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


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;35:00-00. 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. 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. 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). In the nucleus, MRTF acts as a cofactor to the transcription factor serum response factor (SRF), which binds to so-called SRF-binding element (CArG) in the promoter region of smooth muscle markers, resulting in increased transcription. The protein expression of these markers then determines the fate of SMCs and regulates their contractile function.

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. Recently, several novel smooth muscle markers have been identified, primarily by studies performed by Miano and coworkers. 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...
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 to our knowledge has 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,8 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

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.15–19 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 GapmeR, 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; 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 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 including chloride channels Clca1,
Clca2, and Cfr 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. Using bioinformatic analysis, we confirmed the presence of CARG boxes in the Dmd promoter. Furthermore, in the Synpo2 gene, we identified a consensus CARG sequence (CCTTTTAAGG) at position 722 relative to

![Figure 2](http://atvb.ahajournals.org/figure/2.jpg)

**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.
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. 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.

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 SM22α (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 Cica1, Cica2, 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 SM22α, Dmd, and Synpo2 in human renal arteries and SMCs cultured from the same arteries. The expression of SM22α, 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 SM22α in these cells (Figure 4E).

Phenotypic modulation of SMCs is observed in several vascular disease states including restenosis after angioplasty. 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 diluted 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. 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 SM22α (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 Mechano.sensing

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.
As shown in Figure 5A, the contractile response to depolarization by high K⁺ (60 mmol/L) was reduced by 30% (P≤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 of maximal contraction (t½) between wild-type (WT) and mdx arteries (t½±SEM; WT: 251.7±16.9 versus KO: 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 α₁-adrenergic agonist cirazoline. A rightward shift of the dose–response curve for cirazoline was observed (EC50 WT: 37±4.2 versus mdx 52±4.7 mmol/L; P<0.05) and contractile force was significantly reduced at a concentration of 0.1 mmol/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 mmol/L (Figure 5D).

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 ratio 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.

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 SM22α (Figure 6C and 6D). Reciprocal coimmunoprecipitation demonstrated that Synpo2 interacts with α-actin (Figure 6E and 6F).

As shown in Figure 5A, another alternative explanation for this is the presence of actin regulatory proteins associated with the structural contractile machinery. 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.
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 2 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, cardiomyocytes, and SMCs. 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 reduced by 72 hours. Although other mechanisms are likely to be involved, these results suggest that the loss of actin filaments is an important mechanism 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. 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, 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. 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. 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 Ca2+-calmodulin, α-actinin, and smooth muscle myosin. The avian smooth muscle homologue, fesselin, and the Synpo2 gene splice variant, myopodin, which is mainly expressed in skeletal muscle, 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. We found that Synpo2 mRNA and protein were abundant in differentiating 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 gene expression 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. Although Dmd has been suggested to be a marker of differentiated smooth muscle, 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.

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. 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. 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. 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. 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. 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. 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 distension where the portal vein is stretched to its optimal length for force development. In previous work, we have demonstrated that physiological stretch of the portal vein promotes both contractile differentiation and growth of the smooth muscle. 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

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**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 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 hypertrophy and 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.
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**Supplementary Material**

### Genes up-regulated following actin stabilization with jasplakinolide

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**Supplementary figure I.** The 48 most up-regulated genes (>1.4 fold) following actin stabilization with jasplakinolide. Gene expression analysis was performed using Affymetrix mouse gene array on cultured smooth muscle cells treated with the actin stabilizing agent jasplakinolide (100 nM) for 24 hours.
Supplementary figure II. Down-regulation of gene expression by jasplakinolide. Several genes were found to be downregulated by jasplakinolide using micro array analysis. The effect of jasplakinolide (jasp) on some of these genes was confirmed using qPCR including calcium-activated chloride channel regulator 1 and 2 (Clca1 and Clca2), Prominin 1 (Prom1), Decorin (Dcn) and Cystic fibrosis transmembrane conductance regulator (Cftr). *p<0.05, ***p<0.001
Supplementary figure III. Genes that are down-regulated by jasplakinolide demonstrate increased expression in passage 2-4 (p2-4) cultured aortic smooth muscle cells versus intact aorta. These genes include calcium-activated chloride channel regulator 1 and 2 (Clca1 and Clca2), Prominin 1 (Prom1), Decorin (Dcn) and Cystic fibrosis transmembrane conductance regulator (Cftr). Unlike the other genes, decorin was not increased until passage 4 (p4), ***p<0.001
Supplementary figure IV. Stabilization of actin filaments with jasplakinolide partially reverses the down-regulation of dystrophin and synaptopodin 2 in cultured vascular smooth muscle cells. Vascular smooth muscle cells (SMC) were isolated from mouse aorta and cultured to passage 2. Cells were then treated with 100nM jasplakinolide for 24 or 72 hours. Expression levels of dystrophin (Dmd) and synaptopodin 2 (Synpo2) were analyzed by qPCR. n=3-6 *p<0.05, **p<0.01, ***p<0.001 vs SMC Ctrl.
Materials and methods

Ethics statement

All animal work was conducted according to national and international guidelines and approved by The Malmö/Lund ethical committee on animal experiments (M260-11, M113-13). Collection of human vessels was approved by the research ethics committee of Lund University (LU 481-00) and performed after informed consent of the patients. C57Bl/6 mice were purchased from Taconic (Denmark). Mdx-mice (C57BL/10ScSn-mdx/J) were obtained from Jackson Laboratory.

Cell culture and adenoviral transduction

Vascular smooth muscle cells were isolated from mouse aorta by enzymatic digestion and maintained in culture as described previously. Mouse aortic smooth muscle cells from passage 3 to 5 were cultured in DMEM/Ham’s F12 medium (Biochrom, FG 4815) supplemented with 10% fetal bovine serum (Biochrom, S 0115) and 50U/50µg/ml penicillin/streptomycin (Biochrom, A 2212). The cells were treated with 100nM Jasplakinolide (TOCRIS Bioscience, Bristol, UK, cat no. 2792) or 0,1% DMSO as a control for 24 hours. To determine actin depolymerizing effect intact mouse aorta cultured in DMEM/Ham’s F12 medium supplemented with 2% dialyzed fetal bovine serum, 10nM insulin (Sigma, I6634) and 50U/50µg/ml penicillin/streptomycin was incubated with 250nM Latrunculin B (Calbiochem, cat no. 76343-94-7) or 0,05% DMSO as a control for 24h. Overexpression of myocardin and MRTF-A was performed by adenoviral transduction as described previously. Briefly, cells were transduced using cytomegalovirus promoter-driven adenoviral constructs: 20 MOI Ad.MRTF-A, 100 MOI Ad.Myocd and corresponding concentration of empty vector Ad.CMV for 96 hours in DMEM/Ham’s F12 medium supplemented with 10% fetal bovine serum and 50U/50µg/ml penicillin/streptomycin.

Synaptopodin GapmeR transfection

Smooth muscle cells were isolated from mouse aorta by enzymatic digestion and maintained in culture as described previously. Cells in passage 3 and 4 were transfected with Synaptopodin GapmerR 10 nM (Exiqon) or negative control using Oligofectamine transfection reagent (Life Technologies) in OptiMEM media (Gibco, Life Technologies, cat: 11058-021) according to the manufacturer’s instructions. In order to optimize the knockdown efficiency, four separate GapmeR sequences were used at 2.5nM each. The GapmeR sequences were as follows: GM1: ACTTAGACTTTTGCTTC, GM2:CTTCACTCCACTTACA, GM3:GGAATGGATAGGATT, GM4:TTAACGCTTTGAGGT. After 96 hours, cells were harvested for experiments.

Collection and culture of human renal arteries
Human renal arteries belonging to the COLMAH collection of the HERACLES network (http://www.redheracles.net/plataformas/en_coleccion-muestras-arteriales-humanas.html) were obtained from donors at the Clinic Hospitals of Barcelona and Valladolid, with protocols approved by the Human Investigation Ethics Committees of the respective Hospitals. Vessels were divided in two pieces, one of which was placed in RNAlater (Ambion) for RNA extractions and the other in a Dulbecco’s modified Eagle’s medium (DMEM) for cell isolation. VSMCs were isolated from the medial layer of the vessel kept in DMEM after manual removal of both adventitia and endothelial layers under a dissection microscope. Once isolated, the muscle layer was cut in 1 mm² pieces that were seeded in 35 mm Petri dishes treated with 2 % gelatin (Type B from bovine skin, Sigma) in DMEM supplemented with 20 % SFB, penicillin-streptomycin (100 U/ml each), 5 µg/ml fungizone, and 2 mM L-glutamine (Lonza) at 37 °C in a 5% CO₂ humidified atmosphere. Migration and proliferation of VSMCs from the explants was evident within 10-15 days. Confluent cells were trypsinized and seeded at 1/3 density and VSMCs were subjected to several (up to 8) passages in control medium. The composition of this media was DMEM with 5% FBS, penicillin-streptomycin, fungizone and L-glutamine as above, and supplemented with 5 µg/ml Insulin, 1 ng/ml bFGF and 5 ng/ml EGF.

Organ culture of mouse portal vein (PV)

Portal veins were freed from fat and surrounding tissue and mounted on a hook in a test tube containing DMEM/Hams F12 (Biochrom, FG4815) with 2% dialyzed FBS (Biochrom, S0115) and 10nM insulin (Sigma, I6634) as previously described. Vessels were stretched by attaching a 0.3 g gold weight at one end of the vessel. This corresponds to the optimal load for force development. The vessels were incubated in cell culture environment for 24 hours.

Organ culture and balloon injury of human left internal mammary artery (LIMA)

Segments of LIMA were obtained from patients undergoing coronary artery bypass surgery. The vessels were immediately transferred to a tube with ice-cold, sterile calcium-free N-HEPES buffer (composed of 135.5 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl₂, 11.6 mM glucose, and 11.6 mM HEPES, pH 7.4) and dissected free from excessive fat and adventitia. The vessels were then cut into two equal segments (5-7 mm) and placed in a Petri dish containing serum-free DMEM/Ham’s F12 medium (1:1 dilution; Biochrom, FG 4815) supplemented with 50 U/ml penicillin and 50 µg/ml of streptomycin (Biochrom, A 2212) in cell culture incubator at 37°C in a humidified atmosphere of 5%CO₂ as described previously. After overnight equilibration, 2.5x16 mm PTCA balloon dilatation catheter Texas™ Express2™ (Boston Scientific, Natick, MA, USA) was inserted into the lumen of one of the segments and the balloon was inflated to a pressure of 4 atmosphere for 2 min. After 48h the balloon-dilated segment and its corresponding control were collected for further analysis.

In vivo model of proliferation in Sus scrofa coronary arteries.
Coronary intimal lesion (in vivo model) was induced by using a guiding catheter introduced from the right femoral artery to both the circumflex and the anterior descending coronary arteries, where a balloon was inflated three times for 30 s before it was withdrawn. Right coronary arteries were used as control. Four weeks after the surgery the animals underwent euthanasia and arteries samples were collected for RNA/protein extraction with Trizol®. Animal procedures were performed at the animal facilities of the Hospital Clinic of Barcelona, School of Medicine and have been approved by the University of Barcelona Ethics Committee on Animal Experimentation.

RNA isolation and quantitative real-time PCR

Tissues and cultured cells were lysed in 700 ul Qiazol and subsequent RNA isolation was performed using microRNeasy mini kit (Qiagen, 217004) according to the manufacturer’s instructions, including on column DNAse I digestion step. PCR reactions were performed using Quantifast SYBR Green RT-PCR kit (Qiagen, 204156). The reaction conditions were used according to the manufacturer’s instructions. The following Quantitect Primer assays (Qiagen) were used for mRNA detection: Mouse - Mm_Kcnmb1, QT00101500; Mm_Tagln, QT00165179, Mm_Cnn1, QT00105420, Mm_Rn18S, QT02448075, Mm_Lmod1, QT00134463; Mm_Itg8a, QT00170940; Mm_Dmd, QT00161336; Mm_Synpo2, QT01038975; Human – Hs_Rrn18S, QT00199367; Hs_Dmd, QT00085778; Hs_Synpo2, QT00075614; Hs_Tagln, QT01678516. Primers’ sequences are proprietary of Qiagen.

Transcriptome (mRNA) array analysis

mAoSMCs were grown on 6-well plate in 10% FBS (Biochrom, A 2212) containing DMEM/Ham’s F12 medium (Biochrom, FG 4815) to 80% confluency and either vehicle or 100nM jasplakinolide treated (TOCRIS Bioscience, Bristol, UK, cat no. 2792) for 24 hours. The samples were collected and total RNA was isolated using microRNeasy mini kit (Qiagen, 217004). After passing quality control by Agilent Bioanalyzer, total RNA was analyzed by Affymetrix GeneChip® Mouse Gene 1.0 ST Array performed by Swegene Center for Integrative Biology at Lund University (SCI BLU). The microarray data is accessible via the Gene Expression Omnibus (accession number GSE66538, scheduled release on 1 May 2015).

Bioinformatic analysis

Promoter sequences, 5Kb upstream and 1 Kb downstream with respect to the transcription start sites of the corresponding RefSeq genes, were extracted from the UCSC Genome Browser with the build number mm9. CArG box motif sequence transformed to a position weight matrix, which was used to map the CArG box motif to the above promoter sequences. Motif sequences, which have a matrix similarity score <=0.9 were generated using the SMART program 5.

Western blot analysis

Cells grown on 6-well plates were washed twice with ice-cold PBS and lysed on ice directly in the wells with 70-75 µl of 1x Laemmli sample buffer (60 mM Tris-HCl, pH 6.8,
2% SDS, 10% glycerol). Mouse tissues were carefully dissected, weighed and snap frozen in liquid nitrogen (N₂). Frozen tissue was pulverized using liquid N₂ and 100ul of 1x Laemmli sample buffer was added per 20mg of tissue. After protein determination using Biorad DC protein assay, bromophenol blue and β-mercaptoethanol were added to the samples at final concentrations of 0.005% and 5%, respectively. Equal amounts (10-20µg) of protein were loaded in each lane of Bio-Rad TGX 4-15% Criterion gels. Proteins were detected using commercially available primary antibodies: Dystrophin (1:200, Abcam, ab152771), Synaptopodin2 (1:600, Abcam, ab103710), SM22α (1:5000, Abcam, ab14106). Secondary mouse or rabbit HRP-conjugated antibodies (#7074, #7076 1:5000 or 1:10000, Cell Signaling) were used. Bands were visualized using ECL (Pierce West Femto) and images were acquired using the Odyssey Fc Imager (LI-COR Biosciences).

**Immunoprecipitation**

Mouse bladders were dissected, snap frozen and pulverized. Immunoprecipitation was performed using a Pierce Classic IP Kit (Thermo Scientific, 26146). 200 µl of lysis buffer was added to each sample and after protein determination, 200 µg of each sample was used to form the immune complex with 10 µg of the antibodies (Synaptopodin2, Abcam, ab103710 or α-actin, Sigma, A5228). Two samples were used as controls where no antibody was added. The immune complex was eluted and applied on a Bio-Rad TGX 4-15% Criterion gel. Protein detection was performed using the primary α-actin (1:2000) and Synaptopodin2 (1:500) antibodies and mouse or rabbit HRP-conjugated secondary antibodies (#7074, #7076 1:5000 or 1:10000, Cell Signaling).

**F/G actin assay**

Mouse aortas were incubated for 20 minutes in 1mg/ml collagenase (Worthington, LS004176) solution in DMEM/Ham’s F12 media (Biochrom, FG 4815). This step allowed for the removal of adventitia without mechanically destroying smooth muscle. Cells were transfected with synpo2 GM or negative control as described in ‘Synaptopodin GapmeR transfection’ of material and methods. The preparations were snap frozen in liquid nitrogen and stored at -80°C for further analysis. F/G actin assay was performed using G–actin/F –actin in vivo assay kit (Cytoskeleton, Cat.#BK037). Briefly, the frozen samples were homogenized and suspended in lysis buffer provided with the kit, LAS02 containing ATP and protease inhibitor cocktail. Mouse aortic smooth muscle cells grown until passage 2 in 10% FBS, DMEM/Ham’s F12 medium were also collected in LAS02. F-actin was pelleted by centrifugation at high speed (100,000 g) using Beckman ultracentrifuge at 37°C for 1 hour. G-actin was transferred to fresh test tubes. F-actin pellet was dissolved in F-actin depolymerizing buffer and lysed on ice for 1 hour (pipetting every 15 min). Equal volumes of filamentous and globular actin fraction (10ul) were loaded on the gel. Proteins were transferred to nitrocellulose membrane using Trans Turbo Blot device (Bio-Rad) for 10min at 2.5 A. The membrane was then incubated with rabbit smooth muscle alpha-actin antibody (provided with the kit, Cat. # AAN01). Anti-rabbit HRP-conjugated secondary antibody (Cell Signaling, #7076
1:10,000) was used. Bands were visualized using ECL (Pierce West Femto) and images were acquired using the Odyssey Fc Imager (LI-COR Biosciences).

**Isometric force measurements**

Mouse tail arteries were cut in 2 mm rings and mounted on steel wires in a myograph chambers (610M, Danish Myo Technology) in HEPES buffer (135.5 mM NaCl, 5.9 mM KCl, 2.5 mM CaCl2, 1.2 mM, MgCl2, 11.6 mM glucose, and 11.6 mM HEPES, pH 7.4) as previously described\(^6\). The rings were contracted twice for 7 minutes with 60mM KCl-containing HEPES buffer and were interspersed by 25 min relaxation periods. This was followed by cirazoline concentration-response curve. After applying the last concentration of cirazoline, the preparations were relaxed by addition of decreasing concentrations of sodium nitroprusside (SNP). At the end of each experiment, 1µM calyculin A was applied in calcium-free HEPES buffer.

**Statistical analysis**

Quantitative data are presented as mean ± S.E.M. Data were analyzed using GraphPad Prism Software 5. Statistical analysis was performed by student’s t-test or one-way analysis of variance (ANOVA) for multiple comparisons. Differences were considered significant when p<0.05.

**References**