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

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Regulation of smooth muscle dystrophin and synaptopodin 2 expression by actin polymerization and vascular injury

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Abstract

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 up-regulated 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 MRTF-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.

Abbreviations:

CArG box – serum response factor-binding element;
Cnn1 – smooth muscle calponin transcript;
Dmd – dystrophin transcript;
F-actin – filamentous actin;
G-actin – globular actin;
GAPDH – glyceraldehyde-3-phosphate dehydrogenase;
HSP90 – heat shock protein;
Itga8 – integrin alpha8 transcript;
Jasp – jasplakinolide;
Kcnmb1 – β1 subunit of large conductance calcium-activated potassium channel transcript;
LatB – latrunculinB;
Lmod1 – leiomodin1 transcript,
mdx – dystrophin deficient mouse;
MRTF – myocardin-related transcription factor;
Myh11 – smooth muscle myosin heavy chain transcript;
SMC – smooth muscle cells;
SRF- serum response factor;
Synpo2 – synaptopodin2 transcript
ROCK - Rho-associated coiled-coil forming protein kinase;
Tagln – SM22α transcript

Introduction

Vascular smooth muscle cells 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 a number of cardiovascular diseases such as hypertension and post-angioplasty restenosis [1]. The synthetic or proliferative phenotype of smooth muscle cells 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/ROCK-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 so-called CArG elements in the promoter region of smooth muscle markers, resulting in increased transcription [5]. The protein expression of these markers then determines the fate of smooth muscle cells and regulates their contractile function [2].

The classical smooth muscle markers include α-actin and actin binding proteins such as SM22α, calponin and myosin heavy chain. These markers are enriched in smooth muscle cells 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 co-workers [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 co-factors 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 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, which stabilizes actin filaments [17-19]. Two genes, dystrophin and synaptopodin 2, 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, by using dystrophin mutant mice and synaptopodin 2 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 dystrophin and synaptopodin 2 is promoted by jasplakinolide-induced actin polymerization

Actin polymerization is known to promote the expression of established smooth muscle markers. In order to comprehensively clarify the effect of actin polymerization on smooth muscle gene expression we performed a gene array on mouse aortic smooth muscle cells (SMC) treated with jasplakinolide (Jasp, 100nM) or vehicle (Ctrl) for 24 hours. This analysis uncovered 48 genes which were up-regulated at least 1.4-fold by Jasp treatment (Supplementary figure I). A transcription factor binding site analysis of the 135 most up-regulated (>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 up-regulation 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 alpha 8 (*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 actin-binding proteins dystrophin and synaptopodin-2 (Figure 1A). Jasp-induced up-regulation of mRNA expression was confirmed for selected genes using qPCR analysis (Figure 1B-E). 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 dystrophin and synaptopodin 2 are regulated by actin polymerization in smooth muscle and may be novel markers of the contractile phenotype. Several genes were also down-regulated by Jasp including chloride channels *Clca1, Clca2,*and *Cftr* as well as the glycoproteins *Prom1* and *Dcn* (Supplementary figure II).

Dystrophin and synaptopodin 2 are highly expressed in differentiated smooth muscle

In order to clarify the relative smooth muscle specificity of dystrophin and synaptopodin 2 we analyzed the mRNA expression of these genes in aorta and urinary bladder compared with several other tissues (Figure 2A-B). The expression of the smooth muscle marker myosin heavy chain was used as a positive control (Figure 2 C). The results demonstrate a specific expression of both dystrophin and synaptopodin2 in muscle and both genes exhibited higher expression levels in smooth vs. striated muscle tissues.

The results of the qPCR analysis were confirmed on the protein level by western blot analysis using SM22α as a positive control (Figure 2D-G). Protein expression was normalized to Commassie Blue stained total protein content since none of the commonly used endogenous loading controls (GAPDH, HSP90, β-actin, 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 Jasplakinolide-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 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 smooth muscle cells with adenovirus expressing either myocardin (Ad.Myocd) or MRTF-A (Ad.MRTF-A) for 96 hours. Over-expression of either Myocardin or MRTF-A significantly induced the transcription of dystrophin, synaptopodin 2 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-F).

Reduced F/G actin ratio in cultured smooth muscle cells correlates with down-regulation of dystrophin and synaptopodin 2

Isolated smooth muscle cells in culture represent an *in vitro* model of phenotypically modified smooth muscle. These cells share many of the features that characterize smooth muscle cells 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].

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

The reduced actin polymerization in synthetic smooth muscle cells correlated with a substantially down-regulated mRNA expression of dystrophin and synaptopodin 2 (Figure 4B). In fact, the down-regulation 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, D). Interestingly, we found that several of the genes that were down-regulated by Jasp, including *Clca1, Clca2,Cftr, Prom1* and *Dcn* were in fact up-regulated in synthetic smooth muscle cells (Supplementary figure III). The down-regulation of Synpo2 and Dmd in cultured smooth muscle cells could be partially reversed by jasplakinolide (Supplementary figure IV).To address the importance and species generality of our findings, we also analyzed expression of SM22α, dystrophin and synaptopodin 2 in human renal arteries and smooth muscle cells cultured from the same arteries. The expression of SM22α, which again served as a positive control, was decreased in cultured human renal arterial smooth muscle cells by 0.6-fold compared to the intact artery (Figure 4E). Consistent with the results obtained from mouse aortic smooth muscle, the expression levels of dystrophin and synaptopodin 2 were more dramatically down-regulated than SM22α in these cells. (Figure 4E).

Phenotypic modulation of smooth muscle cells is observed in several vascular disease states including restenosis following angioplasty [23]. We thus aimed at determining the effect of balloon dilation of human arteries on the expression of dystrophin and synaptopodin 2. 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 up-regulation of the calcium channel TRPC1, which is involved in smooth muscle proliferation and neointima formation [24]. Herein, quantitative PCR-analysis revealed that the mRNA expression of dystrophin and synaptopodin 2 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 synaptopodin 2 and dystrophin were downregulated at the protein level four weeks after injury. Taken together, these findings show that dystrophin and synaptopodin 2 conform to established patterns of regulation for contractile SMC markers.

Loss of dystrophin results in impaired vascular smooth muscle contraction, relaxation and mechanosensing

The importance of dystrophin for vascular smooth muscle contractile function was investigated usingtail artery rings from control and dystrophin mutant *(mdx)* mice mounted in wire myographs. As shown in Figure 5A, the contractile response to depolarization by high K^+ (60mM) was reduced by 30% ($p \leq 0.001$) in arteries from *mdx* mice compared to 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* smooth muscle cells (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 ($t_{½}$) between WT and *mdx* arteries ($t_{1/2}$ ±S.E.M; WT: 251.7±16.9 vs KO:276.8±33.4). It is therefore likely that the effect involves the structural contractile machinery, but loss of dystrophin did not directly affect the F/G-actin ratio in *mdx* smooth muscle (Figure 5B).

The significance of dystrophin 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±4.2nM vs *mdx* 52±4.7nM; $p<0.05$) and contractile force was significantly reduced at a concentration of 0.1 µM (Figure 5C). To test smooth muscle-dependent relaxation in *mdx* mice, tail arteries were pre-contracted with cirazoline and then stimulated with the nitric oxide donor sodium nitroprusside. Dilatation to sodium nitroprusside was attenuated in *mdx* tail arteries compared to the control vessels with significant effects observed at 10-100nM (Figure 5D). Figure 5E and F show 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 synaptopodin 2 is sensitive to physiological longitudinal stretch. Furthermore thai effect was abolished in dystrophin mutant vessels (Figure 5G).

GapmeR mediated knockdown of synaptopodin 2 results in reduced actin polymerization and contractile differentiation

The avian homologe of synaptopodin 2, fesselin, has previously been demonstrated to bind to G-actin and stimulate actin polymerization [25]. To determine if knockdown of synaptopodin 2 is sufficient to cause actin de-polymerization and loss of smooth muscle marker expression, we transfected cultured smooth muscle cells with synaptopodin 2 GapmeRs. A combination of four different GapmeRs was used to achieve maximal knockdown. After 96 h incubation with GapmeRs, the expression of Synaptopodin 2 was reduced by approximately 62% (Figure 6A). Interestingly, knockdown of synaptopodin 2 caused a dramatic reduction in the F/G-actin ratio in smooth muscle cells 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 dystrophin and SM22α (Figure 6C and D). Reciprocal co-immunoprecipitation demonstrated that synaptopodin 2 interacts with α-actin (Figure 6 E and F).

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 co-workers [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 a number of genes that are transcriptionally activated by actin polymerization and further characterized two of these genes, dystrophin and synaptopodin 2. We show that these genes are highly expressed in differentiated smooth muscle and that their expression is dramatically reduced in phenotypically modulated smooth muscle. Following balloon dilation of human LIMA vessels *ex vivo* and of pig coronary arteries *in vivo*, we found a reduced expression of both dystrophin and synaptopodin 2. In dystrophin 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 smooth muscle cells [10, 28-30]. Herein, we demonstrate that the ratio of filamentous to globular actin is dramatically decreased in phenotypically modulated smooth muscle cells. By stabilizing actin filaments in cultured cells with jasplakinolide, 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 loss of actin filaments is an important mechanisms for the reduced expression of contractile markers during phenotypic modulation of smooth muscle cells.

By screening genes that were induced by actin polymerization, we identified a number of 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 dystrophin and synaptopodin 2. The transcripts levels of both synaptopodin 2 and dystrophin 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 jasplakinolide confirms that a reduction of the G-actin pool is required for MRTF to have its full effect. The expression of synaptopodin 2 and dystrophin is however not solely dependent on actin polymerization, since 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 one or more CC[A/T]6GG motifs, which are SRF binding sites called CArG-boxes. [33] In humans, the dystrophin gene contains one validated CArG-box at -91bp relative to the transcription activation site, and it has been demonstrated that SRF binds to the dystrophin promoter and regulates its transcription in striated muscle [34, 35]. 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 synaptopodin 2 has previously been characterized *in vitro* in rabbit smooth muscle, where two different isoforms were shown to bind Ca^{2+}-calmodulin, α-actinin and smooth muscle myosin [36]. The avian smooth muscle homologue, fesselin [37, 38] and the synaptopodin 2 gene splice variant, myopodin, which is mainly expressed in skeletal muscle [39] 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, 40]. We found that synaptopodin 2 mRNA and protein were abundant in differentiated vascular smooth muscle and dramatically reduced in proliferating smooth muscle cells. Interestingly, the expression of synaptopodin 2 was also significantly reduced in human arteries following balloon dilation, indicating a potential role in restenosis following angioplasty. In accordance with previous reports we found that synaptopodin 2 interacts with actin and plays a key role in actin polymerization. Cells transfected with GapmeRs against synaptopodin 2 exhibited reduced actin polymerization and smooth muscle differentiation. Thus it is likely that synaptopodin 2 is required for proper expression of smooth muscle gene expression via its effect on actin.

The dystrophin protein is a part of the dystrophin associated protein complex, which links the extracellular matrix to the cytoskeleton. As such, dystrophin is an important component of mechanotransduction in striated muscle and mutations in the dystrophin gene result in muscular dystrophy and cardiomyopathy [41, 42]. Although dystrophin has been suggested to be a marker of differentiated smooth muscle [43, 44], its importance for vascular smooth muscle function has not been studied extensively. However, in dystrophin mutant *mdx* mice, it was recently demonstrated that dystrophin deficiency results in accelerated neointima formation following vascular injury [44]. In accordance with these studies we found that dystrophin, was significantly reduced in phenotypically modified smooth muscle cells and balloon-dilated human arteries suggesting that loss of dystrophin could be an important mechanism for the development of vascular disease. It is well known that the lack of dystrophin in *mdx* mice is partly compensated for by its homologue utrophin [45]. Despite this compensatory effect, we found that deletion of dystrophin in *mdx* mice results in abnormal contractile function of vascular smooth muscle and loss of stretch-induced gene transcription of synaptopodin 2. 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 [46].The effect is not due to aberrant calcium signaling or calcium sensitivity since calcium-independent contractile responses to the phosphatase inhibitor calyculin A were reduced to a similar degree. Furthermore, the level of calcium in *mdx* smooth muscle cells has been reported to be the same as in the control smooth muscle [47]. Considering that dystrophin is an actin binding protein it is conceivable that loss of dystrophin 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 smooth muscle cells [48]. This suggests that it is rather the function, than the amount, of actin filaments that is affected in dystrophin mutant mice. Possibly, the absence of dystrophin-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 due to a defect in calcium-independent myosin phosphorylation [49, 50]. 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 smooth muscle cells was negatively affected by loss of smooth muscle dystrophin, which supports the functional importance of dystrophin in smooth muscle vasoregulation.

The importance of dystrophin in mechanosensing has been demonstrated in endothelial cells where it is involved in flow induced dilation of mouse carotid and small mesenteric arteries [51]. Our results indicate that part of this effect may be due to a reduced ability of smooth muscle cells to relax to nitric oxide stimulation, at least during isometric force measurement. However, since dystrophin 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 synaptopodin 2 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 [52]. 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, 52]. The method is fundamentally different from the acute non-physiological stretch applied when using balloon-dilation of human arteries, which results in an injury response in the vessel wall.

In summary, the expression of a number of MRTF-regulated, actin binding proteins including dystrophin and synaptopodin 2, 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 smooth muscle cells.

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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 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. In order 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.

Figure legends

Figure 1. Stabilization of actin filaments promoted the expression of dystrophin and synaptopodin 2. A, The expression of selected genes from an Affymetrix gene array of mouse aortic smooth muscle cells stimulated with 100nM jasplakinolide (Jasp) for 24 hours (n=4). **B-E,** Confirmation of array results of Myh11 (**B**), Kcnmb1 (**C**), Dmd (**D**), Synpo2 (**E**) by individual qPCR reactions (*white bars*). Black bars represent treatment of mouse aorta with actin depolymerizing agent, Latrunculin B (latB, 250nM) for 24h (n=3-5). $***p<0.05$, $**p<0.01$, $*p<0.5$. Myh11, smooth muscle myosin heavy chain; Tagln, SM22α; Cnn1, smooth muscle calponin; Itga8, integrin alpha 8; Lmod1, leiomodin; Kcnmb1, β-subunit of large conductance calcium-activated potassium channel; Dmd, dystrophin; Synpo2, synaptopodin 2, SMC – mouse aortic smooth muscle cells.

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

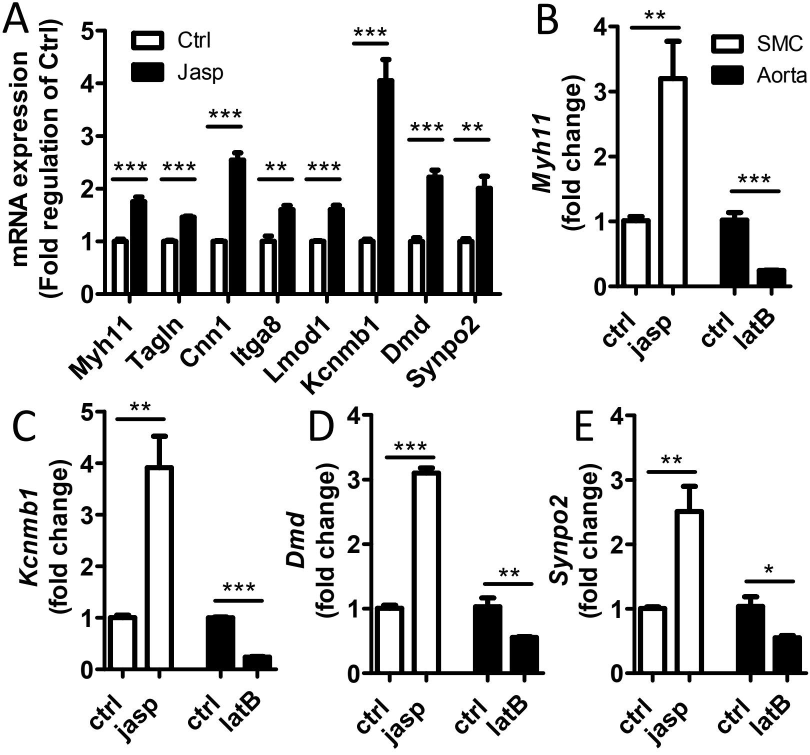
Figure 3. Overexpression of Myocardin and MRTF-A promotes expression of dystrophin and synaptopodin 2. Mouse aortic SMC were transduced with adenovirus expressing either Myocardin (Ad.Myocd, 100 MOI, n=7) or MRTF-A (Ad.MRTF-A, 20 MOI, n=8) for 96 hours. Cells infected with control adenovirus, Ad.CMV (empty vector) 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), dystrophin (Dmd) and synaptopodin 2 (Synpo 2) by qPCR. The effect of actin stabilization by jasplakinolide (Jasp; 100nM) on Ad.LacZ and Ad.MRTF-A transduced cells (20MOI) was evaluated using qPCR for Myh11 (C), Synpo2 (D) and Dmd (E). $**p<0.01$, $***p<0.001$.

Figure 4. Decreased F/G actin ratio in cultured smooth muscle cells correlates with down-regulated mRNA levels of dystrophin and synaptopodin 2. A, Intact mouse aorta without adventitia and cultured mouse aortic smooth muscle cells, with or without jasplakinolide (Jasp, 100 nM) for 24 hours. The F- and G-actin fractions were separated by ultracentifugation and analyzed by western blot. F/G actin ratios and representative blots are shown (n=3). **B**, Quantitative PCR of indicated mRNA and **C,D** Western blot analysis of indicated proteins isolated from intact aorta and cultured smooth muscle cells (SMC, passage2-p2; n=3-6). **C** shows representative blots and **D** shows 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 48h. mRNA levels of SM22α (Tagln), dystrophin (Dmd) and synaptopodin 2 (Synpo 2) 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 dystrophin and synaptopodin 2 in control and balloon-injured pig coronary arteries *in vivo*. Protein analysis was performed 4 weeks after injury and HSP90 was used as loading control. Control (n=2) and injured vessels (n=4) from two separate animals were analyzed *p<0.05, **p<0.01, ***p<0.001.

Figure 5. Dystrophin 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 mM KCl) and calyculin A (1 µM). 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 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** show representative graphs of original recordings of the control (ctrl) and *mdx* mice vascular rings showing response to 60 mM 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 synaptopodin 2 (Synpo 2) was analyzed by qPCR (n=5). *p<0.05, **p<0.01, ***p<0.001, n.s. – not statistically significant.

Figure 6. GapmeR mediated knock-down 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. 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 a-actin was evaluated by immunoprecipitation (IP) of mouse bladder smooth muscle. Representative immunoblots (IB) of a-actin following Synpo2 IP (E) and Synpo2 following a-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.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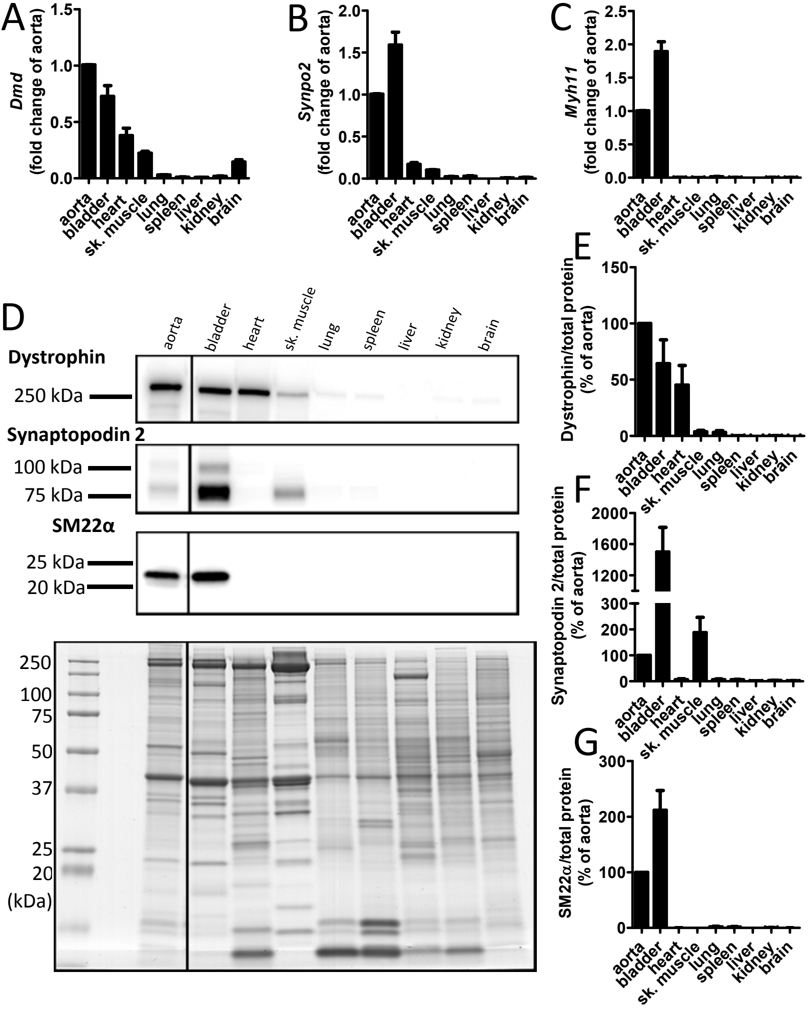
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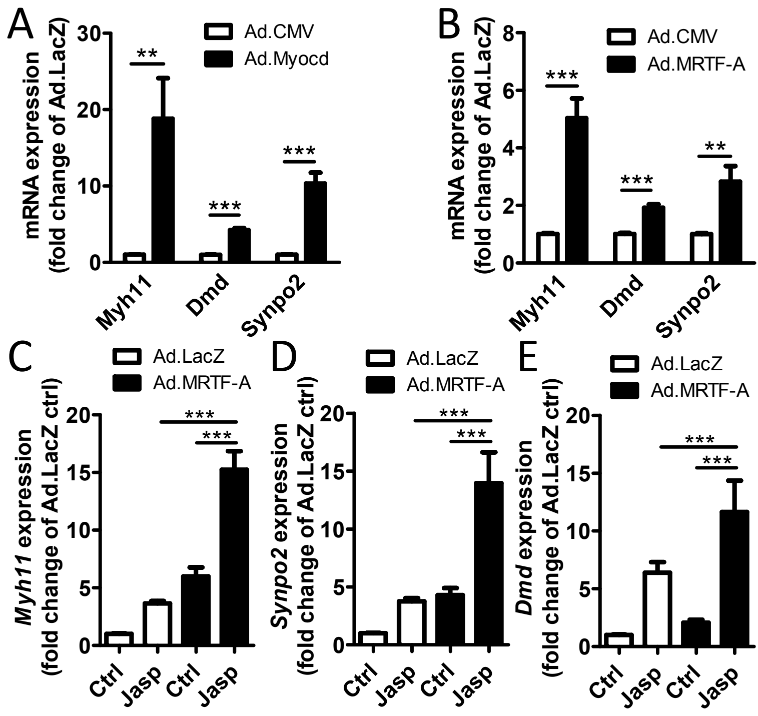
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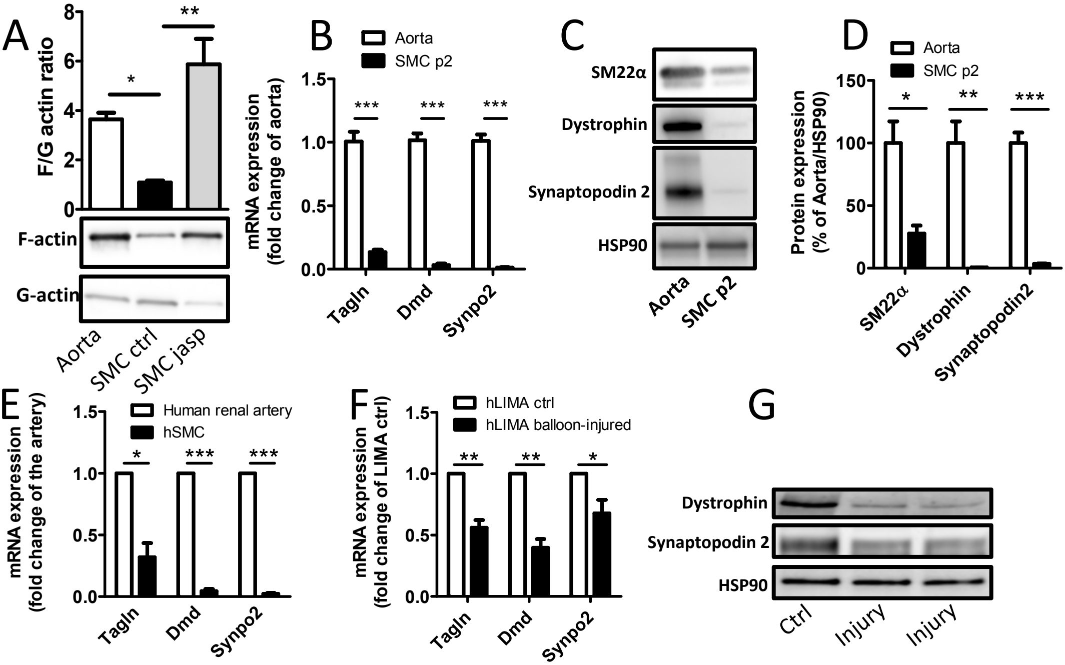
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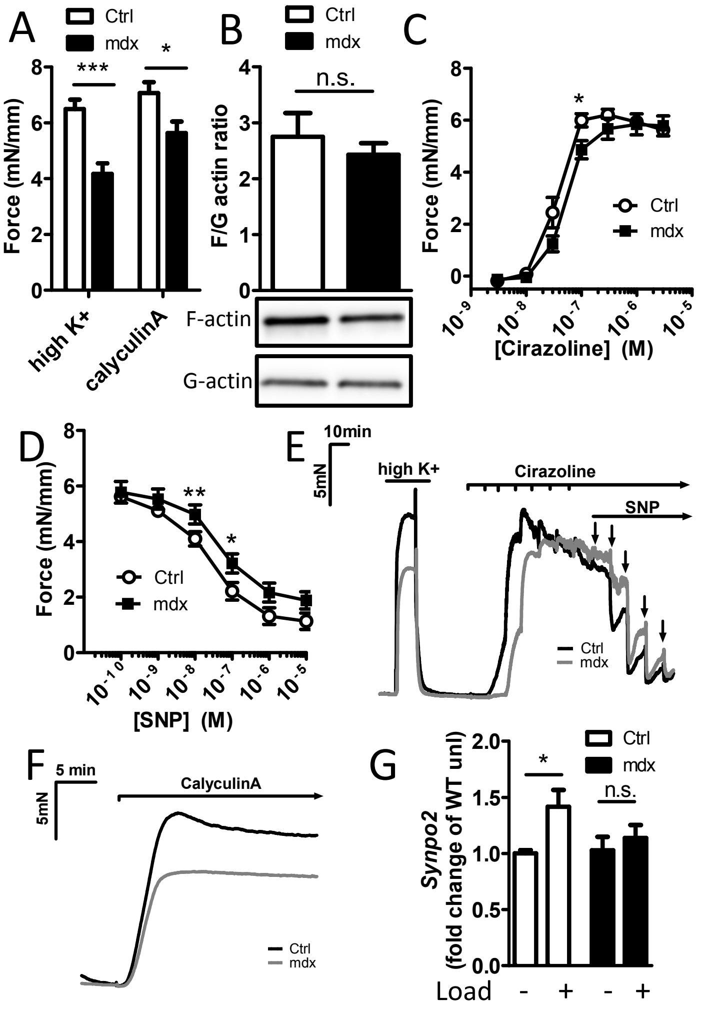
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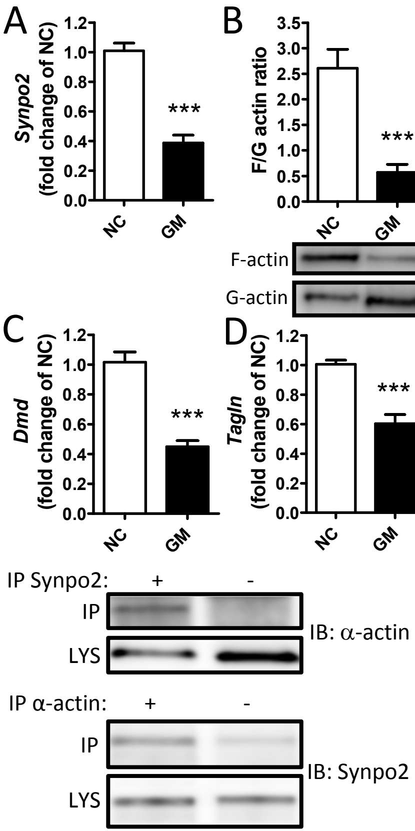
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Materials and Methods

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 [1]. 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. [1, 2] 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 [1]. Cells in passage 3 and 4 were transfected with Synoptopodin 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: ACTTAGACTTTGCTTC, 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 mm^2 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 ºC in a 5% CO_2 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 [3] 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 $MgCl_2$, 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%CO_2 as described previously [4]. 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 Clínic 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_Itga8, 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 (SCIBLU). 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 matix, 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 (N_2). Frozen tissue was pulverized using liquid N_2 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 $p<0.05$.

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