Myocardin is Differentially Required for the Development of Smooth Muscle Cells and Cardiomyocytes

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Abstract

Myocardin is an SRF co-activator exclusively expressed in cardiomyocytes and smooth muscle cells (SMCs). However, there is highly controversial evidence as to whether myocardin is essential for normal differentiation of these cell types and there are no data showing if cardiac or SMC subtypes exhibit differential myocardin requirements during development. Results of the present studies showed the virtual absence of myocardin^{-/-} visceral SMCs or ventricular myocytes in chimeric myocardin knock out (KO) mice generated by injection of myocardin^{-/-} embryonic stem cells (ESC) into wild-type (i.e. myocardin^{+/+} ESC) blastocysts. In contrast, myocardin^{-/-} ESC readily formed vascular SMC, albeit at a reduced frequency as compared to WT ESCs. In addition, myocardin^{-/-} ESCs competed equally with WT ESCs in forming atrial myocytes. The ultrastructural features of myocardin^{-/-} vascular SMCs and cardiomyocytes were unchanged from their WT counterparts as determined using a unique x-ray microprobe transmission electron microscopic method developed by our laboratory. Myocardin^{-/-} ESC-derived SMCs also showed normal contractile properties in an in vitro embryoid body SMC differentiation model, other than impaired thromboxane A2 responsiveness. Taken together, these results provide novel evidence that myocardin is essential for development of visceral SMCs and ventricular myocytes, but is dispensable for development of atrial myocytes and vascular SMCs in the setting of chimeric KO mice. In addition, results suggest that as yet undefined defects in development and/or maturation of ventricular cardiomyocytes may have contributed to early embryonic lethality observed in conventional myocardin KO mice and that observed deficiencies in development of vascular SMC may have been secondary to these defects.
Introduction:

Myocardin was initially identified by an in silico screen for cardiac cell specific genes (40) and was shown to be a powerful SRF co-activator in cardiac and smooth muscle cells (SMCs) (2; 7; 43; 45). However, whether myocardin is absolutely required for normal development of SMCs, or whether its functions can be replaced by the myocardin related transcription factors (MRTF) A and B and/or other genes in vivo is controversial. Olson and co-workers (20) demonstrated that conventional myocardin knockout (KO) mice showed reduced numbers of vascular SMCs in the developing aorta, but this phenotype may be secondary to other developmental defects as KO embryos also showed gross developmental retardation. These mice also had no overt evidence of vascular leakage (e.g. hemorrhage) common to other models of defective SMC-pericyte maturation/differentiation and investment such as KLF2 (17) or PDGF receptor/ligand KO mice (10; 21). Moreover, myocardin KO mice died earlier in development [i.e. 10.5 days post-coitus (dpc)] than would be expected if lethality were due to defective SMC/pericyte investment and differentiation alone. Whereas conventional myocardin KO mice showed no overt defect in heart development, there was evidence that embryonic lethality at 10.5 days post-coitus was secondary to pericardial effusion (20). In addition, these investigators showed that morpholino-induced suppression of myocardin in Xenopus embryos was associated with impaired heart development. Taken together, these observations raise the possibility that failed vascular SMC differentiation in conventional myocardin KO mice may have been secondary to as yet undefined defects in heart function, rather than reflecting a direct absolute requirement for myocardin in SMC differentiation.
Although the preceding results suggest that myocardin may be required for normal heart development, Huang and Parmacek (12) showed that cardiomyocyte-selective KO of myocardin was not associated with any detectable changes in heart development, and KO mice were born at expected Mendelian ratios. However, mice subsequently developed lethal cardiomyopathy post-natally due to impaired cardiomyocyte structural organization, loss of contractile function, and programmed cell death. These results clearly indicate that myocardin is required for maturation and/or maintenance of differentiated function in cardiomyocytes. However, it is important to note that the conditional KO of myocardin in cardiomyocytes in these studies would have occurred after cardiomyocyte differentiation and thus do not address whether myocardin is actually required for initial development of atrial or ventricular myocytes.

Selective knockout of myocardin in neural crest derived cells has been shown to be associated with defective differentiation of SMC within the ductus arteriosus and perinatal lethality due to failed ductus closure (11). These observations are significant in that the ductus functions as a static shunt and requires no SMC contraction in embryos such that there would likely be little or no stimulus for compensatory activation of MRTFs until parturition when it would be too late for compensation to be effective to permit survival. Taken together, results suggest that myocardin is required for development of fully differentiated, SMC within the ductus arteriosus, although a limitation of these studies is that they did not directly assess SMC contractility. As such, it is possible that failed ductus closure could have been due to structural defects rather than impaired SMC contraction secondary to defective differentiation.
Our understanding of the role of myocardin in SMC development was greatly confounded by studies by Olson and co-workers, in collaboration with our laboratories(29), showing that myocardin\(^{-/-}\) ESCs contribute to development of aortic SMC in the setting of chimeric KO mice produced by injection of lineage tagged myocardin\(^{-/-}\)/LacZ\(^{+}\) embryonic stem cells (ESCs) into wild type host blastocysts. Significantly, these myocardin\(^{-/-}\) aortic SMCs expressed a number of SMC differentiation marker genes including SM \(\alpha\)-actin and SM myosin heavy chain (SM-MHC), and were morphologically indistinguishable from SMCs derived from WT ESCs from the host blastocyst at least as observed at the level of low resolution light microscopy. However, there were several limitations in these studies, including the failure to test if myocardin was required for SMC development in other blood vessels or other SMC-containing tissues, whether there were functional or ultrastructural alterations in the myocardin\(^{-/-}\) aortic SMCs, and whether myocardin\(^{-/-}\) ESCs were able to form SMC lineages at a frequency equivalent to WT ESCs. Consistent with the studies in the mouse chimeric injection model, results of our studies in embryoid bodies (EBs) (29), an in vitro development model that bypasses potential embryonic lethality due to failed morphogenesis and organogenesis, showed that myocardin was not required for development of SMC lineages. However, there was evidence of altered kinetics of activation of several SMC marker genes and a quantitative decrease in SM-MHC expression.

Taken together, at present, there is uncertainty as to whether myocardin is absolutely required for development of cardiomyocyte or SMC lineages. It is also unknown whether myocardin dependence differs between different SMC subtypes.
including SMCs derived from distinct embryological origins (25), and/or if there are functional deficits or other abnormalities in myocardin^-/- vascular SMCs that form in chimeric KO mice. The present studies address each of these questions and provide novel evidence showing that myocardin is essential for SMC development in the bladder, gut, and several other visceral SMC tissues in that there was a virtual absence of myocardin-null lacZ-tagged (i.e. myocardin^-/-/LacZ^+ ESCs) derived SMCs in these tissues in chimeric knockout mice. We also confirmed previous observations showing that myocardin^-/- ESC populate vascular SMC tissues (29), but extend previous studies by showing myocardin^-/- ESC are present at a much reduced frequency in vascular SMC tissues indicating they are at a selective disadvantage as compared to the WT host cells. However, of interest, we found that the myocardin^-/- aortic SMCs that did form, had myofilaments and an overall ultrastructural appearance indistinguishable from that of adjacent WT (i.e. myocardin^+/+) SMCs as determined using a unique transmission electron microscopic lineage tracing method developed by our laboratory. Finally, of major significance, we provide completely unexpected results showing the virtual exclusion of myocardin^-/- ESCs from ventricular, but not atrial, cardiomyocytes indicating there is normally an absolute requirement for myocardin for development of these cells, but in the setting of conventional myocardin KO mice, this requirement is somehow circumvented, possibly through activation of compensatory genes such as MRTF A and B.
Materials and Methods:

Chimeric Analyses:
Myocardin\(^{-/-}\)/Gt(ROSA)26Sor-lacZ (LacZ\(^{+}\)) and myocardin\(^{+/-}\)/Gt(ROSA)26Sor-lacZ (LacZ\(^{+}\)) chimeras were generated by both the University of Texas Southwestern Transgenic Core and the University of Virginia Gene Targeting and Transgenic Facility as described, with similar results from each facility over multiple blastocyst injections and n>20 for both heterozygous and null populations(29). Animal experiments were done at 4-5 weeks of age. Animal experiments were approved by the Animal Care and Use Committee Institutional Review Board at the University of Virginia Health Sciences Center and University of Texas Southwestern.

Fixation and 5-Bromo-4-Chloro-3-Indolyl \(\beta\)-D-Galactoside (X-Gal) and 5-Bromo-3-indolyl \(\beta\)-D-galactopyranoside (Bluo-gal) Staining:

Smooth muscle tissues harvested from mice were washed twice in phosphate buffered saline (PBS), fixed in ice cold 4% paraformaldehyde in PBS for one hour or 2% formaldehyde, 0.2% glutaraldehyde in PBS at room temperature for 10 minutes (tissues were stained using two methods to ensure no difference in quality of staining was observed between the protocols used routinely by the Olson and Owens labs). Because of their small size, aortic strips for the histologic assays of percentage chimerism were fixed for 2 minutes at 37\(^{\circ}\)C in 2% formaldehyde, 0.2% glutaraldehyde in PBS to prevent loss of \(\beta\)-galactosidase signal, washed again in PBS and stained for 48 hours using standard X-gal staining solution; 5 mM ferrocyanide, 5 mM ferricyanide, 2 mM MgCl\(_2\), 0.1% NP40,
0.1% sodium deoxycholate, 1 mg/mL X-gal (Sigma, USA) in dimethylformamide. For electron microscopy analysis rings of thoracic aorta were washed with 10 ml Hepes-buffered Krebs solution and then fixed in 2% glutaraldehyde plus 6% sucrose in 0.075 M Na-Cacodylate buffer pH 7.4 (EM-fix) for approximately 25 minutes. The aortic rings were then incubated overnight in EM-Bluo-gal stain solution; 0.1 M Na-Cacodylate, 5 mM ferrocyanide, 5 mM ferricyanide, 2 mM MgCl₂, 6% sucrose, and 1 mg/ml Bluo-gal (Sigma) solubilized in dimethylformamide. The aortic rings were then post-fixed in EM-fix for an additional 4-6 hours post-fixed in osmium tetroxide, followed by uranyl acetate staining and embedding in Spurr’s resin (4). Hearts analyzed at p10 were fixed in 4% paraformaldehyde at 4°C for 1 hr, washed in phosphate buffered saline (PBS) twice and stained overnight in standard X-gal staining solution; 5 mM ferrocyanide, 5 mM ferricyanide, 2 mM MgCl₂, 0.1% NP40, 0.1% sodium deoxycholate, 1 mg/mL X-gal (Sigma, USA) in dimethylformamide. Embryos from 10.5dpc and 12.5dpc were fixed for 30 or 60 mins at 4°C in 2% formaldehyde 0.2% glutaraldehyde washed in PBS twice and stained overnight in standard X-gal staining solution. 14.5dpc embryos were fixed for 60 mins in 2% formaldehyde 0.2% glutaraldehyde at 4°C then decapitated and bisected below the ribcage to allow penetration of fix and stain into the thoracic cavity, fixed for an additional 30 minutes then washed and stained as described above. For electron microscopy hearts were first perfused with 10 ml Hepes-buffered Krebs solution supplemented with 3 mg/ml 2,3-Butanedione monoxime (BDM, Sigma Aldrich, St. Louis MO) and heparin (2.5 Units), and then perfusion-fixed with 10 ml of 2% glutaraldehyde plus 6% sucrose in 0.075 M Na-Cacodylate buffer pH 7.4 (EM-fix). Hearts were then carefully removed under a stereoscopic microscope, and fixed for an additional 25
minutes. Next the atria and ventricles were flushed with and incubated overnight in EM-
Blue-gal stain solution; 0.1 M Na-Cacodylate, 5 mM ferrocyanide, 5 mM ferricyanide, 2
mM MgCl₂, 6% sucrose, and 1 mg/ml Blue-gal (Sigma) solubilized in
dimethylformamide. Hearts were then post-fixed in EM-fix for an additional 4-6 hours
prior to post fixation in osmium tetroxide, staining with uranyl acetate and embedded in
Spurr’s resin (8).

Southern Analysis and Densitometry

A probe complementary to the myocardin locus was generated 3’ to the targeting
insert described in the knockout strategy (20) using the following PCR primers (MWG
Biotech, High Point NC, USA): For – CTGGCATCTAAACACCTACACAAG, Rev –
TTAAACTAGAGCCCCATTCATCATT. The fragment was amplified from mouse
 genomic DNA and cloned using a TOPO-TA kit (Invitrogen, Carlsbad CA, USA),
amplified, and subsequently digested out of the purified plasmid with EcoRI.

Mice were killed by halothane inhalation and dissected tissues placed in DNA
digestion buffer (100 mM NaCl, 50mM Tris pH 8.0, 25mM EDTA pH 8.0, 0.5% SDS,
0.5mg/ml Proteinase K). The remainder of the animal was homogenized in a blender in
200 ml PBS + 25mM EDTA, pelleted at 3000 x g, then digested in 5 times the volume of
DNA digestion buffer. Organs were incubated for 2-3 hours at 55 degrees with rotation
and DNA was purified by phenol:chloroform extraction and precipitation in isopropanol.
10μg of each DNA (~2.5 μg for aorta as this was the maximum amount of DNA that
could be purified from a single aorta) was digested with EcoRI, electrophoresed in a
0.8% Agarose TAE gel, depurinated in 0.25 N HCl for 15 minutes, washed in 1.5 M
NaCl/0.5 N NaOH 30 minutes then transferred to Hybond-N+ nitrocellulose membrane (Amersham, Piscataway NJ, USA) using 0.4 N NaOH overnight. Probe was radiolabeled with α[^32P]dCTP using RediPrime II random-prime labeling kit (Amersham), hybridized to the membrane and exposed to film for 1-5 days. A standard curve using DNA purified from myocardin^-/^ and myocardin^-/^ ESC was used to determine that exposures were in the linear range of the film (r^2 of fit >0.9). We used the Fluorchem AlphaEaseFC software (Alpha Innotech, San Leandro CA, US) to perform densitometry on the films.

**Histological staining**

Tissues for histological analysis were post-fixed in 2% formaldehyde, 0.2% glutaraldehyde overnight, and submitted to the UVA Research Histology Core where they were paraffin-embedded, sectioned and stained with Eosin or Nuclear Fast Red (Vector Labs, Burlingame, CA).

**Percent Chimerism Histologic Assays**

Three rings of thoracic aorta from the same region of the distal end of the vessel of 4-6 week old mice were cut, fixed and stained for X-gal as described above, post-fixed, and sectioned longitudinally. The sections were stained with Nuclear Fast Red according to manufacturer’s protocol. The entire area of three strips was imaged at 100x magnification. Sections of liver were processed similarly and imaged. A student, blinded to the samples, counted positive and negative nuclei for X-gal in 5 images chosen at random from each strip as well as 5 images from each liver as a control measure of chimerism in each animal.
Electron Microscopy and Electron Probe X-ray Microanalysis (EPMA):

Myocardin⁻/⁻/LacZ⁺ cells were definitively identified in aortic sections at the electron microscopic level by spectroscopic detection of the bromine emission peak arising from the reaction product of the Bluo-gal reagent. This reagent gives rise to a fine electron-dense precipitate localized at cell membranes (44). EPMA is based on the generation of x-ray spectra by atomic core shell excitation of elements in a given cellular microvolume, along with the simultaneous measurement of the mass of this microvolume by detection of continuum (background) x-rays. The ratio between the characteristic x-ray count of a certain element and the continuum x-ray count is thus proportional to the concentration of that element in the microvolume being analyzed (33). The accuracy of this technique in measuring subcellular element concentrations has been well documented (37). EPMA was performed with a Phillips CM12 electron microscope operated at 120 keV in transmission mode and fitted with a LaB₆ filament. The microscope was equipped with an ultrathin window energy-dispersive x-ray detector and an XP3 pulse processor (Oxford Link) interfaced to a 4 pi Spectral Engine. X-ray spectra were collected and displayed at 2.5 eV per channel. The electron beam was focused on electron dense deposits found in some of the cells as well as in regions adjacent to the deposits in the same cell and spectra collected. Br peaks (1.48, 1.52 keV, Lα, Lβ-edges respectively) arising from the deposits were used to identify the myocardin⁻/⁻/LacZ⁺. These cells were photographed for morphological analysis and compared with cells lacking deposits.
Embryoid Body (EB) Culture and formation of EB-Derived Purified Myocardin +/- and -/- SMC Lines

We cultured EBs and purified SMC from myocardin<sup>−/−</sup> and myocardin<sup>+/−</sup> ESC (29) as described (34) with the exception that the SMαA-Puromycin plasmid carried a constitutive Zeocin resistance gene instead of neomycin to establish the stable ESC lines since the knockout cells were already resistant to Neomycin from the knockout targeting construct. A detailed protocol is available on request. Stable ESC clones with the SMαA-Puromycin construct were maintained in zeocin selection medium while they were expanded in an undifferentiated state. The cells were confirmed to be undifferentiated by Sox-2 staining with an Abcam (ab#15830, Cambridge MA) rabbit polyclonal antibody per manufacturer protocol (data not shown). EB-derived purified SMC lines (henceforth referred to as “EB-Derived SMCs”) were maintained in puromycin to prevent overgrowth of possible non-SMαA positive cell contaminants after selection.

Contractile Measurements in Chimeric Mouse Thoracic Aortas and Reconstituted Muscle Fibers Generated from Myocardin +/- and Myocardin -/- EB-Derived Purified SMC

Thoracic aortas were carefully removed from the mouse (under a dissection stereomicroscope) and cleaned of periadventitial fat and loose connective tissue in physiologic HEPES-buffered Krebs’ solution. Rings (600 – 800 μm wide) cut from the distal end of the thoracic aorta, were cut again such that a strip was formed. The ends of the strips were attached with silk monofilaments to wire hooks connected a length
adjusting device and a force transducer (AE 801; SensoNor A.S., Horten, Norway, 276 http://sni.nextframe.net/index.html) on a “bubble plate” (36). Once mounted to the force 277 transducer, the strip was stretched to 1.1× its resting length, and allowed to equilibrate in 278 HEPES-buffered Krebs’ for 30 minutes prior to agonist stimulation. Strips were 279 challenged with depolarizing solution (154 mM K⁺) and the stable thromboxane A2 280 mimetic U-46619. All experiments were performed at 37°C.

EB-Derived SMC purified from three independent myocardin⁺/+ and myocardin⁻/⁻ 282 ESC clones were cultured and aggregated into reconstituted muscle fibers according to 283 the methods previously described in Sinha et al. (34). Following 7 days in culture (5% 284 CO₂, 37°C) with EB medium, longitudinal strips (0.75 – 0.80 mm wide, ~1.5 mm long) 285 were cut with a razor knife from the RMF, transferred to HEPES-buffered Krebs’ 286 solution, and mounted to a “bubble-plate” force transducer as previously described (36). 287 All experiments were performed at 37°C.

Western Blotting

Western blotting for smooth muscle differentiation markers was performed as previously 292 described (34) using mouse monoclonal SMαA (clone 1A4, Sigma, USA), rabbit 293 polyclonal SMMHC (BT-562, Biomedical Technologies, Inc., Stoughton, MA). GAPDH 294 (Clone 6C5, Chemicon International, Temecula CA, USA), chemifluorescence was 295 detected and densitometry performed using a Fluorchem 8800 digital imaging system and 296 AlphaEaseFC software (Alpha Innotech, San Leandro CA, US). RhoGDI, Gₐ₁₂, Gₐ₁₃, and 297 Gₐ₉/₁₁ rabbit polyclonal antibodies, as well as anti-RhoA antibodies (clone 26C4) were 298 purchased from Santa Cruz Biotechnology Inc. (Santa Cruz CA). Thromboxane A₂, and 299 the endothelin A and B receptors were detected using rabbit polyclonal antibodies from
Cayman Chemical (Ann Arbor MI) and Abcam Inc. (Cambridge MA), respectively. The rabbit polyclonal anti-calponin antibody was a generous gift from Dr. M. P. Walsh (University of Calgary). ROCK I/β and ROCK II/α mouse monoclonal antibodies were purchased from BD Biosciences (San Jose CA). The C-terminal derived anti-cGMP dependent protein kinase I antibody (which detects both α and β isoforms) was purchased from Assay Designs Inc. (Ann Arbor MI). Primary antibodies were labeled with goat anti-mouse Alexa 680 (Invitrogen, Carlsbad CA) or goat anti-rabbit IRDye800 (Rockland Immunochemicals, Gilbertsville PA) conjugated secondary antibodies, and detected and quantified using an Odyssey Imaging System (Licor, Lincoln NE).

**Real Time RT-PCR**

Reverse transcription and Real Time RT-PCR was carried out as previously described (32). Primers for Real Time RT-PCR will be provided upon request.

**Flow Cytometry**

Flow cytometry for SMαA in EBs was performed as previously described (32), with the exception that a SMαA-Cy3 conjugated 1A4 antibody was used (Sigma, USA), and fluorescence-minus-one controls were used to set the gate for positive cells rather than IgG controls.

**Statistics:**

For significance testing in nuclear counting, densitometry and Real-Time RT-PCR assays we used the Student’s t-test (Microsoft, Excel).
Results:

Myocardin\(^{+/−}/\)LacZ\(^{+}\) ESC Derived SMCs were Virtually Absent within Visceral and Bladder SMC Tissues in Chimeric KO Mice

Particularly striking in the analysis of the myocardin\(^{+/−}/\)LacZ\(^{+}\) chimeric mice was the failure of the myocardin\(^{+/−}\) ESCs to contribute to visceral SMCs while contributing consistently to vascular SMCs and other cell types other than ventricular myocytes (see below). In over 30 bladders from myocardin\(^{+/−}/\)LacZ\(^{+}\) animals from multiple independent blastocyst injections at both the UVA and UT Southwestern facilities, a similar pattern was observed. That is, myocardin\(^{+/−}/\)LacZ\(^{+}\) cells were observed in the SMC layer of bladders from heterozygous control chimeric mice (Figure 1A) but were consistently absent from bladder SMC of the myocardin\(^{+/−}/\)LacZ\(^{+}\) chimeras (Figure 1B). When evaluated in cross-sectional histological sections, the heterozygous knockout myocardin\(^{+/−}/\)LacZ\(^{+}\) ESCs contributed to bladder SMCs (Fig 1C), while myocardin\(^{+/−}/\)LacZ\(^{+}\) ESCs only contributed to the bladder epithelium and interstitial cells, but not SMCs (Figure 1D). In light of this observation, we carefully examined other SMC tissues to see if the contribution by myocardin\(^{+/−}/\)LacZ\(^{+}\) cells was altered. A similar pattern was observed in the stomach with myocardin\(^{+/−}/\)LacZ\(^{+}\) controls showing staining of the SMC layer of the gut (Figure 1E, 1I) while myocardin\(^{+/−}/\)LacZ\(^{+}\) cells did not (Figure 1F). Interestingly, at the transition between skeletal muscle and smooth muscle in the esophagus, the myocardin\(^{+/−}/\)LacZ\(^{+}\) cell contribution rapidly fell off (Figure 1F). Indeed, examination of cells at the transition region showed myocardin\(^{+/−}/\)LacZ\(^{+}\) cells contributing to striated skeletal muscle, but not to non-striated SMCs (Figure 1F, inset), compared to myocardin\(^{+/−}/\)LacZ\(^{+}\) cells which contributed to both muscle types (Figure 1J). It should
also be noted that myocardin$^{-/-}$/LacZ$^+$ cells consistently gave rise to vascular SMC within blood vessels within visceral SMC tissues.

The pattern of myocardin$^{-/-}$ cells failing to contribute to visceral SMC extended throughout the gut. For example, there was little, if any, contribution of myocardin$^{-/-}$ cells/LacZ$^+$ cells to the SMC layers (Figures 1H and 1P) of the large intestine, whereas myocardin$^{+/+}$/LacZ$^+$ cells did contribute to the parasympathetic ganglia. In contrast, SMCs from myocardin$^{+/+}$/LacZ$^+$ ESCs were readily observed in SMC layers of the large intestine (Figures 1G and 1O). Similar to observations of reduced contribution of myocardin$^{-/-}$/LacZ$^+$ ESCs to visceral SMCs, we also observed a marked decrease in these cells investing into SMCs with the lung bronchi (Figures 1K versus L) and uterus (Figures 1M versus N).

Myocardin$^{-/-}$ cells exhibited a marked decrease in investment into cardiac ventricles but contributed normally to coronary blood vessels and atrial myocytes.

Hearts of myocardin$^{-/-}$/LacZ$^+$ chimeric mice showed an unexpected profound decrease in the contribution of myocardin$^{-/-}$/LacZ$^+$ ESCs into the ventricles of the heart at P10 (Figure 2A). Indeed, myocardin$^{-/-}$/LacZ$^+$ cells which persisted in the adult ventricle were confined to a superficial band beginning at the intraventricular septum and wrapping around the right ventricle (Figure 2B). The same pattern was seen in 100% of 30 chimeric mice examined at ten days, 28 days, and at six months of age and across multiple independent blastocyst injections. Longitudinal histological sections through the ventricle showed occasional myocardin$^{-/-}$/LacZ$^+$ cells within the myocardium indicating that at least some myocardin$^{-/-}$/LacZ$^+$ cells were capable of differentiation into ventricular
myocytes (Figure 2C). In contrast, myocardin\textsuperscript{+/−}/LacZ\textsuperscript{+} ESC showed homogenous
distribution throughout the heart (Figure 2A, hearts 1-3, and Figure 2C, upper panel). Investment of myocardin\textsuperscript{+/−}/LacZ\textsuperscript{+} ESCs within the coronary vasculature appeared to be
unaffected indicating that myocardin is not required for differentiation of coronary SMCs
which are derived from the pro-epicardial organ (25) (Figure 2B, Figure 2D). These
latter results extend observations from our previously published studies showing that cell
autonomous myocardin is not required for differentiation of vascular SMC derived from
mesodermal sources(29).

Myocardin\textsuperscript{+/−}/LacZ\textsuperscript{+} ESCs were Underrepresented in Visceral SMC Tissues Based on
Quantitative Analysis of Genomic DNA by Southern Analyses.

Although unlikely, it is possible that myocardin\textsuperscript{+/−}/LacZ\textsuperscript{+} ESCs contributed to
formation of SMC tissues in the gastrointestinal tract, but somehow the LacZ transgene
may have been silenced thus abrogating our ability to detect myocardin myocardin\textsuperscript{−/−}
/LacZ\textsuperscript{+} SMCs. To rule out this possibility, we performed quantitative Southern blot
analyses to detect the relative number of cells present in various tissues of mixed
chimeric mice that were derived from WT ESC (having +/+ myocardin alleles) versus
either the myocardin\textsuperscript{+/−} or myocardin\textsuperscript{−/−} ESC. We analyzed tissues from five myocardin\textsuperscript{+/−}
/LacZ\textsuperscript{+} and four myocardin\textsuperscript{+/−}/LacZ\textsuperscript{+} mice and performed densitometry of the bands. If
myocardin\textsuperscript{+/−} and \textsuperscript{+/−} ESCs contribute equally to all tissues, one would expect the ratio of
−/− and +/− alleles relative to +/+ cells derived from host blastocyst ESC to be equivalent
between a whole animal DNA lysate (designated “whole mouse” in Figure 3) and
individual organ DNA lysate within the same animal. Comparison of the −/− to +/+ and
+/- to +/- ratios from each tissue to the ratios in a whole mouse lysate indicates whether there is an increase or decrease in the relative contribution of the myocardin +/- or myocardin -/- ESCs relative to +/- host blastocyst within any given tissue. Visceral and vascular smooth muscle lysates were compared to whole mouse lysate. Results of the Southern analysis showed that bladder and gut tissues had a reduced contribution of myocardin -/- ESCs as compared to WT host blastocyst ESCs when normalized to overall chimerism using whole mouse lysates. In contrast, results of analysis of liver homogenates showed an equivalent contribution of myocardin -/-/LacZ+ ESCs as wildtype host ESCs indicating no detectable effect of loss of myocardin for development of hepatocytes or other liver cell types (Figure 3B). These results are in full agreement with histological results showing a marked reduction in myocardin -/-/LacZ+ ESCs to visceral SMCs tissues as shown in Figure 1. Southern results from aorta showed a trend towards a decreased contribution of myocardin -/-/LacZ+ ESCs, but this did not achieve statistical significance, perhaps due to the fact these samples contain a substantial proportion of non-SMCs, including adventitial fibroblasts, neurons, and other non-SMCs which are not myocardin dependent.

Myocardin -/-/LacZ+ Derived SMCs were Underrepresented in Vascular SMC Tissues Based on Quantitative Analyses of the Proportion of LacZ Positive Cells

Southern blots are limited in sensitivity due to the tissues being composed of multiple cell types other than SMCs, thus lowering the signal:noise ratio. For example, aortic results are limited by the fact that the aortic samples also include DNA derived from endothelial cells, adventitial fibroblasts, and other non-SMC which would
presumably not show any dependence on myocardin since it is not known to be expressed in these cells at any point in their development. We therefore designed a more sensitive assay to detect quantitative differences in the contribution of myocardin\(^{+/−}\)/LacZ\(^{+}\) ESCs to vascular SMC tissues. We isolated corresponding thoracic aorta segments from five myocardin\(^{+/−}\)/LacZ\(^{+}\), and five myocardin\(^{−/−}\)/LacZ\(^{+}\) chimeric animals, fixed and stained with X-gal, and then sectioned *en face* to allow for easier identification of individual SMCs within the media of blood vessels. Nuclear Fast Red was used to identify nuclei, and an evaluator blinded to the samples counted LacZ\(^{+}\) and LacZ\(^{−}\) nuclei in thoracic aorta and liver controls to determine if there was a difference in contribution to vascular SMCs (Figure 4A). Assuming that the contribution to the liver by either myocardin\(^{+/−}\)/LacZ\(^{+}\) or myocardin\(^{−/−}\)/LacZ\(^{+}\) ESC is not different (Figure 3), the number of LacZ\(^{+}\) cells in the sections of the thoracic aorta was normalized to the liver. These analyses revealed a highly significant, quantitative decrease (p<0.01) in the fraction of myocardin\(^{−/−}\)/LacZ\(^{+}\) SMC within the aortas of chimeric KO mice. Indeed, the myocardin\(^{−/−}\)/LacZ\(^{+}\) cells comprised fewer than 20% of the cells in the aortic media as compared to approximately 50% of the cells in the liver. Myocardin\(^{+/−}\)/LacZ\(^{+}\) cells were present in blood vessels at the same frequency as in liver, indicating that loss of one myocardin allele had no effect on formation of aortic SMCs.

**Myocardin\(^{+/−}\)/LacZ\(^{+}\) Derived Cardiomyocytes were under-represented in cardiac ventricles as determined by quantitative Southern analysis.**

To determine whether the myocardin\(^{+/−}\)/LacZ\(^{+}\) cells persisted in the cardiac ventricles but were not detectable through X-gal staining, we performed Southern blot analysis with a
probe directed towards the 3' region of the myocardin gene locus (Figure 3B). The signal from +/+ and -/- alleles in the whole mouse was compared to the signal from the liver and ventricles. Assuming that the contribution to the liver by either myocardin+/LacZ+ or myocardin-/-/LacZ+ cells is not different (Figure 3), the number of LacZ+ cells in the ventricle was normalized to the liver. Myocardin-/-/LacZ+ cells had a ventricle/whole mouse signal significantly less than the ratio for myocardin+/LacZ+ controls indicating that the contribution of myocardin-/-/LacZ+ cells to the ventricles was significantly less than the contribution to other tissues. The ratios for ventricular samples are also likely to under-estimate the deficiency in myocardin-/-/LacZ+ ESCs forming ventricular myocytes since these samples also contain coronary SMCs, interstitial fibroblasts, endocardial, and epicardial cells which appeared to contain abundant myocardin-/-/LacZ+ cells (see Figures 2B, 2D).

Myocardin-/-/LacZ+ ESCs Formed Aortic SMC within Chimeric Knockout Mice and had Normal Myofilaments and Overall Ultrastructural Appearance Based on Examination by Electron Microscopy and Electron Probe Microanalysis

To determine if the myocardin-/-/LacZ+ cells that populate the aorta are fully-differentiated SMCs, we developed a novel method for identifying and assessing the ultrastructural appearance of myocardin-/-/LacZ+ cells within the aorta. The unequivocal identification was accomplished by staining aortas with bluo-gal (Figure 5A), which forms dense precipitates of non-soluble bluo-gal crystals in the presence of galactosidase activity, that can be readily identifiable by a combination of TEM appearance and detection of bromine in the bluo-gal crystals through Electron Probe Microanalysis.
21 (Figure 5 box inserts in Figures 1A and 1C). The myocardin<sup>−/−</sup>/LacZ<sup>+</sup> SMCs within the aorta had ultrastructural characteristics consistent with a functional contractile apparatus including arrays of myosin filaments that appeared indistinguishable from those present in adjacent WT ESC derived aortic SMCs (large arrows in Figures 5A versus 5B). We should note that while our methods have a high degree of fidelity such that we are confident that a lacZ positive cell is indeed derived from a myocardin <sup>−/−</sup> ESC, due to inherent methodological limitations associated with balancing the need for adequate TEM fixation while retaining the ability to detect LacZ enzymatic activity, we believe there are likely many false negatives with this method. As such, there may be myocardin <sup>−/−</sup> SMC that have not been identified as such. Thus our results show that at least some myocardin<sup>−/−</sup> SMC have ultrastructural characteristics indistinguishable from adjacent myocardin<sup>+/+</sup> SMC but we cannot conclude this is true of all myocardin<sup>−/−</sup> SMC.

Myocardin<sup>−/−</sup>/LacZ<sup>+</sup> ESCs Formed Cells in the Adult Heart that were Phenotypically Normal by Ultrastructural Analyses.

The preceding analyses clearly showed that myocardin<sup>−/−</sup>/LacZ<sup>+</sup> ESCs exhibited reduced investment into ventricular myocytes. However, a small subfraction of myocardin<sup>−/−</sup>/LacZ<sup>+</sup> were present within ventricles (Figure 5C). Blue-gal staining was used to identify myocardin<sup>−/−</sup>/LacZ<sup>+</sup> ventricular myocytes in sections of hearts from myocardin<sup>−/−</sup>/LacZ<sup>+</sup> chimeras. Although myocardin<sup>−/−</sup>/LacZ<sup>+</sup> cells were grossly under-represented within ventricles, those that did invest had an ultrastructural appearance indistinguishable from those derived from wild type host ESCs including a regular lattice of myosin filaments surrounded by actin filaments (Figure 5C, circular inset). These results indicate that cell
autonomous myocardin gene expression is not an absolute requirement for formation of ventricular myocytes. However, the extraordinarily low frequency of myocardin^{−/−} ESC which formed ventricular myocytes, clearly indicates there is a critical myocardin-dependent step in the early development of these cells that coincides with their location along the axis between the ventricles as seen in Figure 2A.

**Myocardin^{−/−}/LacZ^{+} SMCs Exhibited Normal Contractile Properties**

In order to determine if SMCs derived from myocardin^{−/−}/LacZ^{+} ESCs were capable of normal contraction in response to agonists, the contractility of aortic strips was measured. Strips (0.8 wide x 1.5 mm long) cut from both myocardin^{−/−}/LacZ^{+} (n = 15 total, n = 3 strips from n = 5 mice) and myocardin^{+/−}/LacZ^{+} (n = 15 total, n = 3 strips from n = 5 mice) chimeric mouse aortas contracted similarly following stimulation with 154 mM K^{+}, and relaxed back to baseline upon switching to HEPES-buffered Krebs’ solution (data not shown). There were no significant differences in maximal 154 mM K^{+} induced force between myocardin^{+/−}/LacZ^{+} and myocardin^{−/−}/LacZ^{+} chimeric mouse aortas (Figure 6A).

In order to further explore the contractile capabilities of myocardin^{−/−}/LacZ^{+} SMCs, we measured dose response curves to the stable thromboxane A2 mimetic U-46619. Thromboxane A2 signaling is known to result in a large contractile force with a relatively small increase in intracellular calcium ([Ca^{2+}]_{i}) (1, 12). No differences in either maximal force development or thromboxane sensitivity were evident between thoracic aortic strips cut from myocardin^{+/−}/LacZ^{+} and myocardin^{−/−}/LacZ^{+} chimeric mice (Figure 6B).
Because of the reduced fraction of myocardin
\(^{-/-}\)/LacZ\(^{+}\) cells within aortic strips based on histological analysis (Figure 4B), the preceding studies lack sensitivity for detecting changes in contractility within the subset of myocardin\(^{-/-}\) SMC within this tissue. To address this limitation, we generated pure populations of myocardin\(^{-/-}\) SMCs \textit{in vitro} using a unique embryoid body (EB) SMC differentiation model previously described in our laboratories (34). In brief, either myocardin \(+/+\) or \(-/-\) ESCs were aggregated into EBs, induced to differentiate, and SMCs were purified from the heterogeneous cell population. These SMCs (hereafter referred to as EB-derived SMCs) were then embedded into 3-dimensional collagen gels upon which they further differentiate and align along the axis of stress applied to the gel. In this manner, we were able to study contractile properties and agonist sensitivities in strips composed of pure populations of SMCs derived from either myocardin \(+/+\) or \(-/-\) ESCs. All strips cut from both myocardin \(+/+\) \((n = 14)\) and myocardin \(^{-/-}\) \((n = 11)\) reconstituted muscle fibers demonstrated a rapid phasic contraction when stimulated with depolarizing solution \((154\text{ mM K}^{+})\), indicating that not only is the SM contractile apparatus fully functional in myocardin\(^{-/-}\) SMCs, but cells also have all the necessary signaling pathways and functional attachments to the extracellular matrix necessary to generate force. All strips tested were able to maintain \((5 \text{ – } 8 \text{ minute duration})\) 154 mM K\(^{+}\) induced tonic force until extracellular K\(^{+}\) was removed by switching the bathing solution to HEPES-buffered Krebs’ solution, at which time the strips relaxed to near baseline, indicating that a sufficient complement of Na\(^{+}\)/K\(^{+}\)/Ca\(^{2+}\) ATP-(in) dependent exchangers/pumps are present and functional (Figures 6F and 6G). Of major significance, there were no significant differences in maximal 154 mM K\(^{+}\) induced force capacity between myocardin \(+/+\) \((n = \))
As such, these studies provide compelling evidence that fully functional SMCs can develop in the absence of myocardin, at least within the *in vitro* experimental model system tested herein.

To determine if there may be more subtle differences in contractility, we measured responses to various contractile agonists in EB-derived SMC reconstituted muscle fibers derived from myocardin +/+ or -/- ESCs. Myocardin+/- EB-derived SMC fibers exhibited an EC50 of 23.9 ± 3.2 nM to the thromboxane agonist U-46619 (Figure 6E). In contrast, to our surprise, we observed a complete lack of a contractile response to 300 nM U-46619 in myocardin-/- EB-derived SMC fibers (Figure 6F) suggesting a defect in thromboxane A2 receptor (TXA2R) mediated calcium-independent (calcium sensitized) force generation. In contrast, myocardin+/+ and myocardin-/− EB-derived SMC fibers contracted similarly (qualitatively) to 2.5 μM spingosine-1-phosphate (S1P) and 10 nM endothelin-1 (ET-1) (Figures 6F and 6G respectively), indicating that the absence of myocardin does not abrogate the development of either the S1P or ET-1 contractile signaling cascades within the EB-derived SMC fiber model system. Upon further analysis of the ET-1 responses, we found that myocardin-/- SMCs also generated significantly less maximal force (Figure 6H) relative to 154 mM K⁺ induced force (1.25 ± 0.08, n = 3, p < 0.05) as compared to myocardin+/- EB-derived SMCs (4.22 ± 0.28, n = 4).

Reduced Thromboxane-Induced Contraction in Myocardin-/- EB-Derived SMC Fibers Was Not Due to Loss of Expression of the Thromboxane Receptor.
Western blot analyses of TXA2R were performed to determine if the failure of myocardin\textsuperscript{/-} EB-derived SMCs to contract in response to thromboxane was the result of defective receptor expression. Surprisingly, TXA2R expression was increased >2-fold in myocardin\textsuperscript{-/-} EB-derived SMCs as compared to myocardin\textsuperscript{+/+} EB-derived SMCs (Figure 7). In contrast, protein expression levels of both the A and B isoforms of the endothelin receptor showed no significant differences. It has been demonstrated that TXA2R activation in platelets disproportionately signals through G\textsubscript{\alpha13}, with G\textsubscript{\alpha12} activation occurring as a secondary event following the binding of ligand (13). Therefore, we performed Western blots for both G\textsubscript{\alpha13} and G\textsubscript{\alpha12} to determine if the absence of TXA2 response observed in the myocardin\textsuperscript{/-} EB-derived SMCs was due to a defect in TXA2R activation of the heterotrimeric G-proteins responsible for calcium-sensitized force (35). Interestingly, we observed no significant difference in the protein expression of G\textsubscript{\alpha13}, but a significant 2-fold (1.96 ± 0.18, \textit{p} < 0.05, \textit{+/+} \textit{n} = 3 and \textit{-/} \textit{n} = 2 independent clones) increase in G\textsubscript{\alpha12} protein expression in myocardin\textsuperscript{/-} EB-derived SMCs as compared to myocardin\textsuperscript{+/+} EB-derived SMCs. It is possible that the observed defect in thromboxane A2 signaling was due to either the TXA2R becoming desensitized to ligand (30) or to the loss of coupling between the G\textsubscript{\alpha13} subunit to its \beta\gamma subunits, thus inhibiting RhoA activation (26). As cGMP can phosphorylate and inhibit RhoA activity, we next examined the protein expression levels of cGMP dependent protein kinase I (cGKI). There were no significant differences in cGKI expression levels, although we cannot rule out differences in basal cGKI activities within myocardin\textsuperscript{-/-} and \textit{+/+} EB-derived SMCs. We also observed a significant reduction (0.32 ± 0.39 fold change, \textit{p} < 0.05, \textit{+/+} \textit{n} = 3 and \textit{/-} \textit{n} = 2 independent clones) in RhoGDI protein expression, but no significant differences
in the protein expression levels of RhoA, calponin, ROCK I and ROCK II between myocardin\(^{-/-}\) and myocardin\(^{+/+}\) EB-derived SMC. Thus, the lack of response to U46619 in the myocardin\(^{-/-}\) SMCs was not due to an absence of these downstream signaling molecules.

**Myocardin\(^{-/-}\) EB-derived SMCs Showed Reduced Expression of the SM2 Isoform of SM-MHC**

*In vitro* analysis of the role of myocardin in SMC development was also pursued using an EB model of ESC-SMC differentiation previously described by our laboratory (34). qPCR analysis of the SMC differentiation markers and components of the contractile apparatus including SM \(\alpha\)-actin (SM\(\alpha\)A), SM myosin heavy chain (SM-MHC), SM regulatory light chain (SM-Reg-LC), SM myosin light chain kinase (SM-MYLK), and SM essential light chain (SM-Ess-LC) showed no changes between \(+/+\) and \(-/-\) EB-derived SMCs at 10, 15, 20, and 28 days of *in vitro* development, with the sole exception of the SM-MHC gene (Figure 8A, \(n = 4\) experiments in triplicate). Results of Western analyses showed similar changes in protein expression. For example, consistent with the mRNA findings, SM\(\alpha\)A expression was unchanged between the two cell-types, while the SM-1 isoform of SM-MHC was markedly downregulated, and the SM-2 splice variant completely absent in myocardin\(^{-/-}\) cells even at 28 days of *in vitro* differentiation (Figure 8B). This was not a result of selective expansion of myocardin\(^{+/+}\) derived SMCs as flow cytometric analysis showed an equal proportion of SM\(\alpha\)A positive cells in both myocardin\(^{+/+}\) and myocardin\(^{-/-}\) SMC populations at all time points examined (data not shown).
Discussion:

Results of the present studies provide compelling evidence that myocardin requirements differ dramatically between different cardiomyocyte and SMC subtypes. Indeed, our observation that myocardin−/− ESC virtually completely failed to contribute to formation of ventricular myocytes within the setting of chimeric KO mice was completely surprising in light of previous studies in conventional myocardin KO mice which showed no overt heart defects. Similarly, results of the present studies also unexpectedly showed that cell autonomous myocardin was required for development of multiple smooth muscle lineages in that we observed a virtual absence of myocardin−/− ESC derived SMCs within the bladder, small intestine, uterus, and stomach in chimeric knockout mice despite these cells contributing equivalent to +/+ host ESCs in all non-SMC lineages, other than ventricular cardiomyocytes. In contrast, we frequently observed myocardin+/− ESCs within vascular smooth muscle lineages (in multiple blood vessels in all tissues examined) albeit at a reduced frequency as compared to host +/+ ESC. We also present multiple lines of evidence that myocardin−/− SMCs develop normally based on their exhibiting: a) normal morphology by transmission electron microscopy; b) normal myofilament structures; and c) full contractile capabilities, including maximal force development, responsiveness to most contractile agonists and K+ depolarization induced contraction. The few notable deficits detected included failed contractile responsiveness to thromboxane A2 and diminished expression of the late SMC differentiation marker gene, the SM2 variant of SM-MHC, when myocardin −/− ESCs were differentiated in vitro into SMCs in an EB model system. Our current understanding of the genetic and developmental differences between vascular and
visceral smooth muscle is limited. However, of interest, we observed myocardin−/− SMCs in a variety of blood vessels which have different embryological origins including SMCs from pulmonary arteries and cardiac outflow tract blood vessels which are derived from neural crest cells, coronary artery SMCs which are derived from proepicardial cells, and aortic SMC/peripheral artery SMC which are derived from local mesoderm as is the case with visceral SMCs (25). As such, it is unlikely that differential dependence on myocardin for development is a function of the embryological origins of SMCs.

One possibility is that differential myocardin dependence for SMC development is a function of unique environmental cues that exist in blood vessels versus visceral and other SMC tissues. Although the precise nature of these differences is not clear, it is likely that at least part of the mechanism relates to whether the myocardin related genes, MRTF A and B, are activated, and/or if they can fully compensate for myocardin within that SMC subtype. Indeed, although there are numerous overlapping functions of myocardin and the MRTFs (1; 41), there are also notable exceptions including differential control of nuclear localization and Rho dependence (6; 23). Of interest, Huang et al. (11) showed that selective knockout of myocardin in neural crest derived tissues, generated by crossing floxed myocardin mice with either Wnt1 cre or Pax3 cre mice, show 100% perinatal lethality and presented evidence for incomplete differentiation of SMC within the ductus arteriosus and failed ductus closure at birth. These results are informative in that the ductus serves as a shunt that allows blood flow to bypass the fetal lungs, but at birth, contraction of the ductus SMCs is required for its closure and perinatal survival. As such, these results suggest that myocardin is normally required for development of SMCs within the ductus arteriosus, and that MRTF A/B (or
other genes) fail to substitute, presumably because there is no selection pressure to
initiate compensatory activation of these genes until it is too late. Consistent with the
hypothesis that myocardin and the MRTFs have many overlapping, but also distinct,
functions are results of studies by Li et al. (19) showing that selective knockout of MRTF
B in neural crest cells resulted in embryonic lethality between E17.5 and P1 due to
cardiac outflow tract defects. These results indicate that in cardiac neural crest cells
MRTF A and myocardin were incapable of compensating for loss of MRTF B.

The results of the present studies extend our knowledge in this field by showing
for the first time that myocardin is essential for development of multiple SMC lineages
thus implying that MRTF A and B have limited ability to compensate for loss of
myocardin in these SMC subtypes. In contrast, we show that myocardin $^\sim$ ESCs readily
form vascular SMC lineages, although at a somewhat reduced frequency as compared to
WT ESCs. Moreover, myocardin $^\sim$ vascular SMC have a normal morphology and
myofilament structure in vivo suggesting that MRTFs A and B compensate almost fully
in vascular SMC lineages. We also show that myocardin $^\sim$ SMC formed in vitro within
embryoid bodies exhibit nearly completely normal contractile properties. An important
unresolved question is why myocardin $^\sim$ SMCs developed in vitro in the EB model
showed reduced expression of the SM2 variant of SM-MHC. Since SM2 SM-MHC is a
marker of mature SMC (18; 27; 31), it is possible that myocardin is dispensable for initial
SMC differentiation but is required for full maturation. This implies that myocardin, a
transcription factor and potent SRF co-activator, is somehow selectively altering
alternative splicing of the SM-MHC gene, since myocardin $^\sim$ SMCs expressed normal
levels of the SM1 variant of SM MHC. While at least some myocardin $^\sim$/LacZ$^+$ cells
ultimately form mature SMC as determined by the presence of myosin filaments, the majority of myocardin−/−/LacZ+ cells fail to activate the transcription of the contractile proteins required for normal SMC development within the proper temporal window and therefore fail to respond appropriately to extracellular signals and feedback cues. Consistent with this idea, our previous studies have linked contraction in SMCs with myocardin/SRF signaling suggesting that development of the contractile phenotype may be critical for feedback on SMC gene expression and myocardin-mediated hypertrophy (39). MRTFs have been shown to respond to actin dynamics (38) as does the myocardin/MRTF regulated actin and focal adhesion associated protein palladin (14; 15). We previously reported that KO of palladin was associated with reduced expression of SM α-actin and SM-MHC as well as altered SMC contractility and migration. Thus feedback cues such as altered actin dynamics, migration and palladin expression in the myocardin−/− SMCs during development may put them at a disadvantage compared to their wild type (WT) normal neighboring cells.

The precise mechanisms whereby myocardin knockout results in reduced numbers of myocardin−/− SMCs is unknown, as well as why visceral SMCs exhibit greater dependency than other SMC subtypes. However, it is worth noting that chimeric knockout lineage tracing approaches, as employed in the present studies, have remarkable sensitivity in detecting even subtle “cell autonomous” functions of genes that may give WT cells a slight advantage over knockout cells at critical points in development. Specifically, the failure of appropriate morphogenic/local environmental cues to activate myocardin in a presumptive SMC at a critical juncture in development may result in this cell “being left behind.” Adjacent WT cells may progress in their development
eventually altering the local environment so it is no longer conducive to formation of additional SMCs from multipotential embryonic cells. In contrast, within conventional or even conditional cell-selective KO models, there is a much greater possibility for activation of compensatory genes, including the MRTFs, since all cells have the same selective disadvantage.

In summary, whether a given multipotential myocardin-/- embryonic cell differentiates into a SMC or ventricular cardiomyocyte will be a function of complex factors including: 1) the morphogenic and environmental cues that induce differentiation into those cell types and how long these signals persist relative to the rate of activation of compensatory genes; 2) the rate at which WT embryonic cells in proximity to the myocardin -/- cells respond to the morphogenic and environmental cues by differentiating which will eventually result in dissolution of the stimuli that originally induced differentiation of these cell lineages; and 3) the extent to which genes such as MRTF A and B can compensate for loss of myocardin and the kinetics of these compensatory processes. Clearly further studies will be needed to resolve these difficult questions, as well the interesting possibility that myocardin positive cells may secrete factors that could promote differentiation of adjacent myocardin -/- cells through indirect non-cell autonomous mechanisms.

Importantly, SMαA and SM-MHC expression were differentially impacted by the loss of myocardin in our EB SMC differentiation model system indicating that differences are unlikely to be explained simply through alterations in myocardin/CArG/SRF signaling pathways critical for transcriptional control of both of these genes(28). Studies of the role of myocardin during embryonic development suggest
that myocardin has both activator and repressor functions in the differentiation of multiple muscle types through interaction with the skeletal muscle transcription factor MyoD (24). Therefore, another possibility to explain reduced contribution of myocardin-/- ESCs to SMC lineages is that the observed phenotype is due, at least in part, to the absence of repressor functions of myocardin on MyoD which, unopposed by myocardin in the -/- chimeras, may alter the fate of cells originally destined to form visceral SMC. Alternatively, repressors of SRF/CArG interaction such as Klf4 (22), ternary complex factors (42), HERP (5), or Msx1/2 (9) may inhibit SMC differentiation in myocardin-/- cells as their activity is no longer opposed by myocardin. Finally, it is possible that myocardin-/- ESCs form SMCs equally effectively as WT ESCs, but have increased rates of apoptosis and/or reduced proliferative capacity. However, the latter seems unlikely, given the virtual absence of myocardin-/- SMCs within visceral SMC tissues, and observations in cultured cell systems that loss of myocardin promotes, rather than retards, SMC proliferation (45).

An intriguing and completely unexpected observation in the present studies was that myocardin-/- SMCs within our EB model showed selective loss of thromboxane A2 induced contraction and a significant increase in expression of the thromboxane A2 receptor, Gα₁₂, and RhoGDI. Although the CArG/SRF/myocardin dependence of gene expression for the signaling proteins examined has not been tested using standard genetic methodologies to date, it is unlikely that the observed perturbations in protein expression are directly due to a lack of myocardin as the proteins examined are expressed in multiple cell types throughout development. As myocardin+/- and myocardin-/- EB-derived SMCs demonstrated comparable responses to depolarizing solution, S1P, and ET-1 (albeit with
decreased magnitude), defects in Ca\textsuperscript{2+} mobilization following receptor activation and/or depolarization seem unlikely as well. Rather, the smooth muscle specific expression of a guanine nucleotide exchange factor (GEF) specifically activated by the thromboxane A2 receptor could account for the decreased sensitivity to U-46619 in myocardin\textsuperscript{-/-} SMCs without deleterious perturbations in the signaling cascades initiated by either ET-1 or S1P. Although CArG/SRF/myocardin dependent expression of a GEF has not been found to date, RhoGEFs have been shown to be necessary components of TXA\textsubscript{2}R-coupled signaling in pulmonary artery smooth muscle cells (3) and others cell types (8; 16).

Of major interest, our results also showed that myocardin\textsuperscript{-/-} ESCs were at a major disadvantage as compared to host wildtype ESC in contributing to development of ventricular but not atrial myocytes (Fig. 2). Intriguingly, the myocardin\textsuperscript{-/-} ESCs formed a distinct band of superficial cells along the midline of the two ventricles suggesting that the WT cells out compete the myocardin\textsuperscript{-/-} embryonic cells during a critical stage of heart morphogenesis. The precise mechanisms responsible for these effects are unclear. However, of interest, the small number of myocardin\textsuperscript{-/-} ventricular myocytes that do form appear to have a normal ultrastructural appearance as compared to adjacent ventricular myocytes derived from wild type ESCs. One interpretation of these observations is that myocardin is essential for proper migration of presumptive ventricular myocytes during early development but is dispensable for subsequent differentiation and maturation due to activation of compensatory gene networks. Further conditional cardiomyocyte selective myocardin knockout studies will be required to test this possibility.
In summary, results of the present studies provide compelling evidence that cell autonomous myocardin plays a critical role in formation of visceral SMCs and ventricular cardiomyocytes in the developing mouse embryo. The precise mechanisms for defective investment of myocardin−/− ESCs into SMC tissues and into ventricular myocytes are unclear. However, it is interesting to hypothesize that myocardin−/− embryonic cells are at a selective disadvantage in responding to spatial and temporal cues necessary for programming these lineages during early development, and that MRTFs are either not able to compensate, and/or are not appropriately induced within the microenvironments in which SMC and ventricular cardiomyocyte cell lineage programming occurs. Further studies will be needed to address this hypothesis, and to define the environmental cues that contribute to differences in myocardin dependency between different SMC and cardiomyocyte subtypes.

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Figure legends.

Figure 1: Myocardin\textsuperscript{+/−} ESCs fail to differentiate into a variety of visceral SMC types

A variety of visceral smooth muscle tissues from myocardin\textsuperscript{+/−}/LacZ\textsuperscript{+} control and myocardin\textsuperscript{+/−}/LacZ\textsuperscript{+} chimeric mice were dissected, stained with X-gal to identify cells derived from ESCs, and their gross and cross sectional histology evaluated. A- A representative bladder from a myocardin\textsuperscript{+/−}/LacZ\textsuperscript{+} control ESC chimeric mouse in which at least half of the bladder SMCs are LacZ\textsuperscript{+}. Scale bar: 500 microns. B- In contrast, myocardin\textsuperscript{+/−}/LacZ\textsuperscript{+} ESCs are not observed to differentiate into bladder SMCs in spite of significant contributions to bladder epithelial cells (top) and interstitial cells (faint blue staining). Scale bar: 500 microns. C- Histological section of a bladder, stained with Nuclear Fast Red obtained from a myocardin\textsuperscript{+/−}/LacZ\textsuperscript{+} control ESC chimeric animal displaying multiple blue SMC fibers (arrows). Scale bar: 100 microns. D- Histological section of a bladder from a myocardin\textsuperscript{+/−}/LacZ\textsuperscript{+} chimeric animal in which blue SMCs are not observed, despite labeling of all other bladder cell types. Scale bar: 100 microns. lumen of bladder. E – A representative stomach from a myocardin\textsuperscript{+/−}/LacZ\textsuperscript{+} control animal stained with eosin showing staining throughout the SMC layers. Scale bar 200μm. F – A histological section through the stomach and esophagus of a myocardin\textsuperscript{+/−}/LacZ\textsuperscript{+} animal stained with eosin showing the absence of LacZ\textsuperscript{+} cells in the SMC layers. Interestingly, a transition is clearly seen in the esophagus, as the muscular layer transitions from voluntary skeletal muscle with striations to involuntary smooth muscle. Scale 1mM. At the transition between the two layers (inset, scale 100μM) the LacZ\textsuperscript{+} positive cells show striations, while the LacZ\textsuperscript{−} cells do not, indicating myocardin −/− cells
adequately contribute to esophageal striated muscle but fail to contribute to SMC layers.

G – Whole mount preparation of the SMC layer of the large intestine of a myocardin\(^{+/\text{-}}\)\/LacZ\(^{+}\) chimera, which has been dissected away from the epithelial lining, showing LacZ\(^{+}\) SMC fibers in the circular and longitudinal SMC layers. Scale 500\(\mu\)m. H - Whole mount preparation of the SMC layer of the large intestine dissected away from the epithelial lining, from a myocardin\(^{-/\text{-}}\)/LacZ\(^{+}\) animal showing LacZ\(^{+}\) cells contributing to the parasympathetic ganglia, but not to SMC fibers in the muscular layers. Scale 500\(\mu\)m.

I – A cross section from the stomach of a myocardin\(^{+/\text{-}}\)/LacZ\(^{+}\) chimera (corresponding to stomach in E ) showing LacZ\(^{+}\) cells in the epithelial layer and parasympathetic ganglia but not in SMC layers. Scale 200\(\mu\)m. J – A similar section from a myocardin\(^{+/\text{-}}\)/LacZ\(^{+}\) esophagus showing staining in the epithelial layer and SMC layer corresponding to a similar region as shown in the inset in F. Scale 100\(\mu\)m. K – A cross-section of the lungs of a myocardin\(^{+/\text{-}}\)/LacZ\(^{+}\) animal shows LacZ\(^{+}\) cells in the SMC surrounding the bronchi (arrowheads). Scale 100\(\mu\)m. L – A similar section of myocardin\(^{-/\text{-}}\)/LacZ\(^{+}\) animals shows no contribution of \(-/-\) cells to the airway SMC of the lung. Scale 100\(\mu\)m. M – A cross-section of the uterus of a myocardin\(^{+/\text{-}}\)/LacZ\(^{+}\) uterus shows LacZ\(^{+}\) cells contributing to the myometrium. Scale 100\(\mu\)m. N – Cross-sections from myocardin\(^{-/\text{-}}\)/LacZ\(^{+}\) uteruses showed LacZ stained cells within the epithelium but no LacZ\(^{+}\) cells in the myometrium of myocardin\(^{-/\text{-}}\) chimeras. Scale = 100\(\mu\)m. O – A cross section from the large intestine of a myocardin\(^{+/\text{-}}\)/LacZ\(^{+}\) chimera showing LacZ\(^{+}\) cells in the epithelial and SMC layers as well as parasympathetic ganglia. Scale 200\(\mu\)m. P – A similar section from a myocardin\(^{-/\text{-}}\)/LacZ\(^{+}\) large intestine showing staining in the epithelial layer and parasympathetic
ganglia, but not the muscular layer. Scale 200μm. Abbreviations lu=lumen, SMC=smooth muscle cell, Epi=epithelium, Stom=stomach, SkM=skeletal muscle, Pa=parenchyma.

Figure 2: Myocardin −/− ESCs readily formed atrial ventricular myocytes and coronary smooth muscle cells, not ventricular myocytes. Hearts from chimeric animals at 10 days of age were dissected out and stained with X-gal. In (A) the myocardin−/−/LacZ+ ESCs failed to contribute to the ventricles (hearts 4-8) compared to myocardin+/−/LacZ+ cells (hearts 1-3) scale = 1 mm). The myocardin−/−/LacZ+ cells typically formed a superficial band of myocytes extending from the aortic root in the left ventricle, across the ventricular septum and continuing in a band across the right ventricle. (B) A higher magnification of the 4th heart in A shows myocardin−/−/LacZ+ cells contributed to coronary vasculature (see also enlarged image in Figure 2D) and atria. Longitudinal sections (C) showed abundant myocardin+/−/LacZ+ cells contributing to the ventricles (upper panel) compared to myocardin−/−/LacZ+ cells (lower panel). Figure 2D shows a high magnification of a coronary artery of a myocardin−− chimera showing abundant myocardin−/−/LacZ+ cells in a coronary artery (scale = 100μm). Sagittal sections of the right ventricle showed myocardin−/−/LacZ+ cells contributed to a superficial strip across the anterior right ventricle in an inferior (E) and a superior (F) section across the heart (scale 500μm). These results were consistent over 30 myocardin−/−/LacZ+ animals at ages from 10 days to 6 months.
Figure 3: Quantitative Southern analyses showed reduced investment of myocardin 
-/- cells in visceral SMC tissues, bladder, and cardiac ventricles.

The brain (B), aorta (A), small intestine/gut (G), ventricle (V) were dissected from 28 day 
old myocardin +/-/LacZ+ control and myocardin +/-/LacZ+ animals, lysed, DNA purified and 
electrophoresed and transferred to a nylon membrane for Southern analysis. A - 
Representative Southern blots. If cells contributed equally to all tissues the ratio between 
the +/- lane, representing cells derived from ESC from the host blastocyst which have 
myocardin WT alleles, and the -/- lane, representing cells derived from ESC containing 
knockout alleles, would be equivalent in all tissues (i.e. a ratio of 1). The “whole” lysate 
lane represents homogenized tissue from an entire mouse (less dissected organs and 
tissues), and the ratio between the bands in this lane represents the overall contribution of 
chimeric cells in the individual animal assayed (i.e. % chimerism). Analysis of tissues 
from myocardin +/-/LacZ+ chimeras showed a decreased ratio of cells derived from ESC 
with myocardin -/- alleles in visceral SMC tissues and ventricles compared to that 
observed in whole mouse lysates, suggesting myocardin is required for appropriate 
contribution of ESC-derived cells to these tissues. B - Results of densitometric analysis 
of multiple independent Southern blots. Myocardin +/-/LacZ+ chimeric mice showed a 
statistically significant decrease in the contribution of myocardin -/- ESC in the bladder, 
gut and ventricles as compared to WT ESC from the host blastocysts (n=4 myocardin +/-/ 
/LacZ+, n=5 myocardin +/-/LacZ+, * = p <0.05). Heterozygous myocardin +/- ESC 
exhibited no significant change in their contribution to any of these tissues indicating that 
loss of one myocardin allele had no effect.
Figure 4: Histological analysis showed reduced investment of myocardin^{−/−} ESCs into vascular SMC lineages.

The tissues were fixed and stained for LacZ. Aortic strips were prepared en face, sectioned, and LacZ⁺ and LacZ⁻ nuclei were counted to determine the percentage of cells that were ESC-derived in each tissue. A – Cross-sections of aortas prepared en face and examined with Nuclear Fast Red (NFR) staining (left panels) at high magnification showed fewer LacZ⁺ cells in myocardin^{−/−}/LacZ⁺ aortas compared to liver tissue (right panels) relative to myocardin^{+/+}/LacZ⁺ controls. Aorta scale 25μm, liver scale 100μm. B – Quantification of the ratio of LacZ⁺ nuclei in the thoracic aorta relative to the liver shows a markedly decreased contribution of myocardin^{−/−}/LacZ⁺ ESCs to vascular SMCs as compared to the contribution by myocardin^{+/+}/LacZ⁺ ESCs.

Figure 5: The ultrastructural appearance of myocardin^{−/−}/LacZ⁺ adult vascular SMCs and ventricular cardiomyocytes was unchanged from adjacent myocardin^{+/+} host cells.

Thoracic strips were prepared from the aorta of myocardin^{−/−}/LacZ⁺ animals, fixed briefly, stained with Blue-gal for LacZ⁺ and embedded for analysis by EM. LacZ⁺ cells were identified by Electron Probe Microanalysis (EPMA) of the Blue-gal precipitate to ascertain if a given cell was derived from myocardin^{−/−}/LacZ⁺ ESCs versus WT ESC from the recipient blastocyst. No differences in the ultrastructural appearance were observed between WT cells and the -/- cells identified by EPMA. A – Electron micrograph of a LacZ⁺ myocardin-/- SMC in the thoracic aorta of a myocardin^{−/−}/LacZ⁺ chimera showed a normal ultrastructural phenotype including normally appearing myofilaments (arrows).
The boxed region was subjected to EPMA analysis. EPMA (inset) of Bluo-gal precipitates showed a strong bromine peak indicating cells are positive for LacZ, and confirming their myocardin\(^{-/}\)/LacZ\(^{+}\) ESC origin. B – An electron micrograph of an adjacent WT SMC derived from host ESC is shown for comparison. The large arrows denote arrays of myosin filaments which were indistinguishable between SMC derived from myocardin\(^{-/}\) ESC shown in A. Results are consistent with at least some myocardin\(^{-/}\)/LacZ\(^{+}\) ESC derived aortic SMC having a normal functional contractile apparatus. C - Hearts from 28 day old myocardin\(^{-/}\)/LacZ\(^{+}\) were perfusion fixed with EM-fix, stained with bluogal rather than X-gal to enhance detection by electron microscopy, post-fixed and then embedded for EM analysis. The Electron Micrograph shows a myocardin\(^{-/}\)/LacZ\(^{+}\) ventricular myocyte in an adult heart that is positive for bluogal crystals. EPMA (square inset) of the bluogal precipitates shows a strong bromine peak, confirming the presence of bluogal crystals within the cell and myocardin\(^{-/}\)/LacZ\(^{+}\) genotype (inset graph). Myocardin\(^{-/}\)/LacZ\(^{+}\) cardiac ventricular myocytes show a normal ultrastructural phenotype with regularly arrayed myosin filaments surrounded by actin filaments (circular inset).

Figure 6: SMCs derived \textit{in vitro} from myocardin\(^{-/}\) ESCs using a novel ESC-embryoid body (EB) SMC differentiation model system previously developed in our laboratories (34) and in aortas of myocardin\(^{-/}\) chimeric mice exhibit normal contractile properties and agonist sensitivity

A – Comparison of normalized maximal K\(^{+}\) contraction between thoracic aortic strips from myocardin\(^{+/}\)/LacZ\(^{+}\) (n = 14) and myocardin\(^{-/}\)/LacZ\(^{+}\) (n = 15) chimeric mice. B –
U-46619 dose response curves in strips cut from the thoracic aorta of myocardin\textsuperscript{+/−}/LacZ\textsuperscript{+} (◊, EC\textsubscript{50} = 625 ± 150 nM, n = 14 strips from n = 5 animals) and myocardin\textsuperscript{−/−} ROSA26\textsuperscript{+/-} (○, EC\textsubscript{50} = 835 ± 146 nM, n = 15 strips from n = 5 animals) chimeric mice. C – Dose response curves to the stable thromboxane A2 analogue U-46619 in strips cut from the thoracic aorta of C57BL/6 (□, EC\textsubscript{50} = 73.9 ± 4.78 nM, n = 4) and 129SvJ (○, EC\textsubscript{50} = 64.1 ± 11.9 nM, n = 4) strain controls as well as myocardin\textsuperscript{+/−}/LacZ\textsuperscript{+} (◊, EC\textsubscript{50} = 44.4 ± 1.29 nM, n = 4) animals. D – Comparison of normalized maximal K\textsuperscript{+} contraction between myocardin\textsuperscript{+/+} (n = 14) and myocardin\textsuperscript{−/−} (n = 11) SMCs produced in vitro in our ESC-EB SMC differentiation model system (34)(cells are henceforth referred to as “EB-derived SMC”). E – Dose response curve to U-46619 in myocardin\textsuperscript{+/+} EB-derived SMC fibers (◊, EC\textsubscript{50} = 23.9 ± 3.2 nM, n = 3). F – Representative force trace from myocardin\textsuperscript{−/−} (upper) and myocardin\textsuperscript{+/+} (lower) EB-derived SMCs. The traces demonstrate rapid induction and maintenance of contractile force in the presence of depolarizing (154 mM K\textsuperscript{+}) solution, which rapidly relaxes to near baseline once the bathing solution is switched back to Krebs. The myocardin\textsuperscript{−/−} EB-derived SMC failed to respond to 300 nM U-46619, but did respond with force comparable to depolarizing solution when stimulated with S1P. The vertical bars represent times when the Krebs buffer was changed. G – Representative force traces from myocardin\textsuperscript{−/−} (upper) and myocardin\textsuperscript{+/+} (lower) EB-derived SMCs stimulated with 10 nM ET-1. The traces demonstrate that myocardin is not necessary for the development ET-1 contractile responses. H – Myocardin\textsuperscript{−/−} (n = 3) EB-derived SMCs generate significantly less 10 nM ET-1 induced maximal force than myocardin\textsuperscript{+/+} (n = 4) EB-derived SMCs. ET-1 maximal forces were normalized to maximal K\textsuperscript{+} contraction. Bars represent mean ± standard deviation. The level of significance was set at p<0.05.
**Figure 7:** Myocardin$^{-/-}$ ESC-EB-derived SMCs showed a paradoxical upregulation of thromboxane A2 receptor (TXA2) and Ga12 but decreased expression of RhoGDI.

EB-derived SMCs derived from myocardin$^{+/+}$ and myocardin$^{-/-}$ ESCs were grown to confluence, protein was harvested, and analyzed by quantitative Western blot. A – Westerns for three WT lines and 2 -/- lines were compared to determine signaling pathways altered by loss of the myocardin gene. B – Densitometry shows statistically significant down-regulation of RhoGDI signaling molecules, possibly providing an explanation for the observed defect in signaling. Receptors for other agonists and signaling molecules were equivalent between WT and myocardin$^{-/-}$ EB-derived SMCs.

**Figure 8:** Myocardin$^{-/-}$ ESCs showed defective activation of SM-MHC expression within EBs.

Myocardin$^{+/+}$ and myocardin$^{-/-}$ ESCs were aggregated into EBs and RNA and protein were harvested and analyzed at early and late time points of EB development to assess the ability of myocardin$^{-/-}$ cells to form mature SMCs in vitro. A – Real-time RT-PCR analysis of smooth muscle contractile protein genes showed equivalent expression between myocardin$^{+/+}$ and myocardin$^{-/-}$ cells. However, there was a specific failure of SM-MHC to upregulate in differentiating myocardin$^{-/-}$ cells during late EB development (n=4 experiments, each performed in triplicate, ** = p <0.01) B – Western blotting showed global deficits in SM-MHC protein expression in myocardin$^{-/-}$ cells and a distinct absence of the mature SM2 SM-MHC splice variant in myocardin$^{-/-}$ EBs at 28 days of differentiation (each band represents an independent EB experiment for an n=3 for each
cell type at each time point). Consistent with the mRNA findings, SMαA protein expression was unchanged between WT and myocardin \(^{-/-}\) EBs, while SMMHC was downregulated.


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and pericytes during embryonic blood vessel formation in the mouse. 


Ref Type: Journal (Full)


Contribution of Myocardin +/- or -/- ESC Relative to Host WT ESC (normalized to whole mouse DNA extract)

Fig 3
Fig 4

(A) Myocd<sup>+/−</sup>/LacZ+ Myocd<sup>−/−</sup>/LacZ+

(B) Ratio ESC-derived Cells
Thoracic Aorta/Liver

- Myocd<sup>+/−</sup>
- Myocd<sup>−/−</sup>
Myocd<sup>+/-</sup>/LacZ<sup>+</sup>  
Myocd<sup>-/-</sup> EB-SMCs  
Myocd<sup>-/-</sup>/LacZ<sup>+</sup>  
Myocd<sup>+/+</sup> EB-SMCs

U-46619 [M]  
Myocd<sup>+/+</sup> EB-SMCs  
Myocd<sup>-/-</sup> EB-SMCs  
Myocd<sup>-/-</sup> EB-SMCs  
Myocd<sup>+/+</sup> EB-SMCs  
U-46619 [M]

Fig 6
**Fig 7**

(A) Western blot analysis comparing WT EB-SMCs and KO EB-SMCs for various proteins:
- TXA2R
- Gα12
- Gα13
- GAPDH
- cGKI
- Gαq/11
- GAPDH
- ETR A
- GAPDH
- ETR B
- GAPDH
- Calponin
- RhoA
- RhoGDI
- ROCK II (α)
- ROCK I (β)
- GAPDH

(B) Bar graph showing fold change normalized intensity for Myocd +/+ EB-SMCs and Myocd -/- EB-SMCs for various proteins:
- TXA2R
- Gα12
- Gα13
- Gαq/11
- RhoA
- RhoGDI
- Calponin
- ROCK I (β)
- ROCK II (α)
- ETR A
- ETR B
- GAPDH

* indicates statistical significance.