Regulation of Smooth Muscle Dystrophin and Synaptopodin 2 Expression by Actin Polymerization and Vascular Injury

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Objective—Actin dynamics in vascular smooth muscle is known to regulate contractile differentiation and may play a role in the pathogenesis of vascular disease. However, the list of genes regulated by actin polymerization in smooth muscle remains incomprehensive. Thus, the objective of this study was to identify actin-regulated genes in smooth muscle and to demonstrate the role of these genes in the regulation of vascular smooth muscle phenotype.

Approach and Results—Mouse aortic smooth muscle cells were treated with an actin-stabilizing agent, jasplakinolide, and analyzed by microarrays. Several transcripts were upregulated including both known and previously unknown actin-regulated genes. Dystrophin and synaptopodin 2 were selected for further analysis in models of phenotypic modulation and vascular disease. These genes were highly expressed in differentiated versus synthetic smooth muscle and their expression was promoted by the transcription factors myocardin and myocardin-related transcription factor A. Furthermore, the expression of both synaptopodin 2 and dystrophin was significantly reduced in balloon-injured human arteries. Finally, using a dystrophin mutant *mdx* mouse and synaptopodin 2 knockdown, we demonstrate that these genes are involved in the regulation of smooth muscle differentiation and function.

Conclusions—This study demonstrates novel genes that are promoted by actin polymerization, that regulate smooth muscle function, and that are deregulated in models of vascular disease. Thus, targeting actin polymerization or the genes controlled in this manner can lead to novel therapeutic options against vascular pathologies that involve phenotypic modulation of smooth muscle cells. **(*Arterioscler Thromb Vasc Biol*. 2015;35:1489-1497. DOI: 10.1161/ATVBAHA.114.305065.)**

Key Words: angioplasty ■ gene expression ■ vascular diseases

Vascular smooth muscle cells (SMCs) exhibit a remarkable phenotypic plasticity, which allows them to adapt to a changing environment. This so-called phenotypic switching, although being beneficial during blood vessel development and repair, can contribute to pathogenesis of several cardiovascular diseases such as hypertension and postangioplasty restenosis.[1] The synthetic or proliferative phenotype of SMCs is characterized by a reduced level of contractile proteins and by increased extracellular matrix synthesis.

Multiple signaling pathways have been suggested to regulate smooth muscle phenotype including the Rho/Rho-associated coiled-coil forming protein kinase pathway, which in turn promotes actin polymerization.[2] Naturally, an increased polymerization of actin results in an increased amount of contractile filaments. However, the polymerization of actin also results in an increased transcription of genes encoding actin and actin binding proteins, which are known as smooth muscle contractile markers. This effect is mediated by the myocardin-related transcription factor (MRTF), which is bound to globular actin (G-actin) in the cytoplasm and is translocated into the nucleus when G-actin polymerizes into filamentous actin (F-actin).[3,4] In the nucleus, MRTF acts as a cofactor to the transcription factor, serum response factor (SRF), which binds to SRF-binding elements (CArG) in the promoter region of smooth muscle markers, resulting in increased transcription.[5] The protein expression of these markers then determines the fate of SMCs and regulates their contractile function.[2]

The classical smooth muscle markers include α-actin and actin-binding proteins such as SM22α, and myosin heavy chain. These markers are enriched in SMCs but may also be expressed in other mesenchymal cells such as myofibroblasts.[6] Recently, several novel smooth muscle markers have been identified, primarily by studies performed by Miano and coworkers.[7–9] These proteins, similar to the well-established smooth muscle markers, control smooth muscle function, and their deregulation may be involved in the pathogenesis of vascular disease. Most of the smooth muscle markers are regulated by SRF together with its cofactors myocardin or MRTF

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Nonstandard Abbreviations and Acronyms

Cnn1	smooth muscle calponin
Dmd	dystrophin
F-actin	filamentous actin
G-actin	globular actin
Itga8	integrin α8
Jasp	jasplakinolide
Kcnmb1	β1 subunit of large conductance calcium-activated potassium channel transcript
Lmod1	leiomodin1
mdx	dystrophin mutant mouse
MRTF	myocardin-related transcription factor
Myh11	smooth muscle myosin heavy chain transcript
SMC	smooth muscle cells
SRF	serum response factor
Synpo2	synaptopodin 2
Tagln	SM22α transcript
WT	wild-type

and it is thus likely that these markers are also partially controlled by actin polymerization. However, a complete screen of actin-sensitive gene transcription in smooth muscle has to our knowledge not been performed previously.

During recent years, actin dynamics has been recognized as an important factor in the development of cardiovascular disease. For example, altered actin-MRTF signaling has been implicated in aortic aneurysm,[10] vascular retinal disease,[11] and lamin-associated cardiomyopathy.[12] Apart from its role in transcriptional regulation, actin dynamics is known to be crucial for smooth muscle contraction and an abnormal increase in actin polymerization may thus result in hypercontractility of arteries leading to inward remodeling and hypertension.[13,14] Furthermore, we have previously demonstrated that actin dynamics is an important factor for stretch sensing in vascular smooth muscle.[15–17]

In the present study, we hypothesized that genes whose transcription is dependent on actin polymerization are also involved in the regulation of smooth muscle function and vascular disease. The direct effects of actin polymerization were studied using Jasplakinolide (Jasp), which stabilizes actin filaments.[17–19] Two genes, dystrophin (*Dmd*) and synaptopodin 2 (*Synpo2*), were identified as highly sensitive to actin polymerization and their transcriptional regulation was analyzed in different experimental models of smooth muscle phenotypic modulation and vascular disease. Finally, using *Dmd* mutant mice and Synpo2 knock-down, we could demonstrate the importance of these genes for smooth muscle function and contractile differentiation.

Materials and Methods

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

Results

Expression of Dmd and Synpo2 Is Promoted by Jasp-Induced Actin Polymerization

Actin polymerization is known to promote the expression of established smooth muscle markers. To comprehensively clarify the effect of actin polymerization on smooth muscle gene expression, we performed a gene array on mouse aortic SMCs treated with Jasp (100 nmol/L) or vehicle (Ctrl) for 24 hours. This analysis uncovered 48 genes, which were upregulated at least 1.4-fold by Jasp treatment (Figure I in the online-only Data Supplement). A transcription factor binding site analysis of the 135 most upregulated (>1.2-fold) genes revealed a significant enrichment of SRF-regulated genes (data not shown; $P<0.01$). Among these genes were well-established smooth muscle markers such as smooth muscle myosin heavy chain (*Myh11*), calponin (*Cnn1*), and SM22α (*Tagln*; Figure 1A). We also found significant upregulation of recently identified smooth muscle contractile markers such as leiomodin 1 (*Lmod1*),[8] β1 subunit of large conductance, calcium activated potassium channel (*Kcnmb1*),[9] and integrin α8 (*Itga8*[7]; Figure 1A). Furthermore, some additional genes that have not been extensively characterized in smooth muscle were found to be induced by actin polymerization. Two of these were the genes for the actin-binding proteins Dmd and Synpo2 (Figure 1A). Jasp-induced upregulation of mRNA expression was confirmed for selected genes using quantitative polymerase chain reaction analysis (Figure 1B–1E). In addition, depolymerization by latrunculin B treatment of intact mouse aorta resulted in a reduced expression of *Myh11*, *Kcnmb1*, *Dmd*, and *Synpo2*. These results suggested that Dmd and Synpo2 are regulated by actin polymerization in smooth muscle and may be novel markers of the contractile phenotype. Several genes were also downregulated by Jasp

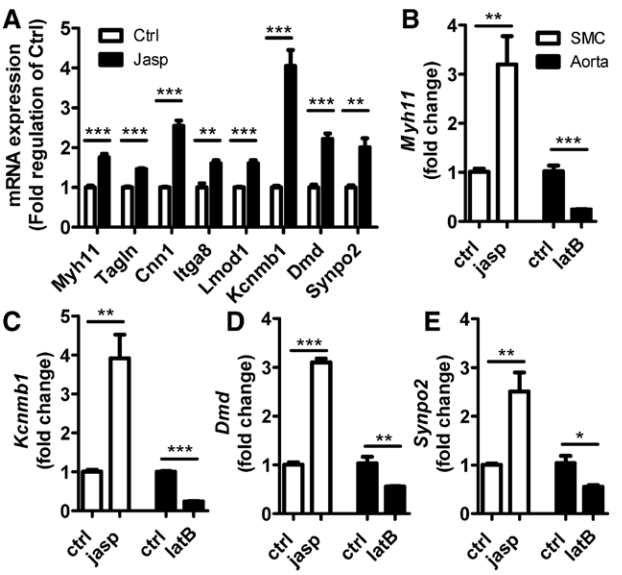

Figure 1. Stabilization of actin filaments promoted the expression of dystrophin (Dmd) and synaptopodin 2 (Synpo2). **A**, The expression of selected genes from an Affymetrix gene array of mouse aortic smooth muscle cells (SMCs) stimulated with 100 nmol/L jasplakinolide (Jasp) for 24 hours (n=4). **B** to **E**, Confirmation of array results of smooth muscle myosin heavy chain (Myh11; **B**), β-subunit of large conductance calcium-activated potassium channel (Kcnmb1; **C**), Dmd (**D**), Synpo2 (**E**) by individual quantitative polymerase chain reaction (white bars). Black bars represent treatment of mouse aorta with actin depolymerizing agent, latrunculin B (latB, 250 nmol/L) for 24 hours (n=3–5). ***$P<0.05$, **$P<0.01$, and *$P<0.5$. Cnn1 indicates smooth muscle calponin; Itga8, integrin α8; and Lmod1, leiomodin.

including chloride channels *Clca1*, *Clca2*, and *Cftr* as well as the glycoproteins *Prom1* and *Dcn* (Figure II in the online-only Data Supplement).

Dmd and Synpo2 Are Highly Expressed in Differentiated Smooth Muscle

To clarify the relative smooth muscle specificity of Dmd and Synpo2, we analyzed the mRNA expression of these genes in aorta and urinary bladder compared with several other tissues (Figure 2A and 2B). The expression of the smooth muscle marker myosin heavy chain was used as a positive control (Figure 2C). The results demonstrate a specific expression of both Dmd and Synpo2 in muscle and both genes exhibited higher expression levels in smooth versus striated muscle tissues.

The results of the quantitative polymerase chain reaction analysis were confirmed on the protein level by Western blot analysis using SM22α as a positive control (Figure 2D–2G). Protein expression was normalized to Coomassie blue–stained total protein content because none of the commonly used endogenous loading controls (GAPDH, heat shock protein 90, β-actin, and α-tubulin) were equally expressed among all of the analyzed tissues. Taken together, actin polymerization regulates genes that are enriched in smooth and striated muscle and many of these genes can play an important role in both cell types.

MRTF-A and Myocardin Promote Expression of Jasp-Induced mRNA

Many of the well-established contractile smooth muscle markers contain a CC[A/T]6GG motif known as CArG box in their promoter region, which allows for myocardin/MRTF-SRF–dependent transcription.[5] Using bioinformatic analysis, we confirmed the presence of CArG boxes in the *Dmd* promoter.[20] Furthermore, in the *Synpo2* gene, we identified a consensus

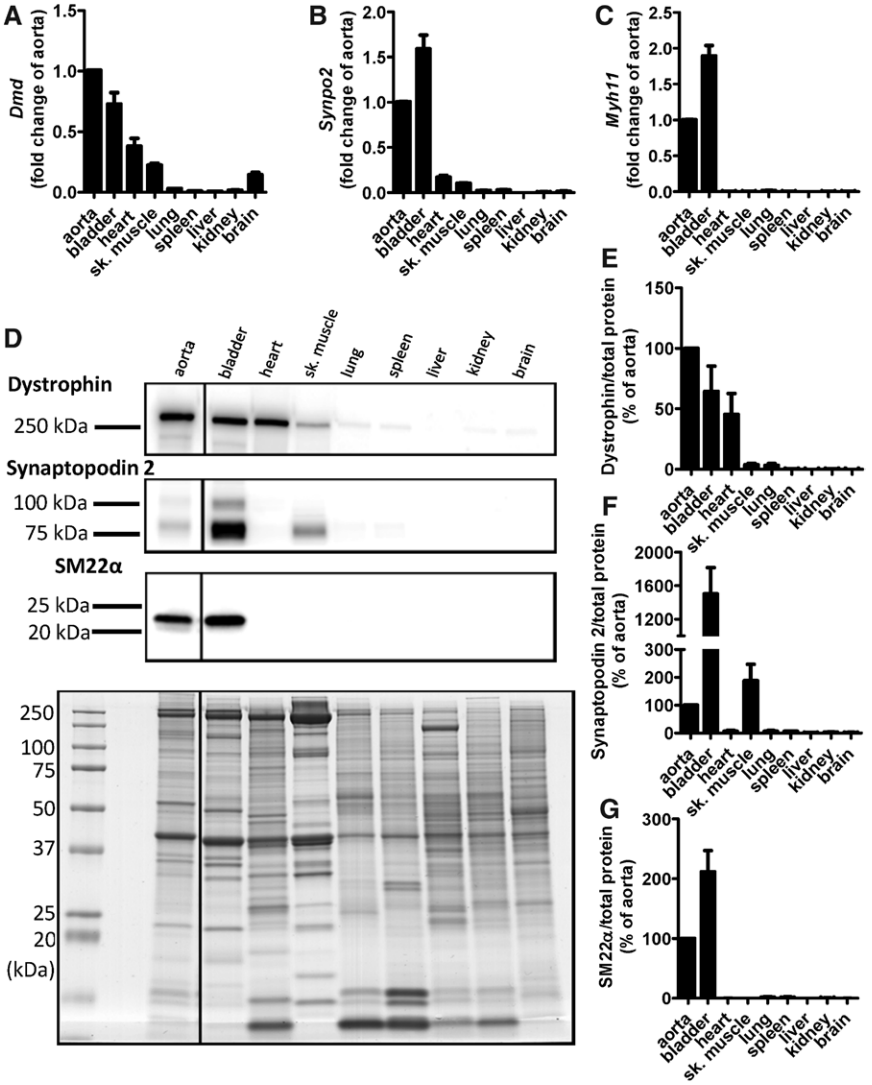

Figure 2. Dystrophin (Dmd) and synaptopodin 2 (Synpo2) are highly expressed in differentiated smooth muscle. **A** to **C**, Different mouse tissues were collected and mRNA expression of Dmd (**A**), Synpo2 (**B**), and smooth muscle myosin heavy chain (Myh11; **C**) was analyzed by quantitative polymerase chain reaction (qPCR; n=3–4). The qPCR data were normalized to 18S used as a reference gene and to aorta as a control group. **D** to **G**, Mouse tissue lysates were analyzed by Western blot using antibodies against Dmd, Synpo2, and SM22α (n=3). Protein expression was normalized to total protein (Coomassie blue–stained gel). **D** and **E** to **G**, Representative blots and summarized data, respectively.

CArG sequence (CCTTTTAAGG) at position 722 relative to the transcription start site. Several CArG-like sequences were also identified but the functional importance of these is uncertain. We therefore transduced mouse aortic SMCs with adenovirus expressing either myocardin (Ad.Myocd) or MRTF-A (Ad.MRTF-A) for 96 hours. Overexpression of either myocardin or MRTF-A significantly induced the transcription of Dmd, Synpo2, and the positive control, myosin heavy chain (Figure 3A and 3B, respectively), further supporting the view that these genes are SRF-dependent contractile smooth muscle markers regulated by actin polymerization. Interestingly, a synergistic effect on contractile marker expression was observed by simultaneous incubation with MRTF-A and jasp (Figure 3D–3F).

Reduced F/G Actin Ratio in Cultured SMCs Correlates With Downregulation of Dmd and Synpo2

Isolated SMCs in culture represent an in vitro model of phenotypically modified smooth muscle. These cells share many of the features that characterize SMCs in vascular lesions, such as increased proliferation and migration, increased matrix production, and reduced expression of contractile and cytoskeletal proteins.[21] Recently, smooth muscle–derived cells in culture have also been observed to transdifferentiate into a macrophage-like phenotype that may play a major role in the disease progression of atherosclerosis.[22]

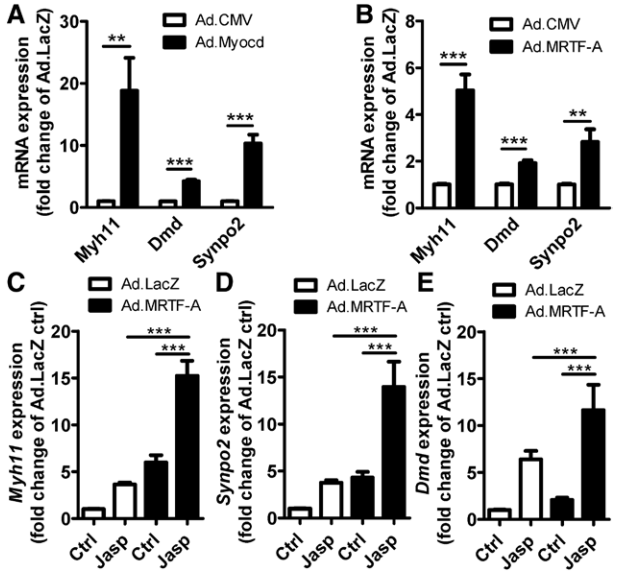

Figure 3. Overexpression of myocardin and myocardin-related transcription factor A (MRTF-A) promotes expression of dystrophin (Dmd) and synaptopodin 2 (Synpo2). Mouse aortic smooth muscle cells were transduced with adenovirus expressing either myocardin (Ad.Myocd; 100 multiplicity of infection [MOI]; n=7) or MRTF-A (Ad.MRTF-A; 20 MOI; n=8) for 96 hours. Cells infected with control adenovirus, containing empty cytomegalovirus promoter (Ad.CMV), were used as a control. **A**, Ad.Myocd and (**B**) Ad.MRTF-A–overexpressing cells were used for mRNA expression analysis of smooth muscle myosin heavy chain (Myh11), Dmd, and Synpo2 by quantitative polymerase chain reaction (qPCR). The effect of actin stabilization by jasplakinolide (Jasp; 100 nmol/L) on Ad.LacZ and Ad.MRTF-A transduced cells (20 MOI) was evaluated using qPCR for Myh11 (**C**), Synpo2 (**D**), and Dmd (**E**). $^{**}P<0.01$ and $^{***}P<0.001$.

To clarify how actin polymerization is affected in phenotypically modulated (synthetic) SMCs, we compared the F/G-actin ratios in quiescent SMCs from intact aorta with proliferating cultured SMCs. As shown in Figure 4A, the F/G actin ratio was significantly reduced in cultured SMCs, suggesting that reduced actin polymerization can be an underlying mechanism of phenotypic modulation of smooth muscle. Jasp-treated cultured SMCs were used as positive control and this substance increased the F/G-actin ratio as expected.

The reduced actin polymerization in synthetic SMCs correlated with a substantially downregulated mRNA expression of Dmd and Synpo2 (Figure 4B). In fact, the downregulation of these genes was more pronounced than that of the positive control Tagln (Figure 4B). Similar results were observed at the protein level as determined by Western blotting (Figure 4C and 4D). Interestingly, we found that several of the genes that were downregulated by Jasp, including *Clca1, Clca2, Cftr, Prom1*, and *Dcn* were in fact upregulated in synthetic SMCs (Figure III in the online-only Data Supplement). The downregulation of Synpo2 and Dmd in cultured SMCs could be partially reversed by Jasp (Figure IV in the online-only Data Supplement).To address the importance and species generality of our findings, we also analyzed expression of Tagln, Dmd, and Synpo2 in human renal arteries and SMCs cultured from the same arteries. The expression of Tagln, which again served as a positive control, was decreased in cultured human renal arterial SMCs by 0.6-fold compared with the intact artery (Figure 4E). Consistent with the results obtained from mouse aortic smooth muscle, the expression levels of Dmd and Synpo2 were more dramatically downregulated than Tagln in these cells (Figure 4E).

Phenotypic modulation of SMCs is observed in several vascular disease states including restenosis after angioplasty.[23] We thus aimed at determining the effect of balloon dilation of human arteries on the expression of Dmd and Synpo2. Healthy human left internal mammary arteries were collected from patients undergoing by-pass surgery. The arteries were then dilated ex vivo for 2 minutes using a percutaneous transluminal coronary angioplasty balloon catheter. The arteries were then incubated in organ culture environment for 48 hours. Previous studies have demonstrated that vascular injury induced by this method results in upregulation of the calcium channel TRPC1, which is involved in smooth muscle proliferation and neointima formation.[24] Herein, quantitative polymerase chain reaction analysis revealed that the mRNA expression of Dmd and Synpo2 was reduced in balloon injured arteries to a similar extent as the positive control Tagln (Figure 4F). Similarly, in an in vivo model of balloon injury in pig coronary artery, both Synpo2 and Dmd were downregulated at the protein level 4 weeks after injury. Taken together, these findings show that Dmd and Synpo2 conform to established patterns of regulation for contractile SMC markers.

Loss of Dmd Results in Impaired Vascular Smooth Muscle Contraction, Relaxation, and Mechanosensing

The importance of Dmd for vascular smooth muscle contractile function was investigated using tail artery rings from control and *Dmd* mutant (*mdx*) mice mounted in wire myographs.

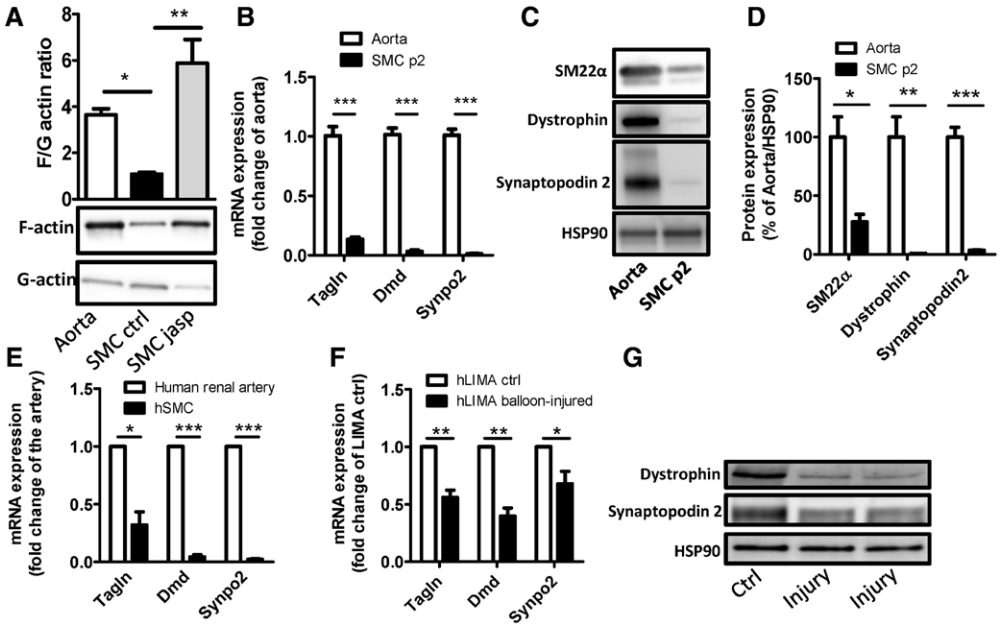

Figure 4. Decreased F/G actin ratio in cultured smooth muscle cells correlates with downregulated mRNA levels of dystrophin (Dmd) and synaptopodin 2 (Synpo2). **A**, Intact mouse aorta without adventitia and cultured mouse aortic smooth muscle cells (SMCs), with or without jasplakinolide (Jasp; 100 nmol/L) for 24 hours. The filamentous (F) and globular (G)-actin fractions were separated by ultracentrifugation and analyzed by Western blot. F/G actin ratios and representative blots are shown (n=3). **B**, Quantitative polymerase chain reaction (qPCR) of indicated mRNA and (**C** and **D**) Western blot analysis of indicated proteins isolated from intact aorta and cultured SMCs (passage2-p2; n=3–6). **C**, Representative blots and (**D**) quantitative analysis of the Western blot data. **E**, Human renal arteries and cultured smooth muscle cells (hSMC; passage 2–5) from the same arterial sample were collected for qPCR analysis of selected mRNA (n=3–4). **F**, Human left internal mammary arteries (hLIMA) were subjected to balloon-injury ex vivo and organ cultured for 48 hours. mRNA levels of SM22α (Tagln), Dmd, and Synpo2 were measured by qPCR analysis (n=5). All the qPCR data were normalized to 18S and show relative mRNA expression to a respective control. **G**, Representative Western blot of Dmd and Synpo2 in control and balloon-injured pig coronary arteries in vivo. Protein analysis was performed 4 weeks after injury and heat shock protein 90 was used as loading control. Control (n=2) and injured vessels (n=4) from 2 separate animals were analyzed **P*<0.05, ***P*<0.01, and ****P*<0.001.

As shown in Figure 5A, the contractile response to depolarization by high K^+ (60 mmol/L) was reduced by 30% ($P \leq 0.001$) in arteries from *mdx* mice compared with the control arteries. Furthermore, the calcium-independent contraction induced by the phosphatase inhibitor calyculin A was significantly decreased in *mdx* mice indicating a defect in the structural contractile machinery of the *mdx* SMCs (Figure 5A). Another possibility for this effect is an altered activity of calcium-independent myosin kinases. To test this, we analyzed the rate of calyculin A–induced force development but found no significant difference in the half time in seconds of maximal contraction ($t_{1/2}$) between wild-type (WT) and *mdx* arteries ($t_{1/2\pm SEM}$; WT: 251.7±16.9 versus *mdx*: 276.8±33.4). It is therefore likely that the effect involves the structural contractile machinery, but loss of Dmd did not directly affect the F/G-actin ratio in *mdx* smooth muscle (Figure 5B).

The significance of Dmd for agonist-specific responses was further tested using *mdx* tail arteries stimulated with the α_1-adrenergic agonist cirazoline. A rightward shift of the dose–response curve for cirazoline was observed (EC_{50} WT: 37±4.2 versus *mdx* 52±4.7 nmol/L; $P<0.05$) and contractile force was significantly reduced at a concentration of 0.1 µmol/L (Figure 5C). To test smooth muscle–dependent relaxation in *mdx* mice, tail arteries were precontracted with cirazoline and then stimulated with the nitric oxide donor sodium nitroprusside. Dilatation to sodium nitroprusside was attenuated in *mdx* tail arteries compared with the control vessels with significant effects observed at 10 to 100 nmol/L (Figure 5D). Figure 5E and 5F shows the representative original recordings of isometric force measurements in response to various stimuli.

We have previously reported that the expression of several smooth muscle markers is sensitive to mechanical stretch. In WT mouse portal veins, we found that the transcription of Synpo2 is sensitive to physiological longitudinal stretch. Furthermore, the effect was abolished in *Dmd* mutant vessels (Figure 5G).

GapmeR-Mediated Knockdown of Synpo2 Results in Reduced Actin Polymerization and Contractile Differentiation

The avian homologue of Synpo2, fesselin, has previously been demonstrated to bind to G-actin and stimulate actin polymerization.[25] To determine whether knockdown of Synpo2 is sufficient to cause actin depolymerization and loss of smooth muscle marker expression, we transfected cultured SMCs with Synpo2 GapmeRs. A combination of 4 different GapmeRs was used to achieve maximal knockdown. After 96-hour incubation with GapmeRs, the expression of Synpo2 was reduced by ≈62% (Figure 6A). Interestingly, knockdown of Synpo2 caused a dramatic reduction in the F/G-actin ratio in SMCs suggesting that this protein plays a key role in the regulation of actin polymerization (Figure 6B). This effect was also associated with a decrease in the expression of Dmd and Tagln (Figure 6C and 6D). Reciprocal coimmunoprecipitation demonstrated that Synpo2 interacts with α-actin (Figure 6E and 6F).

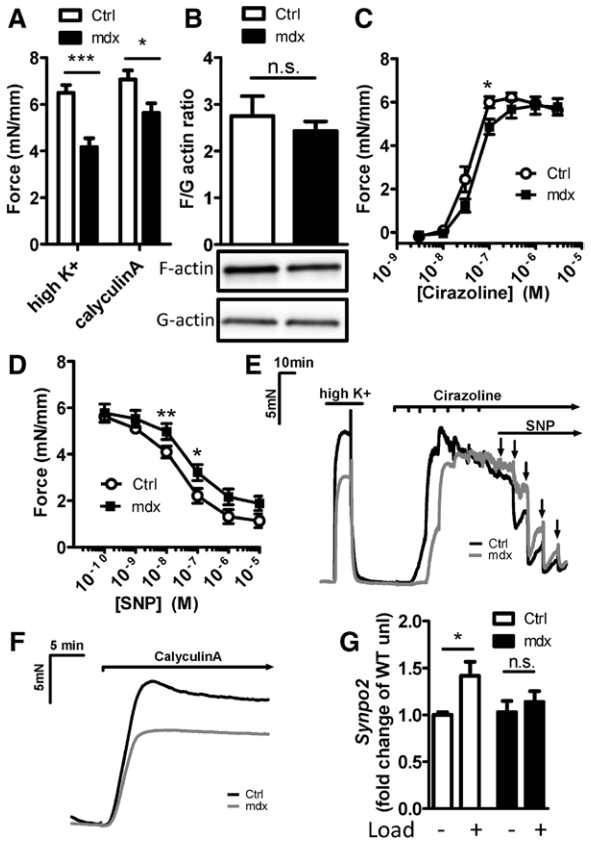

Figure 5. Dystrophin (Dmd) deficiency in mice impairs contractility and stretch-sensitivity. **A**, Tail arterial rings from both control and *mdx* mice were mounted in a wire myograph and stimulated with high K^+ (60 mmol/L KCl) and calyculin A (1 μmol/L). The graph shows calculated force normalized to the length of the corresponding vessel (n=11–12; n=5–6, respectively). **B**, Aortas without adventitia, from control and *mdx* mice, were subjected to filamentous/globular (F/G) actin analysis. F/G actin ratios and representative blots are shown (n=3–4). **C**, Concentration–response curve of cirazoline-induced contraction of tail arterial rings from control and *mdx* mice as measured by developed force/length using wire myograph (n=11–12). **D**, Sodium nitroprusside (SNP)–induced relaxation was performed after contraction to cirazoline (n=11–12). **E** and **F**, Representative graphs of original recordings of the control (ctrl) and *mdx* mice vascular rings showing response to 60 mmol/L KCl, cirazoline, SNP (**E**), and calyculin A (**F**). **G**, The mouse portal vein was organ cultured with or without load for 24 hours. The mRNA expression of Synpo2 was analyzed by quantitative polymerase chain reaction (n=5). **P*<0.05, ***P*<0.01 and ****P*<0.001.

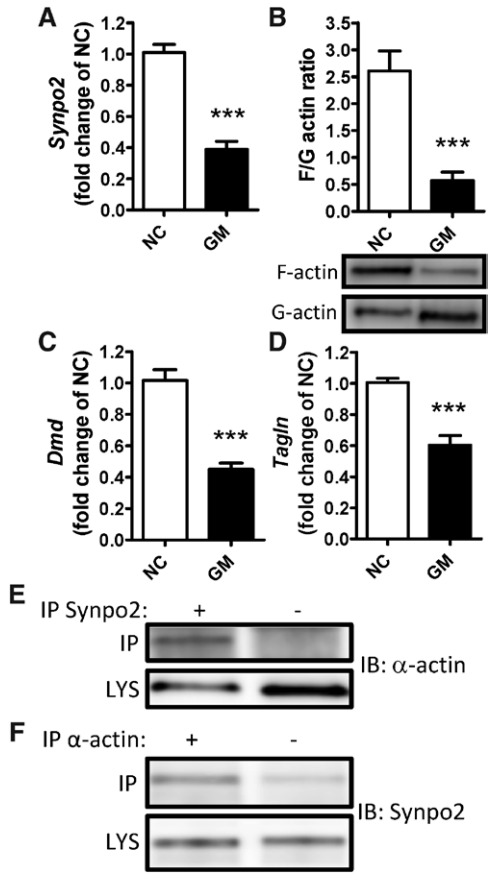

Figure 6. GapmeR-mediated knockdown of synaptopodin 2 (Synpo2) results in reduced actin polymerization and smooth muscle marker expression. **A**, Synpo2 expression is significantly reduced 96 hours after Synpo2 GapmeR transfection of cultured smooth muscle cells compared to negative control (NC). Knockdown of Synpo2 results in reduced F/G-actin ratio (**B**) as well as reduced expression of dystrophin (Dmd; **C**) and SM22 (Tagln; **D**; n=9–11). Interaction between Synpo2 and smooth muscle α-actin was evaluated by immunoprecipitation (IP) of mouse bladder smooth muscle. Representative immunoblots (IBs) of α-actin after Synpo2 IP (**E**) and Synpo2 after α-actin IP (**F**) are shown. Expression levels of the respective proteins in the lysates (LYS) used for IP are shown below the IP blot. Two independent experiments were performed. ****P*<0.001.

Discussion

Although the regulation of smooth muscle phenotype is a complex process, several key discoveries have significantly contributed to our understanding of the underlying mechanisms. One such mechanism is the regulation of MRTF activity by actin polymerization which was initially identified by Treisman and coworkers.[3,4] We could later demonstrate that actin polymerization is essential for stretch-dependent vascular smooth muscle differentiation[15–17,19] and for the effects of the microRNA miR-145 on smooth muscle marker expression.[26] Considering the prominent effect of actin polymerization on the regulation of smooth muscle phenotype, it is likely that this mechanism is involved in the development of vascular disease.

In this study, we have identified several genes that are transcriptionally activated by actin polymerization and further characterized two of these genes, *Dmd* and *Synpo2*. We show that these genes are highly expressed in differentiated smooth muscle and that their expression is dramatically reduced in phenotypically modulated smooth muscle. After balloon dilation of human left internal mammary artery vessels ex vivo and of pig coronary arteries in vivo, we found a reduced expression of both Dmd and Synpo2. In *Dmd* mutant *mdx* mice, we found a significant loss of both smooth muscle contraction and relaxation, thus emphasizing the importance of genes regulated by actin polymerization for smooth muscle function.

The importance of actin dynamics in the pathogenesis of cardiovascular diseases is becoming increasingly appreciated. Actin polymerization as such directly regulates smooth muscle contractility and remodeling in resistance arteries.[13,14,27] Furthermore, the effect of actin polymerization for gene transcription via MRTF has been shown to be involved in multiple

disease states involving endothelial cells,[11] cardiomyocytes,[12] and SMCs.[10,28–30] Herein, we demonstrate that the ratio of filamentous to globular actin is dramatically decreased in phenotypically modulated SMCs. By stabilizing actin filaments in cultured cells with Jasp, the expression of smooth muscle markers can be partially restored already after 24 hours of treatment and further induced by 72 hours. Although other mechanisms are likely to be involved, these results suggest that the loss of actin filaments is an important mechanisms for the reduced expression of contractile markers during phenotypic modulation of SMCs.

By screening genes that were induced by actin polymerization, we identified several previously well-defined smooth muscle contractile markers, verifying the importance of actin polymerization for smooth muscle differentiation. Among the genes that were induced by actin polymerization, but which have not been extensively studied in smooth muscle, were *Dmd* and *Synpo2*. The transcripts levels of both Synpo2 and Dmd were induced by overexpression of either myocardin or MRTF-A suggesting that they are transcriptionally regulated in a manner similar to most markers of the differentiated smooth muscle phenotype.[31] Furthermore, the synergistic effect MRTF and Jasp confirms that a reduction of the G-actin pool is required for MRTF to have its full effect. The expression of Synpo2 and Dmd is however not solely dependent on actin polymerization, because myocardin, which is constitutively localized in the nucleus,[32] also promotes their expression. A majority of the promoter regions of canonical smooth muscle markers contains ≥1 CC[A/T]6GG motifs, which are SRF binding sites called CArG boxes.[33] In humans, the Dmd gene contains 1 validated CArG-box at −91 bp relative to the transcription activation site, and it has been demonstrated that SRF binds to the *Dmd* promoter and regulates its transcription in striated muscle.[20,34] However, the importance of actin polymerization for the regulation of smooth muscle marker genes and development of vascular disease in humans remains to be investigated.

Mammalian Synpo2 has previously been characterized in vitro in rabbit smooth muscle, where 2 different isoforms were shown to bind Ca^{2+}-calmodulin, α-actinin, and smooth muscle myosin.[35] The avian smooth muscle homologue, fesselin,[36,37] and the *Synpo2* gene splice variant, myopodin, which is mainly expressed in skeletal muscle[38] have been studied in more detail. Both fesselin and myopodin have been demonstrated to bind to actin filaments and participate in actin polymerization by formation of actin bundles.[25,39] We found that Synpo2 mRNA and protein were abundant in differentiated vascular smooth muscle and dramatically reduced in proliferating SMCs. Interestingly, the expression of *Synpo2* was also significantly reduced in human arteries after balloon dilation, indicating a potential role in restenosis after angioplasty. In accordance with previous reports, we found that Synpo2 interacts with actin and plays a key role in actin polymerization. Cells transfected with GapmeRs against Synpo2 exhibited reduced actin polymerization and smooth muscle differentiation. Thus, it is likely that Synpo2 is required for proper expression of smooth muscle genes via its effect on actin.

The Dmd protein is a part of the Dmd-associated protein complex, which links the extracellular matrix to the cytoskeleton. As such, Dmd is an important component of mechanotransduction in striated muscle and mutations in the *Dmd* gene result in muscular dystrophy and cardiomyopathy.[40,41] Although Dmd has been suggested to be a marker of differentiated smooth muscle,[42,43] its importance for vascular smooth muscle function has not been studied extensively. However, in *Dmd* mutant *mdx* mice, it was recently demonstrated that Dmd deficiency results in accelerated neointima formation after vascular injury.[43] In accordance with these studies, we found that Dmd was significantly reduced in phenotypically modified SMCs and balloon-dilated human arteries, suggesting that the loss of Dmd could be an important mechanism for the development of vascular disease. It is well known that the lack of Dmd in *mdx* mice is partly compensated for by its homologue utrophin.[44] Despite this compensatory effect, we found that deletion of Dmd in *mdx* mice results in abnormal contractile function of vascular smooth muscle and loss of stretch-induced gene transcription of Synpo2. Specifically, force development to membrane depolarization by high K^+ was reduced in *mdx* arteries. This result is consistent with previous observations in *mdx* portal vein where the amplitude of spontaneous contractions was found to be significantly reduced.[45] The effect is not because of aberrant calcium signaling or calcium sensitivity because calcium-independent contractile responses to the phosphatase inhibitor calyculin A were reduced to a similar degree. Furthermore, the level of calcium in *mdx* SMCs has been reported to be the same as in the control smooth muscle.[46] Considering that Dmd is an actin binding protein, it is conceivable that the loss of Dmd would affect actin filament stability. However, F/G actin ratios were similar in control and *mdx* mice, which is in line with previous observations in cultured SMCs.[47] This suggests that it is rather the function, than the amount, of actin filaments that is affected in Dmd mutant mice. Possibly, the absence of Dmd-mediated physical anchoring of the actin cytoskeleton to the plasma membrane and the surrounding extracellular matrix is detrimental for force development in smooth muscle. We cannot exclude the possibility that the reduced calyculin A response in mdx arteries may be because of a defect in calcium-independent myosin phosphorylation.[48,49] However, the rate of calyculin A–induced force development is similar in WT and *mdx* arteries, suggesting that the activity of calcium-independent myosin kinases is unaffected. Furthermore, the contractile response to depolarization by KCl was also reduced in *mdx* arteries, which suggest a more general defect of the contractile machinery. Interestingly, we found that relaxation of SMCs was negatively affected by the loss of smooth muscle Dmd, which supports the functional importance of Dmd in smooth muscle vasoregulation.

The importance of Dmd in mechanosensing has been demonstrated in endothelial cells where it is involved in flow-induced dilation of mouse carotid and small mesenteric arteries.[50] Our results indicate that part of this effect may be because of a reduced ability of SMCs to relax to NO stimulation, at least during isometric force measurement. However, because Dmd is an important part of the connection between the extracellular matrix and the intracellular cytoskeleton, it is likely that mechanosensing per se is also affected in *mdx* cells. Accordingly, we found a loss

of stretch-sensitive Synpo2 expression in *mdx* portal veins in organ culture. This is a model of physiological distention where the portal vein is stretched to its optimal length for force development.[51] In previous work, we have demonstrated that physiological stretch of the portal vein promotes both contractile differentiation and growth of the smooth muscle.[15–17,19,51] The method is fundamentally different from the acute nonphysiological stretch applied when using balloon-dilation of human arteries, which results in an injury response in the vessel wall.

In summary, the expression of several MRTF-regulated, actin-binding proteins, including Dmd and Synpo2, is promoted by stabilization of actin filaments. Deregulation of actin polymerization in vascular disease states is likely to affect the expression of these proteins and to alter smooth muscle phenotype and function. Thus, targeting smooth muscle actin polymerization or the genes regulated by actin may lead to novel therapeutic options against vascular pathologies that involve phenotypic modulation of SMCs.

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Disclosures

None.

References

1. Owens GK, Kumar MS, Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev*. 2004;84:767–801. doi: 10.1152/physrev.00041.2003.
2. Mack CP, Somlyo AV, Hautmann M, Somlyo AP, Owens GK. Smooth muscle differentiation marker gene expression is regulated by RhoA-mediated actin polymerization. *J Biol Chem*. 2001;276:341–347. doi: 10.1074/jbc.M005505200.
3. Miralles F, Posern G, Zaromytidou AI, Treisman R. Actin dynamics control SRF activity by regulation of its coactivator MAL. *Cell*. 2003;113:329–342.
4. Sotiropoulos A, Gineitis D, Copeland J, Treisman R. Signal-regulated activation of serum response factor is mediated by changes in actin dynamics. *Cell*. 1999;98:159–169.
5. Miano JM. Serum response factor: toggling between disparate programs of gene expression. *J Mol Cell Cardiol*. 2003;35:577–593.
6. Darby I, Skalli O, Gabbiani G. Alpha-smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing. *Lab Invest*. 1990;63:21–29.
7. Kitchen CM, Cowan SL, Long X, Miano JM. Expression and promoter analysis of a highly restricted integrin alpha gene in vascular smooth muscle. *Gene*. 2013;513:82–89. doi: 10.1016/j.gene.2012.10.073.
8. Nanda V, Miano JM. Leiomodin 1, a new serum response factor-dependent target gene expressed preferentially in differentiated smooth muscle cells. *J Biol Chem*. 2012;287:2459–2467. doi: 10.1074/jbc.M111.302224.
9. Long X, Tharp DL, Georger MA, Slivano OJ, Lee MY, Wamhoff BR, Bowles DK, Miano JM. The smooth muscle cell-restricted KCNMB1 ion channel subunit is a direct transcriptional target of serum response factor and myocardin. *J Biol Chem*. 2009;284:33671–33682. doi: 10.1074/jbc.M109.050419.
10. Shen D, Li J, Lepore JJ, Anderson TJ, Sinha S, Lin AY, Cheng L, Cohen ED, Roberts JD Jr, Dedhar S, Parmacek MS, Gerszten RE. Aortic aneurysm generation in mice with targeted deletion of integrin-linked kinase in vascular smooth muscle cells. *Circ Res*. 2011;109:616–628. doi: 10.1161/CIRCRESAHA.110.239343.
11. Weinl C, Riehle H, Park D, Stritt C, Beck S, Huber G, Wolburg H, Olson EN, Seeliger MW, Adams RH, Nordheim A. Endothelial SRF/MRTF ablation causes vascular disease phenotypes in murine retinae. *J Clin Invest*. 2013;123:2193–2206. doi: 10.1172/JCI64201.
12. Ho CY, Jaalouk DE, Vartiainen MK, Lammerding J. Lamin A/C and emerin regulate MKL1-SRF activity by modulating actin dynamics. *Nature*. 2013;497:507–511. doi: 10.1038/nature12105.
13. Moreno-Domínguez A, Colinas O, El-Yazbi A, Walsh EJ, Hill MA, Walsh MP, Cole WC. Ca2+ sensitization due to myosin light chain phosphatase inhibition and cytoskeletal reorganization in the myogenic response of skeletal muscle resistance arteries. *J Physiol*. 2013;591(Pt 5):1235–1250. doi: 10.1113/jphysiol.2012.243576.
14. Staiculescu MC, Galiñanes EL, Zhao G, Ulloa U, Jin M, Beig MI, Meininger GA, Martinez-Lemus LA. Prolonged vasoconstriction of resistance arteries involves vascular smooth muscle actin polymerization leading to inward remodelling. *Cardiovasc Res*. 2013;98:428–436. doi: 10.1093/cvr/cvt034.
15. Zeidan A, Nordström I, Albinsson S, Malmqvist U, Swärd K, Hellstrand P. Stretch-induced contractile differentiation of vascular smooth muscle: sensitivity to actin polymerization inhibitors. *Am J Physiol Cell Physiol*. 2003;284:C1387–C1396. doi: 10.1152/ajpcell.00508.2002.
16. Albinsson S, Nordström I, Hellstrand P. Stretch of the vascular wall induces smooth muscle differentiation by promoting actin polymerization. *J Biol Chem*. 2004;279:34849–34855. doi: 10.1074/jbc.M403370200.
17. Hellstrand P, Albinsson S. Stretch-dependent growth and differentiation in vascular smooth muscle: role of the actin cytoskeleton. *Can J Physiol Pharmacol*. 2005;83:869–875. doi: 10.1139/y05-061.
18. Spector I, Shochet NR, Kashman Y, Groweiss A. Latrunculins: novel marine toxins that disrupt microfilament organization in cultured cells. *Science*. 1983;219:493–495.
19. Albinsson S, Hellstrand P. Integration of signal pathways for stretch-dependent growth and differentiation in vascular smooth muscle. *Am J Physiol Cell Physiol*. 2007;293:C772–C782. doi: 10.1152/ajpcell.00622.2006.
20. Klamut HJ, Gangopadhyay SB, Worton RG, Ray PN. Molecular and functional analysis of the muscle-specific promoter region of the Duchenne muscular dystrophy gene. *Mol Cell Biol*. 1990;10:193–205.
21. Chamley-Campbell J, Campbell GR, Ross R. The smooth muscle cell in culture. *Physiol Rev*. 1979;59:1–61.
22. Feil S, Fehrenbacher B, Lukowski R, Essmann F, Schulze-Osthoff K, Schaller M, Feil R. Transdifferentiation of vascular smooth muscle cells to macrophage-like cells during atherogenesis. *Circ Res*. 2014;115:662–667. doi: 10.1161/CIRCRESAHA.115.304634.
23. Aikawa M, Sakomura Y, Ueda M, Kimura K, Manabe I, Ishiwata S, Komiyama N, Yamaguchi H, Yazaki Y, Nagai R. Redifferentiation of smooth muscle cells after coronary angioplasty determined via myosin heavy chain expression. *Circulation*. 1997;96:82–90.
24. Bergdahl A, Gomez MF, Wihlborg AK, Erlinge D, Eyjolfson A, Xu SZ, Beech DJ, Dreja K, Hellstrand P. Plasticity of TRPC expression in arterial smooth muscle: correlation with store-operated Ca2+ entry. *Am J Physiol Cell Physiol*. 2005;288:C872–C880. doi: 10.1152/ajpcell.00334.2004.
25. Beall B, Chalovich JM. Fesselin, a synaptopodin-like protein, stimulates actin nucleation and polymerization. *Biochemistry*. 2001;40:14252–14259.
26. Albinsson S, Suarez Y, Skoura A, Offermanns S, Miano JM, Sessa WC. MicroRNAs are necessary for vascular smooth muscle growth, differentiation, and function. *Arterioscler Thromb Vasc Biol*. 2010;30:1118–1126. doi: 10.1161/ATVBAHA.109.200873.
27. Walsh MP, Cole WC. The role of actin filament dynamics in the myogenic response of cerebral resistance arteries. *J Cereb Blood Flow Metab*. 2013;33:1–12. doi: 10.1038/jcbfm.2012.144.

28. Long X, Cowan SL, Miano JM. Mitogen-activated protein kinase 14 is a novel negative regulatory switch for the vascular smooth muscle cell contractile gene program. *Arterioscler Thromb Vasc Biol*. 2013;33:378–386. doi: 10.1161/ATVBAHA.112.300645.
29. Zeidan A, Paylor B, Steinhoff KJ, Javadov S, Rajapurohitam V, Chakrabarti S, Karmazyn M. Actin cytoskeleton dynamics promotes leptin-induced vascular smooth muscle hypertrophy via RhoA/ROCK- and phosphatidylinositol 3-kinase/protein kinase B-dependent pathways. *J Pharmacol Exp Ther*. 2007;322:1110–1116. doi: 10.1124/jpet.107.122440.
30. Lagna G, Ku MM, Nguyen PH, Neuman NA, Davis BN, Hata A. Control of phenotypic plasticity of smooth muscle cells by bone morphogenetic protein signaling through the myocardin-related transcription factors. *J Biol Chem*. 2007;282:37244–37255. doi: 10.1074/jbc.M708137200.
31. Yoshida T, Sinha S, Dandré F, Wamhoff BR, Hoofnagle MH, Kremer BE, Wang DZ, Olson EN, Owens GK. Myocardin is a key regulator of CArG-dependent transcription of multiple smooth muscle marker genes. *Circ Res*. 2003;92:856–864. doi: 10.1161/01.RES.0000068405.49081.09.
32. Hayashi K, Morita T. Differences in the nuclear export mechanism between myocardin and myocardin-related transcription factor A. *J Biol Chem*. 2013;288:5743–5755. doi: 10.1074/jbc.M112.408120.
33. Sun Q, Chen G, Streb JW, Long X, Yang Y, Stoeckert CJ Jr, Miano JM. Defining the mammalian CArGome. *Genome Res*. 2006;16:197–207. doi: 10.1101/gr.4108706.
34. Galvagni F, Lestingi M, Cartocci E, Oliviero S. Serum response factor and protein-mediated DNA bending contribute to transcription of the dystrophin muscle-specific promoter. *Mol Cell Biol*. 1997;17:1731–1743.
35. Schroeter MM, Beall B, Heid HW, Chalovich JM. *In vitro* characterization of native mammalian smooth-muscle protein synaptopodin 2. *Biosci Rep*. 2008;28:195–203. doi: 10.1042/BSR20080079.
36. Leinweber BD, Fredricksen RS, Hoffman DR, Chalovich JM. Fesselin: a novel synaptopodin-like actin binding protein from muscle tissue. *J Muscle Res Cell Motil*. 1999;20:539–545.
37. Schroeter MM, Beall B, Heid HW, Chalovich JM. The actin binding protein, fesselin, is a member of the synaptopodin family. *Biochem Biophys Res Commun*. 2008;371:582–586. doi: 10.1016/j.bbrc.2008.04.134.
38. Weins A, Schwarz K, Faul C, Barisoni L, Linke WA, Mundel P. Differentiation- and stress-dependent nuclear cytoplasmic redistribution of myopodin, a novel actin-bundling protein. *J Cell Biol*. 2001;155:393–404. doi: 10.1083/jcb.200012039.
39. Schroeter MM, Orlova A, Egelman EH, Beall B, Chalovich JM. Organization of F-actin by Fesselin (avian smooth muscle synaptopodin 2). *Biochemistry*. 2013;52:4955–4961. doi: 10.1021/bi4005254.
40. Hoffman EP, Brown RH Jr, Kunkel LM. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Cell*. 1987;51:919–928.
41. Kumar A, Khandelwal N, Malya R, Reid MB, Boriek AM. Loss of dystrophin causes aberrant mechanotransduction in skeletal muscle fibers. *FASEB J*. 2004;18:102–113. doi: 10.1096/fj.03-0453com.
42. Lees D, Fabbrizio E, Mornet D, Harricane MC, Travo P. Dystrophin (Xp21), a new phenotype marker of cultured rat aortic myocytes. *Exp Cell Res*. 1994;210:230–235. doi: 10.1006/excr.1994.1034.
43. Rauch U, Shami A, Zhang F, Carmignac V, Durbeej M, Hultgårdh-Nilsson A. Increased neointimal thickening in dystrophin-deficient mdx mice. *PLoS One*. 2012;7:e29904. doi: 10.1371/journal.pone.0029904.
44. Deconinck AE, Rafael JA, Skinner JA, Brown SC, Potter AC, Metzinger L, Watt DJ, Dickson JG, Tinsley JM, Davies KE. Utrophin-dystrophin-deficient mice as a model for Duchenne muscular dystrophy. *Cell*. 1997;90:717–727.
45. Mancinelli R, Tonali P, Romani R, Tringali A, Vargiu R, AZZENA GB. Mechanical properties of smooth muscle portal vein in normal and dystrophin-deficient (mdx) mice. *Exp Physiol*. 1999;84:929–940.
46. Boland B, Himpens B, Casteels R, Gillis JM. Lack of dystrophin but normal calcium homeostasis in smooth muscle from dystrophic mdx mice. *J Muscle Res Cell Motil*. 1993;14:133–139.
47. Harricane MC, Fabbrizio E, Lees D, Prades C, Travo P, Mornet D. Dystrophin does not influence regular cytoskeletal architecture but is required for contractile performance in smooth muscle aortic cells. *Cell Biol Int*. 1994;18:947–958.
48. Deng JT, Van Lierop JE, Sutherland C, Walsh MP. Ca2+-independent smooth muscle contraction. a novel function for integrin-linked kinase. *J Biol Chem*. 2001;276:16365–16373. doi: 10.1074/jbc.M011634200.
49. Weber LP, Van Lierop JE, Walsh MP. Ca2+-independent phosphorylation of myosin in rat caudal artery and chicken gizzard myofilaments. *J Physiol*. 1999;516 (Pt 3):805–824.
50. Loufrani L, Matrougui K, Gorny D, Duriez M, Blanc I, Lévy BI, Henrion D. Flow (shear stress)-induced endothelium-dependent dilation is altered in mice lacking the gene encoding for dystrophin. *Circulation*. 2001;103:864–870.
51. Zeidan A, Nordström I, Dreja K, Malmqvist U, Hellstrand P. Stretch-dependent modulation of contractility and growth in smooth muscle of rat portal vein. *Circ Res*. 2000;87:228–234.

Significance

The vascular smooth muscle cells possess a remarkable ability to alter their phenotype in response to environmental cues. This property allows the smooth muscle cells to adjust to changes in intraluminal pressure and flow and to react to vascular injury. However, excessive changes in smooth muscle phenotype can be detrimental and result in vascular diseases such as hypertension and vascular stenosis. To identify potential targets for therapeutic intervention of these conditions, it is crucial to understand the mechanisms for vascular smooth muscle phenotype regulation including the genes that are involved in determining various aspects of smooth muscle function. This study highlights the important role of actin polymerization in smooth muscle phenotype regulation and identifies novel actin–regulated proteins that control smooth muscle function.