho-associated kinase and its many substrates. In excellent agreement with our previous demonstration that DNmDia inhibited the activities of exogenous SMC-specific promoters,\textsuperscript{14} overexpression of DNmDia inhibited the endogenous expression of multiple SMC-specific marker genes and the nuclear localization of MRTF-A in S1P-treated 10T1/2 cells (Figure 1B–D).

Flag-tagged DNmDia was cloned into a previously described transgene construct in which the constitutive expression of DNmDia (driven by a fragment of the β-actin promoter) is inhibited by the upstream insertion of a floxed EGFP (enhanced green fluorescent protein)-Stop cassette (Figure 2A).\textsuperscript{29,30} DNmDia transgenic mice were generated on a C57BL/6 background and were crossed to a well-characterized SM22Cre line\textsuperscript{31} that expresses Cre in most SMC subsets and in the developing myocardium (Figure 1A in the online-only Data Supplement).\textsuperscript{32} Although many DNmDia+/ SM22Cre+ mice were viable, the number that reached adulthood was \(\approx 20\%\) less than that expected by Mendelian ratios (Table). Several DNmDia+/SM22Cre+ mice died in utero with nonviable embryos beginning to appear at around E15.5. Hemorrhage was detected in 6 of 17 DNmDia+/SM22Cre+ embryos between E15.5 and E18.5 and was most visible near the smaller, more peripheral blood vessels of the head, limbs, and body wall (Figure 2B). Immunohistologic examination of these mice revealed severely dilated blood vessels that were poorly invested with SM-α-actin–expressing cells (Figure 2C). Importantly, SMC coverage of larger blood vessels, such as the aorta and carotid arteries, was not significantly affected in hemorrhagic DNmDia+/SM22Cre+ embryos (Figure 2C).

A subset of DNmDia+/SM22Cre+ mice also exhibited cardiac abnormalities, including ventricular septal defects and hypoplasticity of the ventricular wall and septum at E18.5 (Figure 2D). Because this SM22Cre line can drive Cre expression in the embryonic heart, DRF signaling was likely important for myocardial cell proliferation/survival during this developmental window. Indeed, phospho-histone H3 staining revealed a dramatic reduction in myocardial cell proliferation in DNmDia+/SM22Cre+ mice. A significant number of DNmDia+/SM22Cre+ mice died perinatally and although these mice appeared fairly normal at birth they quickly became runted and did not survive past postnatal day 9. All dead pups had milk in their stomach, suggesting that the runted phenotype was not because a nursing defect. Histological analysis revealed lung congestion and hearts with thin ventricular walls and low phospho-histone H3 staining again suggestive of heart defects in these mice (Figure II in the online-only Data Supplement).

Importantly, the DNmDia+/SM22Cre+ mice that survived to adulthood had no overt phenotype and a normal lifespan. Somewhat surprisingly, heart and vessel morphology and

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**Nonstandard Abbreviations and Acronyms**

- DRF: diaphanous-related formin
- DNmDia: dominant-negative variant of mDia1
- MRTF: myocardin-related transcription factor
- SMC: smooth muscle cell
- SM MHC: smooth muscle myosin heavy chain
SMC marker gene expression were unaffected even though DNmDia was strongly expressed in these tissues (Figure 2E).

**DNmDia Expression Altered SMC Phenotype After Arterial Injury**

It is clear that the activation of compensatory pathways during development can modify the phenotype of genetically modified mice and that the application of acute stresses can unmask previously undetected effects of genetic modifications. The lack of phenotype in adult DNmDia+/SM22Cre+ provided us with an excellent model for examining the effects of mDia signaling on SMC phenotypic modulation after vascular injury. To this end, we subjected DNmDia+/SM22Cre+ and control littermates to a carotid artery ligation procedure that results in a significant neointima by 21 days and a downregulation of SMC differentiation marker gene expression in medial SMCs that peaks at ≈4 to 5 days post injury and eventually returns to normal (Figure 3).33 Notably, we observed no statistical significant difference in vessel diameter between uninjured and ligated arteries in either control or DNmDia+/SM22Cre+ animals.

In excellent agreement with the Western blot data shown in Figure 2E, immunohistochemical analyses revealed no differences in SM α-actin, SM MHC, or calponin expression between uninjured arteries from control and DNmDia+/SM22Cre+ animals (Figure 3C). However, although SMC differentiation marker gene expression was already returning to baseline by day 7 post injury in control mice, it remained downregulated in DNmDia+/SM22Cre+ mice (Figure 3A and 3C). This difference was not apparent at the 21-day time point (Figure 3A), suggesting that the recovery of SMC marker gene expression was delayed but not completely inhibited in this model. Importantly, the differences in SMC marker gene expression at the 7-day time point were accompanied by reductions in MRTF-A nuclear localization (Figure 3D; Figure IV A in the online-only Data Supplement), an effect probably because of decreased actin polymerization in DNmDia-expressing SMC.

The ability of DNmDia to inhibit SMC marker gene expression in this model suggested that these cells were more phenotypically modulated. However, injured vessels from DNmDia+/SM22Cre+ mice had significantly less neointima formation and overall stenosis than controls (Figure 3A and 3B) and reduced collagen deposition (Figure IVB in the online-only Data Supplement). Taken together, these data suggest that mDia signaling has differential effects on SMC phenotype and provide strong evidence that SMC differentiation and growth/migration are not mutually exclusive and are independently regulated.

**Inhibition of mDia Signaling Reduced Directional SMC Migration**

Extensive evidence including the results from elegant lineage tracing studies indicates that the majority of neointima SMC

Figure 1. Dominant-negative variant of mDia1 (DNmDia) expression inhibited smooth muscle cell (SMC)-specific marker expression. A, Schematic representation of full-length and DNmDia. B, 10T1/2 cells transfected with DNmDia or empty expression vector were serum-starved, and treated with sphingosine-1-phosphate (S1P) for 16 hours. Endogenous SMC cell marker expression was detected by immunoblotting. C, 10T1/2 cells expressing mCherry empty vector (EV) or mCherry-DNmDia were serum-starved, treated with S1P for 4 minutes, and then fixed. Localization of endogenous myocardin-related transcription factor (MRTF)-A was determined by immunohistochemistry. D, Quantification of MRTF-A nuclear localization from 3 separate S1P-treated experiments with over 100 cells counted per condition. DAD indicates diaphanous autoinhibitory domain; DID, diaphanous inhibitory domain; FH, formin homology domain; and GBD, GTPase-binding domain. *P<0.05. DAPI indicates 4′,6-diamidino-2-phenylindole.
originate from the medial SMC layer and that injury-induced increases in SMC migration and proliferation are critical for neointima formation. Although it is well known that RhoA signaling has complex and sometimes biphasic effects on these processes, the role of mDia signaling in SMC is less clear. Thus, we performed additional analyses to identify the mechanism for decreased neointima formation in DNmDia+/SM22Cre+ mice.

Although control mice exhibited larger neointimas than DNmDia mice at 21 days post ligation, we observed similar percentages of proliferating cells within the neointima at day 7 (Figure 3E). In agreement with the lack of effect on neointimal cell proliferation we observed in vivo, knockdown of mDia1, mDia2, or both in mouse aortic SMC or 10T1/2 cells had no significant effects on cell proliferation as measured by phospho-histone H3 staining or MTT assays (Figure VA–VC in the online-only Data Supplement). In addition, mDia knockdown or DNmDia overexpression had no effect on cell cycle progression as measured by flow cytometry of 4′,6-diamidino-2-phenylindole–stained cells, and results from TUNEL (transferase dUTP nick end labeling) and lactate dehydrogenase release assays revealed no effects on apoptosis or cytotoxicity, respectively (Figure VD–VG in the online-only Data Supplement). Furthermore, DNmDia+/Rosa26+/SM22Cre+ mice showed uniform expression in the aorta, bladder, lungs, and heart, indicating no significant cell death in DNmDia+ cells in vivo (Figure IA in the online-only Data Supplement).

On the basis of these results and previous studies demonstrating that mDia-mediated actin polymerization was required for filopodia formation and cell migration, we hypothesized that the decrease in neointima size in DNmDia+/SM22Cre+ mice resulted from a decrease in migration from the medial SMC layer. In strong support of this, siRNA-mediated knockdown of mDia1, mDia2, or both (Figure 4C) resulted in a 50% to 70% decrease in SMC migration in transwell assays (Figure 4A) and a significant decrease in scratch wound closure in 10T1/2 cells (Figure 4B). Interestingly, live cell tracking studies demonstrated that subconfluent cells expressing DNmDia-mCherry were capable of random migration and that these cells migrated similar distances in scratch wound assays, directional migration toward the

<table>
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<tr>
<th>Genotype Ratios From the DNmDia+×SM22Cre+ Cross</th>
<th>DN+/Cre+</th>
<th>DN−/Cre+</th>
<th>DN+/Cre−</th>
<th>DN−/Cre−</th>
<th>Total</th>
<th>( \chi^2 )</th>
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<tr>
<td>4-wk-old</td>
<td>84</td>
<td>117</td>
<td>132</td>
<td>121</td>
<td>454</td>
<td>( p=0.0103 )</td>
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<tr>
<td>Dead pups (P1–P9)</td>
<td>17</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>25</td>
<td>( p&lt;0.0001 )</td>
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Figure 2. Dominant-negative variant of mDia1 (DNmDia) expression in smooth muscle SM22-expressing cells resulted in some embryonic/perinatal death. A, Schematic representation of the Cre-dependent transgene construct used to drive DNmDia. B, Gross images of littermate control and hemorrhagic DNmDia+/SM22Cre+ embryos. C, Hematoxylin and eosin (H&E) and SM α-actin staining of the indicated blood vessels from DNmDia+/SM22Cre+ and littermate control mice. Arrows point to a severely dilated vessel with incomplete SM cell (SMC) investment. D, H&E and phospho-histone H3 staining of hearts from DNmDia+/SM22Cre+ and control mice at E18.5. Arrow points to ventricular septal defect. E, Western blot for SMC differentiation marker gene expression in the aorta, bladder, and heart from littermate control (WT) and phenotypically normal 4-week-old DNmDia+/SM22Cre+ (DN) mice.
wound was significantly inhibited in DNmDia-expressing cells (Figure 5B–5C; Movies I and II in the online-only Data Supplement), strongly suggesting that inhibition of mDia signaling in SMC leads to defects in directional migration or cell polarity. Accordingly, DNmDia-expressing cells exhibited significant polarity defects as evidenced by lamellipodia formation in multiple directions (Figure 5D; Figure VIA in the online-only Data Supplement) and failure of the microtubule organizing center to orient toward the scratch wound (Figure 5E–5F; Figure VIB and VIC in the online-only Data Supplement).

mDia Signaling Maintains SMC Differentiation in Adults

The effects of DNmDia expression in SM22-expressing cells during development were incompletely penetrant, and it is likely that stochastic differences in the timing or level of DNmDia expression during a critical developmental window helps explain these results. Compensatory pathways may also play a role and our demonstration that DNmDia had no effect on SMC function at baseline in adults, but delayed the re-expression of SMC differentiation marker gene expression after carotid injury supports this idea. To test this hypothesis further, we crossed DNmDia+ mice with the SM MHC-CreER2 line that allowed us to activate DNmDia expression specifically in SMC in adult mice, effectively by-passing any compensatory pathways activated during development. As shown in Figure IB in the online-only Data Supplement, intraperitoneal injection of tamoxifen for 5 consecutive days activated Cre in the SMC layers of the aorta, bladder, lungs, and stomach in this model although activation of DNmDia expression was somewhat more variable (Figure 6B; Figure VII in the online-only Data Supplement). Importantly, tamoxifen treatment of DNmDia+/SM MHC-CreER2 mice resulted in a significant downregulation of SM MHC gene expression in the aorta, bladder, and stomach and a significant downregulation of all SMC differentiation marker genes in the bladder that exhibited strong and consistent DNmDia expression (Figure 6; Figure VII in the online-only Data Supplement).
Discussion

To our knowledge, these results provide the first in vivo evidence that signaling through the DRFs is critical for SMC differentiation marker gene expression and proper heart development. Our results also suggest that inhibition of mDia signaling inhibits neointima formation by attenuating directed SMC migration from the medial SMC layer of injured vessels.

Although 80% of DNmDia+/SM22Cre+ mice exhibited no detectable phenotype, a significant number died during embryonic development or perinatally. Several embryos exhibited significant hemorrhaging, suggesting that vessel integrity was affected by SMC-specific expression of DNmDia. Although the larger vessels were properly layered with SM α-actin–expressing cells, there appeared to be defects in SMC/pericycle investment of smaller vessels. Carramusa et al demonstrated that mDia1 was required for proper E–cadherin junction formation between MCF-7 cells and inhibition of EC-SMC or SM22α expression in our model occurs only after initial SM22 activation, which suggests that proliferation in striated muscle cell (SMC) migration.

Figure 4. mDia knockdown inhibited smooth muscle cell (SMC) migration. A, siRNA was used to knock down mDia1, mDia2, or both in primary mouse aortic SMC. Equal numbers of mouse SMCs from each knockdown group were plated onto fibronectin-coated transwell inserts and migration was stimulated for 8 hours by addition of 20 ng/mL PDGF-BB (platelet-derived growth factor-BB) to the bottom well. After fixation and staining with Crystal Violet, SMC migration was evaluated in 3 separate experiments and expressed relative to migration of control cells set to 100%. *P < 0.05. B, Confluent cultures of control (NTC [non-targeted control]) and mDia1/mDia2 knockdown 10T1/2 cells were scratched with a P1000 pipette tip and visualized every 3 hours. Shown are time points immediately (0 hour) and 15 hours after scratch wounding. C, Western blot illustrating mDia1/mDia2 knockdown in our mouse aortic SMC and 10T1/2 cell culture models 72 hours after siRNA transfection.

from the apical wall. However, we cannot rule out a potential effect on the endocardial cushions that contribute to the anterior portion of the interventricular septum. Several sarcomeric protein mutations cause cardiac septal defects and it is possible that abnormal sarcomere organization because of defects in linear actin polymerization in DNmDia-expressing cells could be involved. Interestingly, neither knockdown of mDia1 and mDia2 nor expression of DNmDia had significant effects on proliferation in SMC. Gopinath et al reported that mDia1 knockdown in c2c12 skeletal muscle cells inhibited cell cycle progression, perhaps suggesting that proliferation in striated muscles is more sensitive to mDia inhibition. Taken together, our results demonstrate that DRF signaling plays a functional role in normal cardiac development and blood vessel maturation. Nevertheless, additional studies using Cre-driver lines that are more cardiac specific will be needed to determine the precise role of mDia/DRF signaling on cardiomyocyte proliferation and heart development.

We did not detect differences in SMC marker gene expression in the DNmDia+/SM22Cre+ that survived to adulthood. However, we observed developmental defects in SMC investment and vessel integrity and a delay in the re-expression of SMC marker genes after carotid artery in these animals. When coupled with the decrease in SMC marker gene expression on activation of DNmDia expression in adult SMC, these data strongly support our original hypothesis that mDia-mediated actin polymerization plays a role in the regulation of SMC phenotype in vivo. It is important to note that because DNmDia expression in our model occurs only after initial SM22 activation, we do not yet know whether mDia signaling is required for the initial differentiation of SMC or whether it is more important for SMC maturation and maintenance.

DNmDia+/SM22Cre+ mice exhibited reduced neointima formation after carotid artery ligation. These results are in good agreement with a study by Touré et al, who observed reduced neointimal formation in global mDia1 knockout mice. Because SM22α expression has been detected in some myeloid cells and it is possible that abnormal sarcomere organization because of defects in linear actin polymerization in DNmDia-expressing cells could be involved.
Figure 5. Dominant-negative variant of mDia1 (DNmDia) reduced directional migration. A, Subconfluent cultures of 10T1/2 cells were transfected with mCherry empty vector (EV; control) or mCherry-DNmDia. Live-cell imaging was used to monitor random (unstimulated) migration of 8 cells from each group for 8 hours. Individual cell migration paths from a relative starting point (0, 0) are shown and net distance traveled was quantified using ImageJ. B, Confluent cultures of 10T1/2 cells expressing either mCherry-EV (control) or mCherry-DNmDia were subjected to scratch wound with a P1000 pipette tip and then placed on an inverted microscope equipped with a heated, humidified, and O2/CO2 perfused stage. Pictures taken every 5 minutes for 24 hours were assembled into movies using Quicktime. Nine cells near the wound edge from each group were tracked for 24 hours. Graphs are orientated such that migration toward the scratch wound is indicated by a positive value on the x axis. C, Average displacement of cells from B were plotted over time ±SEM. Repeated measurement 1-way ANOVA analysis followed by Bonferroni post hoc test for individual significance demonstrated that DNmDia expression significantly inhibited migration toward the scratch wound (P<0.05). D, 10T1/2 cells expressing either mCherry or mCherry-DNmDia were subjected to scratch wound for 8 hours, fixed, and lamellipodia orientation toward or away from the wound was scored in at least 100 cells from 3 separate experiments. E, 10T1/2 cells expressing either mCherry or mCherry-DNmDia were subjected to scratch wound for 8 hours, fixed, and probed for α-tubulin and stained with 4′,6-diamidino-2-phenylindole (DAPI) to mark microtubule-organizing center (MTOC) orientation. At least 100 cells were counted in 3 separate experiments. F, Primary rat aortic smooth muscle cells expressing either mCherry or mCherry-DNmDia were subjected to scratch wound for 8 hours, fixed, and immunostained for Golgi marker-130 and DAPI to mark orientation. At least 100 cells from 3 separate experiments were counted. *P<0.05.
and macrophage infiltration has been shown to promote neo-intima formation, it is difficult to completely eliminate a role for these cells in our model. However, because the majority of neo-intima cells in our injury model are SMC, we think that mDia signaling plays a SMC autonomous role in neointimal formation most likely by promoting SMC migration from the medial layer. It is well known that mDia1 and mDia2 control linear actin polymerization and are required for filopodia formation and cell migration in cultured cells and in vivo. Our data indicated that DNmDia expression had no effect on overall SMC movement, but instead inhibited directional migration. A growing body of evidence suggests that the DRFs have important effects on microtubule organization and on cell polarity, and our demonstration that microtubule organizing center and lamellipodia positioning were altered in DNmDia-expressing cells strongly supports these results. Thus, it will be critical to identify the signaling mechanisms downstream of the DRFs that regulate cell polarity in SMCs. Interestingly, the reduction in collagen deposition in the DNmDia-expressing mice after ligation suggests that MRTF-A–mediated fibrosis may contribute to the injury response.

In summary, our data demonstrate that DRF-mediated actin polymerization plays a role in proper heart and vascular development, the maintenance of SMC differentiation, and directional SMC migration. These results provide important new evidence that the Rho/actin/MRTF signaling axis plays a critical role in controlling SMC phenotype and support additional studies on this pathway during cardiovascular development and disease progression.

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Disclosures
None.

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Significance

We have previously shown that mDia-mediated actin polymerization regulates smooth muscle cell differentiation in vitro, but comprehensive in vivo studies of this mechanism are lacking. This study is the first to demonstrate that diaphanous-related formin-mediated actin polymerization is critical for normal heart development and for the regulation of smooth muscle cell phenotype following vessel injury.
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Supplemental Figure I. SMC-specific expression in our conditional models. β-gal stained SMC tissues of DNmDia+/SM22Cre+/Rosa+ (A) and tamoxifen-treated DNmDia+/SMMHCCreERT2/Rosa+ mice (B).
Supplemental Figure II. Some DNmDia exhibit a runted, hairless phenotype.

A) Gross images of a runted DNmDia⁺/SM22Cre⁺ mouse, a phenotypically normal DNmDia⁺/SM22Cre⁺ mouse, and a wildtype littermate at P9. B) Representative H&E sections from the heart, lungs, and skin of the above animals. Note the thin ventricular wall, congested lungs, and degenerative hair follicles in the runted, hairless DNmDia⁺/SM22Cre⁺ mouse.
Supplemental Figure III. Immunohistochemistry secondary Ab only.
Supplemental Figure IV. Carotid artery ligation imaging. A) Representative images of MRTF-A localization, quantified in Figure 3D. B) Representative images of Masson's Trichrome stain.
Supplemental Figure V. Inhibition of mDia signaling does not alter cell cycle, death, or proliferation. siRNA-mediated knockdown of mDia1 and/or mDia2 was performed in 10T1/2 or mouse SMCs. A) 48-hr post-knockdown, cells were subjected to indicated media conditions for 24 hr, followed by colorimetric tetrazolium MTT assay. Absorbance at 600nm corresponds to active cell proliferation. N = 3 separate experiments. B) 48-hr post knockdown, cells were fixed, permeabilized, and probed for phospho-histone H3. C) Quantification of pH3+ cells from three separate experiments with over 100 cells counted per condition. D) 48-hr post-knockdown, cells were harvested, briefly stained with DAPI, and subjected to cell cycle analysis by flow cytometry. 50,000+ cells were analyzed in three separate experiments. E) 48-hr post-knockdown, cells were split and re-plated in 1% serum media. After 6hr, media was removed from cells and subjected to colorimetric cytotoxicity assay based upon lactate dehydrogenase activity in the media, which occurs upon plasma membrane rupture, and is measured by absorbance at 490 nm. Negative control = untransfected cells. Positive control = cells treated with 1% Triton X-100. N = 3 separate experiments. F) 48-hr post-knockdown, SMCs were fixed, permabilized, and stained for TUNEL+ cells. Positive control = cells subjected to UV light for 4 hr. G) Quantification of TUNEL+ cells from siRNA-knockdown SMCs and DNmDia-overexpressing 10T1/2 cells. Three separate experiments with over 100 cells counted per condition. * p < 0.05.
Supplemental Figure VI. DNmDia expression inhibited cellular orientation. 10T1/2 cells were transfected with mCherry vector or mCherry-DNmDia, subjected to an 8-hr scratch wound, and fixed. A) Representative images of lamellipodia formation in response to indicated scratch wound. B) Cells were probed with α-Tubulin to mark MTOC, marked by arrows. For DNmDia cell, note the MTOC location at the back end of the nucleus. C) Primary rat aortic SMCs were transfected with mCherry empty vector or mCherry-DNmDia, subjected to 8-hr scratch wound, fixed, and stained for Golgi marker (GM)-130. Representative images are shown with the scratch oriented to the right.
Supplemental Figure VII. DNmDia\(^+\)/SMMHCCreER\(^{12}\+) mice exhibit reduced SM marker expression in the bladder. **A)** Western blot of SM marker expression and DNmDia expression (Flag) in control (WT) and DNmDia\(^+\)/SMMHCCreER\(^{12}\+) (DN) mice. Note the variable transgene expression. **B)** SM marker expression was quantified using ImageJ in 9 controls and 9 DNmDia mice, normalized to tubulin load, and expressed relative to values in control animals, set to 1. *p < 0.05.
VIDEO FILE LEGEND
DNmDia expression prevents directional migration in a scratch wound assay. Confluent cultures of 10T1/2 cells expressing GFP-LifeAct and either mCherry or mCherry-DNmDia were scraped with a P1000 pipette tip and then placed on an inverted microscope equipped with a heated, humidified, and O₂/CO₂ perfused stage. Pictures taken every 5 minutes for 24 hr were assembled into movies using Quicktime. Green channel (LifeAct) movie is shown for better view of cytoskeletal movement. Representative movies for each construct are included. Note that scratch wound is located on the right. 1 sec of movie = 1 hr of live cell imaging.

Video I. Control
Video II. DNmDia
DETAILED MATERIALS AND METHODS

Animal Studies
The DNmDia cDNA variant was cloned into a previously described pBSCX1-LEL plasmid vector (1, 2) (see Figure 2a), and transgenic mice were generated by the University of North Carolina Animal Models Core. All animals were housed in a university animal care facility accredited by the American Association for Accreditation of Laboratory Animal Care, and all procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee. DNmDia mice were bred with either SM22α-Cre mice (Jax) (3) or SM MHC-CreERT2 mice (4) to express DNmDia in a SMC-dependent manner. Some DNmDia mice were crossed to the R26R Cre-recombinase reporter mouse line to visualize where Cre is expressed. To see in vivo LacZ expression, whole tissues were fixed in 4% paraformaldehyde for 15 min, incubated in X-gal staining solution for 16 hr, fixed for another 24 hr, dehydrated in increasing concentrations of methanol, and visualized by X-gal clearing solution. For injury studies, carotid artery ligation was performed as previously described (5). Briefly, a suture was tied around the left common carotid artery just below the bifurcation and the right carotid artery served as a sham control. Animals were sacrificed at various times following injury, and tissues were embedded in either paraffin or OCT. To determine neointimal area, we used ImageJ (NIH) to measure circumference of the lumen, internal elastic lamina (IEL), and external elastic lamina (EEL). Intimal area = IEL area minus lumen area. Medial area = EEL area minus IEL area.

Immunofluorescence/Immunohistochemistry
For IHC in either transfected or knockdown cells, cells were fixed in 4% paraformaldehyde for 20 minutes, permeabilized in 0.5% Triton X-100 for 3 min, blocked in 10% goat serum/3% BSA in PBS for 1 hr, then exposed to antibody in blocking solution for 1 hr. Alexa Fluor secondary antibodies were used for visualization at 1:1000 and DAPI (200 nM) was used to detect nuclei. TUNEL staining was performed with the In Situ Cell Death Detection Kit, TMR red (Roche) as per the manufacturer’s instructions. For DAB staining, HRP-conjugated secondary antibodies (Sigma) were used and signals were visualized by development using ImmPACT DAB Peroxidase Substrate (SK-4105, Vector labs). For MRTFA-DAPI colocalization, a confocal microscope was used to visualize 8 μm sections of ligated and uninjured carotid arteries. The ImageJ Plug-In “Colocalization Threshold” was used to quantify percent pixilation overlap of DAPI and endogenous MRTF-A.

Western Blotting and Antibodies
To examine protein levels, lysates from cells or tissues were prepared by lysing in RIPA buffer with protease and phosphatase inhibitors as previously described (5). The following primary antibodies were used: Flag M2 (F1804, Sigma), SM MHC (ab53219, Abcam), SM α-actin (A5228, Sigma), Calponin/CNN1 (LS-B7497, LifeSpan Bio), SM22/Transgelin (sc-271719, Santa Cruz), α-Tubulin (T6074, Sigma), phospho-histone H3 (6-570, Millipore), and MRTF-A (sc-32909, Santa Cruz). Blots were next incubated with horseradish peroxidase secondary antibodies (Sigma). Blots were visualized after incubation with Luminol Enhancer Solution (Thermo Scientific).
Plasmids, Cell Culture, and Transient Transfections
DNmDia cDNA (amino acids 567–1182, minus the 20AA from 750–770) was subcloned into flag pcDNA3.1 and/or pmCherry-C3 (Clontech). LifeAct (MGVADLKKFESISKEE) was cloned into the N-terminus of pEGFP-C1 (Clontech). 10T1/2 cells were obtained from ATCC and maintained in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal bovine serum (FBS) and 0.5% penicillin-streptomycin. SMCs from rat or mouse thoracic aorta were isolated and cultured as previously described (6) and maintained in DMEM:F12, also with 10% FBS and 0.5% penicillin-streptomycin. For transfections, cells were maintained in 10% FBS media, and transfected 24 hr after plating at 70-80% confluency, using the transfection reagent, TransIT-LT1 (Mirus), as per protocol. For sphingosine-1-phosphate (S1P) treatment, 24 hr post-transfection cells were serum-starved in DMEM with 0.5% FBS for 16 hr and then treated with S1P (Mateya) at 10 µM for 24 hr (Immunoblot) or 4 min (MRTFA localization).

Small Interfering RNA (siRNA) Mediated Knockdowns and in vitro assays
The following short interfering (si)RNAs were obtained from Invitrogen: control (GFP) 5’-GGUGCGCUCCUGACGUAGC-3’, mDia1 5’-GGACCUCUAUUGCCCUCAATT-3’, and mDia2 5’- GCAUGACAAGUUUGUAGATT-3’. 10T1/2 or SMCs were split and replated at 48 hr post knockdown for transwell assays, scratch wound assays, cytotoxicity assays, and tetrazolium MTT assays.

- **Cytotoxicity.** Cells were split and replated at 20K cells/well into 48-well cell culture plates for cytotoxicity assays. Assays were carried out as per protocol of Cytotoxicity Detection Kit: LDH (Roche). Cells were maintained in 1% serum. Negative control = untransfected cells. Positive control = NTC-siRNA cells in media with 1% Triton-X-100. Absorbance at 490 nm was measured in three separate experiments.

- **Proliferation.** Cells were split and replated at 5K/well into 96-well cell culture plates for tetrazolium MTT assays. Cells were serum-starved for 16 hr and treated with either 10% serum, 1 ng/ml TGF-β, or 20 ng/ml PDGF-BB for 24 hr. MTT assays were carried out as per protocol of Cell Proliferation Kit I: MTT (Roche). Absorbance at 600 nm was normalized to serum-starved NTC-siRNA for each cell type for three separate experiments.

- **Flow Cytometry.** 10T1/2 or SMCs were split and resuspended at a concentration of ~1x10^6 cells/ml in 0.1% Triton X-100 in PBS. DAPI was added to cell suspension at 1 ug/ml, and cells were incubated on ice for 30 min prior to cell cycle analysis on a Becton Dickinson LSRII. At least 50,000 cells were scored per siRNA condition, per cell type, in three separate experiments.

Migration Assays
For transwell assays, SMCs and 10T1/2 cells were trypsinized and resuspended in serum-free media or 0.5% media, respectively. Approximately 20K cells were plated on transwell filters (8 µm pore size) precoated with fibronectin, using the same media with 20 ng/ml PDGF-BB as a chemoattractant. After 7 hours, the cells were fixed in 4% paraformaldehyde and the remaining cells in the upper chamber were removed with a cotton swab. Migrated cells were stained with 1% Crystal Violet and visualized by
microscopy. For scratch wound assays, 10T1/2 cells were split and replated post-knockdown at high confluence, scratched with a P1000 pipette tip, and visualized every 3 hr until wound closure. For live cell imaging, 10T1/2 cells were transfected with both LifeAct-GFP and mCherry or mCherry-DNmDia and imaged every 5 min for both unstimulated (random) migration (~8 hr) and scratch wound-stimulated (directional) migration (~24 hr). Cell movement was quantified using ImageJ.

**Statistical Analyses**
All values are presented as means with standard deviation. Comparisons were performed by using unpaired Student t test or $X^2$ analysis, as appropriate. All tests were 2-tailed, and significance was accepted at $P < 0.05$. 
SUPPLEMENTAL REFERENCES


