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Epigenetic regulation of smooth muscle cell plasticity

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Abstract

Smooth muscle cells (SMC) are the major cell type in blood vessels. Their principle function in the body is to regulate blood flow and pressure through vessel wall contraction and relaxation. Unlike many other mature cell types in the adult body, SMC do not terminally differentiate but retain a remarkable plasticity. They have the unique ability to toggle between a differentiated and quiescent “contractile” state and a highly proliferative and migratory “synthetic” phenotype in response to environmental stresses.

While there have been major advances in our understanding of SMC plasticity through the identification of growth factors and signals that can influence the SMC phenotype, *how* these regulate SMC plasticity remains unknown. To date, several key transcription factors and regulatory *cis* elements have been identified that play a role in modulating SMC state. The frontier in understanding the molecular mechanisms underlying SMC plasticity has now advanced to the level of epigenetics. This review will summarize the epigenetic regulation of SMC, highlighting the role of histone modification, DNA methylation, and our most recent identification of a DNA demethylation pathway in SMC that is pivotal in the regulation of the SMC phenotypic state.

Many disorders are associated with smooth muscle dysfunction, including atherosclerosis, the major underlying cause of stroke and coronary heart disease, as well as transplant vasculopathy, aneurysm, asthma, hypertension, and cancer. An increased understanding of the major regulators of SMC plasticity will lead to the identification of novel target molecules that may, in turn, lead to novel drug discoveries for the treatment of these diseases.

Keywords

Smooth muscle phenotype; epigenetics; DNA methylation; chromatin; transcription; TET2

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1. SMC plasticity in health and disease

SMC are a unique cell type in that they are remarkably plastic. They can readily switch between two phenotypic states –contractile and synthetic – depending on environmental cues. Within adult blood vessels, SMC exhibit the contractile phenotype, characterized by low proliferation rates, high levels of cytoplasmic myofilaments, low rates of protein synthesis, and a unique repertoire of contractile proteins including smooth muscle alpha actin (ACTA2), smoothelin, h-caldesmon, calponin, transgelin (TAGLN), and smooth muscle myosin heavy chain (MYH11) [1, 2]. SMC can undergo phenotypic modulation in response to extracellular signals and de-differentiate to the synthetic phenotype. In the synthetic state, SMC express relatively few contractile proteins, re-enter the cell cycle, and become highly proliferative and migratory, and have high rates of protein synthesis and extracellular matrix secretion [3, 4]. SMC can readily switch between these two states when remodelling is required in response to changes in blood flow or when repair is needed following vascular injury. A disruption of this balance, such that the synthetic phenotype predominates, is a major underlying cause of many vascular diseases such as atherosclerosis and aneurysms.

Atherosclerosis is the major underlying cause of myocardial infarction, heart failure, stroke, and peripheral vascular disease. Lipid deposition in the vascular wall alters the integrity of the endothelium, permitting the transmigration of circulating monocytes into the SMC layer where they further mature into macrophages and engulf cholesterol. Growth factors and inflammatory mediators released by activated macrophages promote the de-differentiation of resident medial SMC from a contractile phenotype to the synthetic state. Migratory SMC populate the intima and participate in fibrous cap formation through heightened proliferation and extracellular matrix synthesis [5]. As it is difficult to identify de-differentiated SMC due to loss of their contractile markers, it has been hypothesized that phenotypically modulated SMC may play other critical roles in atherosclerotic lesions as well [5–8].

Severe atherosclerosis can require revascularization procedures, including angioplasty, stenting, or bypass grafts to restore blood flow. Restenosis is the re-narrowing of blood vessels following these procedures that leads to restricted blood flow. This is a major vascular complication caused by SMC phenotypic modulation [9]. Following injury to the vessel wall and the release of multiple growth factors into the microenvironment by platelets and infiltrating inflammatory cells, SMC de-differentiate from a contractile to the synthetic phenotype. These synthetic SMC proliferate and migrate to the intimal space where they actively secrete extracellular matrix proteins, leading to the formation of intimal hyperplastic lesions and the narrowing of the blood vessels [10].

The profound changes in SMC during phenotypic modulation also contribute to aortic aneurysms, which can arise from genetic or environmental causes, including smoking [11]. Aneurysms develop as the SMC undergo phenotypic modulation that is characterized by a decrease in contractile markers such as ACTA2 and MYH11 with increases in the secretion of MMPs, in particular MMP2 and MMP9 [12]. Apoptosis of SMC then ensues; this loss of the primary cells responsible for extracellular matrix synthesis results in the further weakening of the vessel wall and ultimately leads to vessel rupture.

Treatment strategies for these diseases of SMC phenotypic modulation are limited at present. Strategies to prevent progression of atherosclerosis include lifestyle modifications and lipid lowering agents such as statins. Statins also appear to reduce systemic inflammation [13] and, interestingly, can promote SMC differentiation *in vitro* [14]. Ultimately, revascularization procedures are often required for advanced atherosclerosis. Drug-eluting stents (DES) including analogs of rapamycin have helped to prevent restenosis in the coronary arteries for many, but not all patients, with diabetic patients being at particularly high risk for restenosis [10]. DES have proven less effective for peripheral vascular disease, although new studies with drug-eluting balloons have shown promise [15]. Local delivery of rapamycin and its analogs may be particularly effective therapeutic agents due to their potent ability to promote SMC differentiation, in addition to inhibiting proliferation and matrix synthesis [16, 17]. Systemic treatment with rapamycin inhibits transplant vasculopathy in rodent models [18, 19], but systemic rapamycin is not an option for transplant vasculopathy or to maintain patency following bypass grafts in humans due to the very high doses required. For aneurysm, surgery is currently the only treatment, and many aneurysms go undetected until a critical rupture occurs. For these reasons, better treatments for these diseases associated with dysfunctional SMC phenotypic modulation are urgently warranted.

2. Role of epigenetics in regulating SMC plasticity

Many of the genes that characterize SMC phenotypic switching, including the contractile proteins, are regulated at the level of transcription. As such, most research has focused on identifying the transcription factors and promoter elements that drive such responses. Smooth muscle-specific genes are generally regulated by dual CArG elements in their promoter or intronic regions that bind serum response factor (SRF). SRF can also regulate pro-proliferative genes when bound to growth factor-regulated co-factors such as Elk-1 [20]. Smooth muscle-specific gene expression is governed by the smooth muscle- and cardiomyocyte-specific transcriptional co-activator myocardin (MYOCD) [21], which binds to SRF at CArG elements and is sufficient to promote the SMC contractile phenotype [22]. Many studies have focused on other factors that can oppose the actions of MYOCD to promote de-differentiation, including KLF4 [23], FoxO4 [24], and others. Micro RNAs (miRNAs) can also regulate SMC phenotype, including miR-143 and miR-145 which inhibit expression of KLF-4 and KLF-5 [25–27]. New studies have now emerged that are aimed at understanding the epigenetic influences on transcriptional control of SMC phenotype.

Epigenetics refers to heritable changes in gene expression that occur without any changes in the genomic sequence, often as a result of environmental influences. It is becoming increasingly clear that epigenetic mechanisms play an important role in the regulation of chromatin structure and remodelling, and are key mediators of cell type-specific gene expression during development and disease [28]. Chromatin is a dynamic complex composed primarily of genomic DNA and protein. The nucleosome is the fundamental unit of chromatin encompassing 146 base pairs of DNA wrapped around an octamer of histone proteins. This octamer contains two copies each of histones H2A, H2B, H3, and H4. The histone N-terminal tails are not bound to the nucleosome core [29] and frequently undergo modifications including acetylation, phosphorylation, ubiquitination, and ADP-ribosylation

[30]. Epigenetic regulation of histones alters chromatin conformation and the accessibility of transcription factors to DNA, resulting in the activation or silencing of gene transcription. Two of the most extensively studied epigenetic changes are histone modification (which alter the packaging of the chromatin) and DNA methylation (occurring at the 5'-cytosine in CpG dinucleotides).

In recent years, multiple *in vitro* and *in vivo* studies have provided evidence of a key role of epigenetic modification, particularly histone modification, in controlling SMC gene expression during normal cell differentiation versus in disease. A 2012 review elegantly addressed the epigenetics of SMC phenotypic modulation [31]. Herein, we summarize some early pioneering studies, as well as the most recent literature on histone modifications and DNA methylation in regulation of SMC phenotype, with an additional focus on our recent study of DNA methylation in this area.

2.1 Histone modification

The first report describing epigenetic regulation of SMC differentiation arose from a retinoic acid (RA)-inducible A404 cell model of early SMC differentiation [32]. Despite its high expression, SRF was unable to bind to CArG boxes of SMC genes within intact chromatin. However, RA-induced differentiation led to an enrichment of SRF and the hyperacetylation of histones H3 and H4 in SMC CArG-containing regions [32]. Consistent with these results, increasing histone acetyltransferase (HAT) activity with trichostatin A (TSA) treatment or overexpression of CBP/p300 increased the promoter activity and gene expression of SMC genes [33, 34]. In contrast, inhibition of histone acetylation via Twist1, E1A, or histone deacetylase (HDAC) overexpression resulted in the suppression of SMC markers [34–36].

Chromatin is more open at CArG-containing regions of the *ACTA2* promoter in SMC where contractile proteins are highly expressed, compared to endothelial cells, where *ACTA2* and other smooth muscle-specific markers are not expressed [37]. These regions exhibit increased histone methylation and acetylation as demarcated by increased H3K4me2, H3K79me2, H3K9Ac, and H4Ac [37]. The methylation of H3K4 is partially attributed to the recruitment of WDR5 and the associated histone lysine methyltransferase SET/MLL by Pitx2 to SMC promoters in early stages of differentiation [38]. Although the acetylation of histones is diminished during the de-differentiation process, H3K4me2 persists through SMC phenotypic modulation [6, 37]. A novel *in vivo* assay combining *in situ* hybridization and the proximity ligation assay provided concrete evidence that H3K4me2 at the *MYH11* locus is restricted to the SMC lineage in tissue sections [6]. Together, these results suggest that there are cell-specific epigenetic mechanisms that govern the expression of cellular markers during differentiation.

Other stimuli of SMC differentiation have also been shown to induce epigenetic changes that alter chromatin accessibility and hence gene transcription. Overexpression of MYOCD in 10T1/2 cells increased H3Ac at CArG elements at the *ACTA2* and *TAGLN* promoters [35]. TGF β , which stimulates smooth muscle gene transcription, [39, 40] also increased H3Ac and H4Ac at the *SM22 α* locus [34]. Conversely, inducers of SMC de-differentiation, such as PDGF-BB, facilitated the compaction of chromatin at SMC contractile gene loci. PDGF-BB-induced KLF4 recruited HDAC2, HDAC4, or HDAC5 to CArG regions on the

ACTA2 and *MYH11* promoters, reducing histone acetylation and inhibiting the accessibility of this region to the transcription factors MYOCD, SRF, and MRTF [36, 37, 41]. In cerebral SMC, cigarette smoke extract increased the binding of HDAC2 to *ACTA2* and *MYH11* promoters, decreasing H3K4 acetylation [42]. Oxidized phospholipids such as 1-palmytoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC) are present within atherosclerotic lesions and have been shown to be involved in monocyte infiltration [43, 44]. POVPC increased the presence of HDAC2 and HDAC5 at the *ACAT2* and *TAGLN* promoters in a KLF4-dependent manner, resulting in hypoacetylation of histone H4 [45, 46]. The cytokine interferon- γ (IFN- γ) repressed expression of the *COL1A2* gene in SMC, which contributes to destabilization of atherosclerotic plaques. This has been found to occur through IFN- γ dependent recruitment of a repressor complex that includes HDAC2 and the H3K9 methyltransferase G9a [47].

Evidence of histone modifications at SMC gene loci has also been demonstrated in *in vivo* models of SMC de-differentiation. Carotid ligation decreased H3 acetylation at the *TAGLN* promoter where the binding of a complex consisting of KLF4, Elk1, and HDAC2 peaked three days post-injury [46]. Additional studies done in a mutant LacZ transgenic mouse model demonstrated that this complex was contingent on a G/C repressor element found in many CArG-dependent SMC genes [46]. Furthermore, a transient decrease in H4 acetylation occurred at the *ACTA2* and *MYH11* promoters in balloon-injured rat carotids [37]. A specific role for histone modifiers has also been studied with inhibition of HATs or HDACs. Systemic inhibition of class IIa HDACs, including HDAC4 and HDAC5, with the small molecule MC1568 attenuated neointima formation by 50% following carotid ligation [48]. Conversely, local administration of TSA significantly prevented intimal hyperplasia induced by balloon injury in the rat carotid artery [49].

2.2 DNA methylation

DNA methylation is the best characterized epigenetic modification that is linked to gene silencing. In mammalian cells, DNA methylation occurs at the 5' position of the cytosine ring through the actions of the DNA methyltransferases DNMT1, DNMT3A and DNMT3B [50, 51]. Despite being extensively studied in other cell types, very few studies have addressed the role of DNA methylation in regulating SMC phenotype.

Early studies using high performance liquid chromatography (HPLC) to detect genomic 5-methyl cytosine (5mC) content in normal arteries and atherosclerotic lesions reported genomic hypomethylation in human atherosclerotic lesions as well as in lesions from ApoE knockout mice [52]. Furthermore, global hypomethylation was also detected in proliferating intimal SMC of New Zealand white rabbits that had undergone balloon denudation. Global hypomethylation, with concomitant decrease in DNA methyltransferase activity, has also been shown to occur during SMC phenotypic modulation [52] and proliferation [53] in culture.

Several studies have now reported that certain SMC genes are regulated by DNA methylation that in turn could be associated with SMC phenotypic modulation and the development of vascular diseases. Hypomethylation of the extracellular superoxide dismutase gene is associated with the development of atherosclerosis [54]. DNA

methylation of the atheroprotective [55] estrogen receptor- α gene was increased in proliferating synthetic aortic SMC [56]. Interestingly, high-fat diet or LPS challenge downregulated miR-152, a negative regulator of DNMT1, leading to hypermethylation of the estrogen receptor- α gene in human or rat aortic SMC [57]. Phenotypically modulated SMC can also contribute to vascular calcification, and a recent study reported that inhibition of DNMTs with 5-aza-2'-deoxycytidine (5-Aza) facilitated mineralization in cultured human aortic SMC, likely through demethylation of the alkaline phosphatase promoter [58].

Extracellular matrix is well known to affect SMC phenotype [59], and several recent studies suggest that these effects may be mediated in part by DNA methylation. The DNA methylation inhibitor, 5-Aza, was shown to partially inhibit collagen type I and III genes in visceral SMC isolated from neurogenic bladders [60]. Furthermore, 5-Aza attenuated PDGF-induced airway SMC migration and proliferation, and was able to increase cellular contractility [61]. Hypomethylation of collagen type XV alpha 1 gene occurs during SMC proliferation and the consequent increased gene expression may impact SMC phenotype and atherosclerosis development [53]. Rat visceral SMC underwent extensive changes in DNA methylation when plated on native collagen versus denatured collagen. Plating on damaged collagen increased SMC proliferation, which was reversed by 5-Aza. This study further found an increase in nuclear DNMT3 expression in SMC on damaged collagen [62]. Matrix alterations in the maternal vasculature have recently been implicated in preeclampsia. MMP-1 was found to be hypomethylated in omental arteries from preeclamptic women, and 5-Aza treatment induced MMP-1 mRNA expression and protein secretion in cultured human vascular SMC [63].

These and other studies provide evidence that DNA methylation occurs during SMC phenotypic switching and in diseases associated with altered SMC phenotype, but more evidence is required to determine whether DNA methylation plays a causal role in this process.

2.3 TET2 in regulating SMC plasticity

While the mechanisms of DNA methylation have been well studied, the mechanism of DNA demethylation in mammals remains poorly characterized and controversial. Recently, the Ten-Eleven-Translocation (TET) family of enzymes was shown to oxidize 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) in mammalian cells [64–66]. Through the DNA repair pathway and thymine-DNA glycosylase (TDG), 5hmC is then converted to unmethylated cytosine, leading to DNA demethylation and gene activation [67]. As such, the 5hmC modification and the TET enzymes have taken a center stage as activators of gene expression [68, 69]. While the initial studies on the TET proteins were directed to embryonic stem cells, the focus has recently shifted to the role of the TET enzymes and their 5hmC product in adult cell types.

There are currently three identified members of the TET family of proteins (TET1-3) that are expressed in multiple tissues at varying levels [70], and studies from both mouse and human samples have revealed a distinct role of the TET proteins in regulating pluripotency and differentiation in stem cells. TET1 plays a pivotal role in regulating self-renewal and lineage commitment of embryonic stem cells [65, 71, 72] while TET2 plays a major role in

maintaining hematopoietic stem cell differentiation and is found to be frequently mutated in various forms of myeloid malignancies [73–75]. TET3 is highly expressed in oocytes and is the key enzyme that catalyzes 5mC to 5hmC in the paternal genome following fertilization [76].

Our group was the first to demonstrate the presence of a DNA demethylation pathway in myocytes [77]. Out of the three TET family members, we found that TET2 is the highest expressing isoform in human coronary artery SMC cultures, and that TET2 expression is highly enriched in smooth muscle tissues. TET2 expression increased with rapamycin-induced differentiation, and was significantly reduced following PDGF-BB-stimulated de-differentiation. We further showed that TET2 is both necessary and sufficient for SMC differentiation using TET2 overexpression or knockdown approaches: loss of TET2 impaired SMC differentiation and increased expression of SMC synthetic genes such as *KLF4* and non-muscle myosin heavy chain (*MYH10*), while TET2 overexpression promoted all aspects of differentiation in cultured SMC, including induction of many differentiation-specific (*MYOCD*, *ACTA2*, *MYH11*, *TAGLN*) and anti-proliferative genes, reduced proliferation, and an overall differentiated morphology.

To better understand the physiological role of TET2, we examined TET2 expression under normal conditions *in vivo*, as well as in a murine model of intimal hyperplasia. TET2 was highly expressed in the medial SMC layer of uninjured vessels where SMC are in a differentiated state, but was significantly reduced following vascular injury, when the cells are proliferative and synthetic. TET2 expression also declined in proportion to the severity of atherosclerosis in human coronary artery specimens. These results support our initial *in vitro* findings that while high TET2 expression is associated with the mature, contractile SMC phenotype, significant loss of TET2 is a cardinal feature of synthetic SMC. To provide further proof that these observations are due to a direct consequence to changes in TET2 expression, we used a viral gene therapy approach to locally manipulate TET2 expression at the site of vascular injury. Localized TET2 knockdown significantly exacerbated the injury response while TET2 overexpression greatly attenuated intimal hyperplasia, highlighting a key role for loss of TET2 in SMC associated pathologies [77].

The TET proteins and the 5hmC modification have been linked to gene transcription. In embryonic stem cells, 5hmC has been found to be enriched in actively transcribed genes (high in H3K4me2/3) but low in genomic areas marked with H3K9me3 or H3K27me3 [71, 78, 79]. Our study found that TET2 modifies histone methylation at key contractile SMC gene promoters such as *MYOCD* and *SRF*. Using chromatin immunoprecipitation coupled with qPCR, we demonstrated that TET2 bound strongly to contractile promoters of in human SMC induced to differentiate with rapamycin. Concomitant with our observation that TET2 depletion led to significant downregulation of contractile SMC genes, TET2 knockdown decreased the H3K4me3/H3K27me3 ratios at the *MYOCD*, *SRF* and *SM-MHC* promoters, contributing to silencing of these genes. Conversely, TET2 knockdown led to increased H3K4me3 at the *KLF4* promoter [77].

To determine locus-specific expression of 5hmC at gene promoters, we performed hydroxymethylated DNA immunoprecipitation with a newly established 5hmC antibody, as

well as glucosylation-coupled methylation-sensitive qPCR. Consistent with the published literature [79–81], we demonstrated that the 5hmC mark is enriched at actively transcribed gene promoters, and that a loss of 5hmC was associated with a gain of H3K27me3. We report that 5hmC is enriched at the *MYOCD*, *SRF* and *MYH11* promoters in differentiated SMC, and reduced at these promoters following knockdown of TET2. Notably, 5hmC was initially thought to serve only as a transient intermediate in the process of demethylation [64, 82], but our study reveals that this mark persists in quiescent, differentiated SMC in the medial layer of healthy vessels in vivo, suggesting that this epigenetic mark may serve a distinct function in directing gene expression. This is consistent with reports that the 5hmC mark may play a role in self-renewal and lineage commitment [83–85].

Our studies suggest a powerful role for TET2 in the regulation of SMC plasticity. Many questions still remain to be answered, including the function of the 5hmC mark and the mechanism by which TET2 represses expression of *KLF4* and de-differentiation-associated genes. We propose that by understanding the molecular mechanisms and specific targets of TET2 in controlling the overall switch between the synthetic and contractile SMC phenotype, we will gain key insights into the pathogenesis of multiple vascular disease which will ultimately lead to the development of new avenues for therapeutic intervention.

3. Concluding remarks

While the past few decades have identified many key transcriptional regulators of SMC phenotype, recent studies have revealed a key role for epigenetic regulation. Environmental influences have been linked to disease phenotype through modification of the epigenome, although the mechanisms by which extracellular signals are transduced to the level of chromatin are still not well understood. CArG and G/C repressor elements are known to be critical determinants of SMC-specific gene regulation, and will be areas for further focus in understanding the interactions between transcription factor binding and chromatin modifications. In addition, larger studies including genome-wide profiling of epigenetic marks including mC and 5hmC will be helpful in determining how epigenetic changes across the genome affect SMC gene expression and cell phenotype.

Since its discovery, *MYOCD* has been recognized as a master regulator of SMC phenotype. Subsequently, miRNAs were identified as important modulators of SMC phenotype through their ability to indirectly regulate *MYOCD* expression via suppression of KLFs. Most recently, TET2 has emerged as a novel master regulator of SMC phenotype through coordinate epigenetic regulation of key genes including *MYOCD*, *SRF*, and *KLF4*. The functions of the TET proteins, while clearly of major importance, are still poorly understood. Most of the previous work has been limited to embryonic and hematopoietic stem cells. Understanding the mechanisms by which TET2 activates expression of SMC-specific genes while repressing de-differentiation genes will provide important new insights into the coordinated process of phenotypic modulation. Epigenetic modifiers that can alter global gene expression may hold promise for development of new therapies for disease of inappropriate SMC phenotypic modulation.

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Abbreviations

5-Aza	5-aza-2'-deoxycytidine
5mC	5-methylcytosine
5hmC	5-hydroxymethylcytosine
DES	Drug eluting stents
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
HAT	Histone acetyltransferase
HDAC	Histone Deacetylase
IFN-γ	Interferon- γ
KLF4	Krüppel-like factor 4
KLF5	Krüppel-like factor 4
MMP	Matrix metalloprotease
MRTF	Myocardin related transcription factor
MYH11	Smooth muscle-myosin heavy chain
MYOCD	Myocardin
PDGF	Platelet-derived growth factor
POVPC	1-palmytoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine
qPCR	Quantitative polymerase chain reaction
SMA	Smooth muscle actin
SMC	Smooth muscle cell
SRF	Serum response factor
TDG	Thymine-DNA glycosylase
TET	Ten-eleven translocation
TSA	Tricostatin A

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Highlights

- We summarize the role of smooth muscle cell plasticity in disease.
- We review the roles of histone and DNA methylation in smooth muscle phenotype.
- We highlight the key role of TET2 and 5hmC in smooth muscle cell plasticity.

Figure 1.

Composite schematic summary of transcription factor and HDAC binding to *cis* regulatory elements, histone modifications, and DNA methylation (based on studies of the *TAGLN* promoter). While histone acetylation is reduced with phenotypic modulation, the H3K4me2 mark persists. DNA methylation (5mC) is associated with inactive genes. Growth factors inhibit, while rapamycin promotes expression of TET2 and global genomic 5hmC levels in SMC, which correlate with the differentiated state. TET2 catalyzes conversion of 5mC to 5hmC, while TET2 knockdown leads to increased 5mC and reduced chromatin accessibility (decreased H3K4me3/H3K27me3 ratio) at key SMC contractile promoters including *MYOCD*, *SRF*, and *MYH11*.