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JBR

The Journal of Biomedical Research, 2015, 29(1):3-19

Review Article

Myocardin in biology and disease

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Abstract

Myocardin (MYOCD) is a potent transcriptional coactivator that functions primarily in cardiac muscle and smooth muscle through direct contacts with serum response factor (SRF) over *cis* elements known as CArG boxes found near a number of genes encoding for contractile, ion channel, cytoskeletal, and calcium handling proteins. Since its discovery more than 10 years ago, new insights have been obtained regarding the diverse isoforms of MYOCD expressed in cells as well as the regulation of MYOCD expression and activity through transcriptional, post-transcriptional, and post-translational processes. Curiously, there are a number of functions associated with MYOCD that appear to be independent of contractile gene expression and the CArG-SRF nucleoprotein complex. Further, perturbations in *MYOCD* gene expression are associated with an increasing number of diseases including heart failure, cancer, acute vessel disease, and diabetes. This review summarizes the various biological and pathological processes associated with MYOCD and offers perspectives to several challenges and future directions for further study of this formidable transcriptional coactivator.

Cell lineage determination and differentiation require context-dependent extracellular and intracellular signaling events that converge upon the nuclear genome to coordinate specific patterns of gene expression requisite for normal cellular homeostasis. Such programs of gene transcription require cell-restricted and more widely expressed DNA binding transcription factors and their attending co-regulators that fashion the epigenome for appropriate control of gene expression. Skeletal muscle cells were the first cell type shown to arise through the activity of a single DNA binding transcription factor[1]. This factor, named MYOD1, represented a paradigm of cell specification[2], and its discovery triggered a surge in interest to identify similarly acting transcription factors that could contribute to the identity of other distinct cell types such as neurons, cardiac muscle cells, beta cells of the pancreas, and various hematopoietic cells[3–5]. These and other seminal studies further indicate that a cell's phenotype is not fixed; rather, under appropriate conditions, cells may undergo transdifferentiation from one cell type into another[6].

Unlike most of the ~250 distinct cell types that arise from discrete regions of the developing embryo, vascular smooth muscle cell (SMC) types originate from multiple points in the embryo and exhibit considerable phenotypic plasticity during development and disease[7–11]. Throughout the 1990s and early 2000s, investigators utilized such techniques as low stringency degenerate oligonucleotide screening[12,13], mRNA differential display[14,15], and interaction cloning[16–18] to discover transcription factors that could program SMC differentiation in a manner similar to MYOD1; however, none of the identified factors exhibited a MYOD1-like function. A major breakthrough occurred in 2001 when Da-Zhi Wang in Eric Olson's laboratory discovered myocardin[19]. As detailed below, myocardin (MYOCD) is both necessary and sufficient for the development

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Received 20 November 2014, Accepted 10 December 2014, Epub 25 December 2014

The author reported no conflict of interests.

doi: 10.7555/JBR.29.20140151

and differentiation of *most* SMC, suggesting it has some features in common with MYOD1. The purpose of this review is to summarize the initial findings related to MYOCD as a powerful co-activator of serum response factor (SRF)-dependent gene expression in cardiac muscle and SMC and the subsequent literature on its expression, regulation, and function in molecular and pathological processes that extend beyond the cardiovascular system. Part one summarizes the initial discovery, mRNA expression, and major function of MYOCD as a vital component to a molecular switch for SMC differentiation. Part two addresses the transcriptional, post-transcriptional, and post-translational control of MYOCD as well as the growing number of regulatory proteins that alter its expression or activity. In part three, the role of MYOCD in other aspects of cell biology is synopsized including suppression of cell growth, modulation of microRNAs and ion channels, repression of skeletal muscle differentiation, and several CArG-independent functions. Part four will highlight a number of pathological disorders in which MYOCD is suspected to play some role. Throughout each section, future directions for the study of this amazing co-activator of gene expression are offered.

Discovery, expression, and initial characterization of myocardin function

The discovery of MYOCD is a lesson in applying computational biology to gene discovery. Exploiting the wealth of data accumulating from global work on transcriptomics, Wang et al. used the simple BLAST search algorithm to compare expressed sequence tags from embryonic cardiac muscle cDNA libraries with existing sequence data and found one of the 20 novel sequences to correspond to the 3′ untranslated region of MYOCD[19]. Myocardin was so named because of its primarily cardiac muscle-restricted pattern of expression during embryogenesis and in the adult[19]. Initial construction of the mouse *Myocd* cDNA revealed an open reading frame of 807 amino acids with the amino-terminal 300 amino acids consisting of a basic domain, a SAP domain common to transcription factors mediating changes in genomic architecture, and a polyglutamine domain found commonly in transcription factors. Interestingly, expansion of polyglutamine domains in certain transcription factors is implicated in neurodegenerative diseases[20] though such augmentation of polyglutamine tracts in the human *MYOCD* gene have not been reported in neurons. The carboxy-terminal domain of MYOCD shows little homology to other proteins except for a leucine zipper motif that appears to mediate MYOCD homodimerization[21]. Nuclear localization of ectopically expressed MYOCD and GAL4 reporter assays supported the idea that MYOCD exhibits transcriptional activity[19]. Deletion analysis indicated that most of the transcriptional activity of MYOCD is mediated through the carboxy-terminal 300 amino acids. Reporter assays confirmed MYOCD to be a strong transactivator of promoters containing CArG boxes, which are found predominantly in muscle and cytoskeletal genes[22,23]. Depending upon context, the degree of MYOCD transactivation was more than three orders of magnitude above baseline making MYOCD one of nature′s titanic transcriptional co-activators. Finer mapping studies showed that the basic and polyglutamine domain were essential for MYOCD transcriptional activity. Gel shift assays demonstrated MYOCD was unable to bind to a CArG box from the *Tagln* promoter; however, MYOCD was shown to physically bind serum response factor (SRF), a ubiquitously expressed DNA binding transcription factor[24], and together SRF-MYOCD formed a ternary complex over the *Tagln* CArG box[19]. Apparently, MYOCD does not directly contact DNA, though X-ray crystallography experiments of MYOCD bound to SRF over a CArG box should be done to formally prove this point. The association of MYOCD with SRF requires the basic and polyglutamine domains of MYOCD coming into contact with the amino-terminal MADS domain of SRF[19]. The results of this elegant series of experiments proved that MYOCD, like many transcription factors, is modular with respect to its functionality; the amino-terminus serves to bind to SRF and the carboxy-terminus mediates strong transcriptional activation. Deletion of the carboxy-terminal domain of MYOCD resulted in a dominant negative protein that could, if ectopically expressed in developing *Xenopus* embryos, inhibit endogenous cardiac muscle gene expression[19]. This finding suggested that MYOCD was a critical cofactor for the cardiac muscle program and might be necessary for normal cardiogenesis. Two other myocardin-related factors were subsequently discovered and have been reviewed elsewhere[25–28].

Expression profile of myocardin

Initial Northern blotting showed *Myocd* mRNA to be restricted to adult heart tissue and in situ hybridization of developing mouse embryos confirmed robust expression in the cardiac crescent as early as embryonic day 7.75[19]. Levels of *Myocd* mRNA persist in the developing mouse heart over later stages of development and could also be detected in SMC of gut and vasculature[19,29]. *Myocd* mRNA expression could not be detected in skeletal muscle, either in vivo or in

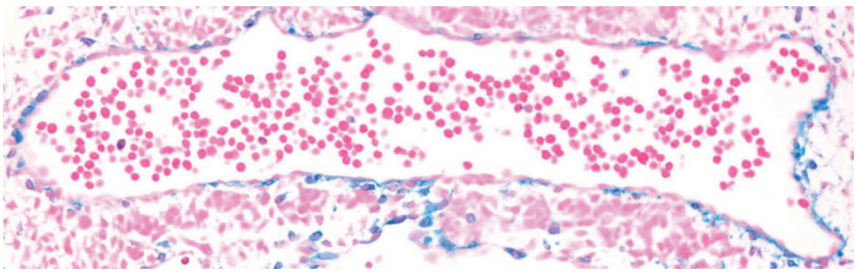

Fig. 1 **Lineage tracing of myocardin in developing mouse aorta.** Beta galactosidase staining (blue SMC) of embryonic day 10.0 mouse aorta. Result was obtained in embryos carrying the R26R reporter gene and Cre recombinase knocked into the endogenous *Myocd* locus.

vitro[19,30]. Further, in situ hybridization failed to detect *Myocd* mRNA in vascular SMC prior to embryonic day 11.5 in the mouse[29]. Lineage tracing data, however, suggest that *Myocd* is present in the dorsal aorta as early as embryonic day 10.0 of development[31] (***Fig. 1***). This suggests that *Myocd* mRNA may exist in early vascular SMC lineages, but is below the level of detection using standard methods. Expression of *Myocd* mRNA in heart and SMC-containing tissues was confirmed in developing *Xenopus*[32] and chicken[33]. RNase protection assay showed robust expression of *Myocd* mRNA in adult rat aortic SMC, with levels approximating those seen in adult heart tissue[30]. More extensive mRNA profiling revealed human and mouse *Myocd* mRNA are expressed highly in many adult SMC-containing tissues[29,34]. *Myocd* mRNA is not detectable in endothelial cells but is induced in a multipotent stem cell line stimulated to become SMC[34]. Moreover, *Myocd* mRNA is elevated following stimulation with all-trans retinoic acid[35] (a potent phenotypic modulator of SMC in vitro and in vivo[36]), potassium chloride[37], stretch[38], thrombin[39], NOX4[40], NRF3[41], and TGFβ1[42]. As discussed below, levels of *MYOCD* mRNA are modulated in association with various human diseases. While many commercial antibodies to MYOCD are available, they do not appear to reliably detect *endogenous* MYOCD protein, perhaps due to low-level expression. Thus, until a more widely accepted and validated antibody to MYOCD is developed, assessing endogenous MYOCD will be largely restricted to mRNA levels. Utilization of proximity ligation assay or knocking in an epitope tag (such as 3xFLAG) into the endogenous mouse *Myocd* locus could circumvent challenges presented with existing antibodies to this cofactor.

Myocardin is an essential cofactor for the SMC differentiated phenotype

SMC are notorious for exhibiting a range of phenotypes both in vitro and in vivo[10]. In general, the contractile phenotype of SMC is reduced when they are cultured or following injury to the vessel wall. This process was originally described as de-differentiation[43], but is more often referred to as phenotypic modulation[44], phenotypic plasticity[45] or phenotypic switching[46]. For decades, the transcriptional processes associated with this change in SMC phenotype remained a mystery. The initial reporting of MYOCD acting with SRF to strongly induce cardiac gene expression[19] launched an effort to define the role of this factor in SMC. The first preliminary study in early 2002 indicated that MYOCD could serve a MYOD1-like role in SMC[47]. A subsequent paper published in the same year reported four novel findings pertaining to MYOCD and the SMC differentiation program[30]. First, levels of *Myocd* mRNA were shown in an RNase protection assay to be lower in immortalized or primary-derived cultures of SMC as compared to SMC within aortic tissue. This result suggested that MYOCD is part of the biochemical de-differentiation program that occurs when SMC are removed from their native milieu and induced to proliferate in a culture dish. Second, overexpression of MYOCD induced the activity of several SMC promoters in a luciferase assay, including the highly specific promoter, *Myh11*. Several other SMC-restricted genes were later shown to exhibit MYOCD-dependent expression and promoter activity including the potassium channel, *Kcnmb1*[48], *Lmod1*[49], and *telokin*[50]. Third, ectopic expression of MYOCD in a cell line that exhibits no detectable expression of *Myocd* mRNA resulted in the stimulation of SMC marker genes such as *Acta2* and *Cnn1*. This MYOD1-like gain-of-function study was the first to clearly demonstrate a role for MYOCD in directing an endogenous SMC differentiation program. Finally, overexpression of MYOCD could blunt cell growth, a key characteristic of differentiated, contractile SMC in the vessel wall. Collectively, these findings – the reduced expression of *Myocd* mRNA in de-differentiated SMC, MYOCD-induced activation of SMC promoters and endogenous SMC marker genes, and the attenuated growth of cells with overexpression of MYOCD – supported a new concept for MYOCD

function, namely, its primacy in directing a SMC differentiation program[30]. These findings were subsequently confirmed and extended in important ways by several independent groups[21,29,34]. MYOCD also appears sufficient for directing a *functionally* competent SMC phenotype based on ultrastructural and physiological experiments (*e.g.*, calcium flux and slow wave contraction)[51–53]. Interestingly, there is in vivo evidence to support ectopic MYOCD converting hepatocytes to a SMC-like state[54]. Thus, whereas the transdifferentiation of a cell type into another distinct cell type often requires a cocktail of transcription factors[5,55], it appears that ectopic expression of a single factor (*i.e.*, MYOCD) is adequate for the conversion of a non-SMC into an SMC-like phenotype. Whether cells transduced with MYOCD first revert to a pluripotency state before differentiating into SMC is unclear. Collectively, in vitro studies have established MYOCD as *the* principal mediator of the normal vascular SMC contractile phenotype.

Further evidence for MYOCD functioning to direct the SMC differentiation program comes from mouse genetic studies. Wang et al. showed first that a pan-knockout of *Myocd* resulted in mid-gestation arrest with little evidence of aortic SMC differentiation; interestingly, however, embryonic vessels were patterned normally and the heart did not appear to display any obvious defect, though there were signs of defects in the yolk sac vasculature[56]. Subsequent studies indicated a strong heart phenotype in mice lacking normal MYOCD[57,58]. The reasons for such disparate cardiac data are unclear, but could relate to the strain of mouse studied. It is also relevant to point out that evidence supports some SMC differentiation in vessels where *MYOCD* is knocked out, suggesting that parallel pathways exist to direct the SMC differentiation program[59]. The weight of evidence, however, clearly supports a dominant role for MYOCD in the establishment and maintenance of a SMC contractile phenotype in vivo[60].

Regulatory control of myocardin expression and activity

Several levels of regulation in MYOCD expression and activity have been studied over the last 10 years, and these can be broadly defined in terms of transcriptional, post-transcriptional, and post-translational control processes. Transcriptional processes include promoter studies and the identification of *cis*-acting elements either activating or inhibiting gene transcription. Post-transcriptional processes encompass splicing and stability with the latter governed by a growing number of microRNAs. Post-translational processes involve various modifications to the MYOCD protein (*e.g.*, phosphorylation) that modulate its expression or activity. Each of these control processes will be reviewed briefly next.

Transcriptional control of myocardin expression

Several in vitro studies have defined regulatory elements controlling *Myocd* promoter activity and, by extension, *Myocd* mRNA expression. The first positive-acting element described was a proximal binding site for the cardiac muscle-restricted transcription factor, NKX2-5[61]. The effect of NKX2-5 on *Myocd* promoter activity was further shown to be modulated either positively or negatively through direct interactions between NKX2-5 and either CDC7[62] or SMAD3[63], respectively. An upstream NFATc3 binding site was demonstrated in the rat *Myocd* promoter using ChIP assays and forced expression of constitutively active NFATc3 could stimulate both *Myocd* promoter activity and mRNA expression[64]. Interestingly, cyclosporine A, which inhibits NFAT activity, reduces *Myocd* mRNA and SMC markers both in vitro and in vivo upon delivery to the vessel wall[65]. Another putative activator of *Myocd* expression is the RNA-binding heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1) factor, which binds to a proximal region of the mouse *Myocd* promoter and stimulates both promoter activity and endogenous mRNA expression in embryonic stem cells[66]. Of note, HNRNPA1 could also bind and stimulate the *Srf* and *Mef2c* promoters and physically interacted with SRF to enhance SMC contractile gene expression[66]. Levels of *Myocd* mRNA are reduced upon inactivation of SRF in a knockout mouse model[67]; however, extensive luciferase assays of conserved CArG elements around the *Myocd* locus have failed to demonstrate significant SRF-dependent activation (author′s unpublished data). Nevertheless, a recent study showed TET2 binding around a CArG box in the human *MYOCD* promoter with expected demethylation as shown by ChIP assays for the repressive methylation mark, H3K27me3[68]. TET2 was proposed to act upstream of the SRF-MYOCD switch, suggesting that it could be a master orchestrator of the vascular SMC contractile phenotype[68]. FOXO3A[69] and KLF4/KLF5[70] bind to regions of the human *MYOCD* promoter and appear to mediate transcriptional repression. Further, embryonic fibroblasts derived from *p53* knockout mice showed elevated *Myocd* mRNA expression, and wildtype p53 could dose-dependently repress a 1-kb human *MYOCD* promoter-driven luciferase reporter[71]. A short hairpin RNA to p53 blunted TGFβ1-induced *Myocd* and its target genes in SMC[71]. In all of the aforementioned studies, experiments were

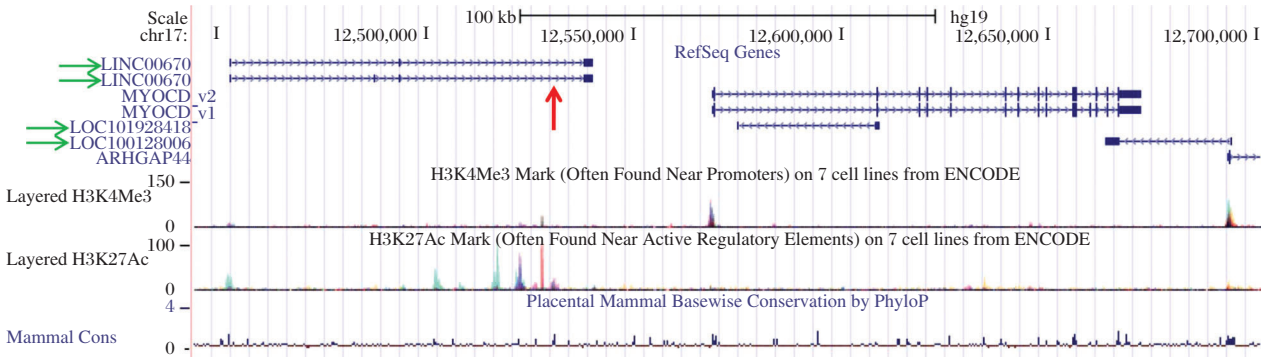

***Fig. 2* Human *MYOCD* locus.** Screenshot of UCSC Genome Browser showing the two human cardiac muscle *MYOCD* isoforms (MYOCD_v1 and MYOCD_v2), plus surrounding long noncoding RNA genes (green horizontal arrows) and the approximate location of the human *MYOCD* enhancer (red vertical arrow). Note the prominent H3K27 acetylation peaks that mark regulatory elements in close proximity to the enhancer and long noncoding RNA and the smaller H3K4 trimethylation peaks that mark promoters at the 5′ ends of both the *MYOCD* and *ARHGAP44* genes.

limited to in vitro analyses and in most cases, the conservation of the putative regulatory element was either not defined or poorly conserved, indicating need for further validation studies.

Remarkably, only one publication has examined *Myocd* promoter activity in the context of a living animal. Creemers et al. performed an elegant series of reporter tiling experiments in mice to show that a distal (~30 kb upstream) enhancer module recapitulated endogenous *Myocd* expression during embryonic and postnatal development[72]. Beta galactosidase reporter gene activity was noted as early as embryonic day 10.0 in the dorsal aorta, consistent with lineage tracing data (***Fig. 1***). The enhancer region was shown by gel shift, expression screening, and luciferase activities to harbor positively-acting binding sites for MEF2C (whose inactivation results in defective SMC differentiation[73]), FOXO4, and TEAD2[72]. No SRF-binding CArG boxes were present in this upstream enhancer region. In this context, MYOCD could activate its own distal enhancer in a MEF2-dependent manner providing the first demonstration of MYOCD acting in a CArG-SRF-independent fashion[72]. It is also interesting to note that the orthologous human distal enhancer lies within an intron of a conserved long intervening noncoding RNA (red arrow in ***Fig. 2***). The functional relationship of the *MYOCD* enhancer module to this uncharacterized transcript is presently unknown.

The study of the transcriptional control of MYOCD is still in its embryonic stages. Several future studies are needed including in vivo validation of key regulatory elements through either BAC recombineering, as shown for the *CNN1* gene[74] or through CRISPR/Cas9-mediated genome editing[75]. Second, several long noncoding RNA genes overlap or are in close proximity to *MYOCD* (green arrows in ***Fig. 2***), and these could act in *cis* to affect MYOCD expression. In this context, a recent study proposed that *SENCR*, a long noncoding RNA expressed abundantly in vascular endothelial cells and SMC, contributes to a SMC differentiated state through undefined effects on *MYOCD* mRNA expression[76]. Finally, it will be instructive to determine whether any SNPs exist in validated regulatory elements that could compromise MYOCD expression and the normal SMC contractile phenotype.

Post-transcriptional control of myocardin expression

Essentially all protein-coding genes undergo alternative splicing and *MYOCD* is no exception. Initial reports defined two isoforms of *MYOCD* that differed by inclusion of a 48 amino acid exon 10a (originally referred to as *MYOCD-A*)[29,61]. siRNA knockdown studies targeting this alternate exon suggest a repressor function[77]. Of note, ERK1/2 mediated phosphorylation of MYOCD was shown previously to diminish MYOCD transactivation[78], though none of the phosphorylation sites map to this exon. However, a putative ERK1/2 phosphorylation site was found in exon 10a, suggesting that there may be novel functions yet to be defined[79]. Subsequent work described inclusion of an exon (exon 2a) containing a premature stop codon resulting in downstream usage of a methionine within exon 4 that generates a SMC-restricted *MYOCD* isoform[80]. Excluding exon 2a results in a longer isoform of *MYOCD* that exhibits a more cardiac muscle-restricted pattern of expression[80]. Thus, there are at least 4 isoforms of *MYOCD* (v1-v4) with *MYOCD*_v1 and *MYOCD*_v2 encoding longer, cardiac muscle-restricted isoforms of amino acid lengths 983 and 935 (***Fig. 2***) and *MYOCD*_v3 and *MYOCD*_v4 encoding shorter, SMC-restricted isoforms of amino acid lengths 904

and 856[79]. Similar splice variants were described in human tissues[81].

Recently, an RNA-binding protein known as Quaking (QK1) was shown to regulate the balance of *MYOCD* isoforms in vascular SMC through direct interactions with the pre-mRNA of *MYOCD*[82]. Normal contractile SMC exhibit little expression of QK1 and express the dominant *MYOCD*_v3 isoform; however, QK1 expression was elevated in vascular lesions and shown to promote expression of the longer, cardiac muscle isoform of *MYOCD* (*MYOCD*_v1)[82]. Interestingly, the *MYOCD*_v3 isoform was more effective in blocking proliferation of SMC and promoting the contractile state than *MYOCD*_v1 suggesting that a delicate balance of *MYOCD* isoforms exists, in vascular SMC to maintain normal vessel wall homeostasis[82]. Formal definition of the function of each *MYOCD* isoform will require precision-guided genome editing using the CRISPR/Cas9 methodology[75].

Another mechanism for the post-transcriptional control of MYOCD expression is through microRNA-mediated mRNA deadenylation and degradation through miR-binding sites in the ~5-kb 3′ un-translated region of *MYOCD*. The miR-143/145 cluster is a major mediator of the SMC contractile phenotype, in part, through its direct or indirect effect on levels of MYOCD[83]. There is a miR-145 binding site in the 3′ un-translated region of *MYOCD* that somehow augments its stability and or translation[83]. Of note, miR-143/145 expression proceeds in a SRF/MYOCD-dependent manner via an upstream CArG box[83,84] and studies in human coronary artery SMC have shown that TGFβ1, a potent stimulus for the SMC differentiated phenotype[85], signals to induce miR143/145 expression via CArG and adjacent SMAD binding sites[86]. Binding sites for miR-145 also exist in the *KLF4* and *KLF5* transcripts, and evidence exists showing conventional repression of these known antagonists of *MYOCD* expression[83,84]. *KLF4* is also targeted by miR-146a[87], miR-25[88], and miR-1[89], leading to an indirect repressive action on *MYOCD* expression. Another mechanism of post-transcriptional control of *MYOCD* is through miR-221-mediated repression of *KIT*, which apparently can activate *MYOCD* gene expression[90].

Recently, a miR-1 binding site was demonstrated in the 3′ un-translated region of *MYOCD*, and levels of *MYOCD*_v3 (SMC enriched) were higher in miR-1 null mice as were several SMC-restricted genes[91,92]. Thus, miR-1 is a negative regulator of SMC gene expression in the heart. This post-transcriptional process of *MYOCD* regulation could explain why there is only transient expression of many SMC-restricted genes in the embryonic heart, only to re-emerge in adult hearts undergoing failure. There are other miR-binding sites in the *MYOCD* 3′ un-translated region including miR-9[64] and miR-135b[93] that effect changes in cardiac hypertrophy and cellular growth, respectively. It will be important to define the dynamic interplay among the known and yet-to-be-defined microRNAs that bind and regulate *MYOCD* expression. In particular, it will be instructive to study the functionality of these binding sites in proper context, such as in a living animal.

Post-translational control of myocardin expression and activity

More than 200 post-translational modifications have been defined that mediate a protein's stability, localization or functional activity, often times through direct protein-protein interactions. A recent review summarized some of the chemical modifications of MYOCD protein[94], and a partial listing of protein-protein interactions associated with MYOCD post-translational modifications or attending activity is provided in the Table. As the Table indicates, in most cases, MYOCD association with another protein alters activity through changes in MYOCD-SRF binding. GSK3B mediates MYOCD phosphorylation at several serine residues; inhibiting GSK3B accentuated MYOCD-dependent cardiac gene activation and hypertrophy of cultured cardiomyocytes, suggesting that MYOCD phosphorylation may be an important control mechanism for pathophysiological cardiac hypertrophy[95]. MYOCD undergoes sumoylation on K445 and this modification appears to increase its transactivation over cardiac muscle-specific genes since a K445R mutant exhibits reduced activity[96]. MYOCD can also be ubiquitinylated and this post-translational change has been linked to reduced levels of MYOCD via the proteasome[97]. On the other hand, UBR5, an ubiquitin E3 ligase, interacts with MYOCD and stabilizes its protein expression, thereby enhancing transactivation of SMC-restricted genes; down-regulation of UBR5 resulted in a decrease in MYOCD-induced SMC gene expression[98]. There may be other ubiquitin ligases that interact with MYOCD in certain contexts to enhance or attenuate expression and/or activity.

MYOCD is known to recruit chromatin remodeling factors such as p300 that acetylate lysine residues in histones tails, leading to augmented gene transcription[99]. More recently, an elegant study demonstrated p300-dependent acetylation of the MYOCD protein. Acetylation occurs at the amino-terminus and appears to enhance the association of MYOCD with SRF over CArG boxes, thereby increasing SMC target gene expression. Acetylation of MYOCD was also associated with displacement of HDAC5, a repressor of gene

expression[100]. Thus, we see how a factor (p300) serves a dual role of acetylating both local chromatin and the MYOCD cofactor itself to mediate gene expression. Whether these processes are mutually exclusive or facultative remains to be sorted out. Interestingly, ERK1/2 phosphorylation of MYOCD as well as serine-to-aspartic acid phosphomimetics of MYOCD reduce the interaction between MYOCD and p300 showing that different modifications of MYOCD can exhibit opposing activities[78]. This implies complex and dynamic, context-dependent changes in MYOCD modifications and subsequent activities necessary to effect moment-to-moment cellular changes in SMC and cardiac muscle cell phenotype. Since MYOCD undergoes phosphorylation, it must also interact with phosphatases that remove phosphate groups from key amino acid residues; however, no studies to date have formally demonstrated dephosphorylation of MYOCD through a specific phosphatase. There are a number of other regulatory post-translational modifications such as methylation, hydroxylation, oxidation, sulfation, and glycosylation (to name just a few) that have yet to be demonstrated in MYOCD. Post-translational modification of MYOCD in normal developmental and pathological conditions is an open area of future investigation and will be important to fully elucidate the biology of this important cofactor of gene expression.

Diversity in function of myocardin

Although much of the literature on MYOCD reports its role as a strong cofactor of SRF directing SMC and cardiac muscle gene expression, there is increasing recognition of its biological activities that are independent of CArG-SRF in these muscle cell types or dependent on CArG-SRF in a non-muscle cell type. For example, a recent study showed that MYOCD interacts with the pancreatic beta cell-specific transcription factor, PDX1, to synergistically activate insulin expression in human mesenchymal stem cells, suggesting that an improved method may exist to generate insulin-expressing beta cells for type I diabetes[101]. Further, a number of unanticipated functions of MYOCD have emerged such as its role in blocking cell growth and inhibiting the skeletal muscle program of differentiation. Thus, like many proteins, MYOCD exhibits a number of disparate biological activities and a brief summary of these follows.

CArG-independent stimulatory functions of myocardin on muscle gene expression

As discussed above, MYOCD appears to enhance its own expression through association with MEF2 over a MEF2-binding site located in an upstream (CArG-less) enhancer region, presumably through the well-defined MEF2 interaction site located in the amino-terminal RPEL domain of MYOCD[72]. MYOCD binds to SMAD3 over a SMAD response element and increases *Tagln* (aka *Sm22α*) promoter activity independent of the upstream CArG elements in this promoter[102]. MYOCD can interact with the DNA-binding domain of GATA4 independent of SRF and stimulate cardiac muscle gene expression. Interestingly, the stimulatory effect of MYOCD-GATA4 does not require the transactivation domain of GATA4, which when present acts to repress cardiac muscle genes[103]. Another report showed that MYOCD can interact with the TBX5 transcription factor to direct cardiac muscle (but not SMC) gene expression through the TBX-binding site in cardiac muscle gene promoters[104]. This study provides some insight into how MYOCD distinguishes between target genes associated with cardiac muscle versus SMC gene programs. *ITGA8*, an integrin alpha subunit highly enriched in SMC, is stimulated by MYOCD independent of SRF or a CArG element[105]. It is unclear as to how MYOCD stimulates this and perhaps other genes that do not otherwise require the CArG-SRF binary complex for gene activation. Further, it will be important to assess the full complement of MYOCD-dependent, CArG-SRF-independent genes in cells where SRF is lacking using ChIP-seq and RNA-seq following MYOCD overexpression. This will require new antibodies to MYOCD such as one recognizing an endogenous, epitope-tagged MYOCD protein.

Growth inhibitory action of myocardin

Given the dominant role of MYOCD in establishing a SMC contractile phenotype, it was perhaps not surprising to find that its overexpression resulted in reduced proliferation of stably-transfected cells[30]. This early finding was corroborated by a series of subsequent studies showing MYOCD-dependent growth inhibition in SMC associated with blunted action of NFκB[106], reduced cyclin D1 expression[51], and miR-1 induction[107]. Interestingly, there is also evidence for loss in MYOCD expression in certain neoplasms, and the expression of MYOCD has been shown to block cancer cell phenotype through its powerful orchestration of the SMC contractile state[108,109]. Future challenges include the elucidation of MYOCD-dependent cyclin D1 repression as well as other genes that are likely to be influenced by the MYOCD cofactor (*e.g.*, induction of tumor suppressor genes).

Repression of skeletal muscle differentiation

SRF is most abundant in all three muscle types where high level expression of SRF-dependent contractile

genes exist[110]. In addition to SRF, there are some SRF-dependent genes that are expressed transiently in each of the three muscle cell types during development (*e.g.*, *Acta2* and *Tagln*)[111]. Importantly, whereas embryonic cardiac muscle and SMC lineages exhibit overlapping patterns of SMC gene expression, there is no such overlap in gene expression between SMC and skeletal muscle (***Fig. 3***). The molecular basis for this lack of overlap in gene expression between skeletal muscle and SMC was not known until the serendipitous finding that MYOCD completely blocks expression of myogenin, a skeletal muscle master regulatory gene[31]. Repression occurred at the level of the *myogenin* promoter via MYOCD binding MYOD1, thereby competitively inhibiting MYOD1 binding to an E-box in the *myogenin* promoter[31]. Consistent with these findings, MYOCD overexpression was shown to inhibit atrogin expression and the skeletal muscle phenotype while knockdown of *atrogin* elevated MYOCD in the L6 skeletal muscle cell precursor[112]. The incompatibility of the SMC and skeletal muscle gene programs is consistent with developmental lineage decisions where common progenitors in the somite give rise to either skeletal muscle or vascular SMC[113]. In this context, lineage tracing studies suggest that MYOCD is transiently expressed within the somite invoking the hypothesis that somitic progenitors with MYOCD expression may be fated for vascular SMC[31,114].

Miscellaneous functions of myocardin

In order for cells to contract, there must be careful transcriptional control of ion currents across cell membranes. Surprisingly, little has been done to link MYOCD to specific activation of ion channel genes. The *KCNMB1* gene, which is highly restricted to SMC, is a direct target of *MYOCD* though two CArG-SRF sites in 5′ untranslated and proximal intronic regions[48]. Genetic proof for the function of these CArG elements was demonstrated in transgenic mouse experiments[48]. Remarkably, ectopic expression of MYOCD can elicit ion current activity in cultured cells[48,53]. Interestingly, ion currents, particularly voltage-dependent calcium entry, can elicit MYOCD expression and various SMC contractile genes[37,115]. These intriguing findings underscore the important

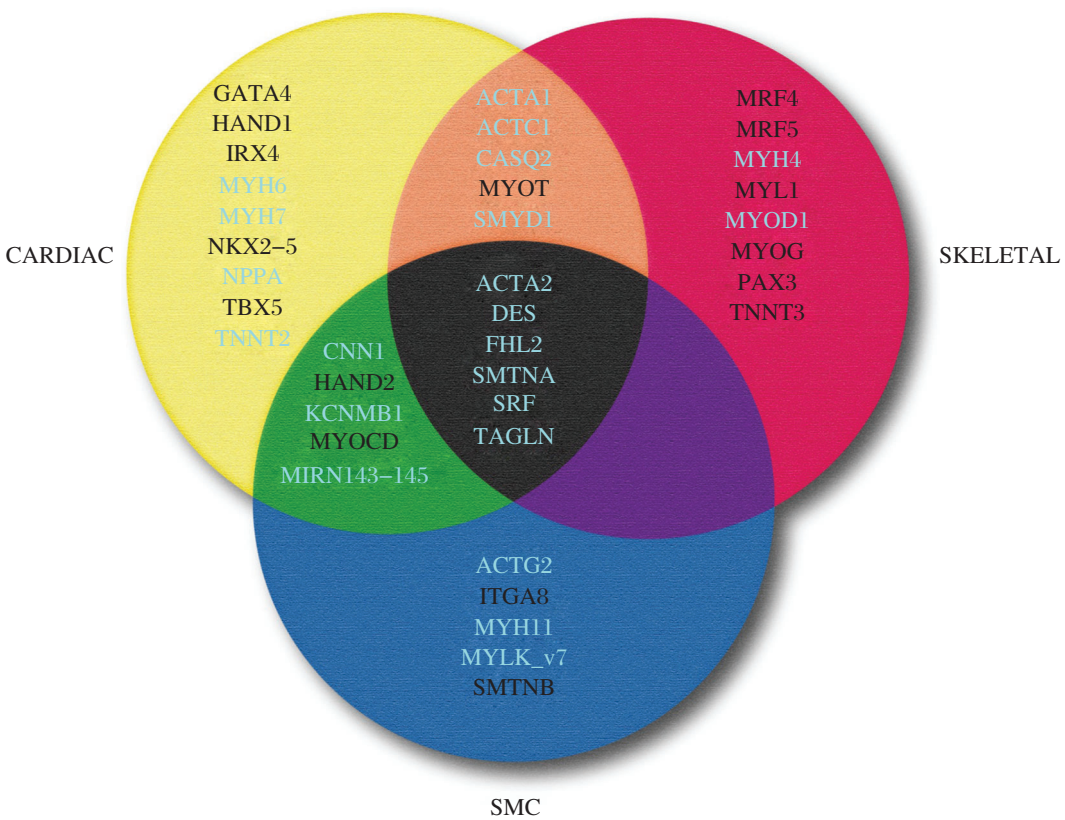

Fig. 3 **3-set Venn diagram of genes across the three muscle cell types.** Shown are the most specific genes unique to each of the indicated muscle cell types. White-labeled genes are those that lack functionally-validated CArG boxes while the genes labeled in light color harbor functional CArG boxes. Genes common to the three muscle cell types during development (center) each have at least one functional CArG box. Whereas several genes are present at the intersection of skeletal and cardiac muscle (orange) and cardiac and SMC (green), no genes are found at the intersection of SMC and skeletal muscle (purple). See the text for discussion.

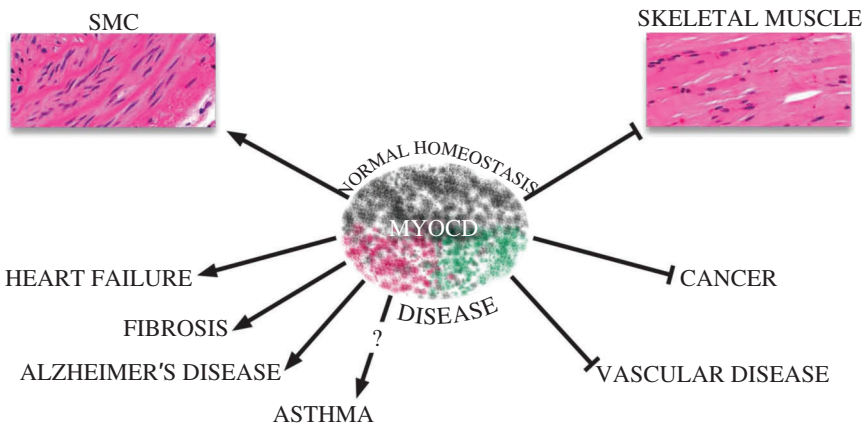

***Fig. 4* Diverse roles of MYOCD in normal homeostasis and disease.** Shaded colors of MYOCD icon reflect its diversity in activity, most of which relates to the maintenance of normal homeostasis (SMC differentiation and repression of the skeletal muscle phenotype). MYOCD may contribute to diseases (shade of red) or attenuate diseases (shade of green). See the text for details.

concept of excitation-transcription coupling[116], and highlight the gap in understanding how channel activity mediates *MYOCD* gene transcription or stability[117]. Given the wide number of ion channels expressed in both cardiac muscle and SMC[118,119], it will be important to delineate those channels whose expression requires MYOCD and whether their expression proceeds in a CArG-SRF-dependent or CArG-SRF–independent manner. There is also a growing appreciation for the role of MYOCD in activation of microRNAs such as miR-145[83] and miR-1[107]. Of note, some microRNAs may be repressed by ectopic MYOCD expression, including miR-199a-3p and miR-214[120]. That MYOCD may repress gene transcription (miRs, myogenin), emphasizing the need for more study of the binding partners of MYOCD in the nucleus that directly or indirectly influence chromatin architecture as well as RNA polymerase II-dependent transcription. Finally, as more RNA-seq experiments are undertaken, it will be informative to further advance our understanding of MYOCD-dependent gene expression.

Pathological processes linked to myocardin

There is a growing literature documenting changes in MYOCD expression in association with a number of diseases. In addition, MYOCD has been shown to attenuate or exacerbate disease phenotypes, highlighting its potential as a therapeutic agent or target for disease prevention (***Fig. 4***). Much of the literature describes altered expression of MYOCD in diseases of the heart or vasculature, but there are other disease states (both clinical and experimental) linked or correlated with changes in MYOCD expression.

Cardiovascular diseases

Heart failure is a pervasive clinical problem in Western countries and is a leading cause of morbidity and mortality. There are several causes of heart failure including ischemic heart disease and hypertension with the terminal stages characterized as dilated cardiomyopathy (DCM). Levels of *MYOCD* mRNA were first shown to be elevated in humans with DCM as compared to normal hearts[81]. Moreover, there have been a series of clinical studies showing increases in *MYOCD* mRNA in circulating cells or cardiac tissue from patients with idiopathic cardiomyopathy[121,122], hypertrophic cardiomyopathy[123], and essential hypertension[124]. Interestingly, variants in the 5′ promoter region of human *MYOCD* were associated with reduced levels of circulating *MYOCD*, which correlated with attenuated left ventricular mass in hypertrophic cardiomyopathy patients[125]. The function of these promoter variants is unknown at this time, but they presumably interfere with the transcriptional regulation of *MYOCD*. A rare missense mutation (K259R) of *MYOCD* has been reported in a congenital heart disease patient, and this mutation alters SRF binding to the cardiac, but not the SMC, isoforms of MYOCD[126]. Increases in *MYOCD* transcripts were observed with elevations in *GATA4* and *NKX2-5* in patients with 3-vessel coronary artery disease[127]. The latter clinical data are congruent with experimental studies showing forced expression of MYOCD provoking impaired left ventricular systolic function and electrical activity in the piglet[128] as well as hypertrophy of cardiomyocytes[122]. Importantly, silencing *MYOCD* via intramyocardial delivery of a short hairpin RNA in a doxorubicin-induced model of heart failure, normalized increases in *MYOCD* and several fetal genes (including various SMC genes)

and attenuated cardiac muscle dysfunction and death[129]. Collectively, these clinical and experimental data suggest that *MYOCD* represents a viable therapeutic target/biomarker in the setting of cardiac hypertrophy and failure.

Whereas MYOCD levels increase in association with cardiac disease, a number of reports have found *MYOCD* mRNA to be transiently down-regulated following acute injuries to the blood vessel wall in different animal models[130–134]. A recent elegant study showed that MYOCD delivery to the vessel wall blocked the neointimal response to vascular injury through microRNA-mediated suppression of the *Pdgfrb* gene[132]. This result suggests therapeutic potential for enhanced MYOCD expression in the context of acute injuries such as those encountered in the clinic where there remains some residual restenosis rates following balloon angioplasty of atherosclerotic lesions. *MYOCD* levels are also reduced in experimental models of atherosclerosis[135] and hypertension[136]. It remains to be studied whether ectopic MYOCD exerts any beneficial effect in these chronic conditions of vascular disease. *MYOCD* mRNA levels were reported to be reduced in venular SMC of experimentally-induced and clinically-diagnosed varicose veins[137]. Interestingly, treatment with Bortezomib, (a proteasome inhibitor) enhanced MYOCD expression and reduced the proliferation and migration of SMC as well as the varicosity of veins in a mouse model[137].

Pulmonary hypertension is associated with hypoxia and the formation of so-called plexiform lesions. Hypoxia induces *Midkine* which has been linked to increases in *MYOCD* in the setting of pulmonary hypertension[138], and these findings are congruent with hypoxia-mediated increases in *MYOCD* in other models of pulmonary hypertension[139,140]. Hypoxia has also been shown to induce *MYOCD* in human cerebral SMC as well as rodent SMC[141]. Hypoxia-induced *MYOCD* in this setting appears to be linked to faulty amyloid beta clearance and the emergence of cerebral amyloid angiopathy, which is pathognomonic of Alzheimer's disease[141]. Of note, a putative hypoxia response element was found in the 5′ promoter region of *Myocardin*[141]; however, formal proof that this element binds to HIF1A and underlies the induction of *MYOCD* by hypoxia awaits further study. Interestingly, increases in MYOCD and the SMC contractile program have also been observed in Alzheimer's disease patient-derived cerebral SMC with consequent hyper-contractility and hypoperfusion, which are additional features of Alzheimer's disease in humans[142]. Thus, Alzheimer's disease and attending neurodegeneration and cognitive decline may result from elevated *MYOCD* expression with poor amyloid clearance and hypoperfusion due to hypercontractile vascular SMC[143]. This would suggest a more vascular-centric (as opposed to neuro-centric) view for the pathogenesis of this devastating neurological disorder. Although the weight of evidence supports hypoxia as a stimulus for MYOCD induction, there are studies that show a negative correlation between *MYOCD* levels and hypoxia, highlighting the context-dependent nature of these responses[144,145]. Finally, there is one report of increased *MYOCD* in calcified aortic valve cusps with accompanying elevations in SMC markers, suggesting that SMC-derived MYOCD could play a role in the pathogenesis of calcific aortic stenosis[146]. There remain other vascular disorders where altered *MYOCD* may be of functional consequence, including peripheral artery disease, aneurysm formation, and transplant arteriopathy. The integration of inducible isoforms of MYOCD in animal genomes using CRISPR-Cas9 technology will be of major interest to further assess the efficacy of MYOCD in thwarting a number of vascular disorders.

Cancer

The consistent finding that MYOCD suppresses normal cell growth is consonant with the increasing evidence supporting a tumor suppressor role for this SRF cofactor in the setting of cancer[147]. One clinical study has shown attenuated MYOCD in several nasopharyngeal carcinoma cell lines, and the decrease in *MYOCD* correlated with hypermethylation of its promoter[148]. Notably, treatment of these cancer cells with the demethylating agent, 5-azacytidine, increased *MYOCD* expression, further suggesting the tumor suppressive nature of MYOCD[148]. Sometimes, the tumor suppressor effect of MYOCD may be indirect. For example, *Maspin* is transcriptionally induced by MYOCD and the up-regulation of *Maspin* leads to apoptosis of breast cancer cells[149]. In uterine leiomyosarcoma cell lines, MYOCD shows clear growth suppressive properties, in part through the activation of the p21 growth inhibitor via SRF-binding CArG element[109], but also because of the induction of several contractile genes such as *CNN1*, which itself has been labeled a tumor suppressor gene[150]. In this context, *CNN1* and other SMC contractile genes have been demonstrated to be reduced in metastatic human tumors and form part of a molecular signature for the metastatic phenotype[151]. Whether reductions in these genes, all of which carry SRF-binding CArG boxes, stem from reduced levels of MYOCD is presently unknown. It must be stressed, however, that there are some instances where levels of MYOCD are elevated in tumor cells[152], further underscoring the

Table 1 **Myocardin (MYOCD)-interacting proteins and functional consequence**

Interacting protein	Function	Reference
ACTR5 (ARP5)	Displace SRF binding; reduced MYOCD activity	[162]
EP300 (p300)	Acetylation; increased MYOCD activity	[100]
EPC1	Increased MYOCD activity; reduced neointimal formation	[163]
FOXF1	Enhances SRF binding; increased MYOCD activity	[164]
FOXO4	Displace SRF binding; reduced MYOCD activity	[165]
GATA4	Specifies MYOCD-dependent cardiac muscle cell target gene expression	[103]
GATA6	Specifies MYOCD-dependent SMC target gene expression	[166]
GSK3B	Phosphorylation of MYOCD; reduced MYOCD activity	[78,95]
HDAC5	Repress SMC gene expression	[99]
HMGXB4 (HMG2L1)	Displace SRF binding; reduced MYOCD activity	[167]
IRF8	Displace p300 binding; reduced MYOCD activity	[168]
IRF9	Displace p300 binding; reduced MYOCD activity	[169]
JUN	Displace SRF binding; reduced MYOCD activity	[170]
KDM3A (JMJD1A)	H3K9 demethylase; increased MYOCD activity	[171]
KPNB1 (Importin β1)	Nuclear localization of MYOCD	[172]
KLF15	Displace SRF binding; reduced MYOCD activity	[173]
NCOA3 (SRC3)	Cofactor of MYOCD; increased MYOCD activity	[174]
PDX1	Increases insulin gene expression in pancreatic β cells	[101]
PLAUR (UPAR)	Proteasomal degradation; reduced MYOCD activity	[175]
RELA (p65)	Displace SRF binding; reduced MYOCD activity	[106]
RUNX2	Displace SRF binding; reduced MYOCD activity	[176]
SMAD3	Increased MYOCD activity in SMC; CArG-independent	[102]
SMARCA4 (BRG1)	Enhance SMC gene expression	[177]
SMARCD3 (BRM)	Enhance SMC gene expression	[177]
SOX9	Displace SRF binding; reduced MYOCD activity	[178]
STUB1 (CHIP)	Ubiquitinylation of MYOCD; reduced MYOCD activity	[97]
TBX5	Increased MYOCD activity in cardiac muscle; CArG-independent	[104]
TDG	Displace SRF binding; reduced MYOCD activity	[179]
TERT	Enhance cardiac and SMC gene expression	[180]
TSHZ3	Displace SRF binding; reduced MYOCD activity	[181]
UBR5	Ubiquitinylation of MYOCD; increased MYOCD activity	[98]
YAP1	Displace SRF binding; reduced MYOCD activity	[182]

complexity of human disease and the need to exercise circumspection when making broad assumptions over the importance of this or any other protein in human pathology. On one final note, it is intriguing to consider the fact that while vascular SMC exhibit phenotypic plasticity, vascular leiomyosarcomas are exceedingly rare cancers[153]. The molecular basis for such scarce tumors is completely unknown, but may well relate to the SMC-restricted isoforms of MYOCD that appear to exert greater growth inhibitory action than the longer, cardiac muscle-enriched isoforms[91].

Diabetes

Type 2 diabetes has ascended to epidemic proportions and will increasingly strain Western health care systems. One of the manifestations of type 2 diabetes is a spike in RAGE with inflammation and calcification. Overexpression of RAGE suppresses *MYOCD* and SMC contractile genes and favors an osteogenic phenotype, which can lead to calcification of arteries[154]. In addition to vascular complications, diabetic patients often present with erectile dysfunction. Experimental models of diabetes have consistently shown a reduced expression of *MYOCD* in the corpus cavernosum[155,156]. From a therapeutic standpoint, ectopic MYOCD was shown to reconstitute normal erectile function in diabetic rats through the conversion of proliferating SMC within the corpus cavernosum to a contractile state[157]. Other complications of diabetes include accelerated atherosclerosis, retinal angiopathy, and peripheral artery disease all of which have yet to be examined in the context of MYOCD.

Miscellaneous diseases

There are several other diseases in which MYOCD has been studied. For example, in a model of intestinal obstruction, *MYOCD* mRNA levels were shown to be reduced concomitant with several MYOCD-dependent SMC contractile genes[158]. In one of the first examples of *Myocd* haploinsufficiency, mice with only one functional *Myocd* allele, exhibited bladder SMC hypersensitivity. This was surmised to result from the reduced expression of miR-1 and a corresponding increase in a known miR-1 target mRNA, *Gja1* or connexin 43. Adult mice with one copy of *Myocd* showed lower bladder capacity consistent with a hypersensitive phenotype[159]. Hepatic stellate cells can undergo transdifferentiation to a myofibroblast-like phenotype in liver diseases associated with fibrosis, and studies have shown *MYOCD* is induced in experimental models of liver fibrosis[160,161]. Interestingly, reducing *MYOCD* with siRNA predictably normalized the myofibroblast phenotype, suggesting that MYOCD (and probably its related MRTFs) could be a novel target for the treatment of liver fibrosis[161].

Future perspectives

The last 10 years of research on MYOCD have focused mainly on its well accepted role as the principal component to a switch for the SMC differentiated state. There have been new insights into MYOCD regulation, its association with other proteins, as well as its expression and potential utility as a marker or target of therapy for several diseases. Work in the next decade should be focused on the development of new reagents (antibodies) and animal models (inducible expression of MYOCD) to further understand this remarkable cofactor′s function in normal and pathological processes. In addition, there should be effort devoted towards fully elucidating transcriptomes under control of MYOCD, particularly the expanding class of long non-coding RNAs. Further, we need to define the mechanisms through which MYOCD functions independently of SRF. What other DNA binding factors does MYOCD interact with and in what contexts? There are SMC-associated diseases in which virtually nothing is known regarding expression and functionality of MYOCD (*e.g.*, asthma). Finally, as more and more human genomes are sequenced, it will be informative to define the functionality of sequence variants such as SNPs both in and around *MYOCD* coding and non-coding sequence space.

Acknowledgment

Dr. Miano is a Fellow at the Collaborative Innovation Center for Cardiovascular Translational Medicine of Jiangsu Province.

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