Rationale: Mutations in myosin heavy chain (MYH11) cause autosomal dominant inheritance of thoracic aortic aneurysms and dissections. At the same time, rare, nonsynonymous variants in MYH11 that are predicted to disrupt protein function but do not cause inherited aortic disease are common in the general population and the vascular disease risk associated with these variants is unknown.

Objective: To determine the consequences of the recurrent MYH11 rare variant, R247C, through functional studies in vitro and analysis of a knock-in mouse model with this specific variant, including assessment of aortic contraction, response to vascular injury, and phenotype of primary aortic smooth muscle cells (SMCs).

Methods and Results: The steady state ATPase activity (actin-activated) and the rates of phosphate and ADP release were lower for the R247C mutant myosin than for the wild-type, as was the rate of actin filament sliding in an in vitro motility assay. Myh11^{R247C/R247C} mice exhibited normal growth, reproduction, and aortic histology but decreased aortic contraction. In response to vascular injury, Myh11^{R247C/R247C} mice showed significantly increased neointimal formation due to increased SMC proliferation when compared with the wild-type mice. Primary aortic SMCs explanted from the Myh11^{R247C/R247C} mice were dedifferentiated compared with wild-type SMCs based on increased proliferation and reduced expression of SMC contractile proteins. The mutant SMCs also displayed altered focal adhesions and decreased Rho activation, associated with decreased nuclear localization of myocardin-related transcription factor-A. Exposure of the Myh11^{R247C/R247C} SMCs to a Rho activator rescued the dedifferentiated phenotype of the SMCs.

Conclusions: These results indicate that a rare variant in MYH11, R247C, alters myosin contractile function and SMC phenotype, leading to increased proliferation in vitro and in response to vascular injury. (Circ Res. 2012;110:1411-1422.)

Key Words: MYH11 • smooth muscle myosin heavy chain • thoracic aortic aneurysms and dissections • smooth muscle differentiation • mouse model

Thoracic aortic aneurysms leading to acute ascending aortic dissections (TAAD) are a common cause of premature deaths. Up to 20% of the patients who present with TAAD do not have an identified genetic syndrome, such as Marfan syndrome, but do have first-degree relatives similarly affected, termed familial TAAD (FTAAD). Heterozygous mutations in genes encoding the major proteins in the smooth muscle cell (SMC) contractile filaments, smooth muscle-specific isoforms of α-actin (ACTA2) and myosin heavy chain (MYH11), respectively, are together responsible for disease in 10% to 14% of TAAD families. In addition to TAAD, ACTA2 mutations also predispose individuals to occlusive vascular disease, including early onset coronary artery disease, stroke, and Moyamoya disease. The vascular pathology in the occluded arteries of patients with ACTA2 mutations is characterized by increased numbers of SMCs in the media or neointima, and aortic SMCs explanted from these patients proliferate more rapidly in culture than control SMCs. These observations have raised speculation that the occlusive vascular diseases associated with ACTA2 mutations...
result from excessive proliferation of SMCs in response to vascular injury.

MYH11 mutations are a rare cause of FTAAD and are identified primarily in families with TAAD inherited in association with a patent ductus arteriosus. Similar to patients with ACTA2 mutations, individuals with MYH11 mutations can also present with early onset occlusive vascular disease and stenotic arteries in the vasa vasorum of the aorta due to increased numbers of SMCs. These observations have raised speculation that MYH11 mutations may predispose to both TAAD and occlusive vascular diseases, ie, confer a predisposition to a similar range of vascular diseases as ACTA2 mutations. The myosin superfamilys is a large class of motor molecules that interact with actin filaments to generate force or exert movement using energy generated through ATP hydrolysis. The vertebrate smooth muscle myosin is a hexameric complex composed of 2 myosin heavy chains (SM-MHC, encoded by MYH11), 2 essential light chains, and 2 regulatory light chains (RLCs). Myosin heavy chains consist of a globular head domain that includes the ATPase activity and filament velocity. We sought to determine if the recurrent rare MYH11 variant R247C disrupts myosin function and alters SMC phenotype to understand its potential for contributing to aortic or occlusive vascular diseases.

Methods
An expanded Methods section is provided in the online-only Data Supplement.

To study wildtype (WT) and mutant R247C myosins, in vitro and in vivo studies were pursued. Animals were cared for according to the NIH Guide for the Care and Use of Laboratory Animals. All animal breeding and experiments were performed under protocols approved by the University of Texas Health Science Center at Houston and University of Texas Southwestern Medical Center and in accordance with NIH guidelines.

Results
MYH11 R247C Mutation Causes Decreased Myosin Motor Activity In Vitro
To determine if the MYH11 R247C rare variant impacted the kinetics of the myosin motor, soluble, 2-headed fragments (HMM fragments) of SM-MHC (SM1A isoform of human smooth muscle myosin) were expressed with and without the R247C mutation. As shown in the Table, the mutation

<table>
<thead>
<tr>
<th>RLC Phos</th>
<th>Maximal Actin-Activated ATPase (µM/Sec±SD)</th>
<th>In Vitro Motility (µM/Sec±SD)</th>
<th>Rate of P1 Release (+ Actin) (Sec−1±SD)</th>
<th>Rate of MgADP Release (+ Actin) (Sec−1±SD)</th>
<th>Duty Ratio (No Load)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MYH11 WT</td>
<td>−0</td>
<td>0</td>
<td>N.D.</td>
<td>N.D.</td>
<td>.05</td>
</tr>
<tr>
<td>MYH11 R247C</td>
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<td>0.38±0.06</td>
<td>1.2±0.3</td>
<td>24±3</td>
<td>.03</td>
</tr>
</tbody>
</table>

RLC indicates regulatory light chain; MYH11, myosin heavy chain; WT, wildtype; P1, inorganic phosphate.

mutations identified in FTAAD families are deletions and missense mutations primarily located in the coiled-coil domain of the protein. MYH11 mutations causing FTAAD differ from mutations in the cardiac-specific myosin heavy chain, encoded by MYH7, that cause hypertrophic cardiomyopathy. MYH7 mutations are primarily missense mutations involving the globular motor domain of the protein. Additionally, MYH7 is a gene that is commonly mutated in familial hypertrophic cardiomyopathy, whereas MYH11 mutations are a rare cause of FTAAD.

The identification MYH11 mutations that cause FTAAD is complicated by the fact that rare, nonsynonymous variants occur in MYH11 in the general population; 0.6% of the individual exomes in the Exome Rare Variant database have such a variant (http://evs.gs.washington.edu/EVS/). A recurrent MYH11 rare variant, R247C, is located in the head region of the myosin heavy chain and disrupts an arginine that is completely conserved across species. This alteration has been identified in patients with thoracic aortic disease without a family history but not in patients with FTAAD (unpublished data). A mutation in the corresponding amino residue in MYH7, R249Q, causes familial hypertrophic cardiomyopathy. The mutation in both the cardiac and smooth muscle isoforms lies near the ATP binding domain, and in vitro assays of MYH7 R249Q have confirmed decreased ATPase activity and filament velocity. We sought to determine if the recurrent rare MYH11 variant R247C disrupts myosin function and alters SMC phenotype to understand its potential for contributing to aortic or occlusive vascular diseases.

Table. ATPase, Motility, and Kinetic Parameters of expressed MYH11 (HMM)

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RLC indicates regulatory light chain; MYH11, myosin heavy chain; WT, wildtype; P1, inorganic phosphate.
resulted in a lowering of the maximal actin-activated ATPase activity when the myosin was phosphorylated on the RLC. However, regulation was not affected in that the mutant, like the WT protein, had no activity in the absence of RLC phosphorylation.

We then examined the rate of inorganic phosphate (P_i) release when the myosin was allowed to bind and hydrolyze ATP and then mixed with actin. This step is rate-limiting for the overall ATPase cycle in WT SM-MHC and is the entry point into the force generating states on actin (see Sweeney and Houdusse for review). From the Pi and ADP release rates, we can ultimately be a determinant of the force that the myosin will ultimately produce. From the Pi and ADP release rates, we can calculate the unloaded duty ratio, making the assumption that the duration of the force-generating states remains unchanged, while the duration of the nonforce-generating states and actin-myosin ATPase cycle rate for both mutant and WT proteins, consistent with it being the rate-limiting step in the overall cycle. We next measured the rate of release of ADP from mutant and WT myosin. The release of ADP from SM-MHC limits the rate of myosin detachment from actin as shown in Online Figure I, and also limits the maximal shortening velocity in the absence of load. The rate of ADP release was lower for the mutant than for the WT as shown in the Table, as was the rate of actin filament sliding in an in vitro motility assay.

The overall time that myosin spends bound strongly to actin during its kinetic cycle is known as the duty ratio and will ultimately be a determinant of the force that the myosin produces. From the P_i and ADP release rates, we can calculate the unloaded duty ratio, making the assumption that all other rates are much faster. This assumption is warranted given that the P_i release rates are only slightly faster than the overall actin-activated ATPase rate for both mutant and WT SM-MHC. As shown in Online Figure I, the rate of P_i release (k_4') controls exit from the nonforce generating states and actin-myosin ADP release (k_4'') controls exit from the force-generating states. Thus the duty ratio = k_4''/(k_4' + k_4''). In the absence of load, the calculated duty ratio of the R247C mutant is almost half of the WT protein (Table). As load increases, k_4'' and the duration of the nonforce-generating states remains unchanged, while the duration of the force-generating states increases (k_4' decreases). Thus it is likely that even under isometric conditions; the duty ratio of the mutant will be decreased as compared to WT due to the reduction in the rate of P_i release. Thus force production and shortening velocity are both likely decreased in vivo by the R247C mutation.

**Generation of the Myh11R247C/247C Mutant Mice**

To investigate the specific physiological and vascular effects of the mutation Myh11 R247C, we generated a targeting vector to flox the Myh11 R247C allele between 2 loxP sites of the backbone, which would knock in the R247C mutation and allow for Cre-mediated conditional inactivation of the Myh11 gene. The targeting vector contains a 4.76 Kb 5’ targeting arm, a floxed 2.04 Kb DNA fragment spanning exons 7 and 8, a neo expression cassette for positive selection, a 6.84 Kb 3’ targeting arm, and a tk expression cassette for negative selection (Figure 1A). The floxed exons 7 and 8 contain the mutation from C to T at nucleotide 846, which corresponds to the amino acid change R247C. In addition, the deletion of exons 7 and 8 would cause a coding-frame shift between exons 6 and 9 and result in disruption of the Myh11 gene (Figure 1A). After the mouse embryonic stem cells were electroporated with the targeting vector DNA and cultured in selection medium containing G418 and FIAU, 288 surviving clones were isolated and DNA samples were prepared from these clones for Southern blot screening. Four clones were positive for recombination by restriction digest analysis (Figure 1B). DNA sequencing confirmed 3 of 4 positive clones containing the mutant Myh11 R247C sequence. Since this targeted allele contains an Frt-flanked neo cassette, this allele is designated as Myh11R247C(f-neo).

Three targeted embryonic stem cell clones were injected into the blastocysts of C57BL/6J mice to produce chimeric mice. A total of 8 chimeric founder mice were identified to have germ line transmission capability. Two of these founder mice (lines A and B) were crossed with female FLPeR mice to remove the Frt-flanked neo cassette in their pups with an Myh11 R247C f-neo+/ FLPeR/+ genotype. As shown in Figure 1, PCR analyses detected WT and targeted alleles in FLPeR-negative Myh11R247C(f-neo)+ mice and WT and floxed alleles in FLPeR-positive and neo-negative Myh11R247C/+ mice (Figure 1C). Subsequently, Myh11R247C/+ breeding pairs were used to generate WT and Myh11R247C/R247C mice that were negative of both neo cassette and FLPeR. DNA sequencing was performed to confirm the presence of the knock-in mutation (Figure 1D). The established mouse colony had a mixed 129SvEv and C57BL/6J strain background. Assessment of the mRNA and protein expression of myosin (Myh11), α-actin (Acta2), and calponin (Cnn1) in aortic tissues using quantitative PCR and immunoblot analyses showed similar expression and protein levels for these contractile proteins between WT, and Myh11R247C/R247C aortas (Figure 1E and F, Online Figure II).

The predicted genotypes based on Mendelian inheritance were observed with breeding of mice heterozygous for the mutation. The average body weights of 1-, 2-, and 8-month-old male and female mice of various genotypes were not statistically different (Online Table II). Up to age 18 months, Myh11R247C/R247C and Myh11R247C/+ mice were indistinguishable from WT mice with regard to life expectancy. Finally, the reproductive capability of Myh11R247C/+ and Myh11R247C/R247C mice with WT mice was not altered (Online Table I). Together, these data suggest that introduction of the R247C alteration into SM-MHC did not affect mouse survival, development, or reproduction.

**Aortic Function and Pathology in the Myh11R247C/R247C Mouse**

Contractile force development in response to phenylephrine was attenuated in ascending aortic rings from Myh11R247C/R247C mice compared to littermate control mice at 5 months (Figure 2A). Similar differences were found in response to 90 mmol/L KCl (data not shown). In contrast, there was no attenuation in RLC phosphorylation (Figure 2B). Therefore, the defective contractile response does not result in changes to signaling modules acting on myosin light chain kinase but appears to be intrinsic to the properties of the mutated myosin.
Figure 1. Generation and validation of the floxed myosin heavy chain (Myh11)R247C/R247C mice. A, The mouse Myh11 gene structure is shown with exons and the restriction enzyme sites used in construction of the targeting vector marked. The targeting vector structure is shown with indicated regions and lengths of its 3' and 5' targeting arms. The loxP sites are indicated by triangles. The Frt sites are indicated by ovals. After DNA is digested with EcoRI, the 5' probe should detect a 9.7-kb band for wild-type (WT) allele and a 5.7 kb band for the targeted allele as indicated. After digesting with PstI, the 3' probe should detect a 5.1 kb band for the WT allele and a 6.8 kb band for the targeted allele as indicated. The floxed allele can be obtained after excision of the Frt-flanked neo cassette through cross to FLPeR transgenic mice. The P7, P8 and P9, P10 primers for PCR based genotyping analysis are indicated (arrow-heads). B, Identification of targeted embryonic stem (ES) clones by Southern blot analysis using the 5' and 3' probes. DNA was digested with EcoRI in the upper panel and with PstI in the lower panel. The genotypes of WT (f/neo) and targeted (f-neo) ES clones are indicated. C, Genotype analysis of mice with WT (f), targeted (f-neo) and floxed (f) Myh11 alleles. Upper panel: both Myh11+ and Myh11R247C(f-neo) alleles were detected by PCR using Myh11 P7 and P8 primers; lower panel: genotype analysis of Myh11R247C mice after the neo cassette is excised by crossing to FLPeR mice. Both Myh11+ and Myh11R247C alleles were detected by PCR using Myh11 P9 and P10 primers. D, Sequence analysis of WT and Myh11R247C mice. The DNA fragment spanning the Myh11R247C region was amplified by PCR and the PCR product was sequenced. E, Q-PCR analysis of Acta2, Cnn1, and Myh11 mRNA isolated from ascending aortas of WT and Myh11R247C/R247C mice at 10 months of age. Gene expression levels were normalized to Gapdh. The expression of these genes was not significantly different between Myh11R247C/R247C and WT aortas. F, Immunoblot analysis of smooth muscle contractile proteins levels in the ascending aortas of WT and Myh11R247C/R247C mice at 1 yr of age. ACTA2 indicates smooth muscle α-actin; CNN, calponin; SM-MHC, smooth muscle myosin heavy chain.
There was no abnormality or enlargement of aorta in these mice apparent by echocardiography and Doppler studies at 7 or 10 months of age (Figure 2C). The Myh11\(^{R247C/R247C}\) mice did not show any dissection, elongation, or tortuosity of the thoracic aorta, and their vessels were morphologically indistinguishable from their WT and Myh11\(^{R247C/H11001}\) littermates (Online Figure III). There were no differences in systolic or diastolic blood pressures between WT and Myh11\(^{R247C/R247C}\) mice (systolic: WT, 98 \pm 3 versus mutant, 95 \pm 7 mm Hg; diastolic, WT, 74 \pm 23 versus mutant, 70 \pm 7 mm Hg). Histological analysis of the ascending and descending aortas of the WT and Myh11\(^{R247C/R247C}\) aortas assessed at various ages up to 10 months of age did not reveal any evidence of vascular pathology (Figure 2D, Online Figures IV and V). There was no evidence of aortic SMC proliferation as detected by anti-PH3 and \(\alpha\)-actin immunostaining at 6 months of age (Figure 2E and F). Additionally, RNA isolated from the ascending thoracic aorta at 10 months of age showed no significant increase in expression of lumican (\(P = 0.81\)), decorin (\(P = 0.46\)), or matrix metalloproteinase 2 (Mmp2; \(P = 0.44\)) expression by Q-PCR (Figure 3G). Therefore, the homozygous Myh11 \(R247C\) allele decreases aortic contractility, but there was no evidence that the presence of mutant SM-MHC causes aortic pathology consistent with thoracic aneurysm formation.

### Proliferation of SMCs in Response to Vascular Injury in the Myh11\(^{R247C/R247C}\) Mice

To test the hypothesis that the Myh11 R247C alteration would lead to increased neointimal formation with vascular injury, we performed carotid artery ligation using the carotid flow cessation injury model and analyzed the vascular injury response in WT and Myh11\(^{R247C/R247C}\) mice at 7, 14, and 21 days after injury. Myh11\(^{R247C/R247C}\) mice exhibited enhanced formation of neointima as compared with WT mice (Figure 3A, Online Figure VI). Neointimal area and intima/media ratio were significantly larger in Myh11\(^{R247C/R247C}\) mice than WT mice at 14 and 21 days (Figure 3B and 3C). Medial areas of injured vessels were increased as compared with uninjured vessels in both Myh11\(^{R247C/R247C}\) and WT mice, but not significantly different from one another at any time point (data not shown). Myh11\(^{R247C/H11001}\) mice were also analyzed but showed no increased neointimal formation compared with WT (Online Figure VII).

To evaluate cellular proliferation in vivo, we quantified proliferating cells in the injured arteries by using anti-PH3

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**Figure 2.** Functional, histological, and morphometric analysis of ascending aortas from wildtype (WT) and myosin heavy chain (Myh11)\(^{R247C/R247C}\) mice. A, Force trace (above) and normalized force development by ascending aortic rings from Myh11\(^{R247C/R247C}\) mice compared with WT mice in response to phenylephrine at age 5 mo. B, Quantification of myosin regulatory light chain phosphorylation in Myh11\(^{R247C/R247C}\) mice compared with WT mice. C, Echocardiography and Doppler studies show no enlargement of the aorta in Myh11\(^{R247C/R247C}\) mice compared with WT or Myh11\(^{R247C/H11001}\) mice at 7 or 10 mo of age. D, Representative architecture of ascending aortic wall in 6-mo-old WT and Myh11\(^{R247C/R247C}\) mice. Cross-sections were stained by H&E, Elastin (VVG), Alcian blue, Sirius red, and Masson’s trichome staining. There is no significant difference in the architecture of the aortic wall, including assessment of the elastic lamellae, and proteoglycans and collagen deposition, between the WT and Myh11\(^{R247C/R247C}\) aortas. E and F, Phospho-histone H3 (PH3) immunostaining shows similar numbers of proliferative cells within the aortic walls from adult WT and Myh11\(^{R247C/R247C}\) mice. G, Q-PCR analysis showed no difference in the expression of Mmp2, Dec, and Lum in RNA harvested from the ascending aortas of WT and Myh11\(^{R247C/R247C}\) mice at 8 wk of age. Gene expression levels were normalized to Gapdh.
staining, a marker of mitosis. There was a significant increase in the number of anti-PH3 positive cells in the arteries of Myh11 R247C/R247C mice compared with WT (Figure 3D and 3E). Thus, the SMC response to vascular injury in the Myh11 R247C/R247C mice is characterized by excessive SMC proliferation and increased neointimal formation.

Carotid artery remodeling in response to vascular injury has been associated with increased matrix metalloproteinase 2 levels. To determine the expression level of Mmp2 in the injured vessels from Myh11 R247C/R247C mice compared with WT (Figure 3D and 3E). Thus, the SMC response to vascular injury in the Myh11 R247C/R247C mice is characterized by excessive SMC proliferation and increased neointimal formation.

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Figure 3. Histological and morphometric analysis of injured carotid arteries from wildtype (WT) and myosin heavy chain (Myh11) R247C/R247C mice. A, Representative H&E and elastin (VVG) stained cross-sections of unligated and ligated carotid arteries at 21 days post surgery from WT and Myh11 R247C/R247C mice. Neointimal formation was accelerated in Myh11 R247C/R247C mice following vascular injury. B and C, Quantification of neointimal area and intima/media ratios of ligated carotid arteries from WT (white bars) and Myh11 R247C/R247C mice (black bars). Carotid arteries were harvested from Myh11 R247C/R247C and WT mice at days 7, 14, and 21 after injury. n=5 mice per genotype and time point. Data represent mean of 5 arterial cross sections ± SD. D, Representative cross sections of PH3 staining at 21 days after carotid artery ligation from WT and Myh11 R247C/R247C mice. E, Quantification of PH3 positive cells of ligated carotid arteries from WT (white bars) and Myh11 R247C/R247C mice (black bars). Carotid arteries were harvested from Myh11 R247C/R247C and WT mice at days 7, 14, and 21 after injury. n=5 mice per genotype and time point. Data represent mean of 5 arterial cross sections ± SD. F, Mmp2 mRNA levels by Q-PCR analysis in injured (inj) and uninjured (un) carotid arteries at days 14 and 21 after carotid artery ligation. Gene expression levels were normalized to Gapdh (n=5 per genotype and time point).

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Proliferation, Dedifferentiation, and Altered Focal Adhesions in Myh11 R247C/R247C SMCs

To further characterize the effect of the Myh11 R247C mutation on SMC phenotype and function, SMCs were explanted from the ascending thoracic aorta; these SMCs are neural crest-derived. Three separate explants were derived and the results described were consistent in all explants. The Myh11 R247C/R247C aortic SMCs proliferated significantly more rapidly than the WT SMCs (Figure 4A). Myh11 R247C+/S SMCs were also explanted; however they did not show increased proliferation compared with WT SMCs (Online Figure VII). Because of the lack of a proliferative response in vitro or in vivo of the heterozygous cells, further analyses were performed primarily on Myh11 R247C/R247C and WT cells.
Increased proliferation of SMCs is typically associated with dedifferentiated SMC phenotype characterized by decreased expression of SMC contractile proteins. Quantitative PCR analysis of message isolated from Myh11\(^{R247C/R247C}\) versus WT SMCs showed decreased expression levels of Acta2, Cnn1, and Myh11 (Figure 4B). Immunoblot analysis performed on protein harvested from the Myh11\(^{R247C/R247C}\) SMCs also demonstrated decreased protein levels of \(\alpha\)-actin, calponin, and SM-MHC (Myh11) (Figure 4C, Online Figure II). Myh11\(^{R247C/R1100}\) cells had decreased expression of contractile proteins (Online Figure VIII). These data suggested that the homozygous Myh11 R247C mutation alters the phenotype of SMC, leading to increased proliferation.

Consistent with the observation that Myh11\(^{R247C/R247C}\) SMCs are dedifferentiated compared to WT cells, immunofluorescence staining of the mutant SMCs demonstrated \(\alpha\)-actin filaments with an altered cellular structure compared to WT SMCs (Figure 4E, Online Figure II). The Myh11\(^{R247C/R247C}\) filaments were thinner and shorter than the WT filaments, and the mutant filaments crossed the cell body but typically did not extend to the periphery of the cell, whereas the WT filaments extended to the cell periphery. Additionally, pools of unpolymerized \(\alpha\)-actin were present in a subset of mutant cells but not in the WT cells. An F/G actin assay confirmed that Myh11\(^{R247C/R247C}\) cells had an increase in the ratio of monomeric \(\alpha\)-actin (G) to filamentous \(\alpha\)-actin (F), whereas WT cells had more F-actin than G-actin (Figure 4F).

In SMCs, the transcription factor serum response factor (SRF) regulates SMC phenotype via association with its cofactors, the myocardin-related transcription factors (MRTFs). MRTF-A is a potent transcriptional coactivator that links changes in actin filaments to gene expression to alter the phenotype of SMCs. Increased pools of monomeric-actin, such as those observed in Myh11\(^{R247C/R247C}\) cells, can sequester MRTF-A in the cytoplasm and prevent its translocation to the nucleus to drive SMC differentiation and contractile gene expression. Assessment of MRTF-A localization in Myh11\(^{R247C/R247C}\) by immunofluorescence revealed a shift from a primarily nuclear localization of MRTF-A in WT SMCs to predominantly cytoplasmic localization in Myh11\(^{R247C/R247C}\) SMCs (Figure 4G, Online Figure IX). This differential localization was...
quantified by computing a Pearson coefficient for the correlation of blue and green staining in the same pixels, confirming a significant decrease in nuclear localization of MRTF-A in Myh11<sup>R247C/R247C</sup> SMCs when compared to WT SMCs.

Because focal adhesion (FA) composition and maturation is dependent on force generation across the cell by cellular type II myosin motors, we also assessed FAs in the Myh11<sup>R247C/R247C</sup> SMCs. Inhibition of these myosin motors in cells using blebbistatin disrupts the maturation of nascent FAs, altering the recruitment of small G protein signaling molecules and leading to an absence of RhoA activators and an enrichment of Rac1 activators. Focal adhesion kinase (FAK), a major signaling molecule at FAs, has been shown to drive actin polymerization as well as proliferation in SMCs. Immunofluorescence using an antibody recognizing phosphorylated Y397 of FAK showed increased staining, and immunoblot analysis confirmed an increase in cellular phosphorylated Y397 of FAK (Figure 5B and C, Online Figure II). FAK activates multiple pathways, including Akt signaling, and phosphorylation of Akt was also increased in Myh11<sup>R247C/R247C</sup> SMCs (Figure 5C, Online Figure II).

Blocking FAK activation using a specific inhibitor (PF537228) decreased proliferation in Myh11<sup>R247C/R247C</sup> SMCs (Figure 5D). However, the FAK inhibitor did not increase nuclear localization of MRTF-A, nor did it stimulate any change in expression of SMC contractile genes (Online Figure X).

Altered FA maturation due to disruption of the type II myosin motors would be expected to alter Rac1 and RhoA signaling. As predicted, Rac1 activity was increased and RhoA activity decreased in Myh11<sup>R247C/R247C</sup> SMCs when compared to WT SMCs (Figure 5E and 5F). Interestingly,
there was no increase in Rac1 activation or decrease of RhoA activation observed in the aortic tissue of Myh11 R247C/R247C mice compared to WT mice (Figure 5G and 5H). We sought to determine if the altered Rac1 and RhoA signaling was responsible for the dedifferentiated phenotype of the Myh11 R247C/R247C SMCs. Inhibition of Rac1 using a specific inhibitor (NSC23766) had no effect on the proliferation of Myh11 R247C/R247C SMCs or on the localization of MRTF-A (Online Figure X). Cellular RhoA activity levels can be activated using an activator derived from the bacterial cyto- toxic necrotizing factor endotoxins, which converts RhoA to a constitutively active form by deaminating glutamine.\(^{18}\) We confirmed that when mutant and WT SMCs were exposed to this compound, CN03, RhoA activity levels were significantly increased (Figure 6A). Further analyses determined that exposure to CN03 increased the nuclear localization of MRTF-A in the Myh11 R247C/R247C SMCs (Figure 6B) and significantly increased expression of smooth muscle specific contractile genes after 4 hours of exposure (Figure 6C). Immunoblot also showed an increased in contractile protein levels in the Myh11 R247C/R247C SMCs after 24 hours of exposure to CN03 (Figure 6D, Online Figure II). Finally, treatment of Myh11 R247C/R247C cells with CN03 for 24 hours increased the polymerization of \(\alpha\)-actin (green) in Myh11 R247C/R247C SMCs. Colocalization of \(\alpha\)-actin (green) and phalloidin (red) stains is quantified on the right (\(P<0.01\), **\(P<0.001\), error bars represent 95% CI). Scale bars 100 \(\mu\)mol/L. ACTA2 indicates smooth muscle \(\alpha\)-actin; SM-MHC, smooth muscle myosin heavy chain.
Discussion

This study sought to determine if a recurrent rare variant, R247C, in the SMC-specific isoform of the myosin heavy chain disrupted myosin function and altered SMC phenotype. The R247C variant was chosen because it is a recurrent rare variant of unknown significance, and mutation of the paralogous amino acid in cardiac myosin heavy chain, MYH7, causes hypertrophic cardiomyopathy. This study demonstrates that MYH11 R247C leads to reduced force production and shortening velocity of SM-MHC in vitro and decreased aortic contractility in vivo. Despite decreased aortic contractility in the Myh11R247C/R247C aortas, RLC phosphorylation responses were not different between the mutant and WT aortas, indicating consistency in activation of signaling pathways leading to RLC phosphorylation. Thus, the defective contractile response does not result from changes to signaling modules acting on myosin light chain kinase and RLC phosphorylation but appears to be due to intrinsic properties of the mutated myosin. The decrease in aortic contractility was not associated with aortic pathology in these mice and suggests that increased aortic contractility alone may not be sufficient to cause thoracic aortic disease. In contrast, vascular injury induced significantly more neointimal formation in Myh11R247C/R247C mice than in WT mice and mutant SMCs proliferated more rapidly in culture than WT cells. SMC hyperplasia associated with this genetic variant may increase the risk of occlusive vascular diseases in individuals with this variant.

Aortic SMCs explanted from the Myh11R247C/R247C mice had an altered phenotype characterized by increased proliferation and dedifferentiation as defined by decreased expression of contractile proteins. The SRF:MRTF axis is a well-characterized pathway linking actin filament formation to SMC proliferation and differentiation. With actin polymerization, MRTF-A moves from the cytoplasm to the nucleus and induces the transcription of SMC-specific contractile genes with the transcriptional coactivator SRF. If actin filaments are disrupted, MRTF-A moves out of the nucleus and binds to monomeric actin, leading to the downregulation of contractile gene expression and allowing SRF to bind to ternary complex factors. The SRF:ternary factor complex activates a subset of SRF-regulated growth responsive genes, leading to cell proliferation. Our results indicate that altering R247 in the myosin motor domain leads to dedifferentiation of the SMCs via the SRF:MRTF axis, based on the decreased expression of contractile proteins, increased expression of c-fos, and increased cytoplasmic localization of MRTF-A when compared to WT SMCs.

Altered FAs in the Myh11R247C/R247C SMCs also contributed to the altered phenotype of these cells. The generation of tension across cells with adhesion to the matrix requires functional cellular actin and myosin and this tension drives FA maturation, composition, and localization. Nascent FAs form at the periphery of cells with the binding of integrins to the extracellular matrix. These nascent FAs recruit FAK, paxillin, and other proteins and initiate the anchoring of actin stress fibers to integrins. The conversion of nascent to mature FAs is dependent on further force generation, which in turn is dependent on cellular type II myosin motors. Recent studies have identified differential recruitment of proteins to FAs in the presence and absence of ATPase inhibitor blebbistatin, which prevents force generation by cellular type II myosin motors. In particular, RhoA activating proteins are absent in the immature focal adhesions of blebbistatin-treated cells, while Rac1 activators are enriched. RhoA-dependent regulation of the actin cytoskeleton has been previously established to regulate SMC differentiation marker gene expression by modulating SRF:MRTF-dependent transcription. Knockdown of RhoA signaling prevents transcription of contractile genes in SMCs, and constitutive activation of RhoA induces both actin polymerization and increases contractile gene expression in SMCs via the SRF:MRTF axis. Our data suggest that the Myh11 R247C variant prevents maturation of FAs, thus decreasing Rho activation and leading to a dedifferentiated SMC phenotype. In fact, activation of RhoA rescued the dedifferentiated SMC phenotype in Myh11R247C/R247C SMCs as assessed by increased MRTF-A nuclear localization and increased contractile protein expression. The heterozygous Myh11R247C/+ cells had intermediate levels of SMC contractile gene expression but no increase in proliferation relative to WT SMCs, which may indicate that the presence of 50% WT myosin provides sufficient force generation to allow the FAs to mature and prevent proliferative pathways from being activated. Although the SMCs contain other cellular myosin motors, including nonmuscle myosin IIA and IIB (encoded by Myh9 and Myh10, respectively), these myosins were not able to compensate for the loss of SM-MHC function to generate sufficient cell tension to drive FA maturation or force generation in aortic SMCs. Therefore, our results demonstrate a previously uncharacterized role for myosin motors and FAs in determining SMC phenotype.

A similar SMC phenotype, characterized by decreased RhoA signaling and SMC dedifferentiation via the SRF:MRTF axis, was reported in the aortas and explanted aortic SMCs from the SMC-specific integrin-linked kinase (ILK) knockout mice. ILK, like FAK, is a kinase that binds to the integrin β1 subunit and plays a critical role in the organization of the actin cytoskeleton through its association with parvin, paxillin, and vinculin. Mice with SMC-specific reduction of ILK exhibit vascular pathologies similar to the vascular diseases observed in FTAAD patients with disease-causing MYH11 mutations, including aneurysms of the ascending aorta and patent ductus arteriosus. Aortic tissue and explanted SMCs from ILK-deficient mice showed similar reductions in the expression of contractile proteins due to aberrant localization of MRTF-A to the cytoplasm, with concomitant decreased levels of RhoA activation.

Although we observed dedifferentiation of SMCs in culture and increased proliferation of SMCs with injury, aortas from the Myh11R247C/R247C had RhoA activity and contractile protein levels similar to WT aortas. In the intact aorta, the interactions between the extracellular matrix and intracellular actin-myosin contractile filaments occur through integrin receptors in dense plaques, which have a similar function to FAs. Unlike FAs in tissue culture, these dense plaques are under continuous biomechanical forces due to pulsatile blood flow. Therefore, other sources, like ILK, may be responsible...
for the normal levels of Rho activation in Myh11R247C/R247C aortas. Alternatively, Rho activation in vascular tissues may be enhanced by signaling brought about by GPCR agonists, such as norepinephrine, that couple to RhoA guanine nucleotide exchange factors.28

The similarity of the phenotype of the SMC-specific reduction of ILK mice and FTAAD patients with MYH11 mutations, specifically thoracic aortic aneurysms with patent ductus arteriosus, could provide insight as to why the Myh11R247C/R247C mice do not have aortic pathology. As described above, the ILK-deficient SMCs are dedifferentiated in vivo, while our data suggest the Myh11R247C/R247C SMCs in vivo are not dedifferentiated. Furthermore, we identified no differences in RLC phosphorylation, suggesting that cell signaling leading to RLC phosphorylation is intact. The majority of the identified MYH11 mutations leading to FTAAD are predicted to disrupt SM-MHC polymerization into thick filaments, which could cause a more severe defect in myosin function and disrupt signaling through dense plaques in the aortic SMCs in vivo. The altered signaling through dense plaques could decrease ILK signaling, therefore predisposing to thoracic aortic aneurysms and patent ductus arteriosus, which is the same phenotype identified in the ILK deficient mouse.

Myh11 deficient mice die shortly after birth from vascular complications, as well as from dysfunction of the bladder and intestine.29 However, Myh11R247C/R247C mice demonstrate no evidence of bowel or bladder dysfunction, normal growth, and survival. Additionally, the reproductive capacity of these mice is similar to WT mice despite the fact that smooth muscle contraction is required for uterine function. Physiologically, myosin light chain kinase (MLCK) is not fully activated nor is RLC phosphorylated in visceral smooth muscles.30,31 The partial decrease in myosin function in the mutant mice may be compensated for by increased nervous and hormonal influences sufficient to maintain visceral functions. Compensation by other type II myosin motors may also explain the lack of contractile problems in other organs.

Mutations in MYH7, the cardiac β-myosin heavy chain, typically cluster in the functional subdomains of the myosin motor head domain, specifically in the ATP-binding and actin binding clefts.7 In fact, studies of the alteration of the paralogous amino acid in MYH7, R249Q, that causes hypertrophic cardiomyopathy have shown decreased ATPase activity and sliding filament velocity, similar to the results reported here for R247C in MYH11.9,10 The MYH11 mutations causing FTAAD are not in the head domain, but rather located in the coiled-coil rod domain and predicted to destabilize myosin filament formation. The one exception is a missense mutation at amino acid 712 near the converter domain, which transduces force from the ATPase motor and allows the flexible movement of the myosin head along actin. Based on these data, it is tempting to speculate that cardiomyocytes are more sensitive to disturbances of myosin motor function than vascular SMCs.

In summary, these data show that the rare variant in MYH11, R247C, can alter SM-MHC function, aortic contraction, and SMC phenotype. These findings raise the possibility that this variant may predispose to vascular diseases in conjunction with other environmental or genetic risk factors. It is clinically important to distinguish between MYH11 variants that cause Mendelian inheritance of TAAD from those variants that are benign or confer a low or no risk for vascular disease and additional studies are needed to fully understand which MYH11 variants confer a high risk for aortic disease. Finally, these data demonstrate a previously unrecognized role of FAs in influencing SMC phenotype.

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Disclosures

None.

References


Novelty and Significance

What Is Known?

- Mutations in MYH11, which encodes the smooth muscle specific myosin heavy chain, cause familial thoracic aortic aneurysms and dissections in humans and may also predispose to vascular occlusive disease.
- A mutation in the cardiac myosin heavy chain, MYH7, at the paralogous amino acid to R247 in MYH11 causes familial hypertrophic cardiomyopathy and reduces force generation and enzymatic activity of the myosin motor.
- Smooth muscle cells retain phenotypic plasticity, and the serum response factor: myosin related transcription factor (SRF-MRTF) axis acts as a switch to promote either a proliferative or a contractile phenotype.

What New Information Does This Article Contribute?

- Rare variants in MYH11, like the R247C variant studied here, may predispose to vascular occlusive disease by causing smooth muscle cell hyperplasia.
- Rare variants in MYH11 can induce de differentiation of smooth muscle cells.
- Focal adhesions play a role in determining smooth muscle cell phenotype via both SRF-MRTF dependent actions (eg, Rho activity at the focal adhesions drives actin polymerization and MRTF nuclear localization) and SRF-MRTF independent actions (eg, activation of focal adhesion kinase drives proliferation).

This study sought to determine the role of rare variants in MYH11 in the pathogenesis of vascular disease, including aortic aneurysms and occlusive diseases. We generated a knock-in mouse model of the most frequently recurrent rare variant, R247C, and showed that this variant decreases force generation in the aorta but does not lead to aneurysms. Knock-in mice had increased neointimal formation in response to vascular injury in vivo and increased smooth muscle cell proliferation in vitro. Cultured smooth muscle cells homozgyous for the R247C mutation were dedifferentiated with immature focal adhesions and decreased Rho activation. A Rho activator rescued the dedifferentiated phenotype of these cells. This study identifies for the first time a key role for focal adhesions in the regulation of smooth muscle cell phenotype, and also suggests that in smooth muscle cells the force generation specifically of smooth muscle myosin rather than cytoskeletal myosin is crucial for the maturation of focal adhesions. Additionally, the data suggests that rare variants in MYH11 in humans may increase the risk of developing vascular occlusive disease.
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Rare, Non-Synonymous Variant in the Smooth Muscle Specific Isoform of Myosin Heavy Chain, *MHY11*, R247C, Alters Force Generation in the Aorta and Phenotype of Smooth Muscle Cells

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Supplemental Methods

Construction of Targeting Vector

The pfrt-loxp parent plasmid contained a neo cassette as a positive selection marker, a tk cassette as a negative selection marker, two loxP sites and two Frt sites. Neo was flanked by a pair of Frt sites. A 2.0 Kb genomic DNA fragment from mouse *Myh11* (GeneBank NM_013607) intron 6 to intron 8 (including exon 7, and exon 8) was amplified by high-fidelity PCR (TAKARA BIO INC, Madison WI) from mouse ES cell DNA with primers containing restriction sites. This DNA fragment was first sub-cloned into a pBlueScript II SK plasmid (Stratagene, La Jolla, CA), and the DNA sequence encoding R247C in exon 7 was changed by PCR based site-specific mutagenesis. Successful mutagenesis was confirmed by DNA sequencing. This mutated DNA was subsequently transferred into the targeting vector at the NotI and KpnI sites within two loxP sites. A 4.4 Kb *Myh11* fragment from intron 8 to exon 9 was amplified by PCR and sub-cloned into the targeting vector between XhoI and BamHI sites to serve as the 3' targeting arm. Another 4.7 Kb genomic DNA from partial intron 4 to intron 6 of mouse *Myh11* gene was amplified by PCR with primers containing restriction sites. This PCR product was sub-cloned into the targeting vector between the PacI and Ascl sites to serve as the 5' targeting arm. All exon sequences and the mutated sequences in the targeting vector were confirmed by DNA sequence analysis (Fig. 1A). The targeting vector was linearized by PacI digestion and transfected into the 129SvEv mouse ES cells by electroporation. Transfected ES cells were cultured in selection medium with G418 (for Neo positive selection) and FIAU (for tk negative selection) and survived clones were isolated for Southern blot analysis (Figure 1B).

Microinjection and Mouse Breeding

The correctly targeted ES clones were microinjected into C57BL6 blastocysts to generate chimeric founder mice. The male chimeric mice were kept for breeding with female C57BL/6 mice to test germ line transmission of the targeted *Myh11* locus. Next, the male chimeric mice with proven germ line transmission ability were bred with the 129SvEv FLPeR mice (stock #003946, Jackson Lab) to remove the Frt-flanked neo cassette. Finally, the FLPeR allele was excluded in the incoming generations by genotype screening (Figure 1C). The wild-type (WT) and *Myh11*<sup>R247C</sup><sup>R247C</sup> knockin mice used in this study are mixed 129 SvJ and C57BL/6 background.

Genotype Analysis

Mouse genomic DNA was extracted from tail tips by following standard protocols (Qiagene INC. Valencia, CA). *Myh11* WT and manipulated alleles were distinguished by PCR using specific primers (Figure 1, Online Table I). PCR amplifications were carried out using HotStar Taq<sup>TM</sup> DNA polymerase (Qiagene INC. Valencia, CA). The PCR program was as follows: 95°C for 5min; 95°C, 30 seconds, 55°C, 30 seconds, 72°C, 30 seconds for 35 cycles; 72°C, for 10min. Genotype analysis of the FLPeR mice was performed as instructed by Jackson Laboratory.

RNA Extraction and Quantitative Real-time PCR

Total mice aortic tissues and primary vascular SMCs cellular RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Reverse transcription reactions were performed using the High Capacity cDNA Archive Kit (Life Technologies, Carlsbad, CA) according to the manufacturer’s protocol. For quantitative real-time PCR analysis of mRNA expression, TaqMan probes were purchased from Applied Biosystems and analyzed using an Applied Biosystems Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol. Experiments were performed in triplicate. *Gapdh* was used as the endogenous control.
Vascular SMCs Isolation and Culture

To isolate primary mouse aortic SMCs, aortas were isolated (from the aortic root to the renal arterial bifurcation) from 6-week old WT and Myh11^{R247C/R247C} knock-in mice. Whole aortas were collected under sterile conditions and put into biopsy medium as described. Ascending aorta/arch and descending aortas were separated after the origin of the left subclavian artery. The adventitia was removed, and the remaining medial layer of aorta was chopped into small pieces and put to digestion overnight for 16 h in 5 ml of aortic biopsy medium supplemented with 0.1 mg/ml of collagenase type I, 0.019 mg/ml of elastase type I and 0.0250 mg/ml of soybean trypsin inhibitor. At the end of incubation, the digestion was stopped with 2.5 ml of fetal bovine serum and 2.5 ml of complete SMC medium as described. Cells and tissue were spun down, resuspended in complete SMC medium and seeded into flasks for further experiments. The identity of these cells as SMCs was verified by staining for smooth muscle α-actin (mouse monoclonal antibody; Sigma-Aldrich, St. Louis, MO) at each passage (95% of cells stained positive for smooth muscle α-actin). SMCs were cultured in complete SMC medium in a 37°C, 5% CO2-humidified incubator.

Three independent cell lines each were explanted from WT mice, Myh11^{R247C/+}, and Myh11^{R247C/R247C} mice, using aortas pooled from three to five mice per genotype per explant. The results presented are representative of at least two independent experiments done on each line of SMCs using passage-matched WT and Myh11^{R247C/R247C} SMCs. All studies were performed on SMCs at less than passage 5.

Immunoblot Analyses

Protein lysates were prepared from mouse aortic tissues or primary vascular SMCs as described. Briefly, aortic tissues or cultured cells were homogenized and lysed in RIPA buffer supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Protein (5µg) for each sample was separated on Tris–HCl gel (Bio-Rad, Hercules, CA) by SDS-PAGE, followed by transfer to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were immunoblotted with primary antibody and the appropriate horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Immunoblots were visualized by the enhanced chemiluminescence technique (GE Healthcare, Piscataway, NJ). Primary antibodies include anti-α-tubulin, phosho-Akt (S473), total Akt (all from Cell Signaling Technology, Beverly, MA), anti-SM myosin (Biomedical Technologies Inc, Stoughton, MA), anti α–SMA, anti-calponin (both Sigma-Aldrich, St. Louis, MO), anti-GAPDH (Fitzgerald Industries, Acton, MA), phosho-FAK (Y397) (Millipore, Bedford, MA), total FAK, MRTFA, Histone H1 (all Santa Cruz Biotechnology, Santa Cruz, CA).

Carotid Artery Ligation

Ten week-old WT, Myh11^{R247C/+}, and Myh11^{R247C/R247C} mice were anesthetized by intraperitoneal injection of 2.5% avertin. The left common carotid artery was ligated near its bifurcation with the use of 5-0 silk. The right carotid artery served as an uninjured control. 7, 14 or 21 days after injury, animals were anesthetized and perfused with 5 ml of 1XPBS, followed by 5 ml of 4% paraformaldehyde for 3 min. Left and right carotid arteries were excised, further fixed for 16 hours and embedded transversely in paraffin. Because lesion thickness varies longitudinally, the entire length of the left and right carotid arteries was sectioned and examined for identification of the apex of the lesion, which displays the smallest lumen. For morphometric analyses, images of hematoxylin and eosin (H&E) and Verhoeff-van Gieson elastin-stained cross-sections were analyzed using Image J. Lumen, intimal and medial area were measured and automatically calculated. Six mice for each genotype and each time point were analyzed.
Contraction, Regulatory Light Chain Phosphorylation and Protein Content of the Ascending Aorta

Ascending aortas were dissected from anesthetized mice and rings (3 mm) were mounted on wire triangles that were fastened to an isometric force apparatus. Rings were equilibrated and stretched to 1.8g with a final resting tension of 1g obtained after 1 hr equilibration for tissues from both wildtype and Myh11R247C/R247C mice. Protocols were performed in physiological salt solution (PSS; in mM: 118.5 NaCl, 4.75 KCl, 1.2 MgSO4, 1.2 KH2PO4, 24.9 NaHCO3, 1.6 CaCl2, 10.0 D-glucose, pre-gassed with 95% O2/5% CO2 at 37°C). Rings were stimulated to contract by depolarization with KCl (PSS with KCl substituted for NaCl for final 90 mM KCl) or by alpha-adrenergic agonist (10 µM phenylephrine). Rings were frozen after treatment of tissues with phenylephrine for 1 min with clamps precooled in liquid nitrogen and stored at -80 °C until processing for phosphorylation measurements.

Tissues were denatured in a frozen slurry of acetone in 10% trichloroacetic acid with 10 mM dithiothreitol for 30 min, then thawed and transferred to Eppendorf tubes. Tissues were rinsed in ether (3x5min), briefly dried (3min) and suspended in 40 µl urea sample buffer 3-4. Proteins were extracted in a Bullet Blender (Next Advance, Inc, Averill Park, NY) (with 2 mm zirconium oxide beads, 4 spins x 3 min each at setting 9). Protein content was determined by Bradford assay. Samples were subjected to urea/glycerol-PAGE at 400 volts for 80 min (RLC) to separate non-phosphorylated and phosphorylated forms. Following electrophoresis, proteins were transferred to nitrocellulose membranes and probed with polyclonal antibodies against smooth muscle RLC. The ratio of monophosphorylated RLC to total RLC (nonphosphorylated plus monophosphorylated) was determined by quantitative densitometry and expressed as mol phosphate per mol protein. Basal phosphorylation levels for RLC in aortic tissues were not significantly different with 0.11±0.016 (WT) vs 0.07±0.28 (Myh11R247C/R247C) mol phosphate per mol RLC. These basal values were subtracted from values obtained in contracting tissues to show net increases in RLC phosphorylation.

Blood Pressure Measurements

Systolic and diastolic blood pressures of conscious mice were measured using a tail cuff blood pressure analyzer designed with volume-pressure recording (VPR) technology (Model Coda, Kent Scientific) as previously described 5-8. Body temperatures of the mice were maintained at 37°C by a heated platform. The blood pressure of trained mice was monitored for 30–40 min and final values were obtained after 15 consecutive readings on each of two occasions with expected values within ±5% of the mean. The mean value for each of these two sets of readings were averaged and taken as the single blood pressure measurement for that animal.

Histological and Immunohistochemical Studies

The excised aorta was fixed in 4% paraformaldehyde and processed for routine paraffin embedding. Aorta tissue cross-sections (6 µm) were stained with H&E and Verhoeff-van Gieson elastin follow standard procedures. Immunohistochemical staining was performed with primary antibodies against Phospho-Histone H3 (PH3, Upstate, Lake Placid, NY) and SM-α actin (Sigma-Aldrich, St. Louis, MO) followed by biotinylated secondary antibody. Staining was treated with peroxidase-conjugated biotin–avidin complex (Vector Laboratories, Burlingame, CA) and visualized by DAB (Vector Laboratories, Burlingame, CA). Slides were counterstained with hematoxylin.
Echocardiography and Doppler Studies

High resolution ultrasound was performed with a Vevo 770 imaging system at 7 and 10 months of age using a 40 MHz 704 probe (VisualSonics, Toronto, Canada). Mice were anesthetized by inhalation of isoflurane (1.5-4%) while ECG and respirations were continuously monitored on a warmed platform. The anterior chest was denuded using depilatory cream prior to application of ultrasound gel and imaging. Images were then exported to Sante DICOM Editor software (SanteSoft LTD, Athens Greece) for further analysis. The dimensions of the ascending aorta (in mm) were measured in a single plane using right parasternal views at four levels (annulus, sinuses of Valsalva, sinotubular junction and ascending aorta). In all studies, at least four animals per group were used. All scanning and analysis was performed by an experienced ultrasound technician who was blinded to the mouse genotype.

Immunofluorescence in Explanted Aortic SMCs

After cells reached confluence, they were seeded onto coverslips in six-well plates with the density of 13 cells/mm² for 24 h. For MRTF-A staining, Cells were starved in 1% serum media for 24 hours, plus/minus inhibitors and fixed with 4% paraformaldehyde for 10 min. Permeabilization was performed in PBS containing 0.1% Triton X-100 and blocking of nonspecific binding sites was performed in PBS containing and 5% donkey serum. Coverslips were then treated with primary antibody (anti-MRTFA (Santa Cruz Biotechnology, Santa Cruz, CA), 1:100) overnight followed by fluorescein isothiocyanate-conjugated secondary antibody (1:100, Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h. Nuclei were counterstained with DAPI (Vector Laboratories, Burlingame, CA), and then randomly chosen fields on each coverslip were imaged by confocal microscopy (Nikon A1R, Nikon Instruments, Melville, NY). For focal adhesion staining, cells were starved in 1% serum media for 24 hours then treated with or without 10ng/mL TGF-β1 for 48 hours. The same fixation and staining protocol described above was used with primary antibodies anti-vinculin (Sigma-Aldrich, St. Louis, MO) and anti-phospho-FAK (Y397) (Millipore, Bedford, MA). For α-actin staining, cells were starved in 1% serum media for 12 hours, followed by 10ng/mL TGF-β1 for 72 hours. CN03 treatment was added for 24 hours after completion of TGF-β1 incubation. Again, the same fixation and staining protocol were used with primary antibody anti α-SMA (Sigma-Aldrich, St. Louis, MO). For phalloidin staining, coverslips were incubated with Texas Red-labeled phalloidin (1:40 in blocking solution) (Molecular Probes, Eugene, OR) for 30 min.

Analysis of Immunofluorescence Images

All analyses were performed using the Nikon NIS Elements software. Pearson coefficients for colocalization of blue (DAPI stained nuclei) and green (MRTFA) pixels were calculated for at least 15 cells per slide on at least 3 slides for each bar shown on the graph. The same protocol was used to obtain Pearson coefficients for colocalization of green (α-actin) and red (phalloidin) pixels for Figure 6. Individual focal adhesion size was calculated for at least 30 adhesions per cell on at least 10 cells per genotype using the Nikon NIS Elements measurement tool.

Cell Proliferation Assays

Proliferation of SMCs was quantified using by the incorporation of BrdU. Briefly, SMCs were seeded in 96-well plates (20,000 cells/ well) and grown for 24 hours in SmBm (see SMCs Isolation and Culture section for details) containing 20% FBS. The cells were serum-starved in SmBm containing and 1% FBS and plus/minus inhibitors for 30 minutes prior to the addition of BrdU reagent. After 24 hours of incubation, BrdU incorporation was quantified by ELISA according to the manufacturer’s instructions (Millipore, Bedford, MA). Inhibitors include NSC23766 (Rac
inhibitor) (Tocris Bioscience, Ellisville, MO), PF537228 (FAK inhibitor) (Tocris Bioscience, Ellisville, MO), and CN03 (Rho Activator) (Cytoskeleton, Denver, CO).

Rho and Rac Activation Assays

RhoA and Rac1 activation were quantified using G-LISA assays (Cytoskeleton, Denver, CO) performed according to the manufacturer’s specifications. Briefly, cells were seeded on 6 cm dishes (200,000 cells/dish) and grown for 24 h in SmBm containing 20% FBS. The cells were serum-starved in SmBm containing 1% FBS for 24 hours, then treated +/-lypophosphatidic acid (Sigma-Aldrich, St. Louis, MO) for 15 minutes. Cells were lysed in the provided lysis buffer and lysates were snap frozen. Protein, (0.5 µg) was loaded per well of the provided ELISA plate, and activated Rac1 and RhoA were quantified by ELISA assay.

F/G actin Assay

Polymerization of actin was assayed using an F/G actin assay (Cytoskeleton, Denver, CO) performed according to the manufacturer’s specifications. Briefly, cells were seeded on 10 cm dishes (900,000 cells/dish) and grown for 24 h in SmBm containing 20% FBS. The cells were serum-starved in SmBm containing 1% FBS for 24 hours, then lysed and homogenized in the provided lysis buffer. Samples were pelleted in an ultracentrifuge (Optima TLX Ultracentrifuge, Rotor TLA-110, Beckman Coulter, Brea, CA) at 55,000 rpm for 1 hour at 37°C. Pellets and supernatant fractions were separately processed and processed by SDS-PAGE for subsequent immunoblot analysis.

Construction, Expression and Purification of Human Smooth Myosin II (MYH11)

The cDNA for human MYH11 (SM1A isoform) was truncated at the codon for threonine 1775 (creates a soluble “HMM” construct), after which a glycine plus FLAG peptide (DYKDDDDK) was appended to facilitate purification. Site-directed mutagenesis was performed using Quickchange XLII kit (Stratagene) to introduce the R247C mutation into the same construct. The constructs were subcloned into the baculovirus transfer vector, p2Bac (Invitrogen). Protein expression and purification were as previously described.

Myosin ATPase Assay and Transient Kinetic Assays

The actin-activated ATPase activity assay was performed at 25°C in buffer 20/20 (KCl 20 mM, Mg²⁺ 5 mM, EGTA 1 mM, MOPS 20 mM pH 7.0), ATP 1 mM final concentration and actin concentration ranging from 0 to 150 µM. Actin was purified from rabbit skeletal muscle and stabilized by phalloidin. Phosphorylation of HMM WT and mutant constructs was performed as previously described. Both unphosphorylated and phosphorylated forms of WT and mutant human smooth myosin 2 HMM constructs were assayed at 0.2 nM final concentration. Curves were fitted with Kaleidagraph software. Triplicate assays were performed with three different preparations of each protein.

Transient kinetic measurements (P_i release from myosin and ADP release from actomyosin) were made in buffer 20/20 at 25 °C with an Applied Photophysics SX.18MV stopped-flow instrument following previously published protocols. Assays were performed with three different preparations of each protein.

In Vitro Motility Assay for Myosin

The sliding actin filament assay was utilized to test the functionality of the recombinant proteins. The assay was performed at 30°C, following published procedures. F-actin was fluorescently labeled by polymerizing 5 µM G-actin in the presence of 6.6 uM Alexa Fluor 546 phalloidin (Molecular Probes) in 10 mM imidazole (pH 7.4), 100 mM KCl, 2 mM MgCl₂ and 10 mM DTT at room temperature for 30 min and then stored at 4°C. The non-ATP-containing
standard assay buffer conditions used in this assay were 60 mM KCl, 25 mM Imidazole (pH 7.4), 1 mM EGTA, 4 mM MgCl₂ and 10 mM DTT. In this assay, WT and mutant myosin proteins at a final concentration of 0.3 mg/ml were phosphorylated in 20 mM NaCl, 10 mM MOPS, 8 mM MgCl₂, 0.1 mM EGTA, 0.2 mM CaCl₂, 0.5 mM DTT, 2mM ATP, 1 µM calmodulin and 1.2 µg/ml skeletal MLCK for 30 min at room temperature. For the HMM constructs, an anti-FLAG antibody (Affinity BioReagents #PA1-984B, Golden, CO) was first introduced into the chamber (nitrocellulose coated cover slip), followed by surface blocking (with bovine serum albumin) and introduction of either phosphorylated or unphosphorylated myosin, following the procedure described by Sellers et al. After 2 minutes, the unbound myosin was washed out of the flow cell with two volumes (1 minute each) of 0.5 mg/ml bovine serum albumin in the standard assay buffer, and then 5nM phalloidin-labeled F-actin filaments (non-fluorescent “black” F-actin) diluted in the standard assay buffer were applied to block rigor-like myosin heads. After an additional 2 minutes, unbound “black” actin filaments were washed out with two volumes of the standard assay buffer. The filaments bound to non-rigor-like myosin heads were then dissociated and washed out of the flow cell with 2 volumes of the ATP-containing assay buffer which contained 2 mM ATP, 25 µg/ml glucose oxidase, 45 µg/ml catalase, 1% glucose, 1 mM creatine phosphate and 0.1 mg/ml creatine kinase in addition to the standard assay buffer. The motility assay was then initiated by applying about 5 nM fluorescently labeled F-actin filaments in the motility assay buffer which contained the above ATP-containing assay buffer plus 0.7% methylcellulose. The fluorescently labeled actin filaments were excited at 543 nm with a GreNe laser and their movement was observed via a heated Leica oil immersed 63x /1.4 objective and digitally recorded with a Leica DMR/ TCS SL confocal microscopy system. The data from ≥20 filaments (per preparation and phosphorylation state) were analyzed with Volocity software (Improvision, England).

**Statistical Analysis**

All values are expressed as means ± standard deviation. Statistical differences between WT and mutant mice or cells were analyzed by a Student’s t-test. Morphometric analysis of carotid artery was done by one-way ANOVA. Differences were considered statistically significant at values of P <0.05. Data for cell culture experiments represent three experiments in triplicates using separate cultures.
Reference List


**Online Figure Legends**

**Online Figure I.** Kinetic scheme for the actin-myosin cross-bridge cycle. In this simplified depiction of the actin-myosin kinetic cycle, myosin (M) is shown interacting either strongly or weakly with actin (A). Myosin hydrolyzes ATP to ADP. P_i (inorganic phosphate) while not bound (dissociated) to actin and subsequently sequentially releases the hydrolysis products, P_i and ADP, once it binds to actin. The release of P_i allows for strong binding and force generation, while ADP is released from an already strongly bound state. Force generation and movement occur while myosin is tightly bound to actin.

**Online Figure II.** Densitometric analysis of all Western blots included in the main paper figures.
A. Densitometry for Figure 1F, aortic tissue proteins. Contractile protein levels were normalized to Gapdh. Two wildtype and three Myh11R247C/R247C samples were combined for this analysis, however additional lysates harvested from mice at other ages were consistent with the findings (total n>10 per genotype, data not shown). B. Densitometry for Figure 4C, contractile protein expression in cell culture. Contractile protein levels were normalized to Gapdh. C. Densitometry for Figure 5C, signaling molecules. Phosphorylated protein levels were normalized to total protein levels for both FAK and Akt. D. Densitometry for Figure 6D, treatment with the Rho activator CN03. Contractile protein levels were normalized to Gapdh.

**Online Figure III.** Representative gross architecture of ascending and descending aortas in 5 month old WT, Myh11R247C/+ and Myh11R247C/R247C mice. Whole thoracic aorta were carefully isolated and examined under dissecting microscope. Macroscopically, there is no significant difference in the architectures of ascending aortas, aortic arches and branches and descending aortas in WT, Myh11R247C/+ and Myh11R247C/R247C mice. Arrow indicated the position of the ascending aorta.

**Online Figure IV.** H&E staining of representative sections of mouse ascending aorta at different developmental age. Cross sections were harvested from the ascending aortas of WT, Myh11R247C/+ and Myh11R247C/R247C mice at 5weeks, 8 weeks, 4months, 6 months and 10 months. Five mice were analyzed per each genotype and each time point. No significant differences in aortic diameter or structure were observed.

**Online Figure V.** Elastin staining of representative sections of mouse ascending and descending aortas from WT, Myh11R247C/+ and Myh11R247C/R247C mice at 6 months. Five mice were analyzed per each genotype. No significant differences in number or morphology of elastin layers were observed.

**Online Figure VI.** Representative images of H&E and Elastin stained cross sections of unligated and ligated carotid arteries from WT and Myh11R247C/R247C mice. Carotid arteries were harvested from WT and Myh11R247C/R247C mice at days 7, 14 and 21 after injury. Five mice were analyzed per each genotype and each time point.

**Online Figure VII.** Photomicrographs are representative images of H&E stained cross sections of unligated and ligated carotid arteries from WT and Myh11R247C/+ mice. Carotid arteries were harvested from WT and Myh11R247C/+ mice at 21 days after injury. Five mice were analyzed per each genotype and each time point. Below are quantified data for neointimal area and intima/media ratio of cross sections in ligated and unligated carotid arteries from WT and Myh11R247C/+ mice. No significant differences in neointimal area or intimal/media ratio were observed between WT and Myh11R247C/+ mice.
Online Figure VIII. Characterization of cellular phenotype of SMCs explanted from the ascending aortas of Myh11<sup>R247C/+</sup> mice. Myh11<sup>R247C/+</sup> cells have an intermediate level of expression of contractile genes (A) and proteins (B) compared with Myh11<sup>R247C/R247C</sup> and WT SMCs. However, no significant increase in proliferation (C) was observed in Myh11<sup>R247C/+</sup> cells.

Online Figure IX. Low-powered images of SM-α-actin and MRTFA in WT and Myh11<sup>R247C/R247C</sup> cells. These images are included to supplement the high-powered single-cell images included in the main Figure 4E and 4G to emphasize the pervasiveness of the described phenotypes in the cell population.

Online Figure X. Inhibitors of Rac and FAK do not alter SMC differentiation. (A) The Rac inhibitor NSC23766 does not prevent proliferation in Myh11<sup>R247C/R247C</sup> SMCs. (B) Neither the Rac inhibitor NSC23766 nor the FAK inhibitor PF537288 significantly alters the nuclear localization of MRTFA. (C) The FAK inhibitor PF537228 does not significantly alter the expression of contractile genes in Myh11<sup>R247C/R247C</sup> SMCs.
Online Tables

**Online Table I.** PCR primers for genotyping *Myh11* R247C knock-in mice.

<table>
<thead>
<tr>
<th>Primers</th>
<th>5'-3' orientation</th>
<th>PCR Size</th>
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<tbody>
<tr>
<td>MYH11-P7</td>
<td>TTTCAAGCTCTCAATGCTCA</td>
<td>WT: 191 bp; f/neo: 261 bp</td>
</tr>
<tr>
<td>MYH11-P8</td>
<td>ACACACACTCCCCAGGTCTC</td>
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<tr>
<td>MYH11-P9</td>
<td>TGCAGGGCACATAGAGTCTG</td>
<td>WT: 208 bp; f: 378 bp</td>
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<tr>
<td>MYH11-P10</td>
<td>TTTCTGCATGGTCAGAACAG</td>
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**Online Table II.** The average body weights of one month old male and female mice of various genotypes.

<table>
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<th>Genotype</th>
<th>Body weight</th>
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<tbody>
<tr>
<td>WT (male)</td>
<td>14.0 ± 1.2 g</td>
</tr>
<tr>
<td>WT (female)</td>
<td>13.5 ± 0.8 g</td>
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<tr>
<td><em>Myh11</em> R247C/+ (male)</td>
<td>14.0 ± 1.0 g</td>
</tr>
<tr>
<td><em>Myh11</em> R247C/+ (female)</td>
<td>13.4 ± 1.2 g</td>
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<tr>
<td><em>Myh11</em> R247C/R247C (male)</td>
<td>13.9 ± 1.5 g</td>
</tr>
<tr>
<td><em>Myh11</em> R247C/R247C (female)</td>
<td>13.4 ± 1.5 g</td>
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**Online Table III.** The number and genotypes of pups derived from the *Myh11* R247C/+ breeding pairs at wean.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Litters per female</th>
<th>Pups per litter</th>
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<tbody>
<tr>
<td>WT</td>
<td>8.5</td>
<td>7.2 ± 2.1</td>
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<tr>
<td><em>Myh11</em> R247C/+</td>
<td>8.7</td>
<td>7.1 ± 2.2</td>
</tr>
<tr>
<td><em>Myh11</em> R247C/R247C</td>
<td>8.3</td>
<td>7.2 ± 1.9</td>
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Online Figure IV
Online Figure V
Online Figure VI

<table>
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<tr>
<th></th>
<th>Day 7</th>
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<th>Day 21</th>
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<tbody>
<tr>
<td>Injured</td>
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<tr>
<td>Uninjured</td>
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<tr>
<td>Injured</td>
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<tr>
<td>Uninjured</td>
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Online Figure VI
**Online Figure VIII**

A

![Graph showing gene expression levels](image)

- Acta2
- Cnn1
- Myh11

Legend:
- WT
- Myh11 R247C/+ (Light grey)
- Myh11 R247C (Dark grey)

B

![Western blot images](image)

- SM-MHC
- Alpha-actin
- Calponin
- Gapdh

C

![Bar graph showing BrdU incorporation](image)

- Untreated
- WT
- Myh11 R247C/+ (Light grey)
- Myh11 R247C/R247C (Dark grey)
Online Figure X