REGULATION OF VASCULAR SMOOTH MUSCLE CELL GENE EXPRESSION BY MYOCYTE ENHANCER FACTOR 2

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ABSTRACT

During development, vascular smooth muscle cells (VSMCs) proliferate and migrate from diverse mesodermal sources to primitive endothelial tubes; while simultaneously inducing the expression of differentiation-marker genes. Furthermore, VSMCs maintain the post-natal ability to proliferate in response to vascular injury. The MADS box protein myocyte enhancer factor 2 (MEF2) plays an essential role in vascular development and phenotype modulation in response to injury; however, the intra-cellular signals and interacting co-factors that toggle MEF2 between differentiation and proliferation-related genes remain unknown. Therefore, the purpose of this body of work was to investigate the molecular underpinnings that regulate MEF2 site-directed transcriptional control in VSMCs.

Protein kinase A (PKA) potently inhibits vascular smooth muscle proliferation and may protect against vascular disease. Therefore, in my first series of experiments, I examined the role of protein kinase A (PKA) in MEF2-dependent regulation of the proto-oncogene *c-jun*. Interestingly, PKA regulates MEF2-dependent *c-jun* expression through inhibition of the HDAC kinase SIK1 (salt-inducible kinase 1). Furthermore, I demonstrate that modulation of *c-jun* expression by MEF2 and HDAC4 is a critical 'switch' that regulates the VSMC phenotype as c-Jun can physically interact with myocardin to repress differentiation marker-gene expression.

Calcium sensitivity in vascular smooth muscle cells (VSMC) is regulated by RhoA/ROCK-mediated inhibition of the myosin light chain phosphatase complex, and alterations in smooth muscle gene expression. In a second series of experiments, I utilized a model of depolarization-induced calcium signalling and found that both MEF2-dependent c-Jun and myocardin expression were increased with this treatment. Interestingly, the induction of c-Jun was inhibited by CaMK inhibition, but the induction of myocardin was attenuated by p38 MAP kinase and ROCK inhibitors. Furthermore, ROCK-mediated phosphorylation of the PP1α inhibitor, CPI-17, at threonine 38 leads to derepression of MEF2C by PP1α and increased myocardin expression.

This work addresses several important aspects of MEF2 regulation in VSMCs and begins to provide mechanistic understanding of the role of MEF2 in vascular development and post-natal vascular disease.

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ABBREVIATIONS

CaMK	calcium/calmodulin-dependent kinase
ERK	extracellular regulated kinase
HDAC	histone deacetylase
IGF-1	insulin-like growth factor 1
MADS	MCM1, Agamus, Deficiens, SRF
MAPK	mitogen activated protein kinase
MEF2	myocyte enhancer factor 2
NFAT	nuclear factor of activated T-cells
PDE	phosphodiesterase
PDGF	platelet-derived growth factor
PKA	protein kinase A
PKC	protein kinase C
PKD	protein kinase D
PP1α	protein phosphatase 1α
ROCK	RhoA associated kinase
SIK1	salt-inducible kinase 1
SRF	serum response factor
TGFβ	transforming growth factor β1
VSMC	vascular smooth muscle cells

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INTRODUCTION

Transcription is the first step in gene expression, and its regulation underlies developmental processes and the cellular adaptation to physiological and pathological stress (1, 2). Transcription occurs in three stages; initiation, elongation, and termination (2). Activator proteins, called transcription factors, binding to DNA sequences, or *cis* elements, that lie upstream of transcription start sites, and are believed to recruit the 'basal' transcriptional machinery, containing the pre-initiation complex to receptive promoters (1).

Gene-targeting studies have highlighted the functional role of the myocyte enhancer factor 2 (MEF2) family of transcription factors in muscle development and post-natal adaptation (3). In vertebrates, there are four *mef2* genes, *mef2a*, *-b*, *-c*, and *–d*; however, the genomes of *Drosophila*, and *Caenorhabditis elegans* contain a single *mef2* gene (4-6). The first MEF2 gene targeted for deletion in mice was MEF2C. Mice homozygous for a *mef2c*-null allele die around E9.5 from cardiac looping defects, an undeveloped right ventricle, and a decreased expression of a subset of cardiac-specific genes (7). In addition, *mef2c*-null mice have a vascular defect, where vascular smooth muscle cells (VSMC) fail to differentiate (8). Consistent with this finding, conditional deletion of *mef2c* within the neural crest results in impaired differentiation of the branchial arch arteries, and post-natal lethality due to a severe craniofacial defect (9). The early lethality of the *mef2c*-null mice has precluded its role in skeletal muscle

development, until recently. Conditional deletion of *mef2c* in skeletal muscle is lethal at 1 day post- natal with abnormal sarcomere assembly and decreased expression of muscle-specific structural genes (10).

Mice deficient in *mef2a* have post-natal dilation of the right ventricle, and deficiency of cardiac mitochondria, consistent with the observation that MEF2A is the dominant *mef2* gene product in the heart after birth (11). Conversely, *mef2d*-null mice are viable, but are resistant to cardiac hypertrophy and pathological remodeling of the heart induced by stress (12). Interestingly, neither the *mef2a*- or *mef2d*-null mice have skeletal muscle defects, which suggests a degree of functional redundancy among the *mef2* genes in this tissue and possibly others (10). However, in *Drosophila*, deletion of the single *mef2* gene results in somatic myoblasts that do not fuse into multinucleated myotubes and fail to express muscle differentiation marker genes (13, 14). Similarly, cardiac cells in the dorsal vessel pattern properly, but do not express contractile genes (13, 14).

Thus, these studies clearly identify a functional role for MEF2 proteins in skeletal, cardiac and vascular smooth muscle cells. A more detailed review of the literature surrounding MEF2 biology published over the last two decades is presented below.

REVIEW OF LITERATURE

MEF2 BIOLOGY

HISTORY AND DISCOVERY

Following the discovery of MyoD, a skeletal muscle-specific transcription factor capable of activating muscle-specific genes in non-muscle cells, muscle biologists began an intensive search for muscle-enriched transcription factors involved in activating differentiation genes (15). Using the well characterized muscle creatine kinase (MCK) promoter, two independent groups identified myocyte enhancer factors involved in MCK expression in cultured skeletal muscle lines (16, 17). These factors were named MEF-1 and MEF-2. Further evaluation of MEF-1 revealed it was likely to be MyoD itself, but MEF-2 was a *bona fide* novel DNA binding factor (18). MEF-2 factors created a conserved 10 bp A+T-rich DNA footprint originally identified in the MCK and myosin light-chain promoter regions, and this binding site was required for full enhancer activity in culture myotubes (17). Within three years of its identification, the first *mef2* genes were cloned, and subsequently named *mef2a* and *mef2b* (19). Analysis of the *mef2* genes revealed that they belong to the recently identified MADS-box family of transcription factors, where MADS represents the first four members of this family, MCM-1, Agamous, Deficens, and Serum Response Factor (SRF). Transcripts for these *mef2* genes are ubiquitous, but more abundant in skeletal muscle, heart, and brain. However, unlike MyoD, MEF2 expression alone was insufficient to induce a complete muscle phenotype

(19). Interestingly, while the first *mef2* genes were being identified, an independent group working on SRF cloned three novel factors based on their similarity to the MADS-box found in SRF (20). These DNA binding factors were named related-to-SRF (RSRF). The RSRF factors bound the identical *cis* element originally described in the MCK enhancer, and were eventually amalgamated into the MEF2 family.

Following the cloning of *mef2a* and *–b*, *mef2c* was cloned from human and mouse tissues (21, 22). Unlike MEF2A, which is more ubiquitous in its expression, MEF2C expression is restricted to skeletal muscle, brain, and spleen; however, MEF2C transcripts could be detected in heart tissue with RT-PCR (21, 22). Like the *mef2a* gene, *mef2c* generates a number of alternatively spliced isoforms (19, 21). Interestingly, one of the MEF2C isoforms is restricted in its expression to brain tissue; however, the functional role of this splice variant remains unknown (21). The fourth *mef2* gene, *mef2d*, was cloned within a year of *mef2c* (23, 24). MEF2D transcripts are widely expressed, including in undifferentiated myoblasts; however, when myoblasts fuse to form myotubes, they cease to express the ubiquitous isoforms and express a skeletal muscle-restricted isoform (23, 24). The functional role of this isoform 'switch' remains unknown in skeletal muscle development; however, removal of the ubiquitous exon by alternative splicing results in the loss of a least one phosphorylation site within MEF2D which has been shown to be a critical repressor of MEF2 transcriptional activity (25).

Further analysis of MEF2 binding from skeletal, cardiac, and brain tissue identified the consensus MEF2 site, which is (C/T)TA(A/T)$_4$TA(G/A) (26). Interestingly,

MEF2A, -C, and –D all bind to this site with high affinity, yet MEF2B fails to bind this consensus site (27). By the mid-1990s, MEF2 consensus binding sites had been identified in the regulatory regions of numerous muscle contractile and structural genes including α-myosin heavy chain, myosin light chains, skeletal α-actin, troponins, desmin, and dystrophin, as well as in the regulatory regions of the muscle-restricted transcription factors, such as myogenin (4). However, it was immediately apparent that MEF2 proteins were also regulators of metabolism, in that MEF2 binding sites were found in the promoter regions controlling the expression of MCK, aldolase, Glut4, myoglobin, and more recently PGC-1 (4, 28). In addition, MEF2 binding sites have been found in the regulatory regions of two immediate-early genes, *c-jun* and *Nur77*, which suggested a proliferative and apoptotic role for MEF2 proteins in some cell types (29, 30). Thus, within a few years of its discovery, MEF2 had been cloned and many target genes had been identified that implicated a functional role in muscle differentiation, but possibly other functions such as cell division and cell death.

STRUCTURE OF MEF2 PROTEINS

The extreme N-terminal region of MEF2 proteins contains their characteristic MADS-box, which is a highly conserved 57-amino acid DNA-binding domain (Figure 1) (4). The MADS-box also mediates homo- and heterodimerization between MEF2 proteins, but it is generally accepted that MEF2 proteins cannot dimerize with other MADS-box proteins, such as SRF (20). Given that dimerization is critical to DNA binding, it is surprising that few reports have evaluated the functional importance of

heterodimerization between different MEF2 proteins and their alternatively spliced isoforms (31). Immediately adjacent to the MADS-box is a 29-amimo acid extension known as the MEF2 domain (4). This region is also highly conserved amongst MEF2 family members, but is not found within other MADS-box proteins, although SRF contains an analogous domain adjacent to its MADS-box (32). The MEF2 domain is believed to be involved in high-affinity DNA binding and dimerization, but also in the recruitment of co-factors that activate or repress MEF2's transcriptional activity (4).

The crystal structure of the MADS-box and MEF2 domain of MEF2A bound to DNA has been solved (Figure 1C) (33). MEF2A binds DNA as a 3-layered dimer, where each monomer contains an N-terminal extension consisting of the first 12 amino acids, and interacts with the minor groove of the DNA double helix through Gly2 and Arg3 (33). The first layer of the structure is formed from a long α-helix (a.a. 14-39) that dimerizes with the other MEF2 monomer to form an anti-parallel coiled coil. The charged side-chains of Arg15, Lys23, Arg24, Lys30, Lys31, and Glu34 form hydrogen bonds within the consensus MEF2 binding site. The exception is Arg15, which hydrogen bonds to an adenine just upstream of the consensus MEF2 site (33, 34). Bound water molecules between the phosphate groups in the DNA and the side-chains of Glu34 and Thr20 mediate additional DNA contact. Interestingly, Thr20 is conserved in SRF and phosphorylation of this residue by PKCδ inactivates SRF by reducing its DNA binding affinity (35). Whether Thr20 of MEF2 is regulated by PKCδ has not been studied.

The second layer of the MEF2 dimer bound to DNA is composed of a four-stranded β-sheet, where each MEF2 monomer donates two strands formed from amino acids 42-59. This β-sheet structure contributes to dimerization directly, and indirectly as the β-sheet of one monomer interacts with an α-helix in the MEF2 domain of the opposite monomer (33). The first two layers of the MEF2 dimer are contained entirely within the MADS-box, whereas, the third layer of the MEF2 structure is produced from the MEF2 domain. The MEF2 domain is structured into a second α-helix formed from amino acids 62-73 that pairs in an anti-parallel manner with the other MEF2 monomer. This structure provides an interface for the binding of MEF2 cofactors, such as the class II HDACs (33, 36). Interestingly, mutational analysis of this region in MEF2C abolishes all transcriptional activity, and the same appears to be true of MEF2D (J.W. Gordon and J. C. McDermott, unpublished) (37). However, the structural reasons for this finding are unclear, but may involve recruitment of a critical cofactor required for transcriptional activation, or alteration of a more complicated structure involving the C-terminal transcriptional activation domain.

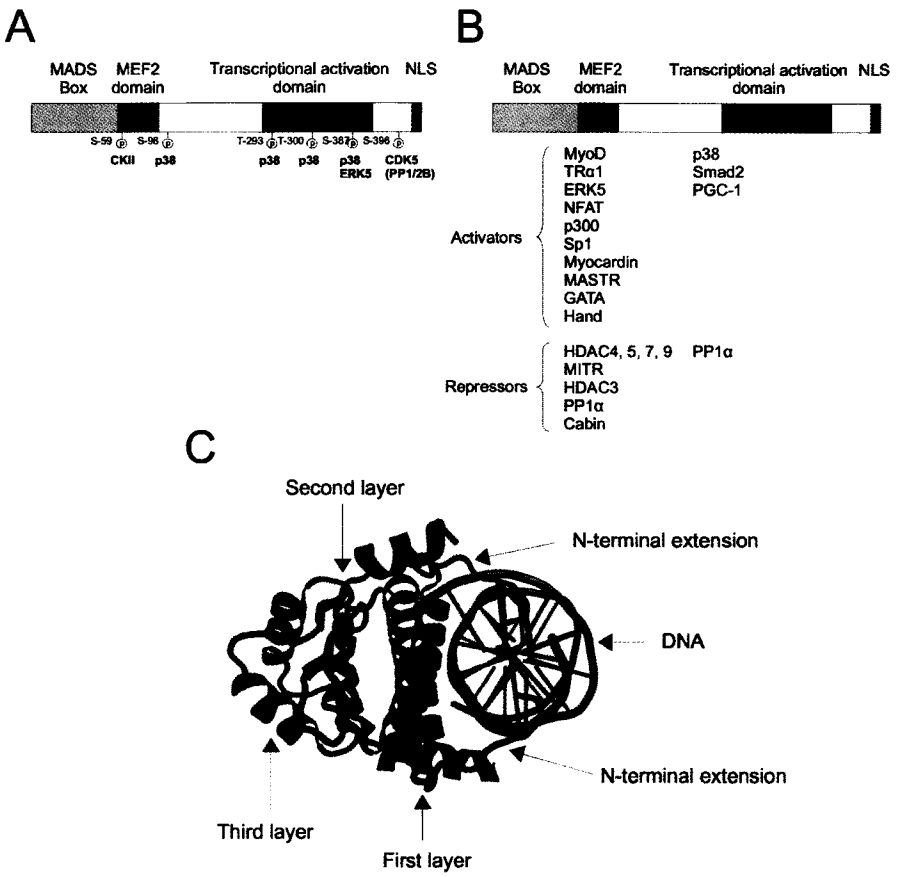

Figure 1. Structure of MEF2 proteins.

A) The structural domains of MEF2C with identified phosphorylation residues known to alter transcriptional activity NLS = nuclear localization domain. B) Numerous MEF2-interacting cofactors and the approximate domains where they interact with MEF2 proteins. C) The crystal structure of MEF2B bound to DNA (Modified from Wikipedia under the Creative Commons Attribution-Sharealike 3.0 Unported License (CC-BY-SA) and the GNU Free Documentation License (GFDL)).

The C-terminal region of MEF2 proteins contains the transcriptional activation domain. Between MEF2 factors there is less than 20% homology in this region, which can undergo substantial alternative splicing, with some exons expressed ubiquitously and other being neuro- or muscle-specific (see above) (3). The major transcriptional activation domains lie between amino acid 274-373 in MEF2A (38). This region of MEF2A is directly phosphorylated by the p38 family of MAP kinases at Thr312 and Thr319 to promote transcriptional activation (39). Indeed, MEF2 proteins are subjected to many post-transcriptional modifications that both increase and decrease their transcriptional activity (see below). The extreme C-terminus of MEF2A, -C, and –D contains a conserved nuclear localization signal at amino acids 472-507, where deletion of this small region switches MEF2 factors from a nuclear to a cytosolic distribution (38).

REGULATION OF MEF2 TRANSCRIPTIONAL ACTIVITY

PHOSPHORYLATION

CASEIN KINASE II

As mentioned above, MEF2 proteins are subject to numerous post-translational modifications that alter their transcriptional activity. The first cellular kinase identified to directly phosphorylate and activate MEF2 proteins was casein kinase II (CKII) (40). CKII phosphorylates Ser59 in MEF2 proteins, which results in a 5-fold increase in MEF2 DNA binding. These findings were prompted by the observation that bacterial translated MEF2 proteins had a significantly reduced DNA binding affinity than reticulocyte-

translated MEF2. In addition, a neutral mutation of Ser59 to alanine resulted in diminished DNA binding and transcriptional activity, determined by CAT assay (40).

P38 MAP KINASE

The first mitogen-activated protein kinase found to directly phosphorylate MEF2C was the p38 MAPK (41). In these experiments, p38 MAPK was identified as a MEF2C interacting partner through a yeast-two hybrid screen, and found to phosphorylate MEF2C at threonine 293, 300, and serine 387 in innate immune system cells in response to lipopolysaccaride treatment (LPS) (41). Furthermore, mutation of these threonine or serine residues to neutral alanines abolished the transcriptional activity of MEF2C and the induction in activity by LPS or p38/MKK6 (41). Additional studies confirmed that MEF2A and MEF2C were directly phosphorylated and activated by p38 MAPK, but MEF2B was not; whereas, MEF2D was phosphorylated to a much lesser extent than MEF2A and -C (39, 42). *In silco* analysis revealed that the p38 phosphorylation sites identified for MEF2C (ie. Thr293, 300, and Ser387) were conserved in MEF2A as Thr312, Thr319, and Ser453, and these site were phosphorylated by p38 *in vitro*. Interestingly, neutral mutation of Thr312 and Thr319 abolished the transcriptional activation induced by p38/MKK6, but mutation of Ser453 of MEF2A had no effect in 293 cells (39). These findings suggest that MEF2A and MEF2C are regulated differently by p38, and possibly in a cell-type specific manner. More recent evidence has challenged the idea that MEF2D is not a relevant target of p38 signalling (43). Indeed,

p38 was found to phosphorylate MEF2D at Thr308 and Thr315 *in vitro*, and this phosphorylation serves to recruit the histone modifier Ash2L to specific genes during skeletal muscle myogenesis (43).

Of the p38 isoforms, p38α is the most potent at phosphorylating MEF2A *in vitro*, followed by p38β and p38δ, while p38γ did not phosphorylate MEF2A (39). Interestingly, the p38 inhibitor SB203580, which inhibits only p38α and p38β, has been shown to potently inhibit MEF2 transcriptional activity in several cell lines (39, 42). In addition, MEF2A and MEF2C also share a conserved MAP kinase docking domain (D-domain) spanning amino acids 266-282 in MEF2A, which has been shown to target p38α and p38β to MEF2 proteins (44). Collectively, this indicates that p38α and p38β are the most physiologically important p38 isoforms that activate MEF2.

In vivo phosphorylation of MEF2A by p38 has been shown to produce a complex pattern of phosphorylation that cannot be explained by phosphorylation of Thr312 and Thr319 alone (42). To address this, Cox et. al. systemically attempted to identify other p38 targeted residues in MEF2A utilizing a combined technique of tandem affinity purification (TAP) and tryptic peptide mass identification with mass spectrometry of purified MEF2A, phosphorylated *in vivo* by ectopic expression of p38/MKK6 (45). This detailed analysis of MEF2A phospho-peptides identified several novel residues both directly and indirectly phosphorylated by p38, as well as three potential residues that could not be fully resolved due to the size of the peptide in which they were contained. Of particular interest, they identified Ser408, which was subsequently shown to be

dephosphorylated by calcineuin and promote MEF2 transcriptional activity (46). This group also identified Ser289, which is an additional CKII phosphorylation site that was indirectly regulated by p38 (45).

ERK5 MAP KINASE/BMK1

In the same year that p38 was found to activate MEF2, another group published that a second MAP kinase directly phosphorylated and activated MEF2C. This MAP kinase was named Big MAP kinase 1 (BMK1) or ERK5 (47). ERK5 was previously shown to be activated by oxidative stress, in a calcium-dependent manner, in VSMCs, but it also responds to serum-stimulation to promote c-Jun expression through the MEF2 *cis* element on the *c-jun* promoter (47, 48). Recent studies also suggest that ERK5, and it's upstream activator MEK5, are critical for full MEF2 activity and induction of c-Jun by epidermal growth factor (EGF) in fibroblasts (49, 50). In addition, ERK5 has also been shown to be potently activated by insulin-like growth factor 1 (IGF-1) in cardiomyocytes, and reduce cardiac apoptosis through MEF2 (51). ERK5 phosphorylates MEF2C at Ser387, but not at the other identified p38 sites, while mutation of Ser387 to alanine renders MEF2C unresponsive to ERK5/MEK5 stimulation (47). Ser387 is a conserved residue in MEF2A and MEF2C, but not in MEF2B or MEF2D, which is consistent with subsequent analysis that demonstrated that only MEF2A and MEF2C are activated by ERK5 stimulation (52). ERK5 was also found to interact with MEF2 proteins in a yeast two-hybrid screen comprised of a VSMC library using a conserved region of MEF2D as

bait (52). Mapping of this interaction demonstrated that ERK5 interacts with MEF2 proteins through the MADS-MEF2 domain and not through the C-terminal D-domain that binds p38 (44, 52). Interestingly, ERK5 is an unique MAP kinase, in that it contains a transcriptional activation domain, which has lead to speculation that in addition to activating MEF2, it might also serve as co-activator (53, 54).

PROTEIN KINASE A

Previous evidence studying skeletal muscle myogenesis demonstrated that protein kinase A (PKA) was able to inhibit differentiation, but the mechanism was unclear (55, 56). In addition, transgenic mice expressing the catalytic subunit of PKA in the heart develop a dilated myopathy with decreased expression of αMHC (57). The reason for this seemingly unpredictable phenotype remains unknown; however, recent evidence from our laboratory has provided some insight into these previous observations in skeletal and cardiac muscle. PKA was found to directly phosphorylate MEF2D *in vitro*, but did not phosphorylate MEF2A or MEF2C to the same extent (25). In addition, purification of TAP-tagged MEF2D from intact cells co-expressing the catalytic subunit of PKA, followed by mass spectrometric determination of phospho-peptides, lead to the identification of Ser121 and Ser190 as the major phosphorylation residues within MEF2D. Indeed, mutation of these serine residues to neutral alanines prevented the PKA-induced repression of MEF2 transcriptional activity and could rescue the inhibition of skeletal muscle differentiation caused by ectopic expression of PKA (25). However, it

remains uninvestigated whether this PKA-resistant MEF2D can rescue the cardiac phenotype of the PKA transgenic mice; although, this line of investigation is particularly interesting given that the MEF2D-null mice are resistant to stress-induced pathological remodelling of the heart (12).

CDK5, CALCINEURIN AND PROTEIN PHOSPHATASE 1α

CDK5 was found to phosphorylate MEF2A in vitro at Ser408, while in the same year, an independent research group identified Ser408 as a target of p38 MAP kinase in intact cells (45, 58). Phosphorylation of Ser408 by CDK5 was found to repress MEF2A transcriptional activity and inhibit the protective effects of MEF2 proteins in neurons from apoptosis (58). Ser408 is conserved in MEF2C as Ser396, and is subjected to alternative splicing; where MEF2C isoforms that contain this phospho-peptide have less transcriptional activity than isoforms that do not (59). Interestingly, the calcium/ calmodulin regulated protein phosphatase calcineurin, has been shown to dephosphorylate MEF2 and increase its transcriptional activity; however, only recently was it discovered, by an independent group, that calcineurin dephosphorylates MEF2A at Ser408, to remove the repressive effects of CDK5 and promote MEF2 transcriptional activity (46, 60). Calcineurin also promotes the nuclear localization of NFAT proteins that physically interact with MEF2 (see below). Protein phosphatase 1α (PP1α) has also been shown to dephosphorylate MEF2A at Ser408; however, the cellular consequences of this

remain unknown since PP1α inhibits MEF2 activity when ectopically expressed in cell culture (61).

INTERACTING COFACTORS

MEF2 proteins are also regulated by a number of interacting cofactors that either increase or decrease MEF2's intrinsic transcriptional activity. A number of these interacting cofactors are also known to be histone modifiers that either promote or inhibit chromatin relaxation.

MYOGENIC REGULATORY FACTORS

Some of the first discovered MEF2 interacting factors were the myogenic family of basic-helix-loop-helix (bHLH) factors, as called myogenic regulatory factors (MRFs, see below) (4, 27, 62). Although MEF2 proteins are unable to induce conversion of non-muscle cells to myotubes in culture, MEF2 proteins act synergistically with the myogenic bHLH factors, MyoD and myogenin, to induce conversion (63). The myogenic family of bHLH factors must dimerize with a ubiquitous class of bHLH factors, such as E12, E47, or HEB (62). Interestingly, MEF2C was found to directly interact with a myogenin/E12 heterodimer, and not with either myogenin or E12 homodimers (63). Mapping of the interaction between MEF2C and the myogenin/E12 dimer revealed that the interaction occurs between the MADS-MEF2 domain of MEF2C and the bHLH region (ie. DNA binding domain) of myogenin/E12, and only the MADS-MEF2 domain of MEF2C was

needed to synergistically convert non-muscle cell to myotubes (63). In addition, it was shown that MEF2C and myogenin/E12 could activate transcription through each others bindings sites, implicating that both binding sites need not be present within a specific promoter region to be activated by this trimeric complex (63).

THYROID HORMONE RECEPTORS AND PGC-1

MEF2A was also found to interact with the thyroid hormone receptor TRα1 (64). When co-expressed, MEF2A and TRα1 synergistically activate the cardiac alpha myosin heavy chain (αMHC) promoter. Mapping of this interaction revealed that the MADS-box of MEF2A and the DNA binding domain of TRα1 mediate this physical interaction, while both the MEF2 *cis* element and the thyroid hormone response element (TRE) are necessary and sufficient for this synergy (64). Further analysis of this interaction in skeletal muscle cultures identified at tripartite cooperation between MEF2, TRα1, and MyoD to induce the expression of the Glut4 promoter (65). The regulation of the Glut4 promoter by MEF2 proteins has become an intensely studied topic, given its relevance to insulin resistance and diabetes mellitus. Interestingly, skeletal muscle cultures express very little Glut4; however, expression of Glut4 can be restored by forced expression of the PPARγ coactivator (PGC-1α) (66). PGC-1α induces Glut4 expression through a physical interaction with MEF2 proteins, where mutation of the MEF2 *cis* element within the Glut4 promoter region abolishes the induction by PGC-1α. Interestingly, PGC-1α interacts with MEF2C in a region spanning amino acids 93 to 174, which lies just outside

of the MEF2 domain (66). Furthermore, the promoter of PGC-1α has two validated MEF2 *cis* elements within it, which suggests that PGC-1α expression is regulated by an autoregulatory loop involving MEF2 proteins (28, 67). Glut4 expression has also been studied in primary rat cardiomyocytes, where IGF-1 treatment activated p38 MAP kinase signalling induces Glut4 expression through MEF2 (68). Interestingly, PGC-1α has also been shown to be activated by p38 MAP kinase signalling; however, it remains to be demonstrated that p38 activation of PGC-1α contributes to Glut4 expression in the heart (69).

GATA4 AND HAND1

The MEF2C-null mouse provided key insight into the role of MEF2 proteins in cardiac development (7). Interestingly, two cardiac marker genes, atrial natriuretic factor (ANF) and cardiac α-actin (αCA), were completely absent in the MEF2C-null heart, yet these genes do not contain consensus MEF2 *cis* elements within their promoter regions. In addition, MEF2C was unable to activate the cardiac αMHC promoter in the absence of co-expressed TRα1 (64). Therefore, cardiac biologists speculated that MEF2C must recruit or be recruited by other cardiac transcription factors to functionally activate cardiac gene expression in a similar manner to the paradigm suggested for MEF2 and the MRFs (4). Indeed, MEF2C was found to be recruited to the ANF and α-CA promoters by GATA4 (70). In this series of experiments, MEF2 proteins were found to bind to a non-consensus A/T-rich region of the ANF promoter; however, mutation of the A/T-rich

region had minimal effect on promoter activity. Interestingly, mutation of an upstream GATA site abolished MEF2 binding to the ANF promoter region. Furthermore, MEF2A and GATA4 were found to physically interact through the MADS-MEF2 domain of MEF2A and the C-terminal zinc finger of GATA4, and functionally synergize on the ANF, α-MHC, and α-CA promoters (70). The same research group later reported that MEF2 is also co-activated by the cardiac Hand factors (71). Hand1 (eHand) and Hand2 (dHand) are bHLH factors involved in left ventricle and right ventricle development, respectively (72). In these experiments, Hand1 was recruited to the ANF promoter through MEF2, and not a ubiquitous E-protein or E-box (71). Both Hand1 and Hand2 synergistically activate the ANF promoter with MEF2, and physically interact with MEF2A, as determined by co- immunoprecipitation and GST-pulldown. Interestingly, the MEF2 and GATA *cis* elements were critical for this synergy, and maximal activation of the ANF reporter-gene was achieved by forced expression of MEF2, Hand1, and GATA4 (71). These results suggest that in cardiac cells a trimeric complex of MEF2, GATA, and Hand factors contribute to cardiac gene expression.

NFATs

The nuclear factor of activated T-cells (NFAT) represents four distinct genes (see below), that are phosphorylated by glycogen synthase kinase 3 (GSK3) and retained in the cytosol (73). When dephoshorylated by calcineurin, NFAT proteins translocate into the nucleus where they direct the expression of NFAT-responsive genes and co-activate a

number of other transcription factors (74). In muscle tissue, NFATs are most famous for their co-activation of GATA transcription factors, where they induce cell hypertrophy (75-77). However, NFATs also interact with MEF2 proteins (78). The first evidence suggesting a functional interaction between NFAT and MEF2 proteins came from the analysis of the myoglobin promoter region in response to ectopic calcineurin expression (79). In this series of experiments, the induction in myoglobin expression was attenuated by mutation of not only the NFAT binding site, but the MEF2 *cis* element as well. In addition, forced expression of calcineurin *in vivo* powerfully activated a MEF2-LacZ reporter gene in skeletal muscle and to a lesser degree in the heart (80, 81). Furthermore, MEF2-LacZ activity induced by exercise training could be inhibited by cyclosporin A treatment or forced expression of an endogenous calcineurin inhibitor, modulatory calcineurin interacting protein 1 (MCIP1) (60). However, given that MEF2 interacts with GATA4, and that calcineurin dephosphorylates MEF2A at Ser408, the exact mechanism or contribution of each mechanism responsible for the *in vivo* activation of the MEF2 reporter-gene by calcineurin remains unknown.

P300 AND CBP

Acetylation of lysine residues within histone tails by histone acetyltransferases (HATs) results in a relaxed nucleosome structure that is permissive to transcriptional activation by allowing access of transcriptional machinery to the regulatory regions of DNA (81). The most studied HATs in muscle tissue are p300 and the CREB-binding

protein (CBP) (82, 83). Both p300 and CBP functionally co-activate MEF2C in transcriptional assays, and p300 has been shown to directly interact with the MADS domain of MEF2C (84). In addition, cardiac-specific induction of the skeletal α-actin gene was mapped to a p300-responsive MEF2 *cis* element, which is consistent with other reports that p300 plays a critical role in agonist-induced cardiac hypertrophy and the transition to heart failure (85-87). However, p300 has also been shown to interact with other important cardiac transcription factors, such as GATA4 and SRF, either directly or indirectly through myocardin (88-90). In addition, MEF2D physically interacts with an Ash2L-containing methyltransferase complex that results in trimethylation of histone H3, epigenetically marking muscle-specific genes for expression during differentiation (43). Furthermore, p38-mediated phosphorylation of MEF2D was shown to modulate this interaction with Ash2L, supporting the role of p38 in skeletal muscle differentiation.

HISTONE DEACETYLASES

The activity of HATs is countered by histone deactylases (HDACs) that catalyze the removal of acetyl groups from histone tails and enhance the electrostatic properties between histones and DNA (82, 83, 91). There are currently three classes of HDACs that are classified based on their similarity to the yeast homologues. Class I HDACs include HDAC1, 2, 3, 8, and 11, and are related to yeast RPD3. Class IIa HDACs are HDAC4, 5, 7, and 9; while class IIb HDACs are HDAC6 and 10. Class II HDACs are related to yeast HDA1 (92, 93). In addition, there is a truncated form of HDAC9 also called MEF2-

interacting transcriptional repressor (MITR) that lacks intrinsic deacetylase activity, but can recruit other HDACs to MEF2 proteins. Lastly, class III HDACs are related to yeast Sir2, and are SirT1-7 (91).

MEF2 was originally shown to interact with class IIa HDACs through a yeast two-hybrid screen engineered to identify novel MEF2 interacting factors (94, 95). Within a year of this discovery, several independent research groups had reported a physical interaction between MEF2 and class IIa HDACs (93, 96, 97). Class IIa HDACs contain a conserved 18 amino acid extension at their N-terminus that mediates the interaction with MEF2 proteins; whereas, other classes of HDACs do not contain this domain and fail to directly interact with MEF2 (83). When bound to MEF2, class IIa HDACs are potent repressors of transcriptional activity; however, this repression can be relieved by ectopic expression of active CaMKs, while the transcriptional activity of MEF2 proteins can also be inhibited by CaMK inhibitors, such as KN-62 and KN-93 (95). CaMKI and IV relieve MEF2 from the repressive effects of HDAC5 by directly phosphorylating this class IIa HDAC at Ser259 and Ser498 (98). These residues are conserved in HDAC4 as Ser246 and Ser467; where phosphorylation of these sites permits HDAC binding to the nuclear chaperone 14-3-3 and disruption of the MEF2-HDAC complex (99, 100). Once bound by 14-3-3, class IIa HDACs are sequestered to the cytoplasm through a CRM1-dependent nuclear export process (98). Phosphorylation-dependent association of class IIa HDACs with 14-3-3 has additional effects, such as blocking the interaction between these HDACs and importin α, which prevents nuclear import, and dissociation of class IIa HDACs from

HDAC3, which would attenuate deacetylase activity (99). The nuclear-cytoplasmic shuttling of class IIa HDACs is made possible through an intrinsic nuclear localization signal (NLS) and nuclear export signal (NES), where the NLS is flanked by the CaMK phosphorylation sites and would be inaccessible to importin α when bound to 14-3-3 (101). However, when dephosphorylated by PP1, class IIa HDACs are free to interact with importin α and translocate to the nucleus (99, 102). In contrast to the NLS, the NES lies at the extreme C-terminus of class IIa HDACs, but is not present in MITR or class I HDACs, and appears to remain inactive until 14-3-3 binding (101, 103). Class IIa HDACs have also been shown to promote a further level of transcriptional repression over MEF2 proteins, in that HDAC4 can potentiate the sumoylation of MEF2C and -D (104). Recent studies suggest that HDAC3 also directly interacts with MEF2 proteins through the MADS-box, where class IIa HDACs interact with the MEF2 domain (105). In functional assays, targeted repression of HDAC3 expression with RNA interference resulted in enhanced MEF2 transcriptional activity and myogenesis; however, forced expression of HDAC3 had little effect on MEF2 activity (105). Interestingly, an independent research group cross-bread mice with a conditional deletion of cardiac HDAC3 with a MEF2-LacZ reporter mouse and observed only slight activation of MEF2 activity (106). Thus, the *in vivo* role of the interaction between MEF2 and HDAC3 remains unclear.

Early studies involving HDAC4 and HDAC5 revealed many similarities in the regulation of these class IIa HDACs; however, some differences were also noted. For

example, HDAC5 was found to be exported to the cytoplasm in cultured skeletal muscle myoblasts as they were differentiating into myotubes (36). However, HDAC4 may follow the opposite pattern, being cytosolic in myoblasts and imported into the nucleus during differentiation (unpublished) (83). Gene targeting studies have begun to identify non-redundant roles for class IIa HDACs. Both HDAC5- and HDAC9-null mice display an increased susceptibility to age-related and/or stress-induced cardiac hypertrophy with increased fetal gene activation, and increased MEF2 activity in response to calcineurin activation (107, 108). However, mice lacking both HDAC5 and HDAC9 develop a lethal post-natal cardiac defect with a thin walled myocardium and septal defects (108). Mice expressing an active CaMKIV in the heart also display a massive hypertrophy with increased MEF2 activity and fetal gene expression (81). Furthermore, mice ectopically expressing a constituently nuclear HDAC5 display a loss of cardiac mitochondria and down-regulation of oxidative genes, which was attributed to HDAC5-MEF2-dependent inhibition of PGC-1 expression (28). Interestingly, HDAC4-null mice display premature ossification of the skeleton during development, which is attributed to a physical interaction between HDAC4 and RunX2 that regulates chondrocyte hypertrophy and skeletogenesis (109). Lastly, HDAC7 was shown to regulate vascular integrity by repressing MEF2-dependent expression of matrix metalloproteinase 10 (MMP10) in endothelial cells, where HDAC7 knock-out mice exhibit vascular dilation and rupture (110).

Recently studies have focused on the different cellular signals that can differentially regulate class IIa HDACs. CaMKII has been shown to specifically phosphorylate HDAC4, and not other class IIa HDACs, by utilizing a unique docking site contained between amino acids 585 to 608 (111). Once docked, CaMKII phosphorylates HDAC4 at Ser467 and Ser 632, in contrast to phosphorylation at Ser246 and Ser 467 that is observed with CaMKI and CaMKIV (111). Furthermore, treatment of cardiomyocytes with endothelin-1 (ET-1), results in inositol triphosphate (IP3)-dependent activation of CaMKII and derepression of HDAC-dependent genes expression. This excitation-transcription coupling pathway was also shown to be physiologically insulated from the excitation-contraction coupling system, in that global calcium transients that cause contraction failed to export HDAC5 to the nucleus (112). Of the CaMKII isoforms expression in the heart, CaMKIIδ appears to play a unique role, in that mice with a genetic deletion of this isoform develop cardiac hypertrophy, with HDAC phosphorylation, in response to cardiac stress in a comparable manner to wild-type mice; however, the CaMKIIδ-null mice fail to transition from a hypertrophic response to cardiac failure (113). These results were attributed to improved calcium handling in the sarcoplasmic reticulum in these mice and indicate that CaMKIIδ serves to enhance ryanodine receptor-mediated calcium leak during the progression to heart failure (113, 114). Although interesting and informative, these studies uncover an apparent discrepancy, in that CaMKII specifically phosphorylates HDAC4, but stimulates the nuclear export of other class IIa HDACs under some cellular conditions. These

discrepancies have been rectified in a recent paper that demonstrated the HDAC4 can physically interact with other class IIa HDACs to confer CaMKII responsiveness (115). This report demonstrated that HDAC4, HDAC5, and MITR can form homo- and hetero-oligomers through a conserved coiled-coil domain upstream of the MEF2 binding domain. In addition, CaMKII docking and phosphorylation of HDAC4 can result in HDAC5 nuclear export (ie. Piggyback phosphorylation), or phosphorylation of HDAC5 with nuclear export, even when HDAC4 is mutated to be constituently nuclear (ie. transphosphorylation) (115). These results add an additional layer of complexity to HDAC regulation of MEF2-dependent genes expression, as class IIa HDACs have been now shown to dimerize with each other, as well as class I HDACs (99).

Another recently identified HDAC kinase is protein kinase D (PKD), which is a downstream effector of novel protein kinase C (PKC) isoforms (116). In this report, PKC inhibitors effectively blocked phenylephrine (PE) and ET-1 induced HDAC5 nuclear export and a constituently nuclear HDAC5 blocked PE induced expression of fetal cardiac genes (116). Furthermore, the PKD inhibitor, Gö-6976, blocked agonist-induced HDAC5 nuclear export; while co-immunoprecipitation experiments indicate that PKD, and not PKC, physically interacts with HDAC5 (116). Interestingly, novel PKC isoforms have also been show to phosphorylate MEF2 *in vitro* and in intact cells; however, the contribution of this mechanism compared to PKD-induced HDAC export in muscle biology remains to be studied (42) (unpublished). The discovery of PKD as an HDAC kinase is critical to cardiac biology, since experiments attempting to link HDAC5

regulation to CaMKI and CaMKIV signalling in cardiomyocytes have been equivocal (91). In unstimulated cells, PKD proteins are localized in the cytosol; however, PKD is imported into the nucleus in response to agonist treatment (91). In addition, all cardiac hypertrophic agonists known to induce HDAC5 nuclear export have also been shown to activate PKD through G protein-coupled receptors that are linked to Gαq (117). In VSMCs, PKD is also activated by angiotensin II and PDGF signalling, where angiotensin II-mediated VSMC hypertrophy is regulated by PKD-induced HDAC5 phosphorylation (118, 119). However, PKD is also capable of phosphorylating the other class IIa HDACs, and we have recently shown that ectopic expression of an active PKD can promote the nuclear export of HDAC4 in cultured VSMCs, and PKCδ inhibition can partially attenuate PDGF induction of c-Jun expression (91, 120). Furthermore, the use of siRNAs targeting PKD1 in cardiomyocytes blunts agonist-dependent HDAC5 export and suppresses cardiac hypertrophy; while *in vivo* evidence has demonstrated that PKD is activated during pathological hypertrophy induced by aortic banding and chronic treatment with norepinephrine (117). Lastly, the use of transgenic mice has clearly demonstrated that forced expression of PKD1 in the heart causes pathological hypertrophy, while mice with a conditional deletion of PKD1 in the heart are resistant to pathological hypertrophy induced by aortic banding (117, 121).

The salt-inducible kinase 1 (SIK1) was originally identified as a serine/threonine protein kinase whose expression was enhanced in the adrenal glands of rats fed a high-salt diet (122). In unstimulated adrenal cells, SIK1 is localized to both cytosolic and

nuclear compartments; however, when adrenal cells are stimulated with ACTH, SIK1 is exported from the nucleus (123). The nuclear shuttling of SIK1 is regulated by direct phosphorylation by PKA at Ser577; where mutation of this serine to alanine results in a nuclear distribution and constitutively active SIK1 activity (123). Furthermore, SIK1 was found to be a HDAC kinase capable of phoshorylating HDAC4 and HDAC5 at Ser246/ Ser467 and Ser259/Ser498, respectively, and promote nuclear export of these HDACs (124). In skeletal muscle, PKA acutely phosphorylates SIK1 at Ser577 to inhibit its catalytic activity and reduce the amount of phosphorylated HDAC5. Congruently, forced expression of SIK1 *in vivo* was able to increase the amount of phosphorylated HDAC5 and reduce the number of necrotic foci in a model of muscular dystrophy (124). Recent evidence from our laboratory suggests that SIK1 is an important regulator of HDAC4-mediated *c-jun* repression in VSMCs (120). In these experiments, forced expression of SIK1 promoted nuclear export of HDAC4 and induced *c-jun* expression in cultured VSMCs. However, co-expression of PKA could overcome SIK1 and return HDAC4 to the nucleus resulting in repression of *c-jun* expression, but not when Ser577 of SIK1 was mutated to alanine. Thus, SIK1 is an important HDAC kinase that regulates MEF2-dependent *c-jun* expression in VSMCs (120).

Recently, PP1α has been shown to inhibit MEF2 transcriptional activity, in part, by recruiting HDAC4 to MEF2A (61). PP1s were originally believed to dephosphorylate class IIa HDACs to promote their nuclear import (99). However, the cellular mechanism responsible for PP1-directed MEF2 repression may involve multiple aspects, as PP1α can

physically interact with MEF2 and can promote nuclear retention of HDAC4 in both a phosphatase-dependent and phosphatase-independent manner (61).

In the past few years, it has become clear that MEF2 proteins are not the only transcriptional targets of class IIa HDACs in the heart, or other tissues (91). HDAC5, but not HDAC4, has been shown to interact with myocardin to repress SRF-dependent gene expression (89). In addition, class IIa HDACs can repress NFAT- and Nkx2.5-dependent genes through bridging cofactors, Mrj and CAMTA2, respectively (Figure 2) (125, 126). Thus, many of the cellular effects attributed to the MEF2-HDAC interaction could in fact be due to class IIa regulation of other transcription factors. A detailed examination of specific class IIa HDAC targeted-genes and their regulatory promoter regions seems appropriate to elucidate the specific mechanisms and transcription factors required in these complex patterns of gene expression.

SP1

The induction of monocyte differentiation by vitamin D3 is also marked by an increased expression of MEF2D, concurrent with a marker of monocyte lineage, CD14 (127). The induced expression of CD14 is achieved by physical interaction of the MADS/ MEF2 domain of MEF2D with Sp1. Interestingly, mutation of the Sp1 binding site alone within the CD14 promoter was sufficient to block the synergy between MEF2D and Sp1, indicating that Sp1 is capable of recruiting MEF2D to this promoter region (127). In cultured muscle cells, mutation of the Sp1 binding site in the myoglobin promoter was

just as effective as mutation of the MEF2 *cis* element at attenuating the calcineurin-induction of this promoter, which further supports the notion of functional synergy between MEF2 factors and Sp1 (79). Furthermore, this research group demonstrated that forced expression of MEF2A and Sp1 synergistically activate the MCK promoter, and that Sp1 could be immunologically detected in a complex with MEF2A in nuclear extracts from muscle tissue, as determined by EMSA (128). Interestingly, the *c-jun* promoter contains both MEF2 and Sp1 binding sites (29, 129). Although not studied directly, circumstantial evidence suggests these elements functionally collaborate to induce *c-jun* expression, as mutation of the MEF2 site attenuates the *c-jun* induction by

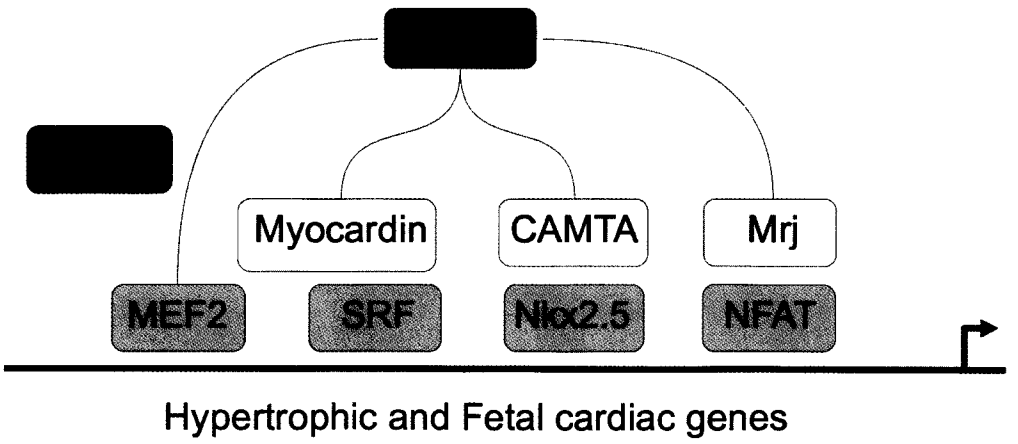

Figure 2. MEF2 and non-MEF2 targets of HDAC5.

In addition to MEF2, HDAC5 has been shown to indirectly repress the transcriptional activity of SRF, Nkx2.5, and NFAT.

EGF, while a shorter reporter-gene that does not contain the Sp-1 is also less responsive to EGF (130). Furthermore, mutation of the MEF2 *cis* element in the shorter reporter-gene results in greater attenuation and nearly a complete loss of basal activity. Lastly, in VSMCs KLF-4 expression is regulated by PDGF induction of Sp1 (131). Interestingly, recent ChIP-on-chip analysis performed to identify novel MEF2-target genes in neurons identified KLF4 as a potential target (132). However, at this time no reports have evaluated the role of the MEF2 and Sp1 synergy in VSMCs gene expression.

SMAD2

The transforming growth factor beta (TGF-β) superfamily plays a critical role in regulating muscle cell differentiation, where cardiac muscle differentiation is induced by the bone morphogenetic proteins (BMPs) and VSMC differentiation is induced by TGF-β (133, 134). This specificity is achieved, in part, by the receptor-Smads activated by these distinct ligands. In cardiac tissue, BMP signalling activates Smad1, which coactivates cardiac gene expression with Tinman (Nkx2.5) and myocardin (135, 136). Whereas in VSMCs, TGF-β signalling activates Smad3 to induce smooth muscle marker gene expression by co-activation of SRF and myocardin (137-139). However, TGF-β signalling also activates Smad2, yet the role of this Smad in target gene expression is less clearly defined. Smad2 differs from Smad3, in that Smad2 lacks the ability to bind DNA efficiently (135). Although, Smad2 may be recruited to specific promoters through the interaction with specific cofactors. A previous report has identified MEF2A and MEF2C

as Smad2 interacting cofactors (140). In these studies Smad2 was found to physically interact with MEF2 proteins by co-immunoprecipitation and *in vitro* GST-pulldown assays. In addition, forced expression of Smad2 and Smad4 increased the activity of MEF2C through the C-terminal transcriptional activation domain, and activated MEF2A to a lesser extent (140). Finally, mutation of Thr293 and Thr300, or Ser387 of MEF2C, previously shown to be targeted by p38 and ERK5 signalling, rendered MEF2C unresponsive to Smad2/4 (41, 47, 140). The physical interaction between MEF2C and Smad2 has not been characterized in other cell types; however, evidence in VSMCs has demonstrated that RhoA/ROCK signalling is required for TGF-β activation of Smad2 and Smad3, and RhoA signalling can enhance MEF2 transcriptional activity through p38 MAP kinase (141, 142). Although these studies are suggestive that MEF2 and Smad2 are functionally collaborating in this cellular context, it has yet to be studied directly. In addition, MEF2 transcriptional activity was only evaluated with artificial reporter-gene constructs is these papers. Further investigation is required to identify specific MEF2 target genes potentially regulated by a RhoA/p38/Smad2 pathway.

MYOCARDIN AND MASTR

Myocardin was originally identified as a SAP-domain SRF cofactor capable of activating cardiac and smooth muscle genes (143). Recently, it was discovered that heart cells express a cardiac-specific isoform of myocardin that physically interacts with both SRF and MEF2 proteins (144). The cardiac isoform of myocardin (myocardin-935)

contains an N-terminal extension that interacts with the MADS/MEF2 domain of MEF2C that is not present in the shorter smooth muscle-specific isoform (myocardin-856) (144). In addition, the expression of myocardin is regulated by a distant upstream enhancer that contains a MEF2 *cis* element (145). Thus, a positive-feedback loop exists in cardiac cells between MEF2C and myocardin that may serve to promote terminal differentiation.

Using the MEF2 interacting domain of the N-terminal extension of myocardin-935, the same research group identified a previously unidentified MEF2 interacting cofactor that is enriched in skeletal muscle (144). This cofactor was named MEF2-activating SAP transcriptional regulator (MASTR). MASTR powerfully co-activates the transcriptional activity of MEF2C and enhances the conversion of non-muscle cells to skeletal muscle when co-expressed with MyoD (144). Interestingly, the *Xenopus* MASTR physically interacts with SRF, and co-activates SRF-target genes (146). In addition, the single *Drosophila* myocardin-related transcription factor only interacts with SRF (147). This indicates that the MEF2-interacting myocardin family members represents a unique evolutionary step in mammals.

THE ROLE OF MEF2 IN SKELETAL MYOGENESIS AND MUSCLE FIBRE-TYPE HETEROGENEITY

MYOGENESIS

Much of the molecular knowledge regarding skeletal muscle development arose from the discovery of the MyoD family of bHLH transcription factors, also known as

MRFs (myogenic/muscle regulatory factors). As noted above, when ectopically expressed in non-muscle cell types, these factors can activate skeletal muscle gene expression (27). During mouse embryogenesis, the MRFs show overlapping, but distinct expression patterns. Myf5 is the first family member to be expressed in the developing somite at E8.0. This is followed by myogenin at E8.5 in the myotome region of the somite, followed by MRF4 at E9.5 and MyoD at day E10.5 (27). MRFs activate skeletal muscle gene expression by binding to a consensus site, called an E-box (CANNTG), but must first heterodimerize with ubiquitous bHLH factors called E-proteins. E-boxes are present in the regulatory regions of most skeletal muscle genes, and are often adjacent to one or more MEF2 *cis* elements. Early observations regarding the MRFs indicated that they could activate transcription of muscle genes that lack E-boxes in their control regions (63). This phenomenon occurs through the formation of a trimeric complex composed of a MRF, MEF2 factors, and a ubiquitous E-protein, that can be recruited to promoter regions that contain an E-box, MEF2 site, or both elements (63). MEF2 appears to be an essential component of this complex as forced expression of a dominant-negative MEF2A successfully blocks myotube formation in cultured C2C12 cells and prevents MyoD-induced conversion of non-muscle cells (148). Similarly, forced expression of HDAC4 or HDAC5 can completely block MyoD-induced conversion through a physical interaction with MEF2 proteins and not MyoD (36).

MEF2 expression in the myotome follows a similar pattern to that of the MRFs. MEF2C appears to be the first member of the family to be expressed in the myotome at

E8.5. This is followed by MEF2B at E9.0, and MEF2A and -D at E9.5 (62). The observation that Myf5 expression occurs at least 1 day before MEF2C has suggested that Myf5 marks the commitment to the skeletal muscle lineage, and MEF2 proteins serve to reinforce differentiation. Indeed, MEF2 proteins and the MRFs form a mutually reinforcing network during myogenesis. For example, the regulatory region of *mef2c* contains a skeletal muscle-specific enhancer that is directly activated by MRFs and MEF2 factors (149, 150). Furthermore, the myogenin promoter contains a consensus MEF2 *cis* element required for proper expression *in vivo* (27). Thus, MEF2 proteins participate with MRFs in a autoregulatory loop to amplify terminal differentiation of skeletal muscle.

Consistent with this role in myogenesis, recent evidence has demonstrated that p38 signalling to MEF2 is critical to myotome development (151). In these studies, treatment of embryos with the p38 inhibitor SB203580 halted somite development and inhibited MEF2 activity. Furthermore, p38 inhibition attenuated myogenin expression, but had no effect on the early commitment marker Myf5. Moreover, p38 signalling to MEF2D has been shown to recruit an Ash-2L-containing methyltransferase complex to the myogenin promoter region to epigenetically mark myogenin for expression during differentiation (43). However, muscle-specific expression of a super-active MEF2C-VP16 fusion protein in mice did not result in premature skeletal muscle differentiation, yet mice habouring a muscle-specific deletion of MEF2C develop a post-natal defect in sarcomere integrity due to insufficient structural protein expression (10, 152).

Interestingly, forced activation of p38 signalling in rhabdomyosarcoma cells is sufficient to halt cell cycle progression and induce terminal differentiation through transcriptional activation of MEF2 proteins and restoration of the myogenic activity of MyoD (153). Collectively, these results indicate that MEF2 proteins are not sufficient to initiate skeletal myogenesis on their own, nor are they able to force a commitment to the myogenic lineage; however, MEF2 proteins are necessary for proper differentiation of myotubes and sarcomeric assembly.

Recently, MEF2 proteins were found to regulate a number of microRNAs (miRNAs) during myogenesis. In particular, miR-1 can target HDAC4 resulting in inhibited expression of this class IIa HDAC (154). This results in down-regulation of HDAC4 during the transition from myoblasts to myotubes, and serves to enhance MEF2-induced myogenin expression (154). However, MEF2C was also found to induce the expression of HDAC9 during skeletal muscle differentiation, thereby creating a negative-feedback loop to modulate MEF2 transcriptional activity after the onset of terminal differentiation (155).

MUSCLE FIBRE-TYPE REMODELLING AND REGENERATION

Mammalian skeletal muscle is composed of heterogeneous specialized myofibres that enable an organism to perform a wide variety of contractile tasks ranging from postural support to ballistic movements. These myofibres are classified based on their expression of the myosin heavy chain genes (MHC I or II) (156, 157). Type I fibres, also

termed slow-twitch or red fibres, are rich in mitochondria and myoglobin, generate force more slowly and are more fatigue resistant; whereas, type II fibres are also called fast-twitch or white fibres, and utilize more glycolytic metabolism, generate force quickly, but fatigue rapidly (157, 158). In addition, type I maintain relatively high levels of intracellular calcium (100-300 nM); whereas type II fibres are characterized by high amplitude calcium transients and lower baseline calcium concentrations (less than 50 nM) (156). These differences in intracellular calcium concentration are believed to be dependent on the pattern of motor neuron stimulation received by the myofibres, where low frequency (10 Hz) stimulation promotes type I formation and high frequency (100 Hz) results in type II fibres (156). Mammalian muscle adapts to increased mechanical energy needs by converting some fast-glycolytic fibres into more slow-oxidative fibres (158). Recent identification of the cellular signalling pathways that regulate fibre-type conversion have focused on MEF2 transcriptional activity and target-gene induction (156).

When ectopically expressed in mice, calcineurin converts fast glycolytic fibres into red oxidative fibres (79). This is achieved by combined activation of NFATs and MEF2 proteins to induce expression of type I-related genes, such as myoglobin, troponin I slow, and PCG-1 (67, 79). Furthermore, exercise-induced fibre-type conversion in mice results in MEF2 transcriptional activation, which is blocked by the calcineurin inhibitor, cyclosporin A, or by co-expression of MCIP-1 (60). Physiologically, calcineurin is

activated by sustained low amplitude intracellular calcium, which further supports its role in fiber-type conversion (159).

Enhanced oxidative capacity following mechanical use is a key characteristic of fibre-type conversion, and is due in part to the dramatic increase in mitochondrial content observed with increased motor activity (158). This mitochondrial biogenesis is regulated largely through the transcriptional co-activator PGC-1. Forced expression of PGC-1 in mouse muscle resulted in increased type I fibres in normally white muscle groups (160). Furthermore, PGC-1 activates type I-specific gene expression synergistically with calcineurin and MEF2D. PGC-1 has also been shown to be a MEF2-target gene, and contains consensus MEF2 *cis* elements within its regulatory region. This lends support to the notion that PGC-1 is autoregulated in muscle through a MEF2-dependent mechanism (67). Interestingly, both MEF2 and PGC-1 are activated by p38 signalling in muscle tissue; however, it is currently unknown whether this signalling pathway contributes to type I fibre conversion (39, 69).

Class IIa HDAC regulation of MEF2 is also important in fibre-type specification. Stimulation of cultured myofibres in slow fibre-type pattern results in nuclear export of HDAC4, which is attenuated by the CaMK inhibitor, KN-62 (102). This inhibitor effectively blocks the activity of CaMKII and IV, and evidence suggests that CaMKIV is not endogenously expressed in skeletal muscle, even though it promotes type I conversion when ectopically expressed. Therefore, these results are interpreted as slow fiber-type activity leads to calcium-mediated activation of CaMKII, which

phosphorlyates and promotes nuclear export of HDAC4 (156). Recently, it was discovered that predominantly red muscle groups, such as the soleus muscle, express very little HDAC4, -5, or -7, compared to white muscle groups (152). Further, forced expression of calcineurin or CaMKIV in mice promotes type I conversion and simultaneously down-regulates class IIa HDAC expression. Through a series of gene-targeting experiments, this research group demonstrated that deletion of any two class IIa HDACs in skeletal muscle increases the percentage of type I muscle fibres. This result was phenocopied by forced expression of a highly active MEF2-VP16 fusion protein, while conditional deletion of MEF2C or MEF2D reduced the number of type I fibres (152).

Given that CaMKIV is not endogenously expressed in skeletal muscle, and HDAC4 expression is decreased during differentiation, the mechanisms responsible for regulating the HDAC-MEF2 interaction in muscle fibres has remained elusive (152, 154). The recent identification of PKD as an HDAC kinase has lead to the finding that PKD enhances MEF2-dependent type I fibre formation (161). PKD was found to be predominantly expressed in type I fibres, and when forcibly expressed in type II fibres, it promotes fibre-type conversion. In addition, mice overexpressing PKD have enhanced MEF2 activity, and are more resistant to fatigue; while, genetic deletion of PKD in skeletal muscle increases the susceptibility to fatigue. Lastly, this group demonstrated that PKD synergistically enhances myoglobin expression in culture skeletal muscle cells, when expressed with an activated calcineurin (161). Collectively, these results indicate

that MEF2 plays a critical role in type I fibre formation when activated by CaMKII, calcineurin, and PKD; however, in the absence of an activating stimulus, class IIa HDACs repress MEF2 activity and promote type II fibre formation.

MEF2 has also been associated with increased gene expression during muscle regeneration (162). Injection of the myotoxic agent, $BaCl_2$ into the gastrocnemeus of mice resulted in enhanced MEF2 DNA binding and transcriptional activity, concurrent with increased expression of cofilin and vimentin. Furthermore, *in silco* analysis of the vimentin promoter region revealed a consensus MEF2 *cis* element. In addition, the $BaCl_2$-induced activation of MEF2 transcriptional activity can be attenuated by injection of the phosphodiesterase inhibitor, IBMX, which activates the MEF2 repressor PKA (25). In addition, viral-delivery of the HDAC kinase SIK1 can rescue the dystrophic phenotype in mice expressing a dominant-negative CREB by activating MEF2 activity (124).

Collectively, these data implicate MEF2-mediated gene expression as a critical aspect of muscle regeneration, in addition to differentiation, and fibre-type specification (figure 3).

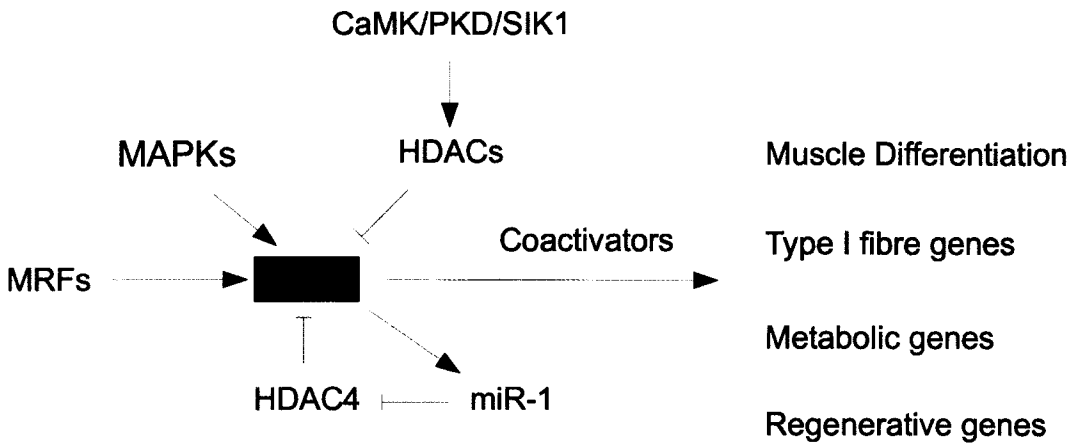

Figure 3. The role of MEF2 in skeletal muscle.

MEF2 proteins form a re-enforcing transcriptional network with the MRFs to promote terminal differentiation of skeletal muscle. In addition, MEF2 proteins are positively regulated MAP kinases, such as p38 and ERK5, and coactivators, such as MASTR. Negative regulation is provided by class IIa HDACs. Furthermore, MEF2 induces the expression of miR-1, which down-regulates HDAC4 to relieve repression. Post-differentiation MEF2 induces the expression of HDAC9 to provide negative feedback control.

INTRODUCTION TO CARDIOVASCULAR DEVELOPMENT

CARDIAC DEVELOPMENT

During development the heart is the first organ to become fully functional. Heart progenitor cells in late gastrulation begin to migrate medially from the lateral plate mesoderm (134). Bilaterally, these cells take on a arched structure referred as the cardiac crescent, which is visible by E7.5 in the mouse and day 15 in humans (134, 163). Migration of these progenitors towards the midline is believed to occur in response to fibroblast growth factor 8 (Fgf8), while specification of these cardiac progenitors occurs in response to bone morphogenetic proteins (BMPs) secreted from the underlying endoderm (134, 164). BMPs induce the expression of the earliest cardiac marker genes, the homeobox Nkx2.5 (ie. tinman in *Drosophila*), and the T-box protein Tbx5. Conversely, secretion of Wnt3A and Wnt8 (Wingless in *Drosophila*) opposes cardiogenesis, and must be inhibited by the endogenous Wnt antagonists Crescent and Dickhopf-1. Less than a day later in mouse embryogenesis (E8.5 or day 21 in humans), the cardiac crescents have merged to form a beating primitive heart tube, which resembles the *Drosophila* dorsal vessel (134, 165). The primitive heart tube has its inflow region located caudally (ie. inferior) and its outflow region located cranially (ie. superior). These regions will become the atria and outflow tracts, respectively (134). The linear heart tube is composed of distinct myocardial and endocardial layers separated by an extracellular matrix, where regions of the primitive myocardium are predetermined to

become left and right atria, left and right ventricles, and outflow arteries (163). The formation of the heart tube is believed to be genetically programmed, as Nkx2.5 increases the expression of GATA transcription factors (ie. GATA4 and -5), which have been shown to play an essential role in this process (163, 166). Between days E9.5 and E10.5 in the mouse (day 28 in humans), the linear heart tube undergoes rightward looping, which is essential for proper positioning of the atria, ventricles, and alignment with the pulmonary and systemic outflow tracts (134, 163). During looping, the four chambers of the heart begin to bulge outward and start to take shape (164). The direction of looping is determined by an asymmetrical signalling gradient composed of Sonic hedgehog (shh) and Nodal in the lateral mesoderm (72, 163). The genetic programming that determines cardiac looping is largely unknown; however, MEF2C and the Hand factors appear to play a critical role in this process, in that targeted inhibition of these genes results in a looping defects, following heart tube formation (7, 167).

As the linear heart tube is looping, around E9.5, a distinct transient structure develops in the area of the sinoatrial junction. This structure is called the proepicardium and it gives rise to the epicardium, coronary vasculature, and the cardiac conduction system (134, 168). Epicardial cells migrate as a continuous sheet on the myocardium to cover the heart's surface (168). The coronary vasculature forms through an epithelial-to-mesenchymal transformation of proepicardial cells, followed by vasculogenesis of endothelial cells and recruitment of VSMCs, which also arise mostly from the proepicardium (see below) (168). This process is stimulated by myocardial secretion of

growth factors that promote vasculogenesis, such as VEGF, FGFs, and angiopoietin-1. Finally, during vascular remodelling, the developing coronary arteries 'invade' the base of the aorta to connect the coronary circulation with the developing systemic circulation (168). As the coronary arteries develop, they secrete endothelin-1 which acts on a subset of ventricular cardiomyocytes that surround the coronaries (163). These myocytes differentiate into tracts of cells that form the purkinje fibres of the cardiac conduction system (169).

Development of the ventricles during and following cardiac looping is regulated by the bHLH transcription factors of the Hand (heart and neural crest derivatives) family (72, 163). Hand1 (eHand) has been implicated in left ventricle development and a down-stream target of Nkx2.5 (72, 163, 165, 170, 171). Conversely, Hand2 (dHand) mutant mice display a hypoplasic and under-developed right ventricle (172). Considerable heart growth occurs during and following looping, which is achieved by a combination of proliferation and differentiation; in contrast to post-natal cardiomyocytes which are terminally differentiated. The most rapidly dividing cardiomyocytes are located along the outer surface of the looping heart and the epicardium appears to serve as a source of mitogens. Retinoic acid (RA) produced from the epicardium is a critical regulator of cardiac growth, as well as atrial specification (163, 164). Growth signals also emanate from the endocardium, such as Neuregulin growth factors, VEGF, and angiopoietin, which are required for trabeculae formation (163, 164). However, the intracellular targets of these growth factors remain ill-defined. Recently, GSK-3 has been implicated in

regulating cardiomyocyte proliferation through β-catenin and Myc, where GSK-3β-null mice display hyperproliferative cardiomyocytes (173). Interestingly, p38 MAPK has also been implicated as a control point for cardiomyocyte proliferation, in that inhibition of p38 allows for proliferation of adult cardiomyoctyes in culture (174). In addition, targeted deletion of SRF in the developing heart (ie. E9.5) leads to decreased expression of *c-fos*, an AP1 factor involved in cell cycle progression (175). In contrast to the regulation of proliferation, a large number of transcription factors have been implicated in differentiation-induced cardiomyocyte growth (ie. hypertrophy) during development. Both MEF2A- and MEF2C-null mice display thin dilated ventricles with reduced expression of cardiac muscle genes before their demise (7, 11). Furthermore, both SRF and the MEF2/SRF-interacting coactivator myocardin have been shown to be essential for cardiac gene expression (175-177). In addition, GATA4 has been implicated as a critical MEF2 and myocardin cofactor that induces cardiac contractile gene expression (70, 90).

By E10.5 in the mouse embryo endocardial cushions (ie. cardiac cushions) begin to form as regional swellings in both the primitive inflow (common atrium and atrioventricluar canal) and outflow (conotruncus and aortic sac) tracts. Under the influence of TGF-β these endocardial cushions differentiate into the fibrous valves that dictate the flow of blood through the heart. The cardiac cushions are also involved in septation of both the atrioventicular canal and the conotruncus as the outflow tract 'wedges' on top of the inflow tract and meets the developing ventricular septum, which is

growing from below (134, 163, 178). The processes of wedging and septation result in the formation of distinct left and right chambers of the heart, as well as the aortic and pulmonary arteries and is completed by E12.5 (134). Improper wedging of the outflow and inflow tracts is often the cause of ventricular septal defects (VSDs) as the wedging tracts fail to contact the ventricular septum growing cranially from the cardiac apex (178).

Connecting the heart to the developing vasculature is largely achieved by migrating cells from the neural crest that originate somewhere between rhombomeres 3-8 (163, 178). These cells are critical for the process of septation of the aorta and pulmonary arteries, and form the VSMCs of aortic/brachial arch arteries that ultimately remodel to form the ductus arteriosus, and the subclavian and carotid arteries (see below) (163).

SECONDARY HEART FIELD

Since the mid-1970s it has been observed that the arterial pole of the heart (ie. outflow tract) was extended during cardiac looping by the addition of extracardiac cells that appear to originate from a source anterior to the heart (179). However, it was not until 2001 that three independent research groups identified the embryonic source of these progenitors (180-182). These studies showed that the myocardial wall of the proximal outflow tract is formed from progenitors that originate from the pharyngeal mesoderm and migrate to the heart following heart tube formation. This second wave of cardiac progenitors has been termed the second heart field (SHF; secondary or anterior

heart field) to distinguish these cells from the first (ie. primary) heart field that is derived from the lateral plate mesoderm and form the cardiac crescent (183). Recent studies using an enhancer trap upstream of the fgf10 gene, which drives lacZ expression in the pharyngeal mesoderm has demonstrated that the proximal outflow tract and most, if not all, of the right ventricle is derived from the SHF in mice (182). These cells are added progressively during cardiac looping and serve to elongate the heart tube and add mass to the developing right ventricle.

The LIM-homeodomain protein Islet-1 (Isl-1) is required in SHF progenitors and for proper formation of the SHF derivatives (183). Isl-1 was named for its role in insulin expression from the β-cells of the Islet's of Langerhans; however, Isl-1 null mice have profound cardiovascular defects. The hearts of Isl-1 mutants fail to undergo looping and do not develop a right ventricle or outflow tract. Interestingly, Isl-1 is not expressed in the right ventricle or outflow tract post-natally, but is expressed and required by the SHF progenitors in the pharyngeal mesoderm (183). A critical target of Isl-1 in the SHF is MEF2C, where Isl-1 and GATA factors activate at SHF-specific enhancer in the *mef2c* regulatory region (184). In addition, this SHF-specific enhancer directs Cre-induced lacZ expression in the right ventricle and outflow tracts in a similar pattern as the fgf10 enhancer trap (185). Furthermore, the *mef2c*-null mice have defects suggestive of an essential role in SHF development (see below) (7). Thus, the genetic program that dictates the formation of the SHF appears to involve an initial Isl-1 activation, followed

by GATA factors that ultimately induce the expression of MEF2C (ie. Isl-1-GATA-MEF2C), and other down-stream transcription factors, such as Hand2 (see below) (183).

Congenital heart defects (CHD) in humans are the most common birth defect, affecting 1% of births and are the leading cause of infant mortality in developed countries; however, another 1-2% of people habour a more subtle anomaly that may become apparent with age (183, 186). Furthermore, severe cardiac malformation are estimated to be the cause of 10% of early miscarriages (187). Despite this major health concern, very little is known regarding the cause of these defects. The most common CHD is the ventricular septal defect (VSD), accounting for 30-60% of all CHD, followed by the patent ductus arteriosus (PDA) which accounts for 10% (163). As mentioned above, the VSD is believed to occur from a failure of cardiac 'wedging' (178). Of the known causes of CHD, trisomy 21 (ie. Down's syndrome) is the most common, followed by DiGeorge's syndrome, which is caused by a deletion mutation of chromosome 22q11; however, at this time the exact genes involved in these syndromes that result in human CHD remain poorly defined (187). In addition, many other CHD are associated with embryonic exposure to teratogens (Heart and Stroke foundation of Canada). Interestingly, about 30% of human CHD are caused by perturbation of the neural crest cells that migrate to meet the SHF and form the cardiac outflow tracts (163). Thus, identification of the molecular genetic programming that regulates cardiac and outflow tract development will be essential to understanding human CHD.

VASCULAR DEVELOPMENT

Vascular development occurs through two interconnected processes; vasculogenesis and angiogenesis. Vasculogenesis is the process of creating new vessels from undifferentiated mesodermal progenitors (ie. *de novo* formation); whereas, in angiogenesis the source of new cells is the endothelium of existing vessels (188). The process of angiogenesis also provides a post-natal mechanism for new vessel growth and remodelling, particularly in response to hypoxia (189). Vasculogenesis has been characterized into four convenient stages (188). 1) The differentiation of endothelial cells from mesodermal progenitors called angioblasts. This stage is largely driven by VEGF and its receptor Flk-1 (VEGFR-2). 2) The formation of lumenless primordial vessels from endothelial cells, a process mediated by platelet/endothelial adhesion molecule (PECAM) and CD34. 3) The formation of endothelial tubes by VEGF and the Flt-1 receptor (VEGFR-1). 4) The formation of primary vascular networks, mediated by the integrin family of receptors. Using the dorsal aorta as a model, vasculogenesis occurs in a cranial-to-caudal gradient (188). Next, these primary networks remodel by angiogenesis, undergoing branching, sprouting, migration, and proliferation to form a more mature network (189). Finally, mature networks recruit mural cells, such as pericytes and smooth muscle cells, through secreted factors like PDGF, angiopoietins, and TGF-β (190).

Gene targeting studies have revealed that targeted disruption of the VEGF loci results in embryonic lethality around E8.5 and mice exhibit defects, including a complete absence of the dorsal aorta; whereas, Flk-1 null animals display a similar lethality with

defects in angiogenic and hematopoietic precursors (188, 189). Furthermore, disruption of the angiopoietin receptor Tie2 leads to embryonic lethality between E9.5-E10.5, where endothelial tubes lack associated mural cells (188, 190).

The embryonic source of endothelial progenitors appears to be mesodermal tissue residing within diverse anatomical regions of the embryo (191). In mice, the first endothelial progenitors appear within blood islands in the extraembryonic mesoderm of the yolk sac, followed by their appearance in the rostral region of the embryo proper around E8 (191). Based on the close association of endothelial progenitors with the hematopoietic lineage, and the phenotype and expression pattern of Flk-1, it has been suggested that these lineages share a common progenitor called the 'hemangioblast' (191). This hypothesis was further supported by the discovery of the bHLH transcription factor TAL1/SCL, and the T-box transcription factor Brachyury, which are expressed in both early hematopoietic and angioblast lineages (188, 192). However, recent evidence from several independent laboratories using lineage tracing techniques, suggests that ES cells isolated from embryos at various time-points can be progenitors for hematopoietic, endothelial, cardiomyoctye, or smooth muscle lineages (192). For example, Kattman et al. isolated ES cells from E7.5 embryos that could give rise to hemangioblasts, cardiomyocytes, or VSMCs in culture (193). Furthermore, Moretti et. al. isolated ES cells at E8.5 that were Isl1-, Nkx2.5-, and Flk1-positive. This finding suggests that both cardiac heart fields (ie. first heart field and second heart field), and the endothelial lineages share a common mesodermal progenitor (194). Interestingly,

MEF2C has been demonstrated to play a critical role in all of these lineages following E9.5 (3, 54).

Although the initial stages of vascular development appear to be genetically determined, environmental factors, such as hemodynamics and hypoxia, appear to modulate vasculogenesis (191). Hypoxia triggers the stabilization of hypoxia inducible factor 1α (HIF-1α), which dimerizes with the constitutively expressed HIF-1β, forming an active transcription factor complex (191). Activation of the HIF-1 complex induces the expression of an array of genes, including the VEGFs, Flk-1, Flt-1, and Tie2, to promote vascular remodelling towards the hypoxic region (189, 191). The onset of circulation often occurs during development while the vascular network it still forming. This creates hemodynamic forces that are believed to influence vascular development (191). For instance, shear forces created by erythrocytes has been shown to induce endothelial nitric oxide synthase (eNOS), which can serve as a modulator of vascular remodelling during development (195).

As noted above, VSMCs are recruited from diverse embryonic origins to developing vascular networks, largely in response to secreted PDGF, TGF-β1, and angiopoietins. In addition, circumstantial evidence has been suggestive of a 'blood flow' hypothesis, where recruitment of VSMCs to primitive vessels is concurrent with onset of blood flow and increasing pulse pressure (188). Primitive VSMCs play an important synthetic role during development, as they are the major source of extracellular matrix components of the vessel wall, including elastin and collagen fibres (196). Thus, this

proliferative phenotype of VSMCs has been classically referred to as the 'synthetic' phenotype. The earliest known marker of VSMC differentiation is smooth muscle α-actin, and its expression is detectable as smooth muscle precursors are recruited into the vessel wall. This is followed by the sequential induction of other smooth muscle-marker genes such as SM22, calponin, and type I SM-MHC. Finally, the latest marker of mature VSMCs is type II SM-MHC (196). The regulation of this differentiation program is complex, but appears to be centred around the transcription factor SRF (see below). Developmental increases in contractile protein expression appears to be inversely correlated to the synthetic functions of VSMC; thus, the two classically defined smooth muscle phenotypes have been called 'contractile' and 'synthetic'. However, these phenotypes are not believed to be mutually exclusive, instead VSMCs appear to reside on a continuum of phenotypes ranging from committed, but undifferentiated 'synthetic' cells to a fully mature 'contractile' phenotype dedicated to blood flow and vascular resistance regulation (196). Interestingly, even fully differentiated VSMCs in mature vessels continue to express matrix components and proliferate, albeit at extremely reduced levels; however, in response to vascular injury, the mature VSMC retain the ability to increase its synthetic functions to promote healing. This ability to modulate the VSMC phenotype appears to be a critical evolutionary step to maintain the integrity of the vascular system; however, it also appears to create a susceptibility to vascular disease later in life when the selective pressures imposed by reproductive success are lower (see below) (133).

The origins of VSMC progenitors during development is remarkably diverse. Fate mapping techniques have recently identified that vascular smooth muscle is a mosaic tissue produced from at least seven developmental origins, where the boundaries between VSMCs of different origins are often distinct even within the same vessel (197). Interestingly, VSMCs of different developmental origins respond in a lineage-specific manner to pathological stimuli, and may provide an explanation to why certain anatomical regions are more or less susceptible to vascular lesions (see below).

As noted above, the descending aorta has served as a convenient model for investigations into vessel wall assembly and morphogenesis (188). Interestingly, the descending aorta develops as two bilateral nascent aortae that fuse in a cranial-to-caudal manner to yield a single tube (188). Using this model, VSMC progenitors can be observed at the site of fusion on the ventral surface, where they migrate around the circumference of the vessel to create an outer smooth muscle layer. Given the success of this model, and evidence from chick-quail chimeras, VSMCs were assumed to be derived from the splanchnic layer of the ventrolateral plate mesoderm (188, 196). However, it is now believed that splanchnic mesoderm-derived progenitors give rise to VSMCs in a restricted region encompassing the abdominal aorta and the iliac arteries (197).

Using chick-quail chimeras and neural crest ablation, several groups identified two fates for migrating neural crest cells in cardiovascular development (178, 197). First, migrating neural crest cells contribute to pharyngeal arch development including differentiating into VSMCs of the aortic arch arteries. Second, a subset of migrating cells

mediate septation of the cardiac outflow tract to produce the aorta and pulmonary trunk (178, 197). Interestingly, lacZ tracing experiments have confirmed the neural crest origin of VSMCs of the aortic arch arteries and there derivatives, yet endothelial cells and adventitia were not labelled and appear to be derived from a source distinct from the neural crest. The aortic arch arteries are transient vascular structures during development and initially appear as six bilaterally paired vessels emerging from the cardiac outflow tract and common carotid arteries (178). Arches 1, 2, and 5 exist only briefly before regressing; however, arches 3, 4, and 6 undergo extensive remodelling to become the major embryonic outflow arteries. Arch 3 will become the internal carotid arteries branching from the common and external carotids. The right fourth arch remodels to become the right subclavian artery, while the left fourth arch becomes the aortic arch. Finally, the right sixth branch regresses and the left sixth arch becomes the pulmonary arteries and the ductus arteriosus (178). Endothelin-1 plays a critical role in recruitment and differentiation of neural crest-derived VSMCs by regulating the expression of Hand factors, and possibly the activity of MEF2C (9, 163). In addition, TGF-β induced calcineurin signalling has been shown to induce smooth muscle differentiation from neural crest-derived stems cells (198). The myocardin family of transcription factors has also been demonstrated to play a role in aortic arch artery development, including the induction of smooth muscle differentiation genes (see below) (199-201). Finally, conditional inactivation of GATA6 in the neural crest results in patterning defects in the

aortic arch arteries and their derivatives, which suggest this GATA factor plays a critical role in the delicate morphogenesis of VSMCs originating from the neural crest (202).

Although neural crest-derived VSMCs populate the aortic arch, they do not extend into the base of the aorta. The origin of these VSMCs appears to be the SHF (197). The SHF consists of two waves of cells that migrate to the arterial pole of the linear heart tube from the pharyngeal mesoderm (see above) (179, 197). The first wave differentiates into cardiac myocytes of the right ventricle and proximal outflow tract, and the second wave differentiates into VSMCs of the more distal outflow tract. Interestingly, this creates two 'seams' in the cardiac outflow tract at the junction of the SHF-derived cardiomyocytes and VSMC, and the SHF-derived smooth muscles cells and the neural crest-derived smooth muscle cells. Both of these 'seams' appear to be a site of vulnerability for dissecting aortic aneurysms (197).

As mentioned above, the origin of smooth muscle cells in abdominal aorta appears to be splanchnic mesoderm; however, the thoracic aorta is populated from somite-derived VSMCs, from both the sclerotome and myotome (197). In addition, mesothelium (ie. developing serous membranes) can be a source of VSMC progenitors, including the coronary arteries (see above) and the mesenteric arteries (197).

INTRODUCTION TO CARDIOVASCULAR DISEASE

CARDIAC HYPERTROPHY AND CONGESTIVE HEART FAILURE

Cardiac hypertrophy is a term used to describe enlargement of the heart due to an increased cardiomyocyte size. Cardiomyocyte hypertrophy occurs as a cellular response to increased biomechanical stress, or in response to humoral stimulation during postnatal development (203, 204). Two forms of cardiac hypertrophy have been described: physiological hypertrophy and pathological hypertrophy (204). Physiological hypertrophy occurs during normal growth and development, as well as in response to the imposed demands of exercise training. Although the nature of physiological hypertrophy can vary depending on the nature of the biomechanical stress (ie. preload, afterload, or both), physiological hypertrophy is not associated with adverse cardiac function, fibrosis, or deterioration to heart failure (204). Conversely, pathological hypertrophy frequently occurs as a sequelae to acute myocyte loss following a myocardial infarction, or as a chronic result of the afterload imposed on the heart by arterial hypertension, and is associated with the deterioration to congestive heart failure, arrhythmias, and mortality (203). Pathological remodelling of the heart is accompanied by increased apoptosis, fibrosis, and alterations in cardiac gene expression. For instance, during pathological hypertrophy there is increased expression of genes involved in embryonic and fetal development with concurrent down-regulation of adult myocardial genes (203). This so-called 'fetal gene activation' involves the increased expression of β-MHC, atrial natriuretic factor, SM22, smooth muscle and skeletal muscle α-actin, with decreased expression of α-MHC and sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) (203, 205). Interestingly, physiological hypertrophy has the opposite effect,

increasing the α/β MHC ratio. Furthermore, there are gross metabolic derangements during pathological hypertrophy where cardiomyocytes decrease their overall oxidative capacity with increased reliance on anaerobic glucose metabolism (206). Consistent with this, PGC-1α and Glut4 expression decrease in pathological hypertrophy, while Glut1 expression increases (68, 206). Again, in physiological hypertrophy the expression of PGC-1α is increased, and cardiac metabolic function preserved (206). Thus, it appears that physiological hypertrophy is a beneficial adaptive response to increased cardiac wall stress; however, pathological hypertrophy is 'maladaptive' with deleterious effects that ultimately culminate in congestive heart failure.

The molecular regulation of pathological gene expression in the heart has been intensely studied over the last decade, with particular focus on the re-activation of developmental genes, the so-called 'fetal gene program'; however, very little is known regarding the mechanism of down-regulation of adult genes. Forced expression of an activated calcineurin in a transgenic mouse is sufficient to induce massive hypertrophy, fetal gene activation, and eventual heart failure (75). Congruently, mice deficient in the calcineurin Aβ gene are resistant to pathological hypertrophy elicited by pressure overload (203). However, the hypertrophy-induced fetal gene activation was not sufficiently attenuated in the calcineurin Aβ-null mice, which suggests that other regulators are involved in the maladaptive phenotype. Recently, it was discovered that miR-208, a micro-RNA encoded within an intron of the α-MHC gene, serves to modulate the ratio of α-MHC to β-MHC in response to thyroid hormone (207). Furthermore, mice

with a null-mutation for miR-208 are resistant to pathological cardiac remodelling and have decreased expression of β-MHC.

Forced expression of CaMKIV in the heart also induces a dramatic hypertrophy with increased expression of ANF and down-regulation of α-MHC (81). As mentioned above, CaMKs phosphorylate class IIa HDAC to relieve their repressive effects on transcription. Consistent with a role in cardiac gene expression, targeted deletion of HDAC5 and/or HDAC9 results in an age-related cardiac hypertrophy and an increased susceptibility pressure overload cardiac remodelling with increased fetal gene activation (107, 108). However, CaMKIV is not expressed in substantial amounts in the heart and experiments designed to identify upstream activators of the CaMK-HDAC pathway during hypertrophy were equivocal and suggested the existence of other HDAC kinases (91). Potent agonists of pathological cardiac remodelling, AngII, ET-1, and α-adrenergic stimulation, all activate Gq/11-coupled receptors, which subsequently activate phospholipase C, and increase fetal gene activation (203, 205). Genetic ablation or forced expression of an dominant-negative Gq in mice can nearly completely block all aspects of pathological hypertrophy (203). Clinical evidence from the HOPE trial also suggests that AngII-blockage with ramipril inhibits adverse cardiac remodelling in humans and provides benefit beyond blood pressure control (208).

It is now evident that all pathological agonists that activate Gq-coupled receptors target novel PKCs and PKD1 as down-stream effectors (Figure 4). As noted above, the PKD family are HDAC kinases that are activated by the novel PKCs, and have been

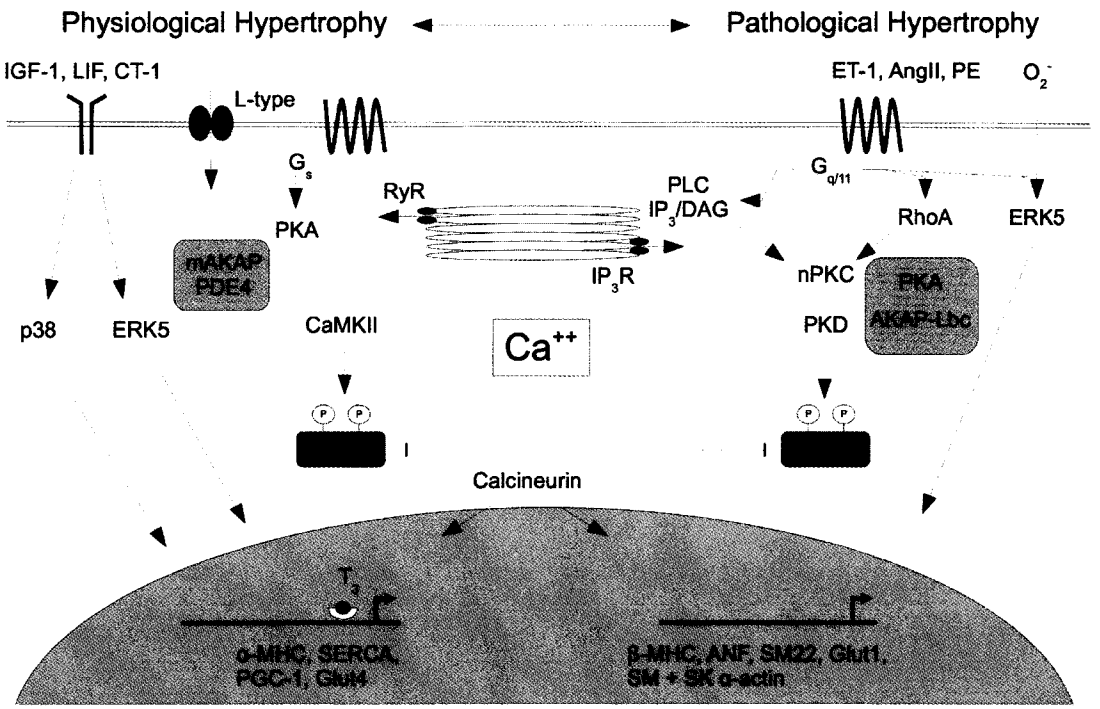

Figure 4. Signalling in Physiological and Pathological Hypertrophy.

The interconnected processes of physiological and pathological hypertrophy, where short-term stimulation of β1-adrenergic receptors, thyroid hormone, IGF-1, or CT-1 induces the expression of adult cardiac genes. Conversely, chronic stimulation with agonists, such as ET-1, AngII, or PE, induces expression of developmental (ie. fetal) cardiac genes and a maladaptive response. Chronic β1-adrenergic alters the expression of AKAPs, which induce apoptosis and promote pathological remodelling. Finally, an interaction between HDAC4 and HDAC5 provides an additional level of cross-talk between physiological and pathological remodelling.

shown to promote nuclear export of HDAC5 in cultured cardiomyocytes (116). Interestingly, PKD is activated by G protein-coupled receptors that activate Gαq, but not by receptors that activate Gαs (91). Furthermore, other studies have suggested a role for the Rho family of GTPases in the regulation of PKD (91). In cardiomyocytes,

knockdown of PKD1 with RNA interference blunts agonist-induced hypertrophy; whereas, conditional deletion of PKD1 in the mice results in improved cardiac function and diminishes hypertrophy in response to pressure overload (117, 121). Activation of the β1-adrenergic G protein-coupled receptors activates Gαs signalling in the heart, and has been associated with physiology hypertrophy and increased contractile function, at least initially (203). However, forced expression of PKA, the downstream effector of Gαs and adenylate cyclase, in the heart results in dilated myopathy, cardiac fibrosis, and decreased expression of α-MHC (57). Furthermore, congestive heart failure in humans has been associated with chronic sympathetic activation of the heart, the so-called 'neurohumoral response' and judicious use of the β-blocker carvedilol in humans with heart failure results in decreased mortality (209, 210). These data suggest that initial activation of Gαs signalling may be physiologically beneficial; however, chronic activation results in pathological remodelling and the progression to heart failure. One mechanism for this pathological cross-talk appears to be the formation of a multi-protein complex containing the PKA anchoring protein AKAP-Lbc, which co-localizes PKA, PKC, with PKD1 (211). Phosphorylation of AKAP-Lbc by PKA promotes the release of PKD1 and subsequent up-regulation of pathological cardiac genes (Figure 4) (211, 212). Furthermore, prolonged activation of Gαs signalling by treatment of cultured cardiomyocytes with the β-agonist isoproterenol results in decreased expression of the phosphodiesterase 3A (PDE3A) and PKA-mediated apoptosis, which may also contribute to pathological cardiac remodelling (213).

CaMKII has been shown to play a critical role in cardiac excitation-contraction coupling in response to β-adrenergic signalling (91, 214). However, recent evidence suggests that CaMKII plays an additional role in pathological cardiac remodelling in response to endothelin-1 induced IP_3 signalling (112). In addition, CaMKII specifically targets HDAC4 to promote fetal genes activation following treatment of cultured cardiomyocytes with the α-adrenergic agonist phenylephrine (111). Furthermore, mice deficient in CaMKIIδ in the heart are protected from pathological hypertrophy and fetal gene activation following aortic banding (113, 215). However, CaMKII may alter fetal gene activation through the formation of a HDAC4-HDAC5 heterodimer (see above) (115).

IGF-1 signalling has also been shown to play an important role in cardiac hypertrophy. For example, IGF-1 activates p38 signalling in cardiomyocytes to promote expression of Glut4 (68). Furthermore, IGF-1 activates ERK5 signalling which can appose isoproterenol-induced cardiac apoptosis (51). The MEK5-ERK5 pathway has also been shown to play a unique role in cardiac hypertrophy, in that forced expression of an active MEK5 in cardiomyocytes produces an elongated hypertrophic response with serial assembly of sarcomeres which resembles mammalian eccentric (ie. preload-induced) hypertrophy (216). In addition, forced expression of active MEK5 induced the expression of a subset of fetal cardiac genes. *In vivo*, forced expression of MEK5 induces a post-natal eccentric hypertrophy which readily degrades to a fatal cardiac dilation. Interestingly, the cytokines leukemia inhibitory factor (LIF) and cardiotrophin-1 (CT-1)

also induce an eccentric hypertrophy response which can be blocked by forced expression of a kinase-dead MEK5 (216). However, LIF and CT-1 induced hypertrophy can also be blunted by a dominant-negative STAT3 (203). ERK5 activity is also induced *in vivo* by ischemia, and in cultured cells by oxidative stress through Gq-signalling (48, 217). Thus, ERK5 appears to protect against pathology remodelling under some cellular circumstances and participate in pathological hypertrophy in others. Further insight into the role of ERK5 in cardiac hypertrophy has been recently uncovered with the identification of ERK5 in an mAKAP containing complex that orchestrates a cAMP negative feedback loop (218). In this series of experiments, cAMP-induced PKA activation resulted in phosphorylation of and activation of PDE4 within the mAKAP complex. This serves as a negative-feedback to terminate PKA signalling when cAMP reached micromolar levels (218). In addition, micromolar concentrations of cAMP can activate Epac1 (exchange protein directly activated by cAMP), which is also contained within the mAKAP complex, resulting in inhibition of ERK5-induced hypertrophy. However, when cardiomyocytes are stimulated with LIF, ERK5 activation induces cardiac gene expression while simultaneously inhibiting PDE4 to promote prolonged cAMP-induced PKA activation, and presumably result in pathological cardiac remodelling (218). Thus, LIF-ERK5 activation may prove to be a critical molecular switch that toggles PKA from regulating physiological excitation-contraction coupling to promoting pathological hypertrophy (Figure 4).

ATHEROSCLEROSIS AND RESTENOSIS

Atherosclerosis is a chronic inflammatory disease that results in the formation of an atheroma, or plaque, within the arterial intima and media which eventually narrows the lumen to cause downstream ischemia (219). Acute disruption of a plaque is the leading cause of acute coronary syndromes and results in occlusive thrombus formation leading to unstable angina and myocardial infarction in the heart, or cerebral infarction in the brain. The process of atherogenesis has been hypothesized to be a response to chronic injury, which was originally proposed to be a result of endothelial denudation, especially at bending or bifurcations in the arterial tree where shear stress was low and turbulent flow more likely (219). A more recent version of this hypothesis involves endothelial dysfunction as a result of oxidative or free radical stress. Possible sources of endothelial injury include elevated and oxidized low density lipoprotein (LDL), and free radicals produced from cigarette smoking, hypertension, elevated serum homocysteine, and diabetes mellitus (219). In addition to intimal injury, free radicals and reactive oxygen species inactivate and neutralize the vascular protective effects of nitric oxide generated by the endothelium (220).

Intimal injury also alters the endothelium's adhesiveness and/or permeability to monocyte-derived macrophages, T lymphocytes, and platelets. In addition, the endothelium becomes more procoagulant in its properties, produces vasoconstrictors, like angiotensin II and endothelin-1, and generates plasminogen activator inhibitor type 1 (PAI-1), as well as other cytokines and growth factors (219, 220). Recruitment of

monocyte-derived macrophages results in internalization of oxidized LDL that may have been trapped within the vessel wall. This internalization is believed to be initially protective against the injuring effects of modified LDLs; however, it also stimulates the conversion of macrophages into foam cells which form the initial 'fatty streak' lesion during atherogenesis (219). Activation of macrophages within an arterial lesion also leads to secretion of matrix metalloproteinases (MMPs), cytokines, chemokines, and growth factors like PDGF-BB and IGF-1 (219). As a result, the inflammatory response stimulates proliferation, migration, and the synthetic function of VSMCs from the media of the vessel to become intermixed within the lesion (219). Furthermore, activation of tissue macrophages results in increased activity of the NADH/NADPH oxidase system, which produces superoxide anions to increase to local oxidative stress (220). Thus, there appears to be cyclic aspect to atherosclerosis (Figure 5).

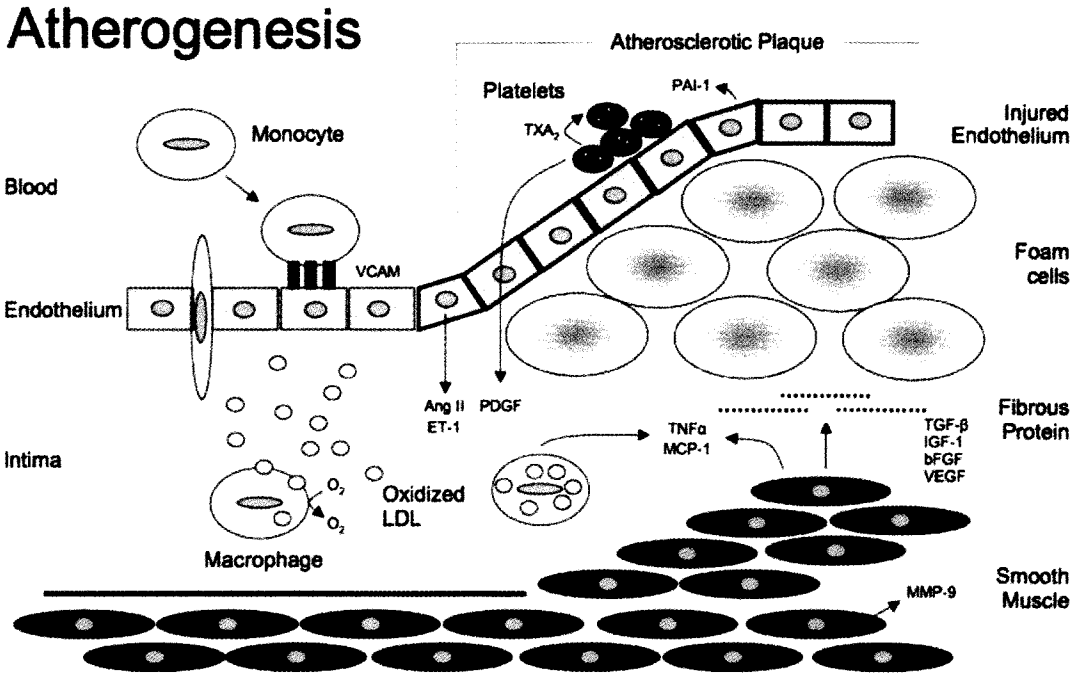

Figure 5. Atherogenesis.

The three interconnected processes of atherogenesis. First, macrophages are recruited to consume oxidized LDL and become trapped foam cells within the arterial wall. Second, platelets adhere to dysfunctional endothelium and release growth factors and thromboxanes. Third, VSMCs alter their phenotype, proliferate, and migrated into the plaque, contributing to plaque bulk, foam cell mass, and formation of a fibrous cap.

As the process of atherosclerosis progresses, a VSMC-containing fibrous cap forms overlying a lipid-laden and often necrotic core. This lesion is now considered an advanced plaque. The mechanism resulting in plaque necrosis is not completely known; however, it appears that the increased proliferation rate of VSMCs, macrophages, and T cells stimulates compensatory angiogenesis from the vasa vasorum. Interestingly, intra-plaque hemorrhaging is a common finding within advanced plaques (219). As a plaque continues to develop, erosion or uneven thinning of the fibrous cap occurs, often at the shoulders where macrophages enter (219). Degradation of the fibrous cap is believed to be due to continued secretion of MMPs from activated macrophages and results in plaque instability, hemorrhage, and rupture with occlusive thrombus formation (219).

Tissue angiotensin II is believed to play a important role during all stages of atherogenesis, including the development of plaque instability (220). Tissue angiotensin is produced from the endothelium under conditions of oxidative stress and promotes many aspects of atherosclerosis including vasoconstriction, inflammation, vessel remodelling, and thrombus formation. Angiotensin stimulates vessel constriction both directly, and indirectly by inducing the release of endothelin-1 and noradrenaline. To promote inflammation, angiotensin II induces the expression of monocyte chemoattractant protein-1 (MCP-1), as well as endothelial adhesion molecules and tumour necrosis factor (TNFα). In addition, angiotensin II activates the NADH/NADPH system to promotes vascular oxidative stress (220). Angiotensin is also a direct growth factor for VSMC, but also induces the expression of PDGF, bFGF, IGF-1, and MMPs.

Lastly, angiotensin II stimulates the production of PAI-1 from the endothelium to promote platelet aggregation and thrombus formation (220). Interestingly, human clinical trials with ACE inhibitors to reduce angiotensin II production suggest that these medicines exert additional vessel protection in addition to their hemodynamic effects (208).

Role of VSMCs in the development of atherosclerosis has been intensely studied over the past three decades, yet it is often overlooked as a mechanism of this disease process when compared to endothelial cells or macrophages. In the adult, most VSMCs reside in the media of the vessels as mature 'contractile' cells. However, mature VSMC do exist in within the intima in areas known as eccentric intimal thickenings (221). Eccentric intimal thickenings form at arterial bifurcations and areas of turbulent flow, and are present in all humans by one year of age, and correlate with locations of advanced atherosclerotic lesions later in life (221). Furthermore, *in vitro* and *in vivo* studies have demonstrated that VSMCs modify their phenotype from contractile to synthetic in response to atherogenic stimuli such as fibronectin and type IV and VIII collagen, PDGF, low shear stress or mechanical strain, reactive oxygen species, and modified lipids (221-223). Once activated by these stimuli, VSMCs from the media are believed to migrate into the developing lesion. However, this notion has been recently challenged by evidence that suggests that extreme vascular injury results in recruitment of circulating cells, derived from the bone marrow, into the vascular lesion, and these cells may transdifferentiate into VSMCs (133). The role of these circulating smooth muscle

progenitors in experimental atherosclerosis has recently been disproven utilizing sex-mismatched bone marrow from transgenic GFP+ mice transplanted into lethally irradiated apoE-null mice (224). These studies confirmed the abundant literature from the 1970s and 1980s that concluded the source of intimal VSMCs in an atherosclerotic lesion is the underlying media (225).

The phenotype of intimal VSMCs during atherogenesis includes increased DNA synthesis and expression of cell cycle markers, decreased expression smooth muscle marker-genes (ie. SM-MHC and SM α-actin), and changes in cell morphology such as replacement of myofilaments with rough endoplasmic reticulum and Golgi, and rounding of the cell from its typical elongated spindle shape (133). In addition to contributing to plaque volume, the intimal VSMCs have been demonstrated to play a maladaptive functional role in the initial stages of atherosclerosis, such as lipid uptake through LDL- and scavenger-receptors resulting in a VSMC contribution to the foam cell population within a plaque, inflammatory cytokine production (eg. PDGF, TGF-β, and MCP-1), altered production of extracellular matrix components, and retention of macrophages through the production of adhesion molecules (221). Furthermore, several reports have suggested that the embryonic origin of the VSMCs within a vessel dictates the susceptibility of the artery to atherosclerotic lesion development (197, 219). However, an interesting dichotomy has been observed, in that VSMCs may be maladaptive in the early stages of atherosclerosis, yet may be adaptive in the formation of the fibrous cap which

can stabilize the plaque and prevent or delay the appearance of acute rupture events that result in occlusive thrombus formation and downstream infarction (133).

In 1977, the German cardiologist Andreas Gruentzig performed the first successful coronary angioplasty to physically remodel an atherosclerotic lesion and enhance the coronary lumen diameter. Although this procedure revolutionized interventional cardiology, approximately 20-40% of patients who receive this therapy will develop clinically significant restenosis of the treated artery within 6-months, even with deployment of a metal stent designed to retain the coronary lumen diameter (226, 227). Restenosis is defined as the arterial wall's healing response to mechanical injury, and is comprised of two stages, neointimal hyperplasia and vessel remodelling (228). Neointimal hyperplasia is believed to be triggered by platelet aggregation and inflammatory cell infiltration that result in the release of cytokines and growth factors that ultimately recruit phenotypically modified VSMCs from the media (228). The resultant neointima consists of synthetic VSMCs, extracellular matrix, and macrophages recruited over the course of a few weeks. During vessel remodelling, there is a greater production of extracellular matrix components, and reendothelialization occurs (228). Using animal models of vascular injury, neointimal VSMCs were found to down-regulate the expression of SM-MHC isoforms at 2-3 weeks after injury, but retained their expression of SM α-actin; however, by 6-months following arterial injury VSMCs had recovered their expression of SM-MHC (133). Experimental evidence also suggests that VSMC proliferation is critical to neointima formation, where medial cells reach a peak

proliferation rate 5-7 days following injury (228). This has become the biological basis for drug-eluting stents, which can release rapamycin and block G1-S phase transition. Furthermore, arterial injury has become a convenient model in which to study VSMC phenotype modulation *in vivo*.

THE ROLE OF MEF2 IN CARDIOVASCULAR DEVELOPMENT AND DISEASE

CARDIOVASCULAR DEVELOPMENT

Drosophila has been extensively used as a model system in which to study the genetic regulatory networks that dictate cardiac development (165). In *Drosophila*, the NK2 homeobox transcription factor *tinman* (Nkx2.5 in mouse) is critical for the specification of the cardiac lineage. *Tinman* induces the expression of the core cardiogenic transcription factors, including *dMEF2*, *pannier* (GATA in mouse), Tbx, and the single *Drosophila Hand* gene. In addition, these factors induce each others expression, and many physically and functionally interact to create a reinforcing network that promotes cardiac morphogenesis and differentiation (165). Loss of function mutations of the single *dMEF2* gene abolishes the expression of cardiac contractile genes, but does not affect cardiac patterning or lineage specification (Figure 6) (3).

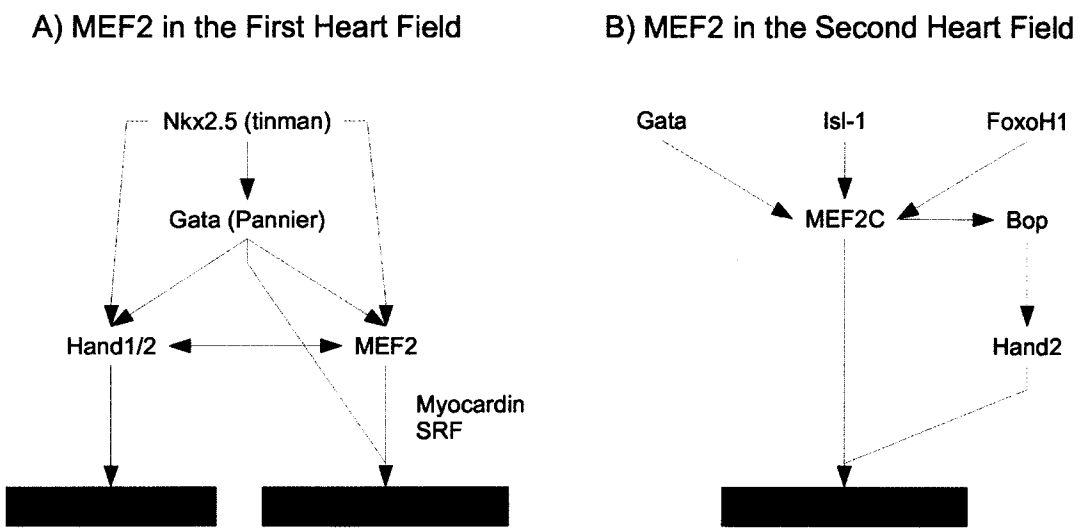

Figure 6. The role of MEF2 in cardiac development.

A) MEF2 is a component of the core cardiac transcription factors, being induced by Nkx2.5 and Gata factors, and promoting the expression of differentiation genes and patterning genes, such as Hand2. B) In the second heart field, MEFC expression is induced by Isl-1, Gata, FoxH1, and TGF-β. MEF2C promotes the expression of Hand2; as well as differentiation genes.

Functional redundancy between the four mouse *mef2* genes has precluded a complete analysis of role of *mef2* genes during mammalian cardiac development; however, cardiac-specific expression of a MEF2C-engrailed repressor fusion protein blocks cardiac differentiation and inhibits the expression of GATA and Nkx transcription factors, confirming the role of MEF2 as a core cardiac transcription factor (229). Interestingly, null-mutations in the individual *mef2* genes has revealed certain non-redundant roles. For instance, *mef2c*-null mice die a E9.5 from a cardiac looping defect, fail to form a future right ventricle, and have a decreased expression of certain embryonic contractile genes, such as myosin light chain 1A and SM22 (7, 8). MEF2A is expressed later in the course of embryonic development, and the *mef2a*-null mice survive until early postnatal life and suffer from right ventricle dilation, myofibril disorganization, and decreased expression of α-skeletal actin and mitochondrial genes (11). However, *mef2b*- and *mef2d*-null mice are viable, which suggests their absence can be compensated for by either MEF2A or MEF2C during cardiac development (7, 12). MEF2 proteins have also been shown to drive the expression of myocardin during cardiovascular development, which places MEF2 upstream of SRF-dependent cardiac genes (see below) (145).

The evolutionary acquisition of a four-chambered heart has been achieved through the addition of a second source of embryonic progenitors from the anterior (ie. second) heart field, as well as an extension to the existing cardiac regulatory gene network. Evidence for MEF2's involvement in the genetic programming of the second heart field originated from the phenotype of the *mef2a*- and *mef2c*-null mice, which both

display a right ventricle defect. Furthermore, *mef2c*-null mice have decreased expression of dHand (Hand2), a critical transcription factor involved in the right ventricle patterning (Figure 6) (7). However, the regulation of dHand expression by MEF2C has been elusive, and may occur through an indirect mechanism involving the histone methyltransferase BOP, which is a direct MEF2-target gene (3). MEF2C expression is regulated in the second heart field through two distinct enhancers. The first contains Foxh1-, Nkx2.5-, and Smad-binding elements and is TGF-β responsive (230). The second contains *cis* elements for GATA factors and Isl-1 (184). Given that Isl-1 is expressed early in SHF progenitors and not in the mature heart, it is believed that Isl-1 is responsible for early activation of *mef2c*, and GATA4 sustains the activation of this enhancer throughout development.

The *mef2c*-null mice also display vascular defects, in addition to cardiac defects (8). These defects include an absent or malformed dorsal aortae and failure of VSMCs to differentiate, as indicated by SM22-lacZ expression. The molecular basis of these findings remained a long-standing mystery in developmental biology given that most smooth muscle contractile genes are SRF-dependent and do not contain MEF2 *cis* elements within their regulatory regions. However, it now appears that MEF2C induces VSMC differentiation by regulating the expression of myocardin, a critical and powerful SRF cofactor that is required for VSMC differentiation (see below) (145). In addition to VSMC defect, *mef2c*-null mice display disorganized endothelial tubes within the yolk sac and embryo proper (8). However, endothelial cells express normal level of differentiation

genes, such as PECAM, VEGF, Flt-1, Flk-1, Ang-1, and Tie-2. This phenotype has been attributed to the loss of a MEF2C-HDAC7 repressor complex that inhibits the expression of MMP10 to promote endothelial tube integrity (110). Mice harbouring a null mutation of the *dHand* (*Hand2*) gene display vascular defects remarkably similar to the the *mef2c*-null mice, which suggest MEF2C is genetically upstream of dHand, or that dHand and MEF2C are obligatory cofactors in VSMCs (231).

Endothelin-1 has been demonstrated to control neural crest-derived VSMC migration and differentiation during development (3, 163). Furthermore, the homeodomain transcription factors DLX5 and DLX6, as well as Hand2 have been shown to be downstream targets of endothelin-1 during brachial arch development. Recent studies have further demonstrated that conditional knockout of *mef2c* in the neural crest substantially reduces the expression of DLX5/6 and Hand2 within the neural crest-derived brachial arch arteries, heart, and outflow tracts (Figure 7) (9). MEF2C controls DLX5/6 expression through a conserved enhancer which contains up to four MEF2 *cis* elements, and is synergistically activated by forced expression of MEF2C and DLX5. However, it remains unknown whether MEF2C expression or transcriptional activation are regulated by endothelin-1.

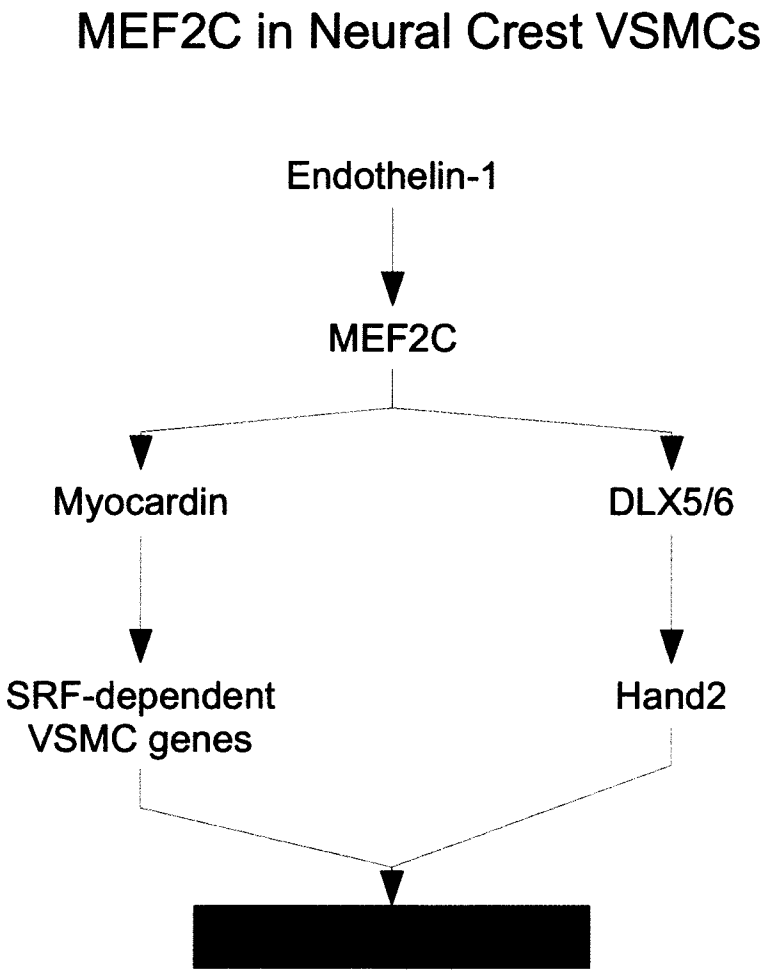

Figure 7. The role of MEF2C in neural crest-derived VSMCs.

Endothelin-1 induces the differentiation of neural crest-derived VSMCs. MEF2C plays a central role in differentiation inducing the expression of myocardin, as well as DLX5/6, that in turn activate Hand2 expression.

As noted above, MEF2C regulates VSMC differentiation by inducing the expression of the 'master' smooth muscle transcriptional coactivator, myocardin (see below) (145). Interestingly, mice habouring a null myocardin mutation in the neural crest develop a post natal patent ductus arteriosus, with decreased expression of SRF-dependent smooth muscle genes (199). This phenotype resembles human syndromes associated with mutation in the myocardin/SRF-target gene for smooth muscle myosin heavy chain.

MEF2 AND CARDIAC HYPERTROPHY

MEF2 proteins were originally implicated in pathological cardiac remodelling in a transgenic mouse line where an activated CaMKIV was ectopically expressed in the heart through the αMHC promoter (81). As described above, these mice develop post-natal cardiac hypertrophy with fetal gene activation. When these mice were crossed with a MEF2-lacZ reporter mouse, marked MEF2 activation was observed. Furthermore, the same group crossed the MEF2-lacZ mice with the calcineurin transgenic line and reported MEF2 transcriptional activation, but not to the same extent as in the CaMKIV transgenic mouse (81). Mice homozygous for a null mutation in the HDAC9 gene develop an age related cardiac hypertrophy and are more sensitive to hypertrophy induced by aortic banding. Crossing these mice with the MEF2-lacZ reporter mouse has also revealed MEF2 activation, which is greatly enhanced when crossed with the calcineurin transgenic mouse (107). In addition, aortic banding performed in the MEF2-

lacZ mouse line results in substantial MEF2 activation during the development of cardiac hypertrophy (J.C. McDermott, unpublished observation). Further evidence supporting the role of MEF2 proteins in pathological hypertrophy has been recently published. Forced expression of MEF2A, -C, or -D in the heart of transgenic mice results in marked chamber dilation, increased susceptibility to pressure overload, and increased fetal gene activation (12, 232, 233). Conversely, forced expression of a dominant-negative MEF2 in calcineurin transgenic mice preserved cardiac contractility and cardiac dimensions (232). Consistent with this finding, MEF2D-null mice display a resistance to pathological cardiac remodelling with decreased expression of fetal cardiac genes following aortic banding or isoproteronol administration (12). However, the MEF2A-null mice have revealed a greater degree of complexity than the previous findings would predict, in that these mice display an increased fetal gene expression and MEF2-lacZ activity (11). Thus, it appears that MEF2C and MEF2D serve to activate fetal cardiac genes; whereas, MEF2A serves a postnatal role to repress fetal cardiac genes and activate adult and mitochondrial gene expression.

Despite numerous *in vivo* observations implicating MEF2 transcriptional activation during pathological cardiac remodelling, the precise molecular mechanisms underlying MEF2 target-gene activation or repression remain unknown. For example how do some MEF2 target-genes, such as ANF and α-skeletal actin, get induced following a hypertrophic stimulus while others, such as αMHC get repressed? In addition, there appears to be a functional interaction between MEF2 proteins and other

core cardiac transcription factors like SRF and GATA factors during the development of cardiac hypertrophy. The first evidence implicating a genetic interaction between MEF2 and SRF demonstrated that myocardin, a powerful SRF coactivator, was a direct MEF2-target gene during cardiovascular development (145). Myocardin expression is increased during cardiac hypertrophy and activates many SRF-dependent fetal cardiac genes (see below)(Figure 8). Second, MEF2 also directly increases the expression of STARS (striated muscle activator of Rho signalling) during pathological remodelling of the heart (234). STARS is an actin binding protein that induces the nuclear translocation of the myocardin-related SRF-coactivators MRTF-A and MRTF-B in response to RhoA-induced actin polymerization. When forcibly expressed in the heart, STARS enhances the hypertrophic response to aortic banding and increases fetal cardiac gene expression by promoting the nuclear localization of MRTF-A (234). Furthermore, myocardin and MRTF-A have been shown to dimerize through their leucine zipper domains and synergistically activate SRF-dependent genes (235, 236). The cardiac isoform of myocardin can co-activate both SRF- and MEF2-dependent cardiac genes (144, 177). Therefore, a complex genetic and functional interaction between MEF2 and SRF proteins exists in cardiac tissue and this intricate interaction is a critical event modulating pathological fetal gene activation during cardiac hypertrophy.

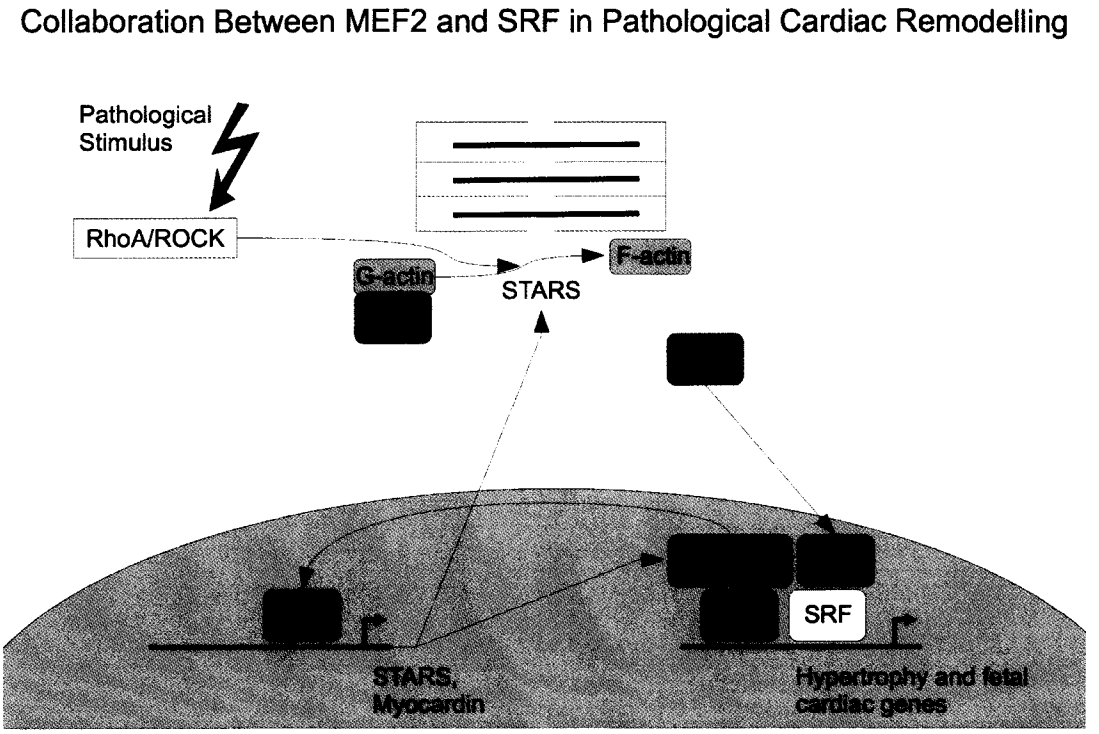

Figure 8. Genetic interaction between MEF2 and SRF during cardiac hypertrophy.

MEF2 induces the expression of STARS and myocardin. STARS promotes the formation of F-actin, in combination with RhoA signalling. This releases MRTF-A and -B from G-actin to coactivate SRF-dependent genes. Myocardin can coactivate with both MEF2 and SRF, and dimerize with MRTF-A and -B, forming a reinforcing transcriptional network for pathological remodelling of the heart.

Recent publications have identified the molecular mechanism connecting pathological β-adrenergic stimulation and PKA activation to MEF2-dependent pathological remodelling. As noted above, AKAP-Lbc forms a multi-protein complex containing PKA, PKC, and PKD (211). Furthermore, the expression of AKAP-Lbc is increased in primary cardiomyocytes following treatment with pathological agonists, such as phenylephrine, and RNA interference designed to reduce AKAP-Lbc expression blunted the hypertrophic response to agonist stimulation (212). Furthermore, forced expression of AKAP-Lbc in cultured cells increased MEF2 transcriptional activity concurrent with nuclear export of HDAC5.

MEF2 AND VASCULAR DISEASE

MEF2 proteins were first implicated in the activated VSMC phenotype when it was noted that serum stimulation of cultured A10 cells resulted in greater MEF2 transcriptional activity than in quiescent cells (237). Further studies demonstrated that the expression of MEF2A, -B, -C, and -D was increased in the neointima of balloon injured arteries, when compared with medial VSMCs (238). In addition, angiotensin II, a known inducer of the activated VSMC phenotype, induces c-Jun expression in cultured VSMCs, and this induction was found to be attenuated by mutation of the MEF2 *cis* element within the *c-jun* promoter, or by inhibiting the p38 MAP kinase pathway with SB203580 (239). Angiotensin II was also found to activate Smad2, an identified MEF2 co-activator, in a p38 MAP kinase-dependent manner in cultured VSMCs (240). More recently, MEF2

was found to regulate the expression of the chemokine, monocyte chemoattractant protein 1 (MCP-1) in VSMC through a consensus MEF2 *cis* element (241). Null mutation of the MCP-1 receptor, CCR2, protects apoproteinE-null mice from atherosclerotic lesion formation (242). Collectively, these data implicate MEF2 in the activated VSMC phenotype. However, the recent characterization of the promoter regions of two smooth muscle target genes has revealed the necessity of a consensus MEF2 binding site for proper expression *in vivo* (145, 243). These genes are myocardin, a master regulator of smooth muscle gene expression, and the histidine-rich calcium binding protein (HRC). These data suggest that MEF2, like SRF, and can play a dual role in both VSMC differentiation and the activated smooth muscle phenotype during vascular disease, yet the molecular regulation of these disparate roles remains poorly defined.

THE ROLE OF SRF IN CARDIOVASCULAR DEVELOPMENT AND DISEASE

THE SERUM RESPONSE FACTOR STRUCTURE AND ISOFORMS

The serum response factor (SRF) is a founding member of the MADS domain transcription factors. Like the MEF2 family, SRF contains a MADS domain near its N-terminal region, which allows it to efficiently bind DNA, dimerize, and recruit regulatory co-factors that increases or decreases its transcriptional potency (32, 244). The MADS domain of SRF lies within an extended region between amino acids 137 to 224.

Alignment of the core 57 amino acids of SRF with the MADS domain of the MEF2 transcription factors, the only other known mammalian MADS-box proteins, reveals remarkable conservation. SRF binds DNA as a homodimer, and the structure of crystallized DNA-bound SRF has been solved. The N-terminal portion of the MADS-box assumes an α-helix structure that forms an antiparallel coiled coil between the SRF dimer pair as it contacts DNA. This is followed by a four-stranded antiparallel β-sheet which is the central component for dimerization (245). C-terminal to the MADS-box of SRF is a region thought to be important for the binding of regulatory cofactors (see below). Interestingly, MEF2 proteins also contain a conserved region C- terminal to their MADS-box, known as the MEF2 domain, which functions to receive cofactors involved in transcriptional regulation (see above) (4).

The C-terminus of SRF (ie. amino acids 225 to 508) contains a transcriptional activation domain, which is believed to be regulated by phosphorylation upon serum stimulation (246). Interestingly, SRF is not a potent transcriptional activator on its own, as it requires binding of a coactivator within the extended MADS domain to achieve high levels of transcriptional activation (143, 247). In addition to full-length SRF, three shorter isoforms have been identified (32, 248, 249). All of these isoforms involve splicing exclusion of the fifth exon (SRFΔ5), which encodes a portion of SRF's transcriptional activation domain. Each isoform retains an intact MADS-box, and is capable of binding DNA; however, without adequate transcriptional activation, it is believed that SRFΔ5 acts as an endogenous dominant-negative (248). Interestingly, the ratio of full-length SRF to

SRFΔ5 is highest within smooth muscle containing tissues, which suggests a high level of expression of SRF-dependent genes. At this time, however, it is not known whether this ratio is altered during conditions of smooth muscle phenotype modulation, such as vascular injury, when smooth muscle cells assume a more proliferative phenotype (32).

DNA BINDING: THE SERUM RESPONSE ELEMENT AND CARG BOX

In 1985, Treisman identified an enhancer region within the promoter of the immediate-early gene, *c-fos*, that was responsive to serum stimulation in cultured 3T3 cells (250). This enhancer subsequently became known as the serum response element (SRE) (251, 252). The first identified protein that bound the SRE was a 508 amino acid polypeptide and was called the serum response factor (SRF) (253, 254). SRF binds to the core sequence of the SRE, CCATATTAGG. Additional studies involving the cardiac α-actin promoter identified the consensus SRF binding site as $CC(A/T_6)GG$, which has become known as the CArG box (255). Within the SRE, an additional *cis* element, GGAT, lies adjacent to the CArG box. This site binds the ETS family of transcription factors, such as Elk-1 (256). Thus, the serum induction of the SRE requires the interaction of SRF and Elk-1 through their respective *cis* elements (see below).

The nucleotides most critical for SRF binding to the CArG box are the G residues at the 3' end of the element, which reside in the major groove of the DNA (32). These residues contact the MADS-box coiled coil of the SRF dimer. DNA binding is stabilized by an N-terminal extension of the MADS domain penetrating the A/T-rich minor groove

(143, 245). SRF DNA binding is disrupted by mutation of the GG residues, or by insertion or deletion of residues within the A/T rich core that alter the topology of the GG residues from the major groove. Interestingly, a single G or C substitution within A/T region reduces DNA binding, but does not completely disrupt it (32). Such naturally occurring CArG degeneracy might provide a mechanism of SRF site-directed control in smooth muscle cells (see below) (257, 258).

SRF REGULATION OF GROWTH AND MUSCLE DIFFERENTIATION GENES

An interesting dichotomy became immediately evident in the study of SRF-dependent gene expression. How could the same transcription factor activate both a growth-dependent proto-oncogene like *c-fos*, and cell type-specific promoter like α-actin (254, 255)? This long-standing issue has been particularly interesting to smooth muscle biologists, since smooth muscle cells do not terminally differentiate like skeletal and cardiac muscle. Instead, smooth muscle cells can modulate their phenotype becoming more contractile or proliferative in response to environmental stimuli (133, 196, 247). In addition, nearly all smooth muscle marker genes identified to date are dependent on one or more CArG elements found within their promoter or first intron (133). Thus, it appears that smooth muscle phenotype modulation is dependent largely on SRF site-directed control.

In *Drosophila*, SRF is required for tracheal and wing intervein development; however, functional analysis of SRF in mammalian muscle was precluded by the fact that

SRF-null mice die at gastrulation from a failure to form mesoderm (259-261). Recently, tissue-specific SRF ablation has been completed in all three muscle types. In skeletal muscle, SRF inactivation results in a myopathic phenotype where muscle fibres form, but fail to undergo hypertrophic growth resulting in perinatal fatality (262). Cardiac ablation of SRF results in embryonic lethality at E11.5 with thin dilated myocardium and poor trabeculation (175, 176). Furthermore, forced expression of SRF in the heart results in pathological cardiomyopathy with fetal gene activation and fibrosis (263). Recently, the results of an conditional SRF-null mouse have been published where a floxed-SRF line was crossed with a Nkx2.5-Cre. This early cardiac inactivation of SRF prevented the development of beating myocytes and the formation of sarcomeres (264). Furthermore, these conditional SRF-null hearts did not express Hand1 or miRNA1. Interestingly, miRNA1 has been shown to target Hand2 for degradation, and collectively suggests that SRF may be a key transcription factor regulating primary (ie. first) heart field development (265). Indeed, some authors have suggested that SRF should be renamed the Sarcomeric Regulatory Factor (266).

Inactivation of SRF in vascular smooth muscle results in decreased recruitment to the dorsal aorta with attenuation of smooth muscle marker gene expression (176). Interestingly, RNA interference to SRF in cultured vascular smooth muscle cells mimics the proliferative effects of platelet-derived growth with equal reduction in both CArG-dependent smooth muscle genes and immediate-early genes (267). Thus, it appears that SRF-dependent genes are dispensable for proliferation in smooth muscle, but

indispensable for smooth muscle differentiation. SRF, and possibly MEF2, also appear to regulate the expression of miRNA133, which serves to repress smooth muscle gene expression in favour of cardiac gene expression in the heart (268, 269). A null-mutation of miRNA133 in mice results in aberrant smooth muscle gene expression in the heart and increased cardiomyocyte proliferation (268). Interestingly, expression of miRNA133 has also been shown to prevent pathological cardiac remodelling through inhibition of RhoA signalling (270). Lastly, SRF has been recently shown to regulate the expression of the miRNA143/145 gene cluster that modulates the VSMC phenotype by targeting KLF4 expression (271).

A number of mechanisms have been proposed to explain how SRF can distinguish between the opposing smooth muscle phenotypes associated with growth and differentiation. First, SRF expression is higher in smooth muscle cells than in non-muscle cells, and SRF expression is induced by smooth muscle differentiation factors, like TGF-β (137, 272). These data indicate that at high levels of expression, SRF may favour the activation of smooth muscle-specific genes. However, SRF levels do not appear to be rate-limiting in early stages of VSMC differentiation (133). Second, there may be regulation of SRF-DNA binding to specify the appropriate target promoter. CArG boxes within many smooth muscle promoters have a reduced binding affinity for SRF compared with the CArG box of *c-fos* (273). This reduced binding is a result of evolutionary conserved single G or C substitutions within the A/T-rich core of the smooth muscle CArG boxes, which has been termed CArG degeneracy (133). Interestingly, substitution

of the smooth muscle α-actin CArG boxes with the *c-fos* CArG box had no effect on smooth muscle-specific expression in a transgenic reporter mouse. However, these substitutions substantially attenuated the repression of SM α-actin following vascular injury, suggesting that a reduced SRF binding is critical for proper smooth muscle-specific gene expression during phenotype modulation (257). Third, most smooth muscle-specific genes have two or more CArG boxes within their regulatory regions; whereas, the *c-fos* promoter contains only a single CArG box (32, 133). In fact, a number of these smooth muscle-specific enhancers have been evaluated with *in vivo* reporter gene analysis, and it appears that the interaction between CArG elements and their spatial relationship with one another contributes to smooth muscle-specific expression (274-277). However, there are notable exceptions to this mechanism. The telokin gene is restricted in its expression to smooth muscle tissue and contains a single CArG box within its promoter (278). In addition, the vascular smooth muscle-restricted gene smoothelin B appears to contain no conserved CArG box and is thought to be SRF-independent (279, 280). Also, the SRF-dependent immediate-early gene Egr-1, has multiple CArG boxes in its promoter (32). Fourth, SRF activity appears to be regulated through RhoA-dependent actin treadmilling (281, 282). Interestingly, this mechanism was originally shown to induce the *c-fos* promoter in non-muscle cells; however, studies in smooth muscle have now demonstrated that RhoA stimulates transcription of CArG-dependent smooth muscle genes, while having no effect on the *c-fos* promoter (283, 284). Lastly, site-directed transcriptional control of SRF has been demonstrated by interaction

with specific coactivators. As noted above, the *c-fos* CArG box is flanked by a binding site for ETS domain transcription factors, such as Elk-1. However, the majority of smooth muscle CArG boxes do not lie adjacent to ETS binding sites (32, 133). A classical pathway has evolved in the literature, in that growth factor-induced activation of mitogen activated protein (MAP) kinase signalling results in Elk-1 phosphorylation and recruitment of SRF to promote *c-fos* expression through the SRE (285). However, substantial evidence now suggests that in the absence of mitogen stimulation, SRF is bound to smooth muscle-selective coactivators that promote the expression of smooth muscle marker genes (32, 247, 286, 287).

SRF, GATA AND NK FACTORS

Early studies involving the cardiac α-actin promoter revealed an interaction and functional cooperation between SRF and the homeodomain protein Nkx2.5 (288). The same research group later showed that the cardiac-restricted zinc fingered transcription factor GATA4 also interacted with SRF to drive α-actin expression (289). Both Nkx2.5 and GATA4 are restricted in their expression to cardiac tissue; however, homologous NK and GATA family members are expressed in smooth muscle cells. They are Nkx3.2 and GATA6, respectively. Studies involving the smooth muscle-specific promoters for α1 integrin and SM22 revealed that the triad of SRF, GATA6 and Nkx3.2 provided synergistic transcriptional activation (290). In addition, these factors formed a ternary complex on a CArG-box containing oligonucleotide, and physically interacted with SRF, as determined by co-immunoprecipation. Interestingly, the combination of SRF, Nkx3.2,

and GATA6 failed to activate the *c-fos* promoter. Thus, interaction with smooth muscle-restricted coactivators Nkx3.2 and GATA6 appears to preferentially activate smooth muscle marker genes and provide a mechanism for SRF site-directed control.

Recently, the cysteine-rich LIM protein, CRP2 was found to act as a bridging molecule that associates SRF with GATA6 (291). The combination of SRF-CRP2-GATA6 potently activated smooth muscle-specific promoters, and was sufficient to convert pluripotent 10T1/2 fibroblasts into smooth muscle cells. Thus, it appeared that CRP2 plays a critical role in promoting smooth muscle differentiation; however, CRP2 deficient mice display normal vascular patterning without altered expression of smooth muscle marker genes (292). Interestingly, when the CRP2-null mice were exposed to vascular injury, they developed enhanced intimal thickening and had accelerated smooth muscle migration in response to platelet derived growth factor (292). Thus, it appears that additional SRF coactivators can compensate for the deficiency of CRP2 during development, but CRP2 is required to re-engage a smooth differentiation program following vascular injury.

SRF AND MYOCARDIN

Substantial progress has been made in the last few years in regards to SRF-dependent cardiac and smooth muscle gene regulation with the identification of the SRF coactivator myocardin (143, 247, 286, 287). Myocardin was discovered in a bioinformatics screen designed to identify unknown cardiac-specific genes; however, myocardin is also highly expressed in smooth muscle (177). Myocardin interacts with the

MADS domain of SRF and forms a ternary complex on a CArG-box containing oligonucleotide, but only in the presence of SRF. Consistent with this, mutation of the CArG-boxes in the SM22 promoter abolishes myocardin's potent activation of this reporter gene (177). SRF interacts with myocardin through an N-terminal region of basic amino acids and a glutamine-rich region, where disruption of either of these regions prevents the formation of a ternary complex with SRF (177). Myocardin also contains a 35 amino acid SAP (SAF-A/B, Acinus, and PIAS) domain, which has the potential to bind DNA, and a leucine zipper-like domain that allows myocardin to homodimerize (143). Myocardin shares significant homology with the myocardin-related transcription factors, MRTF-A (also called MAL, MKL-1, and BSAC), and MRTF-B (also called MKL-2). These factors also co-activate with SRF, but their expression is much more ubiquitous than myocardin (143). Interestingly, targeted disruption of the single MRTF gene is *Drosophila* results in a phenotype virtually identical to the SRF-null flies, with impaired tracheal and wing intervein development (147). Furthermore, in *Xenopus* the transcriptional coactivator MASTR is a cofactor for SRF and not MEF2. In this species, MASTR coactivates SRF- dependent skeletal muscle with the MyoD family (146).

Early studies with myocardin demonstrated its ability to activate smooth muscle marker genes and induce a smooth muscle contractile phenotype in a variety of cells types including L6 myoblasts and embryonic stems (ES) cells, and its ability to convert 10T1/2 fibroblasts into smooth muscle cells (293-297). Interestingly, myocardin overexpression alone was unable to activate cardiac-specific genes in 10T1/2 fibroblasts,

but it was later found that sumoylation of myocardin would allow for transactivation of cardiac genes in this pluripotent cell type (295, 298). In addition, overexpression of a dominant-negative myocardin or underexpression of myocardin with siRNA, resulted in reduced smooth muscle gene expression in smooth muscle cells lines and primary cultures (294-296). Mice homozygous for a myocardin loss-of-function mutation die at E10.5 and display no evidence of vascular smooth muscle differentiation in the dorsal aorta (299). Surprisingly, myocardin-null mice have no apparent decrease in cardiac gene expression. The authors of this report speculate that the MRTFs are able to compensate for the loss of myocardin in the heart, but not in the developing aorta. At this time, there are no reports on combined myocardin- and MRTF-null mice to confirm or refute this speculation; however, inactivation myocardin in *Xenopus* is sufficient to block cardiac development during the formation of the cardiac crescent (177, 300). Cardiac-specific deletion of myocardin has been recently published using a floxed-myocardin gene and crossing these mice with the αMHC-Cre mice. These mice succumb to a dilated cardiomyopathy within their first year due to increased cardiomyocyte apoptosis and loss of sarcomeric organization (301).

Myocardin has also been linked to cardiac hypertrophy, where myocardin's expression is increased following aortic banding or following phenylephrine or LIF-1 treatment of cultured cardiomyocytes (302). Furthermore, a dominant-negative myocardin can block agonist-induced hypertrophy and fetal gene activation in culture. Like the myocardin-null mice, the MRTF-B-null mice do not have a cardiac defect, but

do display a smooth muscle defect in the brachial/aortic arch arteries (200, 201). Collectively, these data are quite interesting, in that smooth muscle cells from the dorsal aorta are derived from lateral mesoderm, and smooth muscle cells in the brachial arteries are derived from ectodermal neural crest cells. This suggests that smooth muscle cells from differing embryonic origin rely differently on myocardin or the MRTFs for proper smooth muscle gene expression *in vivo*.

Myocardin's ability to activate smooth genes is regulated, in part, through its interaction with SRF. In quiescent smooth muscle cells, myocardin is found complexed with SRF, but stimulation with PDGF results in Elk-1 phosphorylation and displacement of myocardin from SRF in favour of SRE activation (303). The opposing influences of myocardin and Elk-1 on smooth muscle phenotype are mediated through competition for a common binding site on SRF. Thus, it appears that growth and development signals modulate smooth muscle gene expression by regulating the interaction of SRF with opposing cofactors (303, 304). In addition, myocardin has also been shown to interact with GATA4 to induce expression of CArG-dependent smooth muscle and cardiac genes (90). Recently, a phosphorylation site within SRF's MADS box has been shown to regulate its ability to distinguish proliferative and myogenic genes (305). A phospho-mimetic mutation of serine-162, a consensus PKCα residue, completely inhibits SRF binding and activation of the α-actin genes. However, SRF-dependent *c-fos* activation was preserved through its interaction with Elk-1 (305). Thus, growth factor induced activation of PKCα may be able to target SRF to growth- responsive genes and prevent

activation of SRF-dependent smooth muscle genes. In addition, myocardin's transcriptional activity is regulated by its interaction with the forkhead transcription factor, Foxo4 (306). Foxo4 binds to the basic-region and SAP domain of myocardin and inhibits it's transcriptional activity (306). Insulin-like growth factor 1 (IGF-1) is known to induce smooth muscle differentiation through activation of PI3' kinase-Akt signaling. To achieve this, IGF-1 stimulates the nuclear export of Foxo4, thereby relieving myocardin from its inhibitory influences and promoting smooth muscle differentiation (306).

Myocardin's ability to activate SRF-dependent smooth muscle genes is also regulated by physical association with histone acetyltransferases (HATs) and deacetylases (HDACs) (89). Myocardin is believed to recruit the HAT, p300, to smooth muscle regulatory elements, resulting in H3 acetylation and destabilization of chromatin structure. p300 increases myocardin's ability to convert 10T1/2 fibroblasts to smooth muscle cells and enhances the transcriptional activation of smooth muscle promoters (89). Subsequent studies have now shown that myocardin increases SRF enrichment at smooth muscle CArG boxes and increases SRF association with modified histones (307). Conversely, HDAC5 inhibits 10T1/2 conversion by myocardin, and inhibits transcriptional activation of smooth muscle promoters (89). In primary VSMCs, angiotensin II has been shown to activate PKD and relieve myocardin from the repressive effects of HDAC5 (119). Since its discovery, myocardin has been hailed as the 'master' smooth muscle regulator, being both necessary and sufficient for smooth muscle differentiation, and the most potent SRF coactivator characterized to date.

SRF AND SMADS

TGF-β, and its downstream intracellular signalling molecules, the Smads, are known to promote smooth muscle differentiation (196). Early studies demonstrated that TGF-β can induce the SM α-actin promoter, but this induction was dependent on two CArG boxes within this promoter (137). This suggested that there was a link between the TGF-β/Smad pathway and SRF. Subsequent studies have now shown that Smad3 directly interacts with SRF and can cooperate with SRF to induce the SM22 promoter (138, 308). In addition, the inhibitory Smad7 can compete for SRF with Smad3 to inhibit TGF-β induction of the SM22 promoter (309). Interestingly, myocardin has also been shown to interact with Smad3 in VSMCs, and to synergistically activate the SM22 promoter, even when the CArG boxes have been mutated to prevent SRF binding (139). Furthermore, myocardin's activity is modulated by a physical interaction with Smad1 in cardiomyocytes, which is stimulated by BMP-2 signalling, and induces the expression of CArG-dependent cardiac genes (136). TGF-β signalling also activates the p38 MAP kinase pathway in smooth muscle cells through RhoA/PKN-dependent signalling, and inhibition of p38 by SB203580 attenuates TGF-β induction of smooth muscle marker promoters and a SRF-dependent reporter gene (141, 142). Recently, the zinc-fingered transcription factor δEF1 has been shown to be inducible by TGF-β signalling, and enhance the expression of smooth muscle marker genes in a CArG box-dependent manner (310). δEF1 interacts directly with SRF and Smad3 to synergistically activate the SM α-actin promoter, and δEF1 knockout mice exhibit an exaggerated neointima

formation following vascular injury; whereas, viral-mediated overexpression of δEF1 inhibits neointima formation (310). Thus, it appears that TGF-β stimulation induces the formation of a SRF-Smad3-δEF1 complex that selectively activates smooth muscle genes.

CALCIUM SIGNALLING IN VASCULAR SMOOTH MUSCLE CELLS

CALCIUM AS A SIGNALLING MOLECULE IN MUSCLE: EXCITATION- CONTRACTION AND EXCITATION-TRANSCRIPTION COUPLING

In vivo, vascular smooth muscle cells (VSMCs) have an average intracellular concentration of free calcium that is several orders of magnitude lower than the extracellular calcium concentration (311). As in striated muscle, the rise and fall of intracellular calcium initiates smooth muscle contraction and relaxation, respectively. However, smooth muscle does not contain thin-filament-associated troponin to bind the rising intracellular calcium and activate contraction. Instead, smooth muscle contraction is activated by calcium binding to calmodulin (CaM), which directly activates the myosin light-chain kinase (MLCK) (312). Activated MLCK then phosphorylates serine-19 of the regulatory myosin light-chain (MLC20), which allows the myosin ATPase to be activated by actin and contraction to engage (313). A fall in intracellular calcium, inactivates MLCK and allows MLC20 to be dephosphorylated by the myosin light-chain

phosphatase (MLCP); thus, deactivating myosin ATPase and permitting muscle relaxation (313, 314). The events that result in elevated intracellular calcium and activation of contraction are termed excitation-contraction coupling (EC-coupling). In smooth muscle, EC-coupling is achieved by two, often interconnected processes: electromechanical coupling and pharmacomechanical coupling; where electromechanical coupling involves changes in the cell's membrane potential resulting in activation of contraction, and pharmacomechanical coupling involves ligand activation of membrane-bound receptors that activate contraction (312).

The resting membrane potential of smooth cells is negative with respect to the extracellular space, ranging from -40 to -70 mV (312). In electromechanical coupling, depolarization of the membrane results in opening of voltage-gated calcium channels, notably the L-type calcium channel, and calcium influx to trigger contraction. As in cardiac muscle, calcium influx through voltage-gated channels is believed to induce calcium release from the internal sarcoplasmic reticulum (SR) stores (312). Thus, activation of the contractile apparatus occurs both directly from calcium influx and from calcium release from the SR.

In pharmacomechanical coupling, ligands (ie. hormones or neurotransmitters) can release calcium from the sarcoplasmic reticulum through the generation of a second messenger (eg. Inositol 1,4,5 triphoshate, IP3), or modulate calcium sensitivity by regulating MLCP activity (313, 314). Lowering the global intracellular calcium concentration to allow relaxation is the combined result of hyperpolarizing the cell

membrane to limit calcium influx through voltage-gated channels, extrusion of calcium through the sarcolemma, and uptake of calcium by the sarcoplasmic/endoplasmic reticulum calcium ATPases (SERCA) (315). Localized intracellular calcium transients, called calcium sparks, are caused by the coordinated opening of ryanodine-sensitive calcium channels on the sarcoplasmic reticulum (316). Interestingly, in smooth muscle cells, calcium sparks have been shown to induce muscle relaxation (317). Calcium sparks illustrate how a localized release of calcium can result in a different physiological effect than a global change in intracellular calcium. A calcium spark results in a discrete release of calcium between the superficial sarcoplasmic reticulum and the sarcolemma. This localized increase in subsarcolemmal calcium activates the large-conductance Ca^{2+}-activated K^+ channels (BKCa), promoting K^+ efflux and membrane hyperpolarization, which favours relaxation (315). Thus, sparks may serve as an inhibitory pathway to prevent excessive smooth muscle contraction, or as a way to distinguish calcium-mediated contraction events from calcium-mediated transcriptional events (315, 316).

Excitation-transcription coupling (ET-coupling) is the process by which signalling pathways that regulate EC-coupling also regulate transcriptional-mediated alterations in gene expression (315). This concept is particularly interesting, in that mechanisms that regulate short-term vascular tone can translate into long-term alterations in smooth muscle phenotype and vascular remodelling. ET-coupling has been studied intensively in terminally differentiated cells, such as neurons, skeletal muscle, and cardiac muscle; whereas, it is only an emerging field in vascular smooth muscle cells which can modulate

their phenotype in response to changes in the local environment. Nonetheless, there is an abundance of published literature focused on the transcriptional regulation of smooth muscle growth and differentiation genes in the past decade. Most of this literature has centred around the transcriptional regulation of SRF and the cofactors that modulate smooth muscle phenotype by targeting SRF to smooth muscle-specific promoters or growth-related immediate early gene promoters (see above) (133, 247, 286, 287). Thus, a fundamental gene regulation paradigm has been established in which to study ET-coupling in smooth muscle cells by examining calcium-mediated signalling pathways that converge on SRF and other transcription factors, such as MEF2, GATA6, and NFAT, known to regulate smooth muscle gene expression.

CALCIUM/CALMODULIN KINASE SIGNALLING

The calcium-calmodulin complex (Ca^{2+}-CaM) regulates a number of intracellular enzymes involved in both EC- and ET-coupling. As noted above, Ca^{2+}-CaM activates the MLCK to phosphorylate MLC20 and activate contraction. Ca^{2+}-CaM also activates the Ca^{2+}-CaM-dependent protein kinases (CaMKs). Smooth muscle cells *in vivo* and in culture express CaMKII and CaMKIV, where CaMKII is largely cytosolic and is believed to play a role in EC-coupling; whereas, CaMKIV is predominantly nuclear and believed to regulate ET-coupling (318-320). The role of CaMKII in smooth muscle EC-coupling is largely inferred from established literature using cardiomyocytes as a model (214). However, CaMKII likely plays a role in modulating MLCK sensitivity to calcium, regulating L-type calcium channels, and SERCA activity (315). Calcium-dependent

phosphorylation of MLCK in cultured smooth muscle cells decreases its sensitivity to elevations in cytosolic calcium (321). Phosphorylation of MLCK is blocked by the addition of the CaMK inhibitor KN62 or an inhibitory peptide to CaMKII. This mechanism may serve as negative-feedback to prevent excessive phosphorylation of MLC20 (321). CaMKII has long been known to facilitate and enhance calcium influx by regulating the L-type calcium channels (322-326). Recently, this effect has been shown to be mediated by direct interaction with and phosphorylation of serine-1512 and serine-1570 of the C-terminus of the Cav1.2 (α1C) subunit of the L-type channel (325, 327). An additional report demonstrated that CaMKII binds to the β2A subunit of the L-type calcium channel and phosphorylates threonine-498. Mutation of threonine-498 to a neutral alanine prevents CaMKII-mediated facilitation of the L-type channel (324).

CaMKII may also play a role in ET-coupling. Evidence from neuronal models suggests that CaMKII can directly phosphorylate the cAMP response element binding protein (CREB) at serine-142 and negatively regulate its transcriptional activity (328). In addition, CaMKII can directly phoshorylate the class II histone deacetylase 4 (HDAC4) in cardiomyocytes resulting in HDAC4 nuclear export, derepression of HDAC-dependent genes, and cardiac hypertrophy (111). However, the role of CaMKII in regulating CREB and HDACs in smooth muscle tissue remains largely unknown; however, evidence from our laboratory suggests that CaMKII can rescue *c-jun* from HDAC4 repression where CaMKI and CaMKIV can not (Gordon and McDermott, unpublished). Lastly, CaMKII has been previously implicated in the process of smooth muscle migration in response to

platelet derived growth factor (PDGF) treatment (329). PDGF is believed to activate calcium signalling in VSMCs through the PDGF β-receptor, which physically associates and activates phospholipase C-γ (PLC-γ) (330). Once active, PLC-γ catalyzes the hydrolysis of phosphoinositol bisphosphate into inositol triphosphate (IP3) and diacylglycerol (DAC); where IP3 liberates calcium from internal stores and DAC is a potent activator of PKC signalling. Evidence from our laboratory suggests that inhibition of both CaMK and PKC signalling is required to repress the down-stream expression of c-Jun by PDGF-stimulation of cultured VSMCs (120). Furthermore, it appears that PDGF activation of CaMKII is dependent on activation of MEK1, the integrin receptor αvβ3, and basic fibroblast growth factor (bFGF) for full activation (331-333). In these studies, both the MEK1 inhibitor PD98059 and the CaMK inhibitor KN62 inhibited PDGF-induced smooth muscle migration, but the combined effects of these inhibitors was not additive (333). In addition, PDGF activation of CaMKII was inhibited by PD98059, which suggests some level of cross-talk between these two signalling pathways. The functional interaction between the CaMKII and MEK1 signalling may be mediated indirectly by the αvβ3 integrin receptor and bFGF, in that neutralizing antibodies to these molecules can inhibit PDGF-induced smooth muscle migration and prevent PDGF activation of CaMKII. In both of these studies, the effects on smooth muscle migration were rescued by a calcium ionophore or forced expression of an activated CaMKII (331, 332). These results are consistent with our previous findings that combined inhibition of CaMKs and PKCδ can completely prevent the induction of c-Jun by PDGF (120).

A fundamental paper in smooth muscle ET-coupling was published in 2000 by Nelson's research group. They evaluated smooth muscle calcium signalling in intact cerebral arteries treated with 60 mM KCl. This treatment raises the cell's membrane potential increasing the opening probability of L-type calcium channels, and elevating cytosolic calcium. Results from this study demonstrated that calcium influx through L-type calcium channels resulted in phoshorylation of CREB and induction of the immediate early gene *c-fos*. In addition, this response was attenuated by the CaMK inhibitor KN93 (334). These results are interesting, in that previous studies in other cells types have shown that CaMKIV can directly phosphorylate CREB at serine-133 to increase its transcriptional activity, and that the *c-fos* promoter region contains a cAMP response element (CRE) 3' to the CArG box; whereas, CArG-dependent smooth muscle marker genes do not contain consensus CRE sites in their promoters (315, 335). Thus, CaMK-mediated activation of CREB appears to be an important mechanism regulating the expression of immediate early genes, like *c-fos*, during ET-coupling. However, at this time there are no published studies utilizing a *c-fos* reporter gene, *in vivo* or *in vitro*, to evaluate the contribution of the CRE in response to membrane depolarization in vascular smooth muscle cells. Indeed, previous literature in other cells types has shown that CaMK can directly phosphorylate SRF at serine-103, and that the serum response element (SRE) contributes to calcium-mediated induction of the *c-fos* promoter (336).

CONVENTIONAL PROTEIN KINASE C SIGNALLING

Protein kinase Cs (PKCs) transduce signals from G protein-coupled receptors, tyrosine kinase receptors, and non-receptor tyrosine kinases (337). Three general classes of PKC isozymes have been established base on the signalling molecules that activate these intracellular kinases. Classical or conventional PKCs (α, β, and γ) are activated by diacylglycerol liberated by phospholipase C (PLC) and elevation of intracellular calcium; whereas, novel PKCs (δ, ε, η, θ) are activated by diacylglycerol. Much less is known regarding the activation of the atypical PKCs (ζ, ι, λ) (337). Conventional PKCs have been implicated in EC-coupling by regulating calcium sensitivity through the activity of the MLCP (313). PKCs phosphorylate threonine-38 of a potent inhibitor of the MLCP catalytic subunit, called CPI-17 (338-340). Activation of CPI-17 by PKCs enhances its inhibition of MLCP and increases calcium sensitivity and the contractile state (314). Treatment of smooth muscle cells, either in culture or within intact vessels, with angiotensin II, histamine, or phenylephrine increases calcium sensitivity and the phosphorylation of CPI-17, where these effects were blocked with administration of the PKC inhibitor GF109203X (341-343).

It has long been known that conventional PKCs play a role in smooth muscle ET-coupling following application of a growth stimulus, like the vasoconstrictor angiotensin II, to induce *c-fos* expression (344). Indeed, in other cell types, phorbol ester induction of *c-fos* has been found to involve PKCs and require the serum response element within this promoter region (345, 346). Furthermore, PKCα and PKCε have been shown to activate the serum response element through Elk-1 transactivation, where PKCδ and PKCζ did

not (347). In addition, PKCα has been implicated in skeletal myoblast proliferation and not differentiation (348). The mechanism by which conventional PKCs active SRF-dependent immediate early genes, like *c-fos*, but not SRF-dependent myogenic genes has remained unknown until recently. A novel consensus PKC phosphorylation site, serine-162, within the MADS box of SRF has been identified that can selectively target SRF to growth-responsive genes containing an adjacent Elk-1 binding site, and prevent SRF binding to muscle-specific genes, including SM α-actin (305). Taken together, it appears that contraction agonists, like angiotensin II, increase calcium sensitivity in quiescent smooth muscle cells by activating conventional PKCs, which directly phosphorylate CPI-17 to inhibit MLCP. However, in proliferative smooth muscle cells angiotensin II-activated PKCα targets SRF to proliferative genes, like *c-fos*, and inhibits the expression of smooth muscle marker genes.

RHOA SIGNALLING

The GTPase RhoA has been implicated in both EC- and ET-coupling in smooth muscle cells. RhoA has been shown to regulate calcium sensitivity during EC-coupling through the downstream Rho-kinase (ROCK), which can inhibit the MLCP (313). In smooth muscle, the MLCP is composed of three subunits: a catalytic subunit comprised of protein phosphatase 1 (PP1); a regulatory subunit called M110 or MYPT1; and a subunit of unknown function called M21 (313, 314). The M110 subunit is believed to act as a myosin binding subunit to target the trimeric MLCP to the MLC20. Activation of RhoA/ROCK results in M110 phosphorylation and inactivation of phosphatase activity

(313, 314). This would effectively increase the phosphorylation of MLC20 and enhance calcium sensitivity and contraction. G protein-coupled receptors known to activate RhoA-induced calcium sensitivity in smooth muscle include: α-adrenergic, muscarinic, prostanoid, thrombin, angiotensin, endothelin, and oxytocin (313). Agonists for most of these receptors are also known release IP3 from membrane phospholipids and stimulate SR- mediated calcium release. Thus, RhoA/ROCK activation serves an important role in pharmacomechanical EC-coupling.

RhoA has also been shown to regulate the transcriptional activation of SRF through a mechanism known as 'actin treadmilling' (281, 282, 349, 350). ROCK is known to induce the formation of actin filaments (F-actin) from actin monomers (G-actin). Reduction in the cytosolic free G-actin pool, or an increase in F-actin formation results in activation of SRF-dependent genes (32, 143). In smooth muscle cells, RhoA activation has been shown to activate smooth muscle-specific reporter genes, such as SM22 and SM α-actin (284). This induction of smooth muscle promoters was substantially inhibited by the ROCK inhibitor Y-27632, or the actin polymerization inhibitor latrunculin B (284). Until recently, the mechanism responsible for transcriptional activation of SRF-dependent genes by RhoA remained unknown. However, Treisman's research group has now shown that RhoA-mediated F-actin formation promotes the nuclear localization of the potent myocardin-related SRF coactivator MAL (myocardin related transcription factor A [MRTF-A], or megakaryoblastic leukemia 1 [MKL-1]) (351). MAL directly associates with G-actin,

which promotes its cytosolic retention. However, RhoA-induced actin polymerization results in redistribution of MAL from the cytoplasm to the nucleus and subsequent activation of SRF-dependent genes (351, 352). Recently, it has been demonstrated that RhoA signalling in VSMC also activates Diaphanous 1 and 2, which serve to increase the nuclear localization of both MRTF-A and -B to enhance SRF-dependent smooth muscle gene expression (353). This effect was dependent on actin polymerization.

In addition to agonist-mediated activation of RhoA, membrane depolarization in cultured smooth muscle has also been shown to activate RhoA-dependent signalling (354). Treatment of cultured vascular smooth muscle cells with 60 mM KCl resulted in translocation of RhoA to the membrane fraction, which is associated with activation of RhoA-dependent signalling (354). Depolarization-dependent translocation of RhoA was blocked by the L-type calcium channel blocker, nifedipine. In addition, this report by Owen's group demonstrated that calcium influx through L-type channels increases the expression of both SRF-dependent smooth muscle marker genes, like SM α-actin, SM22, and SM-MHC, and the SRF-dependent immediate early gene *c-fos*. Interestingly, the induction of smooth muscle marker genes by membrane depolarization was blocked by the ROCK inhibitor Y-27632, while *c-fos* induction was blocked by the CaMK inhibitor KN-93 (354). These results indicate that calcium signalling is capable of activating both growth and differentiation genes in smooth muscle cells; however, the downstream calcium-mediated signalling pathways that regulate these genes are distinct from one another. Lastly, this paper showed that the calcium-mediated induction of smooth muscle

marker genes was inhibited by a siRNA to myocardin (354). Taken together, these data suggest a convergence of calcium/RhoA/ROCK signalling on myocardin/SRF-dependent smooth muscle gene expression.

RhoA signalling has also been shown to activate another cellular protein kinase independent of ROCK. This kinase has been called protein kinase N (PKN) (355-360). In smooth muscle cells, RhoA and PKN have been shown to promote differentiation by increasing the expression of smooth muscle marker genes (141, 142). Forced expression of an active PKN increased the promoter activity of SM α-actin, SM22, and SM-MHC, as well as cancatomerized reporter genes for SRF, GATA, and MEF2 transcription factors (141). In addition, under-expression of PKN by siRNA inhibited the expression of SM α-actin, SM22, and SM-MHC (141). Interestingly, the activation of smooth muscle reporter genes by PKN was attenuated by overexpression of a dominant negative p38 MAP kinase, which suggests that PKN may lie upstream of p38 signalling in smooth muscle cells (141).

In addition, a degree of cross-talk may occur between PKC-mediated calcium sensitivity and RhoA-mediated calcium sensitivity in that ROCK and PKN have also been shown to phosphorylate CPI-17 at threonine-38 (361, 362). The physiological significance of this cross-talk remains in question given the success of PKC inhibitors in preventing CPI-17 phosphorylation. However, it now appears that both PKC and RhoA signalling play an overlapping role in mediating CPI-17 phosphorylation (363, 364). Recently, our research group has shown that CPI-17 is a critical regulator of MEF2-

dependent gene expression in VSMC (unpublished). Activation of CPI-17 by RhoA signalling serves to relieve MEF2C from the repressive effects of PP1α and induce myocardin expression. Furthermore, p38 MAPK activation induced by RhoA/ROCK signalling results in phosphorylation of MEF2C at Ser98, enhancing transcriptional activation.

CALCINEURIN SIGNALLING

Calcineurin, also known as protein phosphatase 2B (PP2B), is a serine/threonine phosphatase regulated predominantly by intracellular calcium (74). Calcineurin regulates a family of transcription factors known as the nuclear factors of activated T cells, or NFATs. Four ubiquitously expressed NFAT genes have been characterized, designated NFATc1 (NFAT2/c), NFATc2 (NFAT1/p), NFATc3 (NFAT4/x), and NFATc4 (NFAT3) (74, 315, 365). Inactive NFATs exist in the cytosol as phosphoproteins. When activated by Ca^{2+}-CaM, calcineurin dephosphorylates NFAT proteins which unmasks their nuclear localization signal, resulting in nuclear accumulation and transcriptional activity (74). When the calcium signal is removed, nuclear NFATs are believed to be re-phosphorylated by GSK3, which promotes nuclear export (74). Smooth muscle cells express all four NFAT genes; however, NFATc3 appears to predominate (366). NFATc3 and –c4 have been shown to play a critical role in vascular development, in that mice habouring disruptions of these genes die at E11 with a defect in vessel assembly (367). Nelson and colleagues have shown that PDGF signalling promotes the nuclear accumulation of NFATc3 through an L-type calcium channel-mediated mechanism; however,

depolarization alone is insufficient to accumulate NFATc3 in the nucleus (366). In addition, NFATc3 is entirely nuclear in intact pressurized vessels, where L-type channel blockade promotes its nuclear export (368). Supporting the role of the calcineurin/NFAT pathway in promoting smooth muscle differentiation, NFATc1 has been previously found to interact with GATA6 and cooperatively increase the expression of the SM-MHC reporter gene through consensus NFAT and GATA binding sites (77, 369). In addition, blockade of calcineurin signalling with cyclosporin A or FK506 down-regulated SM-MHC expression in differentiated smooth muscle cells (77). Activation of the calcineurin pathway has also been shown to be sufficient to drive neural crest stem cells to a smooth muscle fate, and that TGF-β1 is an important activator of calcineurin in this conversion assay (198). Lastly, a conserved NFAT binding site has recently been identified in the SM α-actin promoter, which overlaps a well established CArG-box (370). These authors demonstrated that NFATc3 interacts with SRF and that the two factors and theirs binding sites cooperate to express SM α-actin (370).

SUMMARY

The literature presented above serves to illustrate that MEF2 proteins serve a well-studied functional role in skeletal, cardiac, and vascular smooth muscle cells. Furthermore, the transcriptional activity of MEF2 proteins is modulated by a number of intracellular signalling pathways that target MEF2 directly, or indirectly through

interacting co-factors. Finally, it has become evident in recent years that MEF2 and SRF are regulated by a genetic interaction, where MEF2-target genes often regulate the transcriptional activity of SRF. The most studied example of this genetic interaction occurs in cardiac and vascular smooth muscle cells, where MEF2 regulates the expression of myocardin, which activates many SRF-dependent contractile genes.

STATEMENT OF PURPOSE

Despite the vascular phenotype of the *mef2c*-null mice, surprisingly little published research has focus on the role of the *mef2* genes in VSMCs. Indeed, until recently there were no identified muscle-specific MEF2-target genes in VSMCs (145, 243). However, MEF2 proteins do regulate the expression of some ubiquitous genes, such as the immediate-early gene *c-jun* (29).

Therefore, the purpose of this work was to identify signalling pathways that regulate MEF2 transcriptional activity and target-gene expression in VSMCs and begin to connect this to the VSMC phenotype. This would ultimately contribute to the field of vascular biology by describing the molecular machinery that underlies the phenomenon of smooth muscle phenotype modulation that contributes to vascular diseases, such as atherosclerosis and restenosis. This purpose will be addressed experimentally through specific hypotheses:

1) MEF2 and HDAC4 serve to repress *c-jun* expression in quiescent VSMCs.
2) Platelet-derived growth factor derepresses MEF2 through CaMK and PKCδ signalling.
3) PKA promotes *c-jun* repression through inhibition of SIK1.
4) MEF2-dependent myocardin expression is regulated through an interaction with PP1α.

5) RhoA-signalling activates myocardin expression through p38 and the PP1α inhibitor, CPI-17.

The evaluation of these hypotheses is presented in Manuscript 1 and Manuscript 2, below.

MANUSCRIPT 1

RATIONALE

This first manuscript was published in the Journal of Biological Chemistry and focuses on MEF2-dependent *c-jun* expression in VSMCs. The immediate-early gene *c-jun* is used as a cellular marker of the proliferative VSMC phenotype, as previous studies have strongly implicated MEF2 in the response to vascular injury (238). This manuscript addresses the first 3 experimental objectives outlined in the Statement of Purpose, which are: 1) MEF2 and HDAC4 serve to repress *c-jun* expression in quiescent VSMCs; 2) Platelet-derived growth factor derepresses MEF2 through CaMK and PKCδ signalling; and 3) PKA promotes *c-jun* repression through inhibition of SIK1.

These objectives are evaluated primarily in an immortalized VSMC-line (A10 cells), but also in a transgenic mouse line that habours a MEF2-driven reporter gene (described in Naya, et. al. 1999)(371).

PKA REGULATED ASSEMBLY OF A MEF2/HDAC4 REPRESSOR COMPLEX CONTROLS C-JUN EXPRESSION IN VASCULAR SMOOTH MUSCLE CELLS

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CP: Immunoblotting, HDAC4/MEF2 COIP, siHDAC4 time-course, wound-scrape immunofluorescence (Figures 3A, 4D, 6C, 8A, and -C, S3A).
JS: Immunofluorescence of HDAC4 with inhibitor treatments (Figures 2B and 6A).
MD: Nuclear localization of HDAC4.
JJA: Luciferase assays (Figure 5B and S3C).
JZ: Immunofluorescence and X-gal from injured arteries (Figure 1A).
GH and MPB: Carotid injury (Figure 1A).
RLP: MEF2-VP16 constructs (Figures 1D and 5C).
QD and DC: Immunofluorescence of human and rat specimens (Figure 3B, -C, and -D)
JCM: Advisor and manuscript editor.

ABSTRACT

Vascular smooth muscle cells (VSMCs) maintain the ability to modulate their phenotype in response to changing environmental stimuli. This phenotype modulation plays a critical role in development of most vascular disease states. In these studies, stimulation of cultured vascular smooth muscle cells with platelet derived growth factor (PDGF) resulted in marked induction of *c-jun* expression, which was attenuated by protein kinase C delta (PKCδ) and calcium/calmodulin-dependent protein kinase (CaMK) inhibition. Given that these signaling pathways have been shown to relieve the repressive effects of class II histone deacetylases (HDACs) on MEF2 proteins, we ectopically expressed HDAC4, and observed repression of *c-jun* expression. Congruently, suppression of HDAC4 by RNA interference resulted in enhanced *c-jun* expression. Consistent with these findings, mutation of the MEF2 *cis* element in the *c-jun* promoter resulted in promoter activation during quiescent conditions, suggesting that the MEF2 *cis* element functions as a repressor in this context. Furthermore, we demonstrate that protein kinase A (PKA) attenuates c-Jun expression by promoting the formation of a MEF2/ HDAC4 repressor complex by inhibiting salt-inducible kinase 1 (SIK1). Finally, we document a physical interaction between c-Jun and myocardin, and we document that forced expression of c-Jun represses myocardin's ability to activate smooth muscle gene expression. Thus, MEF2 and HDAC4 act to repress c-Jun expression in quiescent VSMCs, PKA enhances this repression, and PDGF derepresses c-Jun expression through CaMKs and novel PKCs. Regulation of this molecular 'switch' on the *c-jun* promoter may

thus prove critical for toggling between the activated and quiescent VSMC phenotypes.

Key words: Vascular smooth muscle cells, MEF2, HDAC4, c-Jun, PKA, phenotype modulation.

INTRODUCTION

Vascular smooth muscle cells (VSMCs), unlike their skeletal and cardiac counter parts, do not terminally differentiate, but can modulate their phenotype under conditions of growth or differentiation (133). Differentiated smooth muscle cells express high levels of contractile proteins and other muscle-specific genes, a phenotype that has been termed 'quiescent' or 'contractile'. However, in response to vascular injury, VSMCs down-regulate muscle-specific genes, increase their proliferation rate and migration capacity, and actively secrete matrix proteins. This proliferative phenotype has been called the 'activated' or 'synthetic' phenotype (133). Although proliferative VSMCs are undoubtedly required for vascular development and during vascular repair, this activated phenotype also plays a role in multiple smooth muscle diseases, such as atherosclerosis and restenosis following angioplasty (133). Therefore, the molecular mechanisms whereby VSMCs modulate their phenotype between the quiescent and activated states is of particular interest for our understanding of smooth muscle cell biology under physiological and pathological conditions.

The MADS-box transcription factor, serum response factor (SRF) plays a critical role in smooth muscle phenotype modulation. SRF binds to a cognate *cis* element termed

the CArG box, which can be found in multiple copies in many smooth muscle structural genes (32). Conversely, SRF is also involved in smooth muscle proliferation by binding to a single CArG box in the proximal promoter of *c-fos*, a growth responsive immediate-early gene (32). This dual role for SRF is largely regulated by recruiting coactivators, such as myocardin (287), to activate smooth muscle genes, or ternary complex factors (TCFs), such as Elk-1, to activate immediate-early genes (303).

Another mammalian MADS-box transcription factor, known as myocyte enhancer factor 2 (MEF2) is functionally important in cardiac, skeletal and smooth muscle cells. Recent studies have identified two smooth muscle marker genes that require a consensus MEF2 binding site in their respective promoter regions for expression in VSMCs *in vivo*. These genes encode myocardin, a master-regulator of smooth muscle differentiation (145), and the histidine-rich calcium binding protein (HRC), a sarcoplasmic reticulum protein expressed in skeletal, cardiac, and smooth muscle (243). In addition, gene-targeting studies have revealed that MEF2C is required for proper vascular patterning and vascular smooth muscle differentiation (8). However, despite this emerging evidence supporting the role of MEF2 proteins in vascular smooth differentiation, MEF2 has also been associated with the activated, proliferative smooth muscle phenotype (238).

Analogous to SRF's activation of the *c-fos* gene, MEF2 can increase the expression of the immediate-early gene, *c-jun*, which is known to act as a down-stream target of the smooth muscle mitogen, platelet derived growth factor (PDGF) (372, 373). It is currently not known whether PDGF's induction of *c-jun* is mediated through MEF2;

however, evidence from other cell lines suggests the involvement of MEF2 in the serum-induction of *c-jun* (29). To date, very little is known regarding MEF2's role in smooth muscle phenotype modulation, but it appears that both SRF and MEF2 proteins have a regulatory role in smooth muscle proliferation and differentiation.

The transcriptional activity of MEF2 proteins is regulated by post-translational modifications, such as phosphorylation and sumoylation, and a number of interacting protein co-factors. The cellular consequences of the interaction between MEF2 and class II histone deacytylases (HDACs), and its regulation by calcium/calmodulin kinases (CaMK) and PKCδ/PKD signaling, has not thus far been elucidated in VSMCs. Interestingly, PDGF signaling is known to activate CaMKs and PKCδ/PKD during VSMC migration (332, 374, 375), and we have previously shown that the novel PKC isoforms, PKC δ and ε, can activate MEF2 proteins in HeLa and COS cells (42). Therefore, we speculated that PDGF induction of *c-jun* in VSMCs might be mediated by PKCδ- and CaMK-mediated derepression of MEF2.

Protein kinase A (PKA), the cyclic AMP-dependent protein kinase, potently inhibits vascular smooth muscle proliferation and may protect against vascular disease (376). In VSMCs, PKA is activated by prostacyclin (PGI_2) and β-adrenergic agonists. Interestingly, in humans, reduced production of PGI_2 by cyclooxygenase II inhibition is associated with increased cardiovascular risk (377). One mechanism by which PKA has been shown to inhibit smooth muscle proliferation is to inhibit the expression of *c-jun* (378). In addition, recent evidence from our laboratory, and others, suggests that PKA can

promote HDAC4 repression of MEF2-dependent transcriptional activation in other cell types (25, 124, 379). Therefore, we evaluated the role of PKA signaling on MEF2-dependent *c-jun* expression in VSMCs.

In this report, we demonstrate that a MEF2 *cis* element in the *c-jun* promoter serves as a repressor element in quiescent VSMCs, and that this repression is largely abolished during conditions of cell growth. Consistent with this finding, HDAC4 is exported from the nuclear compartment during growth conditions or by exogenous expression of CaMK or PKD, while PDGF induction of *c-jun* is prevented by CaMK and PKCδ inhibition. In addition, gain and loss of function manipulation of HDAC4 levels reveal its involvement in regulation of *c-jun* expression in VSMCs, making this the first report to document that class II HDACs regulate immediate-early gene expression in connection with a proliferative phenotype in VSMCs. Furthermore, PKA promotes MEF2/HDAC4 repression of *c-jun* expression by inhibiting the activity of salt-inducible kinase 1 (SIK1). Finally, forced expression of c-Jun inhibits myocardin's ability to activate smooth muscle gene expression, illustrating the fundamental importance of c-Jun regulation by MEF2 and HDAC4 during smooth muscle phenotype modulation.

MATERIALS AND METHODS

Plasmids. MEF2 and c-Jun reporter constructs (pJC6, pJSX, pJTX) in pGL3, and expression vectors for MEF2A, MEF2C, MEF2D, the MEF2A-VP16 fusion, the Gal4-

MEF2A and Gal4-MEF2D fusions, and c-Jun have been described previously (25, 42, 380). Mouse CaMKIV was cloned by RT-PCR, and an activated construct was generated by truncation at amino acid 275. PCR products were ligated into the NotI-XbaI (CaMKIV) site of pcDNA3 for mammalian expression. An expression vector for rat CaMKII deltaB was kindly provided by A. Hudmon, and a constituatively active mutation was made by replacing threonine 287 with an aspartic acid residue by PCR-based mutagenesis. Expression vectors for the activated PKD and myocardin were generous gifts from E. Olson, and expression vectors for Flag-tagged HDAC4 and HDAC5 were provided by S. Schreiber. The HDAC4-EGFP fusion and HDAC4 L175A vectors were kindly provided by X-J Yang. pSVL-SIK1 and pSVL-SIK1 S577A were kindly provided by H. Takemori. The Gal4-c-Jun fusion proteins were a kind gift from E. Yeh. The SM-MHC promoter was a gift from S. White, and the smooth muscle alpha-actin and calponin reporter genes were generously provided by J. Miano. The cardiac promoters for alpha-cardiac actin and alpha-myosin heavy chain were generously provided by M. Nemers, the PGC-1 promoter was purchased from Addgene, and the MMP-9 promoter was a gift from D. Boyd. The HRC promoter was provided by B. Black, and subcloned into pGL4.10 (XhoI-HindIII). The 350 bp myocardin enhancer described by Creemers et. al. (145), was PCR amplified from mouse genomic DNA with KpnI and and BglII restriction sites incorporated into the primers. The resulting DNA fragment was ligated with the *c-fos* minimal promoter (BglII-NcoI), described previously (380), into pGL4.10 (KpnI-NcoI). The MCP-1 luciferase construct was kindly provided

by A. Garzino Demo. An expression vector containing the catalytic subunit of PKA (pFC-PKA) was purchased from Stratagene.

Cell Culture and Treatment of VSMCs. Rat A10 myoblasts (ATCC; CRL-1476) were maintained in growth media consisting of 10% fetal bovine serum (FBS). Quiescence was obtained by refeeding the cells with either 1% or 0% FBS in DMEM overnight. C3H10T1/2 mouse embryonic fibroblasts (ATCC; CCL-226) and COS7 cells (ATCC) were maintained in standard DMEM with 10% FBS, and refed in 5% horse serum (HS) to achieve quiescence. For conversion assays, C3H10T1/2 were grown to confluence and made quiescent for 4 days prior to harvesting.

Luciferase and β-Galactosidase Assays. Transient transfections of A10 and C3H10T1/2 cells were performed by a modified calcium phosphate-DNA precipitation with pCVM-β-galactosidase serving as an internal control for transfection efficiency (381). Luciferase and β-galactosidase activities were measured as described previously (382).

Immunoblot Analysis. Protein extractions were achieved using an NP-40 lysis buffer described previously (381). Protein concentrations were determined by Bradford assay, and 15 μg were resolved using SDS-PAGE and transfed to an Immobilon-P membrane (Millipore, Inc.). Immunoblotting was carried out using appropriate primary antibody in 5% powdered milk in PBS. Appropriate horseradish peroxidase-conjugated secondary antibody (Bio-Rad, 1:2000) was used in combination with chemiluminescence to visualize bands.

Nuclear/Cytosolic Fractionation. Nuclear and cytosolic fractions were obtained using a Pierce Biotechnology kit. Fractions were subjected to SDS-PAGE and immunoblotting as described above.

Immunofluorescence. A10 VSMCs, cultured as described in the Figure Legends, were fixed, permeabilized, and incubated with a primary HDAC4 antibody (Sigma), and TRITC-conjugated secondary antibody. Cells were visualized using standard fluorescence techniques or confocal microscopy.

siRNA Oligonucleotides. Sense and anti-sense siRNA oligonucleotides specific for mouse and rat HDAC4 (5'- GATCCACTGGTGCTTAACATTTGATTCAAGAGATCAAATGT TAAGCACCAGTTTTTTTGGAAA-3') were purchased from Sigma Genosys, annealed, and ligated into pSilencer 3.0 H1 (Ambion). The siRNA for HDAC4 or a nonspecific scrambled control were transfected into A10 cells with Lipofectamine reagent (Invitrogen) according to the manufacturer's protocol. Transfected cells were enriched by puromycin selection (0.5 μg/mL) for 3 days prior to harvesting for protein extracts.

Carotid Injury of the MEF2 'Sensor' Mouse and Sprague-Dawley rat. Wire-injury of mouse carotid arteries, and balloon-injury of Sprague-Dawley rats, was described previously (383, 384). Immunofluorescence and X-gal staining of mice harbouring three tandem MEF2 consensus DNA binding sites driving a LacZ reporter-gene which was described previously (151).

Human aortic tissues. Human abdominal aortic aneurysms (AAA) segments were obtained from patients undergoing elective repair (n=4, all men). The average age was

70.4 years. The average size of the aneurismal lesions estimated by CT scan and /or angiography was 6.95 cm. During graft replacement for AAA, macroscopically normal adjacent normal aortic (Con) segments were carefully excised from 4 patients and used as controls. Immediately after procurement, segments were placed in sterile normal saline and transported to the laboratory. The protocol of this study was approved by the Clinical Research Ethics Committee at the St. Michael's Hospital and University of Toronto. Written informed consent was given by all patients. Samples were frozen in liquid nitrogen and stored for RNA analysis or was embedded in OCT compound, immediately frozen in liquid nitrogen, and stored at -80 °C.

Laser Capture microdissection (LCM). Cryostat sections (~ 8 μm) were mounted on membrane based microdissection slides (Acutrus Engineering, Mountain View, CA) and fixed for 2 minutes with cold acetone. After washing twice 5 seconds each with DEPC-treated PBS, PH:7.6, the sections were incubated with fluorescein isothiocyanate (FITC)-conjugated mouse anti-human smooth muscle(SM) α-actin antibody (Abcam Inc., Cambridge, MA, 1:20) for 5 to 8 minutes at room temperature. The sections were washed rapidly three times for 1 minute each with DEPC treated PBS followed by dehydrated in graded ethanol solutions (70% 1 time, 1 min, 95% 1 time, 1 min, 100% 2 times, 1 min each) and cleaned in xylene (2 times, 5 min each). After air-drying for 5 minutes, LCM was performed under direct microscopic visualization on the SM α-actin-positive stained areas. The Leica LCM system (Leica Microsystem, Wetzlar GmbH,

Germany) was set to the following parameters: laser diameter, 15 μm; speed, 1.5ms; and amplitudes, 40 mW. A total of 500 to 3000 target cells were captured for each sample.

Total RNA isolation and amplification. Total RNA from LCM captured cells was isolated by using the RNeasy micro RNA isolation kit (Qiagen). T7-based RNA amplification was performed by using the RiboAmp kit (Arcturus Engineering, Mountain View, CA) according to the manufacturer's instructions.

Analysis of gene expression by quantitative real-time RT-PCR. Total RNA extracted either from laser-captured SMC or from alternating whole sections was reverse transcribed using omniscript first-stand synthesis kit (InVitrogen) under conditions described by the supplier. cDNA was amplified by quantitative real-time PCR (ABI prism 7700 Sequence Detection System, Applied Biosystems, Foster city, CA) using SYBR Green PCR Master Mix Reagent (Qiagen). The primer pair sequences for each reaction was performed in duplicated by using equal amount of cDNA from each sample as template. The primer sequences of genes used in this study were: HDAC-4: F: 5'-GGTTTGAGAGCAGGCAGAAC-3', R: 5'-CAGAGAATGAGGCCAAGGAG-3'; GAPDH: F: 5'-GAAGGTGAAGGTCGGAGTC-3', R: 5'-GAAGATGGTGATGGGATTTC-3'. Thermal activation was initiated at 95 °C for 10 minutes, followed by 40 cycles of poloymerase chain reaction (melting for 15 seconds at 95 °C, and annealing/extension for 1 minute at 60 °C). Relative quantitations of gene expression were calculated using standard curves and normalized to GAPDH in each sample.

Immunostaining analysis of aortic tissue. Frozen segments from AAA and adjacent NA tissues were sectioned in 10-μm-thick sections, briefly dried, and fixed in acetone. The sections were incubated in normal horse serum (Sigma, St. Louis, MO) for 1 hour, followed by a 1-hour incubation with the primary antibody rabbit anti-human HDAC4 (1:200, Sigma). With intervening washes in PBS, sections were then incubated for 30 minutes with biotin-conjugated horse anti-rabbit secondary antibody (1:200, Vector Laboratories, Burlingame, CA), followed by a 1-hour incubation with Alexa fluor 488 - conjugated streptavidin (1:200, Sigma). The sections were washed, mounted, and analyzed with confocol microscope (Leica Microsystem Inc, Exton, PA).

RESULTS

MEF2 Expression and Transcriptional Activation Following Carotid Injury. Previous studies have implicated MEF2 proteins in the activated smooth muscle response (238). Although, MEF2 transcriptional activation following vascular injury has not, as yet, been reported. To this end, we utilized the MEF2 'sensor' mouse, that we, and others have previously used to evaluate MEF2 transcriptional activation during development (151, 371). As shown in figure 1A, carotid injury elicited a widespread increase in MEF2A expression, consistent with previous reports (238). MEF2 transcriptional activation, as indicated by X-Gal staining of the MEF2 Lac Z derived arteries, was observed at the site of injury (Figure 1A). In addition, we observed an increased expression of the MEF2-

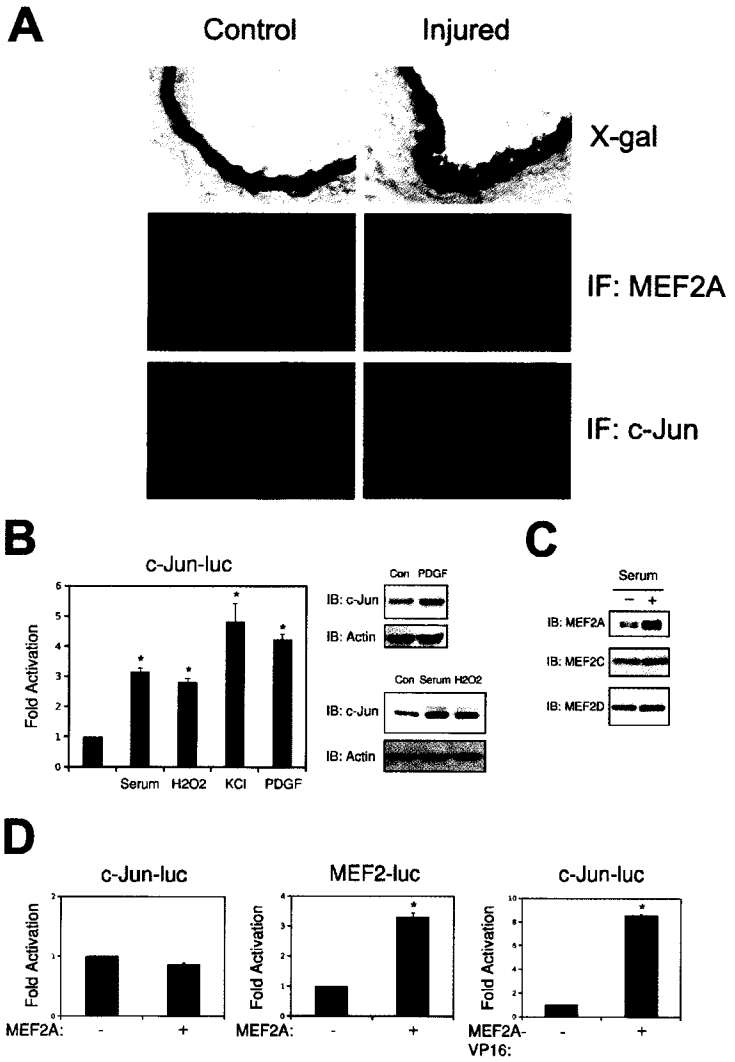

Figure 1. MEF2 activity and expression in VSMCs in vivo and in vitro.
Common carotid arteries of MEF2-LacZ mice were injured by inserting a 2mm wire into the external carotid. Contralateral arteries were used as the control. A) X-gal staining and immunofluorescence for MEF2A and c-Jun 14 days following injury. B) A10 cells were transfected with the wild-type *c-jun* promoter (c-Jun-luc). Following recovery, cells were serum starved overnight, and treated with 20% FBS, 100 μM H_2O_2, 60 mM KCl, or 10 ng/mL of PDGF for 4 hours for luciferase extracts or 2 hours for protein extracts. C) Growth phase A10s in 10% FBS (+) or serum-free media (-) were harvested for protein subjected to immunoblotting for MEF2A, -C, -D. D) A10 cells were transfected with the *c-jun* or MEF2 reporter-genes and MEF2A or MEF2A-VP16. (* $p<0.05$ was considered statistically significant).

target gene, *c-jun*, at the site of injury (Figure 1A). Since the induction of MEF2A expression was not accompanied by a widespread increase in MEF2 transcriptional activation, we further studied the role of MEF2 proteins in the regulation of smooth phenotype. In particular, we analyzed the regulation of the *c-jun* promoter, a previously characterized MEF2-target gene that has been implicated as a key regulator of VSMC proliferation control. In the context of quiescent cultured smooth muscle cells, we found that the *c-jun* promoter, as predicted, was induced by serum stimulation, oxidative stress, depolarization, and PDGF treatment (Figure 1B). These treatments resulted in corresponding increases in c-Jun protein expression; whereas, treatment with transforming growth factor β1 (TGF- β1) had no effect (Figure S1). To evaluate MEF2's role in *c-jun* expression, we first ectopically expressed MEF2 proteins with the *c-jun* reporter-gene. Interestingly, and in contrast to other cell types, we found that MEF2 proteins were unable to activate *c-jun* expression in A10 smooth muscle cells (Figure 1D and S1). However, MEF2 proteins were able to activate an artificial MEF2 reporter-gene (MEF2-luc), a myocardin enhancer-based reporter-gene, and the HRC promoter (HRC-luc) in this context (Figure 1D and S1). In addition, a fusion protein consisting of the MEF2A DNA binding domain fused to the VP16 transcriptional activation domain was able to activate the *c-jun* promoter (Figure 1D). Collectively, these data suggest that MEF2 is capable of binding to both the *c-jun* and muscle-specific reporter regions in cultured smooth muscle cells, but the transcriptional responses of these target genes is divergent.

c-Jun Expression is Regulated by CaMK, PKCδ and HDAC4 in Smooth Muscle Cells. To examine the potential signaling pathways that regulate *c-jun* expression by PDGF, we utilized common pharmacological inhibitors in our culture model. As shown in figure 2A, inhibition of PKCδ by rottlerin, inhibition of CaMKII and IV by KN-62, or inhibition of MEK1 by PD98059 all resulted in a modest reduction in c-Jun protein, while inhibition of PI3' kinase by LY294002 had no effect. However, combination of rottlerin and KN-62 resulted in marked reduction in c-Jun, below levels observed in quiescent cells. In addition, activation of CaMK or PKC signaling by A23187 or PMA, respectively, also increased c-Jun protein expression (not shown). Given that previous studies have implicated the CaMKs and the novel PKCs in the regulation of class II HDACs, we next evaluated the role of KN-62 and rottlerin on the subcellular localization of HDAC4 (83, 116). Figure 2B shows that HDAC4 is distributed throughout the cell during growth conditions, as determined by immunofluorescence. However, combined treatment with KN-62 and rottlerin resulted in nuclear accumulation of HDAC4. Furthermore, we utilized an HDAC4-GFP fusion protein, and observed that it was primarily localized in the nucleus during serum-free quiescent conditions, but was exported to the cytosol during low-density growth conditions. This result was confirmed by nuclear and cytosolic fractionation studies that demonstrate that PDGF treatment promotes nuclear export of HDAC4 (Figure 2D and E). Interestingly, when smooth muscle cultures were allowed to reach confluence, the HDAC4-GFP fusion protein was again primarily nuclear (Figure 2D).

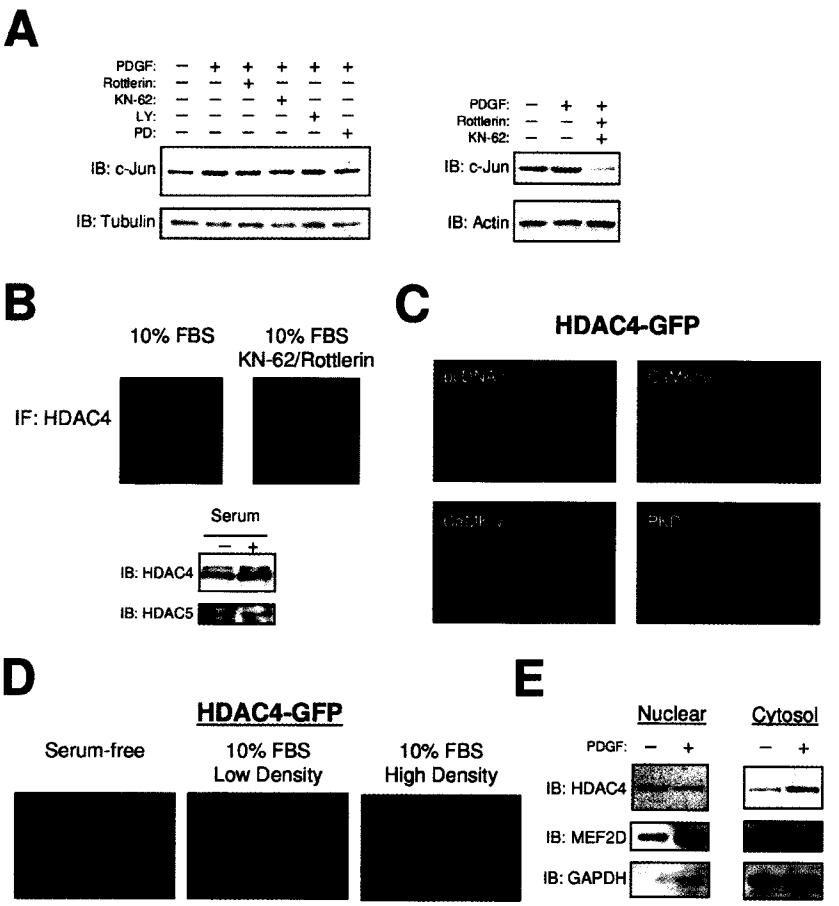

Figure 2. PDGF induction of c-Jun is mediated by CaMK, PKCδ, and MEK.
A) Serum starved A10 cells were treated with PDGF (10 ng/mL) for 2 hours following 15 minute pretreatment with rottlerin (5 μM), KN-62 (5 μM), LY294002 (10 μM) or PD98059 (10 μM). Protein extracts were immunoblotted with a c-Jun antibody (H79, Santa Cruz). B) Growth phase VSMCs were treated with rottlerin (5 μM) and KN-62 (5 μM) followed by fixation with 4% paraformaldahyde. Fixed cells were then subjected to immunofluorescence with an HDAC4 primary antibody (Sigma). C) VSMCs were transfected with an EGFP fusion protein containing full-length human HDAC4 (HDAC4-GFP), and either activated CaMKII deltaB, CaMKIV, or PKD. Following serum starvation micrographs were obtained by standard fluorescent techniques. D) A10s were transfected with HDAC4-GFP. Micrographs were obtained in serum-free media, low density growth media (10% FBS), and high density growth media (10% FBS). E) Nuclear and cytosolic extracts were made from cultured VSMCs treated with 10 ng/mL PDGF for 2 hours. Extracts were subjected to SDS-PAGE and immunoblotted for HDAC4, MEF2D, or GAPDH.

Lastly, ectopic expression of activated CaMKs and activated PKD, a downstream HDAC kinase of PKCδ, resulted in a distribution of HDAC4-GFP to the cytosol (Figure 2C). Together, these results indicate a growth-responsive role for HDAC4 that is regulated by PDGF activation of CaMKs and novel PKCs.

To validate this role for HDAC4 in vascular disease models, we utilized a scratch-wound assay of VSMC migration. As shown in figure 3A, in confluent A10 cells that are positive for both HDAC4 and the Dapi nuclear stain, the HDAC4 signal is confined to the nuclear region. In contrast, in cells migrating into the wound, HDAC4 fluorescence is cytosolic. In addition, we utilized a rat model of carotid injury, since this animal model of vascular disease is more prone to neointimal formation than the mouse that habours the MEF2-LacZ reporter gene (385, 386). Figure 3B shows HDAC4 staining is increased within the neointima of injured rat carotid arteries, where the HDAC4 immunofluorescence is more diffuse than the nuclear stain. This result is suggestive of a HDAC4 cytosolic distribution following vascular injury. Lastly, previous evidence has suggested a causal link between the JNK-c-Jun pathway and the development of aneurysms (387). Therefore, we evaluated HDAC4 expression in human aortic aneurysms to evaluate whether this mechanism might be responsible for heightened c-Jun activity in an aneurysm. As shown in figure 3C, immunofluorescence of HDAC4 is reduced in abdominal human aneurysms.

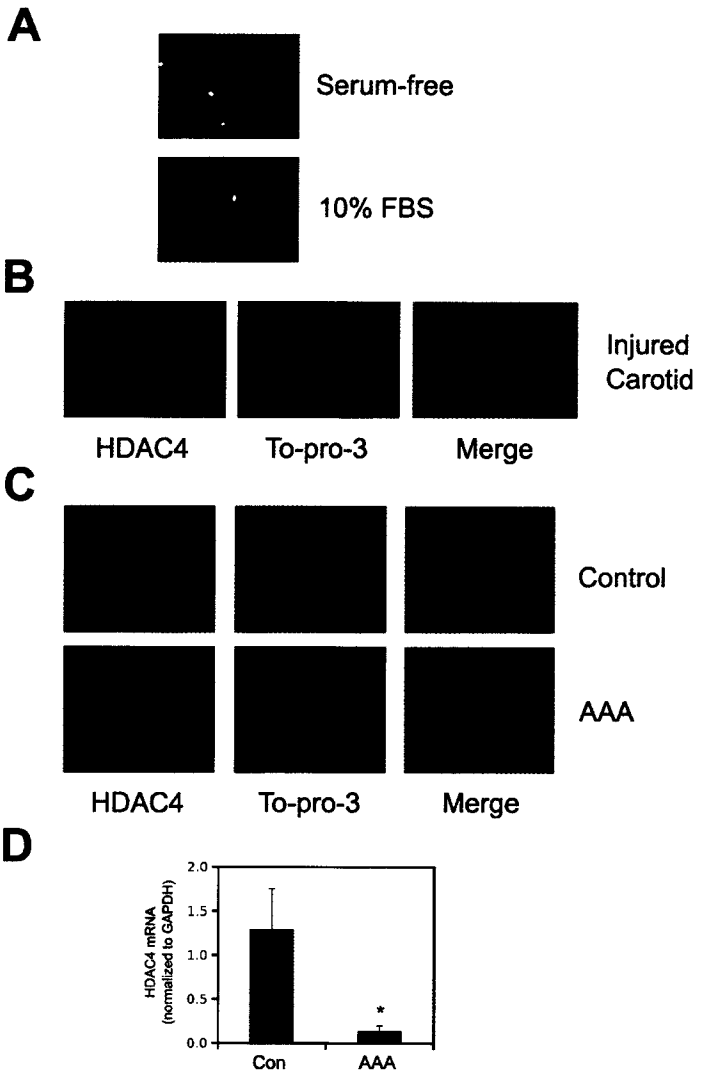

Figure 3. HDAC4 expression in models of vascular disease.

HDAC4 expression in models of vascular disease. A) A10 cells were grown to confluence and scraped with a standard 200 μL pipette tip. Cells were re-fed either serum-free media or media containing 10% FBS overnight, then fixed for immunofluorescence. Red = HDAC4, Green = Dapi (ie. Nuclear). B) Sprague-Dawley rats were subjected to balloon-injury of the carotid artery. Following 14 days of recovery, arteries were fixed and harvested for immunofluorescence. Green = HDAC4, Blue = To-pro-3 (ie. Nuclear). C) Human aortic aneuysms, or a non-diseased control specimen were harvested during elective surgical reconstruction, and fix for immunofluorescence. Green = HDAC4, Blue = To-pro-3 (ie. Nuclear). D) Human control and aortic aneuysms sections were immunostained for smooth muscle alpha-actin and subjected to laser microdissection. Total RNA was isolated from collected cells and subjected to quantitative PCR for HDAC4 and GAPDH. (n=4, * $p<0.05$ was considered statistically significant).

To validate that this reduction occurred in VSMCs, we utilized a technique of laser microdissection of smooth muscle α-actin-positive cells to purify RNA and perform quantitative PCR. Figure 3D illustrates that HDAC4 expression is in fact reduced in VSMCs in human aortic aneurysms. However, we were unable to detect an increased c-Jun mRNA expression in this model (not shown). This finding is consistent with other reports, which have indicated that c-Jun expression may not increase until rupture of an aneurysm (388). In this case, the down-regulation of HDAC4 may proceed an increase in c-Jun, which could occur with an appropriate rupture-induced stress signal. Together these results indicate that HDAC4 may be an important regulator of c-Jun expression in stenotic vascular diseases characterized by VSMC proliferation and migration; however, in arterial aneurysms, characterized by VSMC degeneration, down-regulation of HDAC4 is not sufficient to induce c-Jun expression.

In order to dissect the function of the MEF2 *cis* element within the *c-jun* promoter, we evaluated a *c-jun* reporter-gene construct with a mutation in the MEF2 binding site under growth and quiescent conditions. As shown in figure 4A, mutation of the MEF2 *cis* element site under growth conditions resulted in modest promoter activation, while mutation in the AP1 site had no effect. Interestingly, mutation in the MEF2 *cis* element under quiescent conditions resulted in much greater promoter activation. These data suggest that the complex assembled at the MEF2 *cis* element serves to repress *c-jun* expression under quiescent conditions.

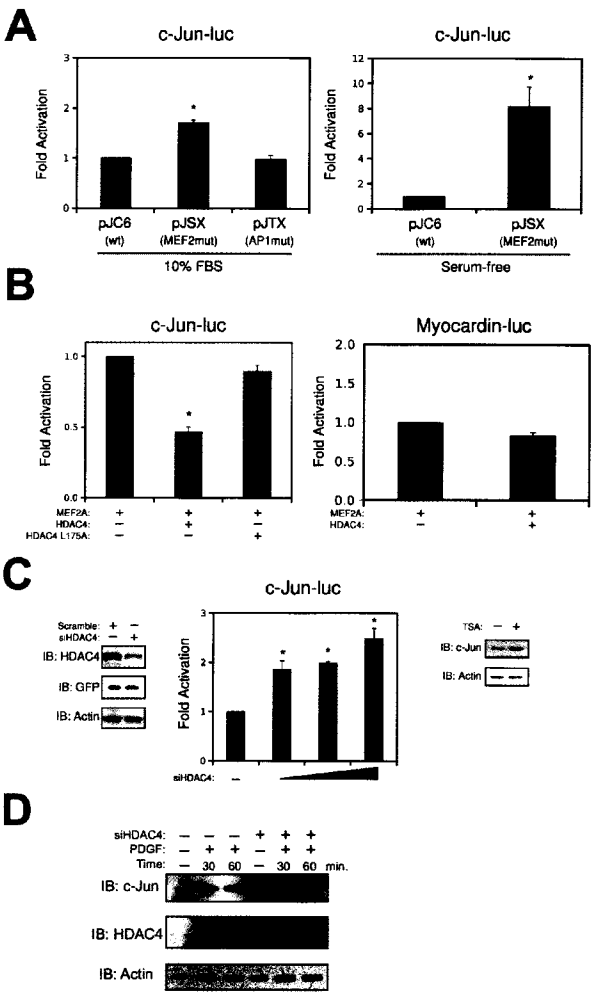

Figure 4. The MEF2 cis element in the c-jun promoter acts as a repressor element in quiescent VSMCs.

A) A10 cells were transfected with a wild-type *c-jun* promoter (pJC6), a *c-jun* reporter with the MEF2 binding site mutated (pJSX), or a *c-jun* reporter gene with the AP1 site mutated (pJTX). Cells were harvested for luciferase under growth conditions (ie. 10% FBS) or in serum-free DMEM. B) A10 cells were transfected with wild-type c-Jun-luc or myocardin-luc, with MEF2A, HDAC4, or HDAC4 L175A, as indicated. C) VSMCs were tranfected with a specific siRNA targeted to HDAC4 (siHDAC4) or a scrambled non-specific oligonucleotide in pSilencer H3 (Ambion). Following transfection, positive cells were selected using puromycin, followed by immumoblot analysis. For luciferase, increasing amounts of siHDAC4 were transcfected with wild-type c-Jun-luc. Growth arrested A10 cells were treated with TSA (1 μM, Sigma) for 2 hours prior to harvesting. D) A10 cells were transfect siHDAC4 or scrambled control. Following recovery, positive cells were selected using puromycin, following by overnight quiescence in serum-free media. Cells were stimulated with 20 ng/mL of PDGF, as indicated and subjected to immunoblot analysis. (* $p<0.05$ was considered statistically significant).

This was not the case for other MEF2-dependent reporter-genes, as mutation of the MEF2 *cis* element in the myocardin and HRC promoters did not result in activation (Figure S2). Furthermore, ectopic expression of HDAC4 resulted in enhanced repression of the *c-jun* promoter, whereas ectopic expression of a mutant HDAC4 that cannot bind MEF2 proteins (HDAC4 L175A) or HDAC5 had no effect (Figure 4B and S2). This repressive effect appears to be specific to *c-jun*, in that exogenous expression of HDAC4 had no effect on the myocardin and HRC promoters (Figure 4 and S2). Consistent with these observations, suppression of HDAC4 expression by specific siRNA resulted in dose-dependent activation of the *c-jun* promoter, while treatment of quiescent smooth muscle cells with the deacetylase inhibitor, trichostatin A (TSA), resulted in an increase in c-Jun expression (Figure 4C). Lastly, we evaluated the effect of the HDAC4 siRNA on endogenous c-Jun expresion. Figure 4D demonstrates a modest increase in c-Jun expression in quiescent VSMCs; however, when A10s cells were stimulated with PDGF, we observed an accelerated induction of c-Jun. Collectively, these data implicate MEF2, in conjunction with HDAC4, in the repression of the *c-jun* gene in quiescent conditions.

PKA Represses c-Jun Expression by Promoting the Nuclear Accumulation of HDAC4. We have recently documented that PKA inhibits MEF2 transcriptional activity in skeletal muscle cells, in part, by promoting the nuclear accumulation of class II HDACs (25). In addition, thrombin induction of c-Jun has been shown to be inhibited by cAMP in VSMCs, yet the mechanism for this phenomenon has not been completely elucidated (378). Therefore, we evaluated whether cAMP-mediated PKA activation could inhibit c-

Jun induction by PDGF. As shown in figure 5A, the *c-jun* promoter is inhibited by combined treatment with the β-adrenergic agonist isoproteronol and phosphodiesterase (PDE) inhibitors. In addition, pretreatment with isoproteronol, and the PDE3 inhibitor milrinone, completely prevented the induction of c-Jun by PDGF in cultured VSMCs. This suppression of c-Jun expression could be rescued with the addition of PKA inhibitors, Rp-cAMPS and H89 (Figure S3); however, these pharmacological inhibitors were somewhat toxic in this cell line, similar to previously published work in A7r5 VSMC treated with the β-adrenergic receptor antagonist, propanolol (389). In addition, ectopic expression of the catalytic subunit of PKA, reduced the expression of the wild-type *c-jun* reporter-gene, but not when the MEF2 *cis* element was mutated (Figure 5B). This effect was specific to *c-jun*, in that PKA failed to inhibit the expression of other smooth muscle marker genes, such as smooth muscle myosin heavy chain, myocardin, and HRC (Figure 5D). Furthermore, figure 5B demonstrates that a MEF2-driven luciferase reporter is attenuated by a cAMP analog, milrinone, isoproteronol, and forskolin. Similar results were also obtained by ectopic expression of PKA (Figure S3).

In order to identify a mechanism underlying PKA's inhibition of MEF2-dependent *c-jun* regulation, we utilized Gal4- and VP16-fusions of MEF2A and -D. As shown in figure 5C, PKA could not inhibit the Gal4-MEF2 fusion proteins that lack the N-terminal class II HDAC binding domain, but readily attenuated the activity of the MEF2A-VP16 fusion proteins that contain the class II HDAC binding domain.

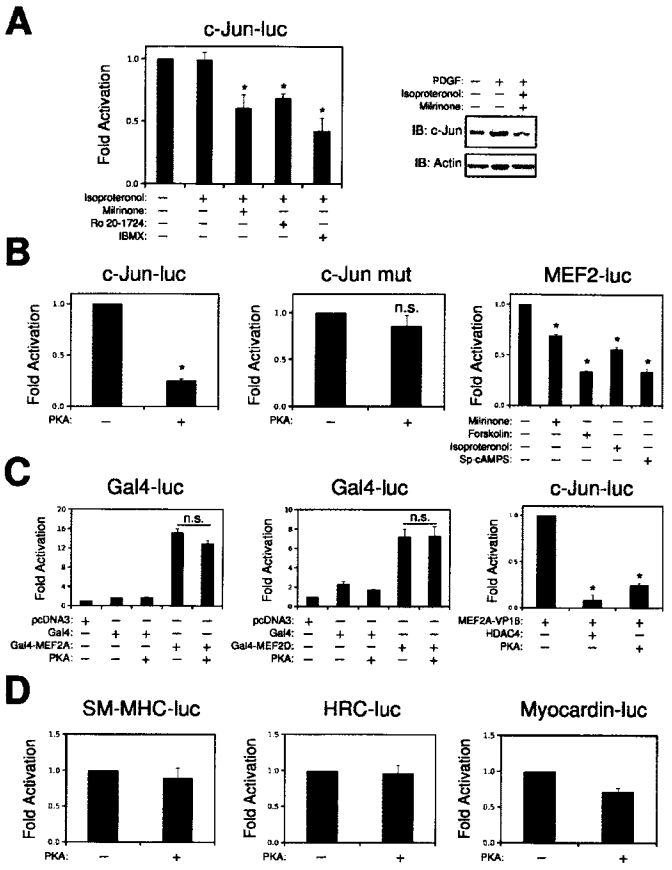

Figure 5. PKA inhibits induction of the c-jun promoter through a MEF2-dependent mechanism.

A) A10 cells transfected with c-Jun-luc were treated with isoproteronol (1 μM), milrinone (10 μM), Ro 20-1724 (10 μM), or IBMX (500 μM) as indicated overnight. Serum-starved A10s were pre-incubated with milrinone (10 μM) and isoproteronol (1 μM) for 15 minutes, then treated with PDGF (10 ng/ml), for 2 hours. Protein extracts were prepare and immunoblots preformed for c-Jun. B) A10 cells were transfected with wild-type c-Jun-luc or a construct with a mutation in the MEF2 *cis* element (c-Jun mut), and the catalytic subunit of PKA (pFC-PKA, Stratagene), as indicated. A10 cells were transfected with a reporter containing a consensus MEF2 binding site (MEF2-luc). 24 hours prior to harvesting cells were treated with 20 μM cAMP analog (Sp-cAMPS, Sigma), 10 μM milrinone, 10 μM forskolin, or 1 μM isoproteronol, as indicated. C) A10 cells were transfected with a Gal4- luciferase (Gal4-luc), a Gal4 DNA binding domain (Gal4), a Gal4-MEF2A or -MEF2D fusion containing the C-terminus of MEF2A or -D, with or without PKA, as indicated. c-Jun-luc was transfected with the MEF2A-VP16 fusion with HDAC4, or PKA, as indicated. D) A10 cells were transfected with the smooth muscle myosin heavy chain (SM-MHC), HRC, or myocardin enhancer reporter-genes and pFC-PKA, as indicated. Cells were harvested for luciferase 24 hours after recovery. (n.s., not significant, * $p<0.05$ was considered statistically significant).

In addition, figure 6A and 6B demonstrate that activation of PKA increases the nuclear localization of HDAC4, as determined by immunofluorescence and nuclear/cytosolic fractionation. In addition, our previous work has shown that ectopic expression of PKA enhances the interaction between MEF2 and HDAC4, determined by CO-IP in COS7 cells (25). Figure 6C demonstrates that activation of endogenous PKA by treatment with isoproteronol and milrinone increases the association of HDAC4 with MEF2A in A10 VSMCs. Lastly, Figure 6D demonstrates that HDAC4 is required for PKA's inhibition of the *c-jun* promoter, in that reduced expression of HDAC4 by siRNA targeting prevented the attenuation of the c-Jun reporter-gene by the catalytic subunit of PKA.

PKA Enhances the Nuclear Accumulation of HDAC4 by Inhibiting the HDAC-Kinase SIK1. Recent studies in other cells types have identified the salt-inducible kinase 1 (SIK1) as a potential PKA regulated HDAC-kinase (123, 124). Therefore, we evaluated SIK1's role in MEF2-dependent *c-jun* expression in VSMCs. As shown in figure 7A, ectopic expression of SIK1 in quiescent VSMCs resulted in nuclear export of a HDAC4-GFP fusion protein. Furthermore, forced expression of SIK1 resulted in activation of the *c-jun* reporter-gene (Figure 7B). However, the addition of the catalytic subunit of PKA resulted in attenuation of SIK1's induction of *c-jun*. PKA has been shown to inhibit SIK1 by direct phosphorylation of serine 577, and a neutralizing mutation of this residue to alanine (SIK1 S/A) is sufficient to eliminate this effect (123).

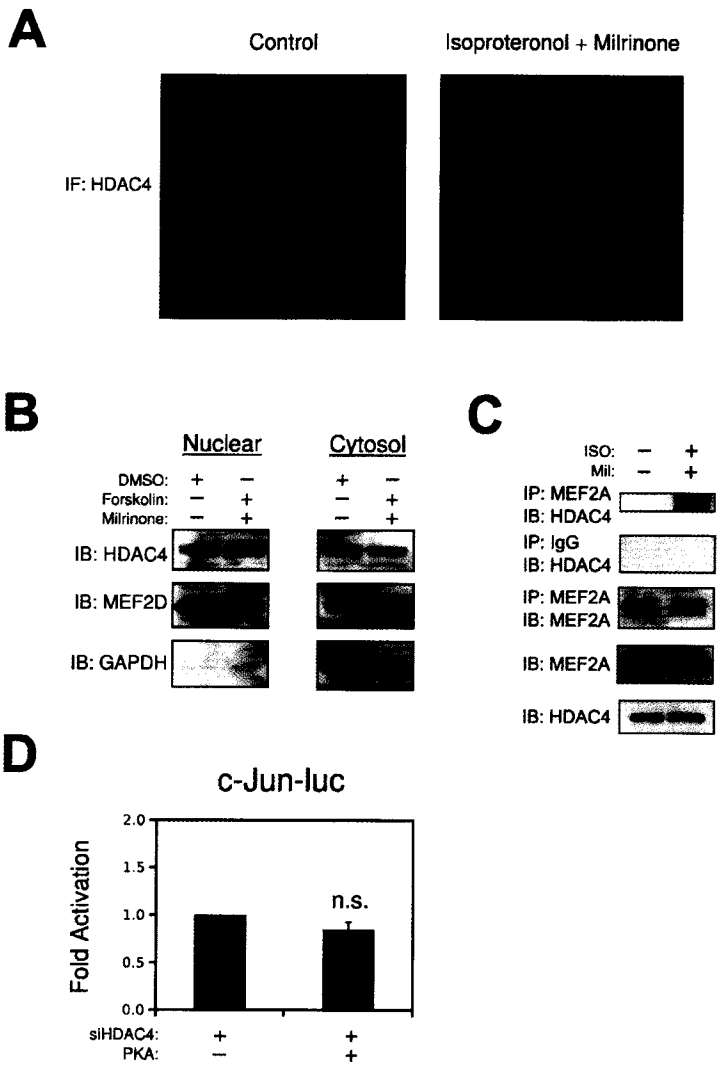

Figure 6. PKA inhibits c-jun expression through HDAC4.

A) Growth phase VSMCs were treated with milrinone (10 μM, Sigma) and isoproteronol (1 μM) for 2 hours followed by fixation with 4% paraformaldahyde. Fixed cells were then subjected to immunofluorescence with an HDAC4 primary antibody (Sigma). B) A10 cells were serum-starved and pretreated with forskolin (10 μM) and milrinone (10 μM, Sigma), or DMSO. Nuclear and cytosolic extractions were immunoblotted for HDAC4, MEF2D, and GAPDH. C) A10 cells were transfected with MEF2A and HDAC4 and treated for 2 hours with isoproteronol and milrinone. Protein extracts were subjected to immunoprecipitation (IP) and immunoblotting (IB) as indicated. D) VSMCs were transfected with c-Jun-luc, siHDAC4, or PKA, as indicated. (n.s., not significant)

As shown is figure 7B, the SIK1 mutation is still capable of activating the *c-jun* reporter-gene; however, PKA is not able to inhibit this mutated SIK1. Consistent with this finding, PKA could not inhibit the nuclear export of HDAC4-GFP by the mutated SIK1 in COS7 cells (Figure 7D). Therefore, these data indicate that PKA inhibits *c-jun* expression in VSMCs by inhibiting SIK1 and promoting the nuclear accumulation of HDAC4.

Exogenous Expression of c-Jun Prevents Myocardin's Induction of Smooth Muscle Marker Genes. Although numerous studies have evaluated the role of c-Jun on the activated smooth muscle phenotype, to our knowledge, no such reports exist evaluating the role of c-Jun on smooth muscle differentiation. A recent study has highlighted the role of insulin-like growth factor 1 (IGF-1) and PI3' kinase/AKT signaling in the promotion of smooth muscle differentiation by activating the transcriptional activity of myocardin (306). Therefore, we utilized this model of VSMC differentiation to evaluate *c-jun* expression. As shown in figure 8A, treatment of VSMCs with IGF-1 resulted in increased expression of smooth muscle alpha-actin (SMA). Interestingly, IGF-1 treatment simultaneously downregulated *c-jun*, where this effect was dependent on the MEF2 *cis* element. Therefore, we speculated that c-Jun could negatively modulate smooth muscle differentiation. In support of our hypothesis, constitutive expression of c-Jun attenuated myocardin's induction of smooth muscle reporter-genes for smooth muscle myosin heavy, smooth muscle alpha-actin, and calponin (Figure 8B).

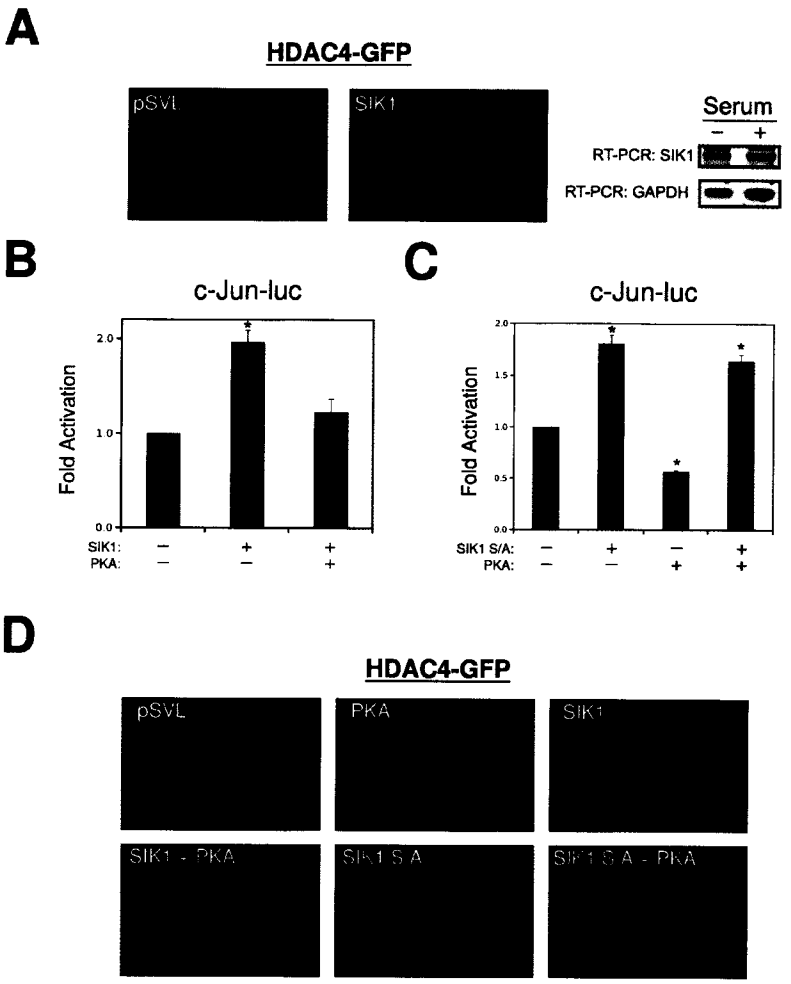

Figure 7. PKA inhibits HDAC4 nuclear export through SIK1.

A) VSMCs were transfected with HDAC4-GFP and SIK1 or empty pSVL. Following serum starvation micrographs were obtained by standard fluorescent techniques. Growth phase A10s in 10% FBS (+) or serum-free media (-) were harvested for total RNA and subjected to RT-PCR for SIK1 and GAPDH. B) and C) A10 cells were transfected with c-Jun-luc, SIK1, SIK1 S577A, or PKA, as indicated. D) COS7 cells were transfected with HDAC4-GFP, and SIK1, SIK1 S577A, or PKA, as indicated. Micrographs were obtained following 24 hours of recovery.

In addition, we utilized a 10T1/2 conversion assay as a model to evaluate the role of c-Jun in smooth muscle differentiation. As shown in figure 8C, ectopic expression of the smooth muscle isoform of myocardin (Myocardin 856) was sufficient to induce the endogenous expression of smooth muscle α-actin and smooth muscle myosin heavy chain, a definitive marker of the smooth muscle lineage (390). However, when c-Jun was co-expressed with myocardin, expression of these smooth muscle marker genes was attenuated. We hypothesized that c-Jun might attenuate the activation of myocardin by competing for a common co-activator. Previous, studies have shown that both c-Jun and myocardin interact with the histone acetyltransferase and coactivator, p300 (89, 391). However, ectopic expression of p300 could not substaintially rescue myocardin's transciptional activity once repressed by c-Jun (not shown). Therefore, we choose to evaluate whether c-Jun might inhibit myocardin through a physical interaction. This hypothesis seemed reasonable, given that c-Jun has previously been shown to physically interact and repress the transcriptional activation of other muscle-restricted transcription factors, like MyoD (392, 393). Figure 8D demonstrates that myocardin immunoprecipitated with an antibody targeted to c-Jun when co-expressed in COS7 cells. The antibody to c-Jun resulted in a greater immunoprecipitation of myocardin that a control rabbit IgG (not shown). To validate this interaction between c-Jun and myocardin, we performed a mammalian two-hybrid assay in 10T1/2 cells using Gal4-c-Jun and myocardin-VP16 fusion proteins.

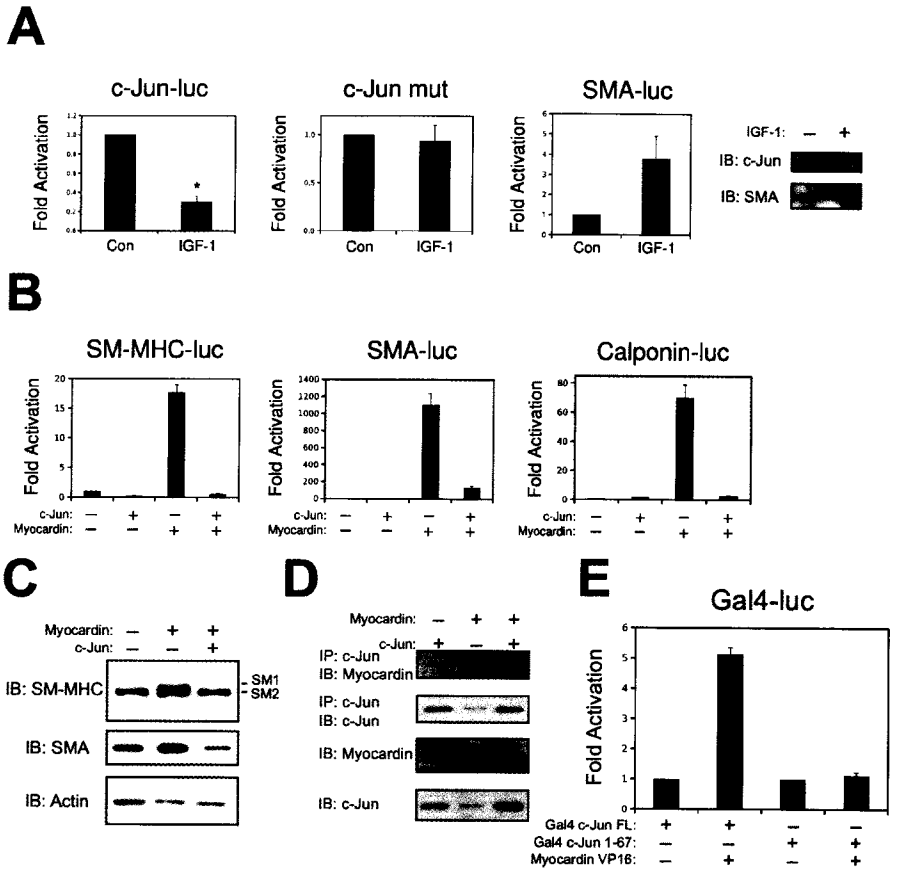

Figure 8. Downregulation of c-Jun is critical for VSMC differentiation.

A) VSMCs were transfected with wild-type c-Jun-luc, c-Jun-luc containing a mutation in the MEF2 binding site (c-Jun mut), or a smooth muscle alpha-actin reporter gene (SMA-luc). Quiescent cells were treated with 50 ng/mL of IGF-1 overnight and havested for luciferase extracts. Protein extracts from overnight treated IGF-1 A10 cells were subjected to immunobloting for c-Jun or smooth alpha-actin (SMA; Sigma) B) 10T1/2 fibroblasts were transfected with smooth myosin heavy chain (SM-MHC-luc), smooth muscle alpha-actin (SMA-luc), or calponin (Calponin-luc) reporter genes with expression vectors for c-Jun and the smooth muscle isoform of myocardin (myocardin 856), as indicated. Cells were havested for luciferase 24 hours after recovery. C) 10T1/2 cells were transfected with myocardin 856 and c-Jun, as indicated. After a 24 hour recovery, cells were re-fed in 5% horse serum, and allowed to differentiate for 4 days before harvesting for protein extracts and immunoblotting for SMA or SM-MHC (Biomedical Science). D) COS7 cells were transfected with c-Jun or myocardin 856, as indicated. Protein extracts were subjected to immunoprecipitation (IP) and immunoblotting (IB), as indicated. E) 10T1/2 cells were transfected with a Gal4 reporter-gene, and Gal4-c-Jun fusion proteins containing full-length c-Jun (FL) or amino acids 1-67, with a myocardin-VP16 fusion protein, as indicated. Extracts were subject to luciferase assay.

As shown in figure 8E, myocardin-VP16 could activate the Gal4 fusion protein containing full-length c-Jun, but not a Gal4 fusion protein containing the N-terminal transcriptional activation domain of c-Jun (1-67). This fusion protein lacks the B-zip domain of c-Jun which has been shown to be critical for protein-protein interaction (394). Next, we speculated that if c-Jun can modulate the transcriptional activity of myocardin by physical interaction, myocardin might inhibit AP-1 dependent transcription. To evaluate this, we ectopically expressed myocardin with the AP-1-dependent promoter for matrix metaloprotease 9 (MMP-9) (395). Previous studies shown that MMP-9 is involved in both proliferative VSMC disease and degenerating disease, such as aneuysm (387, 396, 397). As shown in figure 9B, myocardin can repress the MMP-9 promoter in A10 VSMCs. Taken together, these data support the hypothesis that c-Jun and myocardin are mutual co-regulators that modulate VSMC phenotype in response to growth factor stimulation, such PDGF and IGF-1.

Myocardin was originally identified as an activator of cardiac gene expression, and has been shown to induce cardiac hypertrophy (177, 302). Interestingly, c-Jun expression can be induced by cardiac wall stress and hypertrophy *in vitro* and *in vivo* (398, 399). Therefore, we speculated that the interaction between c-Jun and myocardin might be an important regulator of myocardin-induced activation of cardiac gene expression. As shown in figure 9A, the cardiac isoform of myocardin (myocardin 935) potently activated the promoters for alpha-cardiac actin and alpha-myosin heavy chain.

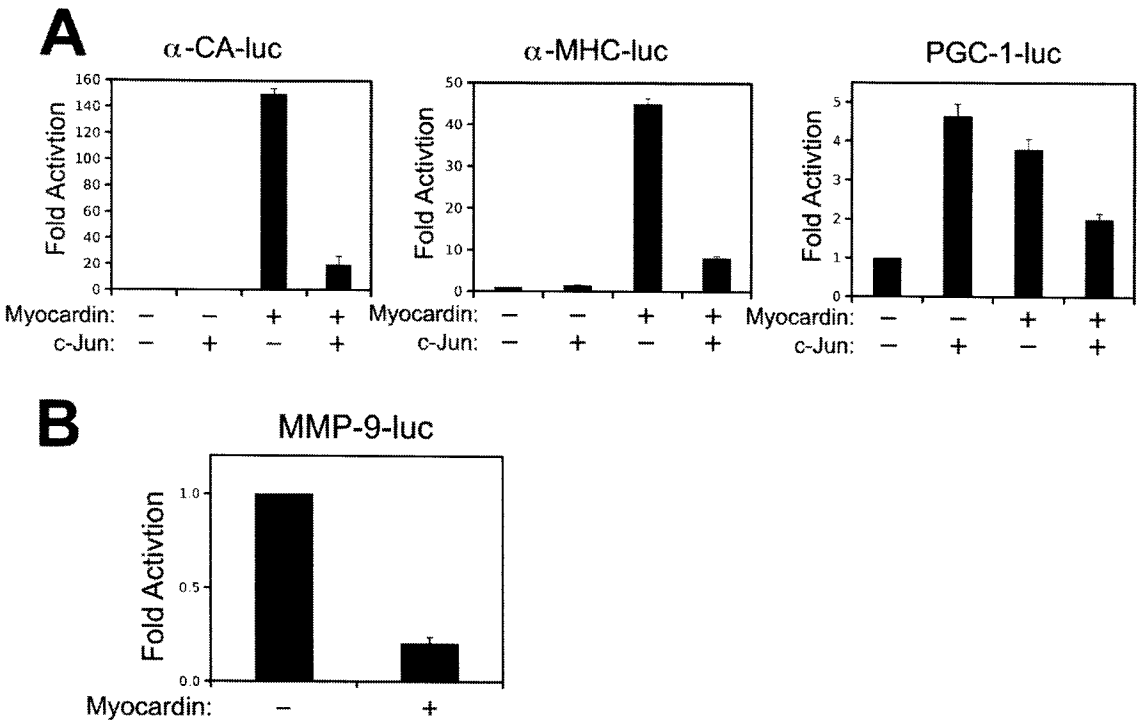

Figure 9. Myocardin and c-Jun are mutual co-regulators.

A) 10T1/2 cells were transfected with the cardiac alpha-actin promoter (α-CA-luc), the alpha-myosin heavy chain promoter (α-MHC-luc), or the PGC-1 promoter (PGC-1-luc), c-Jun, and the cardiac isoform of myocardin (myocardin 935), as indicated. Extracts were subjected to luciferase assay. B) 10T1/2 cells were transfected with the MMP-9 promoter (MMP-9-luc) and myocardin 856, as indicated. Extracts were subjected to luciferase assays.

This induction was nearly completely attenuated by co-expression of c-Jun. The mitochondrial regulator, PGC-1 is induced during cardiac hypertrophy, but is thought to be down-regulated during the progression of heart failure (206). Interestingly, the PGC-1 promoter was induced by both c-Jun and myocardin 935, but co-expression of these transcription factors resulted in attenuation of the induction. Therefore, the interaction of c-Jun and myocardin may have implications to both vascular and cardiac disease.

DISCUSSION

Vascular diseases, such as atherosclerosis and restenosis involve smooth muscle activation characterized by proliferation and migration to sites of injury. In the quiescent non-proliferating state, VSMCs are acted on by protective vasodilators, such as prostacyclin produced from the intact endothelium, and β_2-adrenergic stimulation. Indeed, reduced prostacyclin production in humans by cyclooxygenase inhibition increases the risk of cardiovascular events (377). Prostanoids, like prostacyclin, activate PKA signaling and oppose growth factor-induced VSMC proliferation. However, vascular injury is known to increase the expression of phosphodiesterases (PDEs), which may counteract PKA activation, and allow growth factor-induced proliferation (376, 400). Thus, cAMP-dependent PKA activation may function as a signaling conduit controlling the phenotype of VSMCs. We report that these dilators can also function at the level of regulation of gene expression, and demonstrate a novel role of PKA signaling to

modulate MEF2-dependent repression of *c-jun* expression, a critical regulator of VSMC proliferation.

MEF2 proteins have been most extensively studied in striated muscle, where they are intimately involved in muscle development and various postnatal phenotypes (3). The role of MEF2 in vascular smooth muscle cells is less well characterized although a role for MEF2C in VSMC differentiation and vascular ontogeny has been invoked (8). VSMCs represent an interesting model in which to study MEF2 site-directed gene expression, since VSMCs maintain the ability to modulate their postnatal phenotype in response to environmental stimuli, unlike other MEF2-dependent tissues such as striated muscle and neurons. Given the importance of the MEF2-target genes, myocardin and *c-jun* to their respective quiescent and activated smooth muscle phenotypes, understanding the regulation of MEF2-dependent gene expression will be key in understanding smooth muscle phenotypic modulation in vascular disease. Like SRF, MEF2 activity is modulated by recruiting co-activators or co-repressors to promoter regions. Thus, it remains likely that site-directed transcriptional control of MEF2 is modulated by the unique combination of *cis* elements present within these promoter regions that constitute a specific promoter architecture that serves to recruit a precise combination of co-factors and transcriptional regulators. Indeed, the regulation of MEF2 proteins by class II HDACs has not been established in VSMCs; however, HDAC5 has been shown to regulate the transcriptional activity of myocardin, and angiotensin-induced smooth hypertrophy is mediated through nuclear export of this histone deacetylase (89, 119).

In this report, we demonstrate that the MEF2 *cis* element in the *c-jun* promoter acts as a repressor element in quiescent VSMCs, where growth factor mediated activation of CaMK and PKC promotes nuclear export of HDAC4 to relieve MEF2 proteins from repression. This observation likely explains the absence of widespread MEF2 activation *in vivo* following vascular injury. Of the various CaMKs, CaMKIIδ appears to be the most likely kinase involved in c-Jun induction, given that recent evidence has demonstrated a critical role of this isoform during neointima formation; whereas, CaMKIV has been implicated in VSMC differentiation (318, 401). In addition, we demonstrate that PKA can enhance the repression of *c-jun* by increasing the nuclear localization of HDAC4 through inhibition of SIK1. This repression of *c-jun* is of fundamental importance for VSMC differentiation, in that forced expression of c-Jun inhibits myocardin's ability to activate smooth muscle-dependent gene expression.

PKA has been previously implicated in inhibition of VSMC proliferation and migration through inhibition of the MEK/ERK MAP kinase signaling pathway (376). In addition, PKA has been implicated in promoting VSMC differentiation and increasing the expression of smooth muscle marker genes, such as SM-MHC (402). Our data suggests that PKA does not directly increase the activity of smooth muscle promoters (Figure 4), but promotes competence for smooth muscle differentiation through down-regulation of c-Jun.

PKA signaling is terminated by PDE enzymes that hydrolyze cyclic nucleotides to 5' nucleotide monophosphates that do not activate PKA (400). Numerous studies have

implicated PDE3 and PDE4 isoforms as the dominant cAMP metabolizing enzymes in VSMCs, and there is reported synergism between adenylate cyclase activators, PDE3 and/or PDE4 inhibitors in terms of VSMC relaxation, and inhibition of proliferation and migration (400). This is consistent with our data, in that combined treatment of isoproteronol and milrinone completely inhibited PDGF induction of c-Jun (Figure 4).

PKA signaling has also been shown to have anti-inflammatory effects in VSMCs, where inhibition of PDE3 by cGMP signaling inhibits tumor necrosis factor-α (TNFα)-induced activation of NFκB-dependent gene expression (403). Interestingly, MEF2 proteins have been shown to play a role in VSMC inflammation through a consensus MEF2 *cis* element in the promoter of the monocyte chemoattractant protein (MCP-1) gene (241). Indeed, our preliminary evidence suggests that PKA inhibits the activity of a MCP-1 reporter-gene (figure S3). Thus, it appears that PKA mediated repression of MEF2-dependent gene expression will inhibit multiple components of the activated smooth muscle phenotype.

Interestingly, the phenotypic alterations mediated by PKA signaling differs between striated and VSMCs. Recent evidence from our laboratory has demonstrated that PKA can directly phosphorylate MEF2 proteins *in vivo* to inhibit skeletal muscle differentiation (25). In addition, transgenic mice expressing the catalytic subunit of PKA in the heart develop a dilated myopathy with downregulation in MEF2-dependent cardiac-marker genes (57).

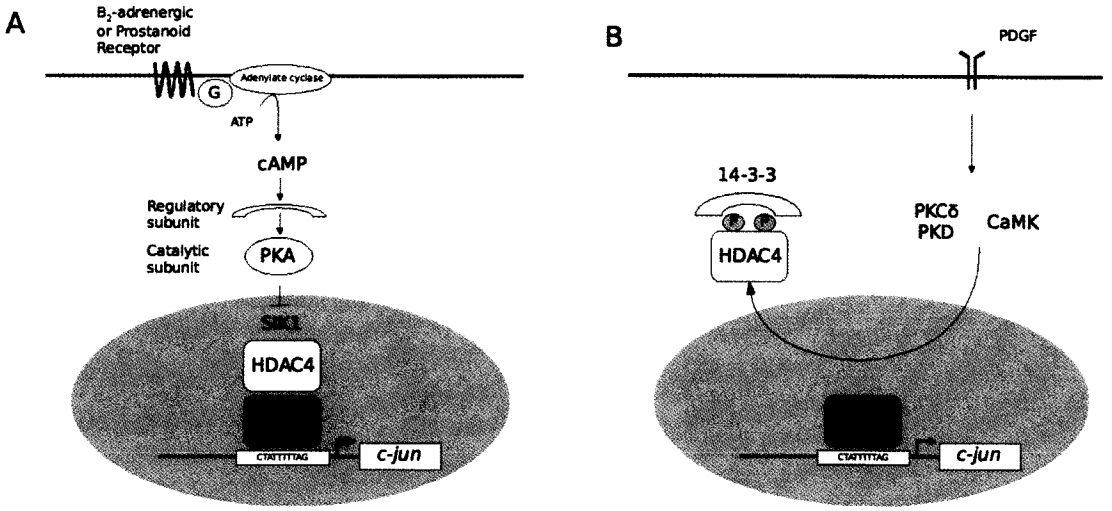

Figure 10. Model of c-jun regulation in VSMCs.

A) In quiescent conditions, *c-jun* expression is repressed by a MEF2/HDAC4 complex, which is promoted by PKA-induced inhibition of SIK1. B) Growth factor (ie. PDGF) stimulation of VSMCs results in PKCδ/PKD- and CaMK-induced derepression through HDAC4 nuclear export, and MEK/ERK-dependent activation of *c-jun*.

However, in VSMCs, PKA inhibits proliferation, and, in contrast to striated muscle, may enhance smooth muscle differentiation. Therefore, based on our work, and the work of other laboratories, we propose that PKA regulated inhibition of MEF2-dependent gene expression can result in different outcomes depending on the cellular context.

In summary, these studies support a novel link between MEF2 and the growth responsive *c-jun* gene in quiescent VSMCs (Figure 10), in which repression of *c-jun* expression is promoted by agents that elevate cellular cAMP such as prostacyclin or β_2-adrenergic stimulation. This effect involves a mechanism in which PKA activation promotes the assembly of a MEF2/HDAC4 repressor complex. In view of the fundamental role of c-Jun as a modulator of VMSC differentiation, it will be important to determine whether MEF2 can mediate a protective effect of clinical relevance for vascular injury and disease.

ACKNOWLEDGEMENTS

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SUPPLEMENTAL FIGURES FOR MANUSCRIPT 1

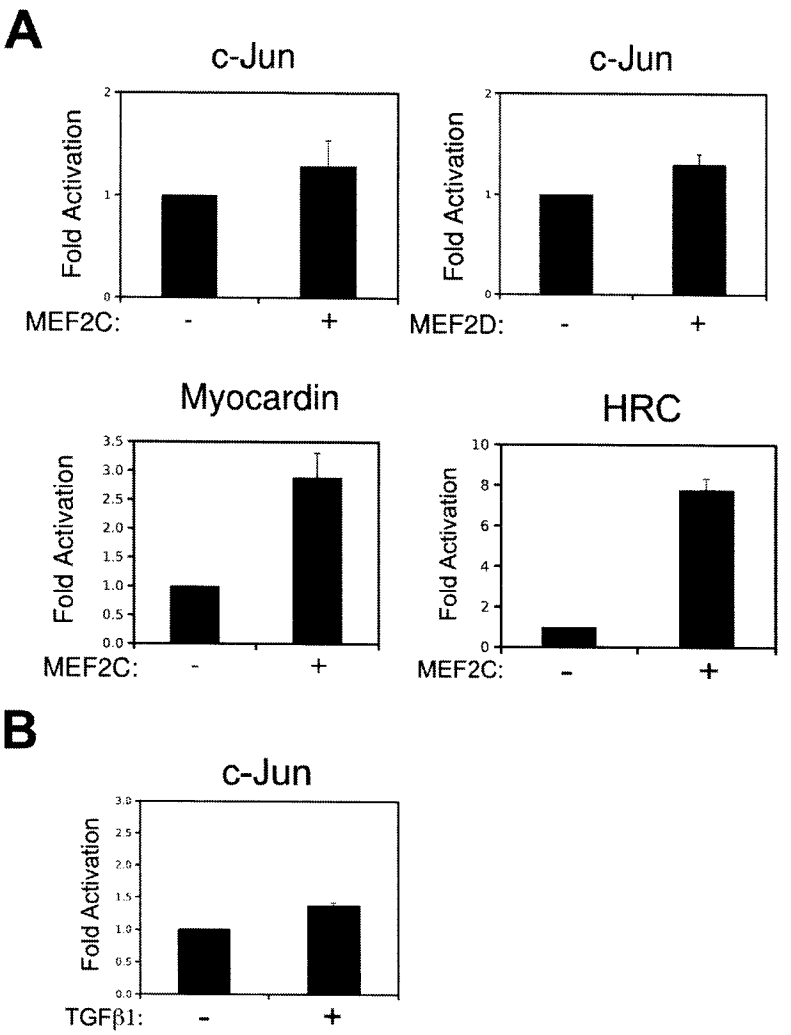

Figure S1. MEF2 activates of the myocardin reporter gene but not c-jun in VSMCs. A) A10 cells were transfected with the c-jun, HRC, or myocardin enhancer reporter-genes and either empty pcDNA3 or MEF2C, or MEF2D. Cells were harvested for luciferase 24 hours after recovery. B) A10 cells transfected with the *c-jun* reporter gene were treated overnight with TGF-β1 (2 ng/mL) and harvested for luciferase assay.

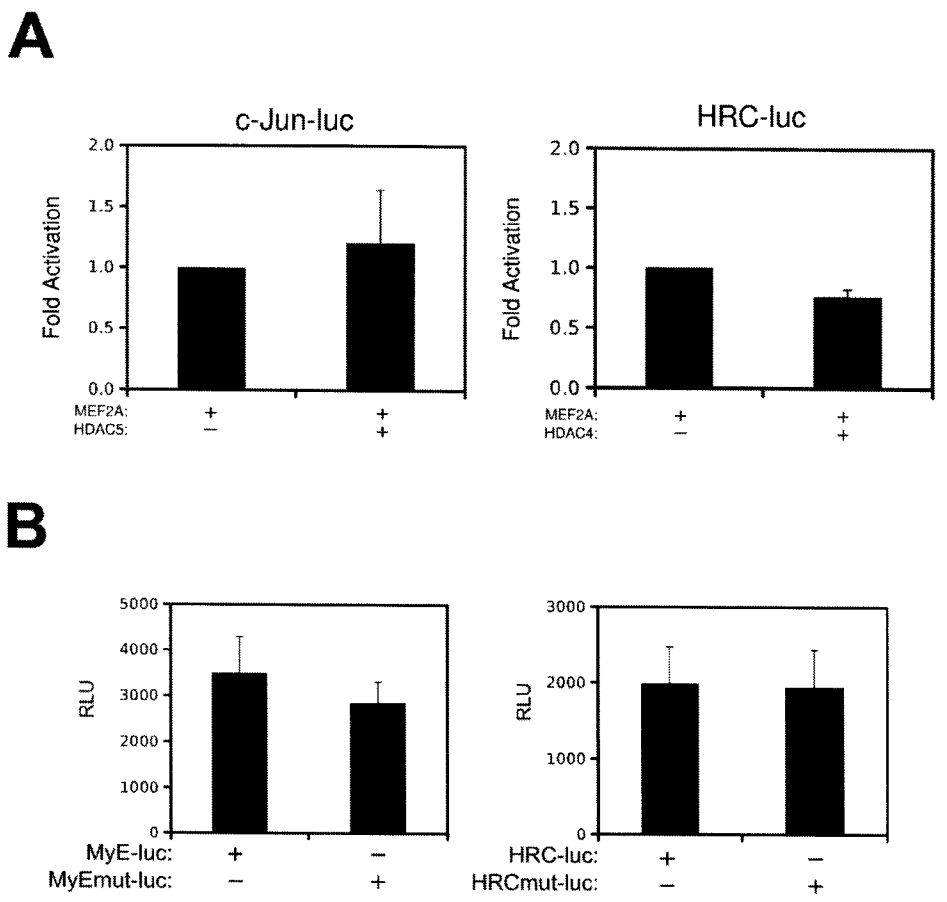

Figure S2. HDAC5 does not inhibit the c-jun promoter and HDAC4 does not inhibit the HRC promoter.

A) A10 cells were transfected with the HRC reporter-gene and pMT2-MEF2A and/or Flag-HDAC4, or Flag-HDAC5, as indicated. Cells were harvested for luciferase 24 hours after recovery. B) A10 cells were transfected with the myocardin-enhancer reporter gene (MyE-luc), a myocardin-enhancer with the MEF2 binding site mutated (MyEmut-luc), the HRC promoter (HRC-luc), or the HRC promoter with a mutation in the MEF2 binding site (HRCmut-luc). Cells were harvested for luciferase assay.

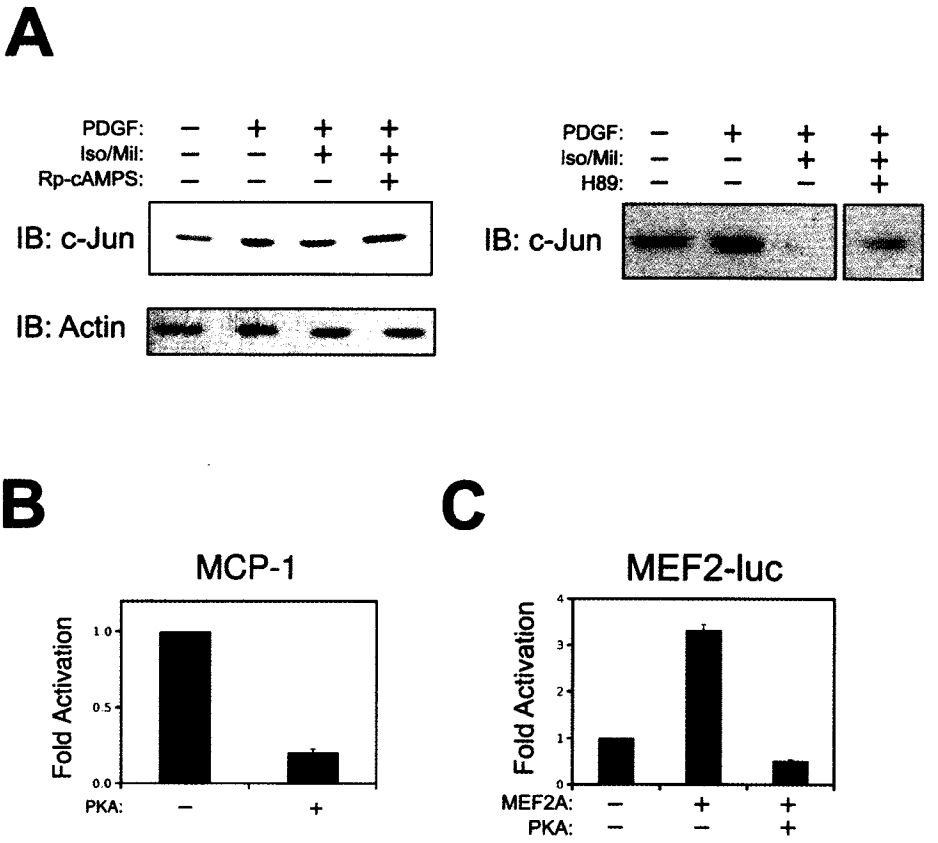

Figure S3. Effect of PKA on c-Jun expression and the MCP-1 promoter.
A) Quiescent A10 cells were pretreated with isoproteronol (1 μM), milrinone (10 μM), Rp-cAMPS (10 μM), or H89 (10 μM), as indicated, and stimulated with PDGF (20ng/ mL) for 1 hour. Protein extracts were subjected to immunoblotting. B) A10 cells were transfected with the MCP-1 reporter-gene and pFC-PKA, as indicated. Cells were harvested for luciferase 24 hours after recovery. C) A10 cells were transfected with a MEF2 reporter gene (MEF2-luc), pMT2-MEF2A, or pFC-PKA, as indicated. Cells were harvested for luciferase assay.

MANUSCRIPT 2

RATIONALE

This second manuscript focuses on MEF2-dependent myocardin expression in VSMCs. While working on Manuscript 1, it was discovered that myocardin, a SRF co-activator, was a transcriptional target of MEF2, making the genetic connection between MEF2 and SRF-dependent smooth muscle differentiation genes (145). This finding also served to ratify the phenotype of the MEF2C-null mouse, in which SRF-dependent smooth muscle genes fail to be expressed and VSMC do not differentiate (8). This manuscript addresses the last two objectives identified in the Statement of Purpose, which are: 1) MEF2-dependent myocardin expression is regulated through an interaction with PP1α; and 2) RhoA-signalling activates myocardin expression through p38 and the PP1α inhibitor, CPI-17.

These objectives are addressed primarily using cell culture models, such as the A10 cell line and rat primary VSMCs.

RHOA SIGNALING REGULATES MEF2-DEPENDENT MYOCARDIN EXPRESSION IN VASCULAR SMOOTH MUSCLE CELLS THROUGH P38 MAPK AND CPI-17.

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CP: Immunoblots, luciferase assays, immunofluorescence, and COIPs (Figures 1C, 2, 4A and -B, 5A, -C, and -D, S1A, and -D, S2C, and -D, S3).
JWG: Designed experiments, wrote the manuscript, luciferase assays, immunoblots, cloned myocardin enhancer, site-directed mutatgenesis, PCR primer design, RT and qPCR, ImageJ analysis.
MD and KWS: Mass spectrometry (Figure 3B).
JCM: Advisor and edited the manuscript.

ABSTRACT

Calcium sensitivity in vascular smooth muscle cells (VSMC) is regulated by RhoA/ROCK-mediated inhibition of the myosin light chain phosphatase complex. Previous studies have demonstrated that this signaling pathway functions in parallel to increase the expression of SRF-dependent smooth muscle-marker genes through regulation of the myocardin-family of SRF co-activators. In concert with SRF, MEF2 fulfills a critical role in VSMC gene expression and can also regulate the expression of myocardin in cardiac and VSMC, leading us to investigate whether the RhoA/ROCK signaling cascade might regulate MEF2-dependent VSMC gene expression. Depolarization-induced activation of calcium signaling in cultured VSMCs increased the expression of myocardin, and the MEF2-dependent immediate early gene, *c-jun*. Interestingly, depolarization-induced expression of myocardin was sensitive to ROCK and p38 inhibition; whereas, induction of c-Jun was sensitive to CaMK inhibition. We have previously identified protein phoshatase 1α (PP1α) as a potent repressor of MEF2 transcriptional activation. In VSMCs, PP1α is the catalytic subunit of the myosin light chain phosphatase, and is regulated by RhoA/ROCK signaling. Furthermore, treatment of VSMCs with the PP1α inhibitor, calyculin A, resulted in increased expression of myocardin, while ectopic expression of PP1α inhibited the induction of myocardin by MEF2C. Consistent these data, exogenous expression of the ROCK-regulated PP1α inhibitor, CPI-17, resulted in rescue of PP1α repression of myocardin expression. Finally,

we identify a novel p38 MAPK (p38) phosphorylation site in MEF2 proteins that confers RhoA-responsiveness to MEF2C. These data provide evidence of a novel signaling cascade that links RhoA-mediated calcium sensitivity to MEF2-dependent myocardin expression in VSMC through a mechanism involving p38 and PP1α regulation of MEF2 proteins. This knowledge could have important implications for both vascular disease and cardiac outflow tract defects.

INTRODUCTION

During development vascular smooth muscle cells (VSMCs) migrate to primitive endothelial tubes while simultaneously executing a program of differentiation (188). Once invested, VSMCs become quiescent and primarily regulate vascular tone (196). However, unlike terminally differentiated striated muscle cell types, VSMCs retain a capacity, referred to as the activated phenotype, to proliferate post-natally in response to vascular injury. This activated phenotype is of particular clinical interest, since it plays an important role in most stenotic vascular diseases described to date (133). The MADS-box (MCM-1, Agamus, Deficiens, Serum response factor) proteins serum response factor (SRF) and the MEF2 family (MEF2A-D) play critical roles in the phenotypic modulation of VSMCs, as these transcription factors are known to regulate both immediate early genes involved in proliferation and migration, and smooth muscle marker genes involved in the contractile phenotype (32, 120, 145). The cellular signals that direct SRF to these

distinct sets of genes have been intensely studied, where SRF physically interacts with the myocardin family of co-activators in contractile VSMCs to induce smooth muscle marker gene expression (287). However, in response to proliferative growth factor stimulation, myocardin is displaced from SRF, in favor of an Elk-1 interaction, to target immediate early gene expression, such as *c-fos* (303). Recently, calcium signaling induced by depolarization has been shown to increase the expression of both SRF-dependent immediate early genes and smooth muscle marker genes (354). Interestingly, the induction of *c-fos* in this model was prevented by calcium/calmodulin dependent kinase (CaMK) inhibition, and the induction of VSMC marker genes was attenuated by RhoA-associated kinase (ROCK) inhibition (354). These results suggest that distinct calcium-mediated signaling pathways regulate these seemingly opposing SRF-dependent genes.

Much less is known regarding the regulation of MEF2-dependent gene expression in VSMCs. Like SRF, MEF2 regulates the expression of immediate early genes, such as *c-jun*, and recent studies have suggested that *c-jun* expression in VSMCs is CaMK-dependent (120). However, MEF2C has also been shown to be genetically upstream of myocardin and of critical importance to VSMC differentiation (8, 145). Yet, the signaling pathways that regulate MEF2-dependent myocardin expression in VSMCs remain unknown. We recently identified protein phosphatase 1α (PP1α) as a potent trans-dominant repressor of MEF2 activity (61). Interestingly, in VSMC PP1α serves as the catalytic subunit of the myosin light chain phosphatase and is regulated by RhoA

signaling to control calcium sensitivity during contraction (313). In this report we document a novel signaling pathway in VSMC that links RhoA-mediated regulation of calcium sensitivity to MEF2-dependent expression of myocardin. This pathway involves the depression of MEF2 from PP1α inhibition by a two-step mechanism involving a novel p38 MAP kinase (p38) phosphorylation site on MEF2C, and by ROCK-mediated activation of the PP1α inhibitor CPI-17. Thus, this is the first report to identify a dominant signaling cascade that regulates myocardin expression in VSMCs, which may prove critical to our understanding of vascular development and stenotic vascular disease.

MATERIALS AND METHODS

Plasmids. MEF2, PP1α, and luciferase constructs were described previously (61, 120). The RhoA L63 and C3 transferase expression vectors were kindly provided by A. Hall, while the CPI-17 expression vector was a generous gift from A. Aitken. Thr38 and Ser98 mutations in CPI-17 and MEF2, respectively, were generated by site-directed mutagenesis.

Primary VSMC and Immortalized Cell Cultures. Primary rat aortic smooth muscle cells were isolated by enzymatic cell dispersion, as described in Hou et. al. (383). Culturing, transient transfections, and luciferase assays of A10 VSMCs, C3H10T1/2 fibroblasts, and COS7 was described previously (120). All luciferase assays were performed in triplicate.

Immunoblot analysis, immunofluorescence, and quantitative RT-PCR. Protein extracts were resolved with SDS-PAGE and transferred to an Immobilon-P membrane, as described previously (120). Primary antibodies included, rabbit myocardin (Santa Cruz), c-Jun (H79, Santa Cruz), p38 (NEB), and CPI-17 (Santa Cruz). Immunofluorescence of culture cells was previously described (61, 120). Total RNA was isolated from primary VSMCs using a Cell-to-cDNA kit (Ambion), and quantitative PCR was performed, as described previously (120). All immunoblots were repeated at least once.

RESULTS

Depolarization enhances MEF2-dependent gene expression through distinct calicium-mediated signaling pathways in VSMCs.

To determine the effect of depolarization-induced calcium signaling on VSMC marker gene expression, cultured VSMCs were treated with 60 mM potassium chloride (KCl). This treatment, in both primary cultures and the A10 cell line, resulted in a nifedipine-sensitive increase in the expression of myocardin, the MEF2-dependent immediate early gene, *c-jun*; as well as SRF-dependent genes (Figure 1A and S1). Although the nifedipine-sensitive induction in myocardin RNA, evaluated by qPCR, was not statistically significant (Figure 1A), these data show a consistent trend with the data shown in Figure S1.

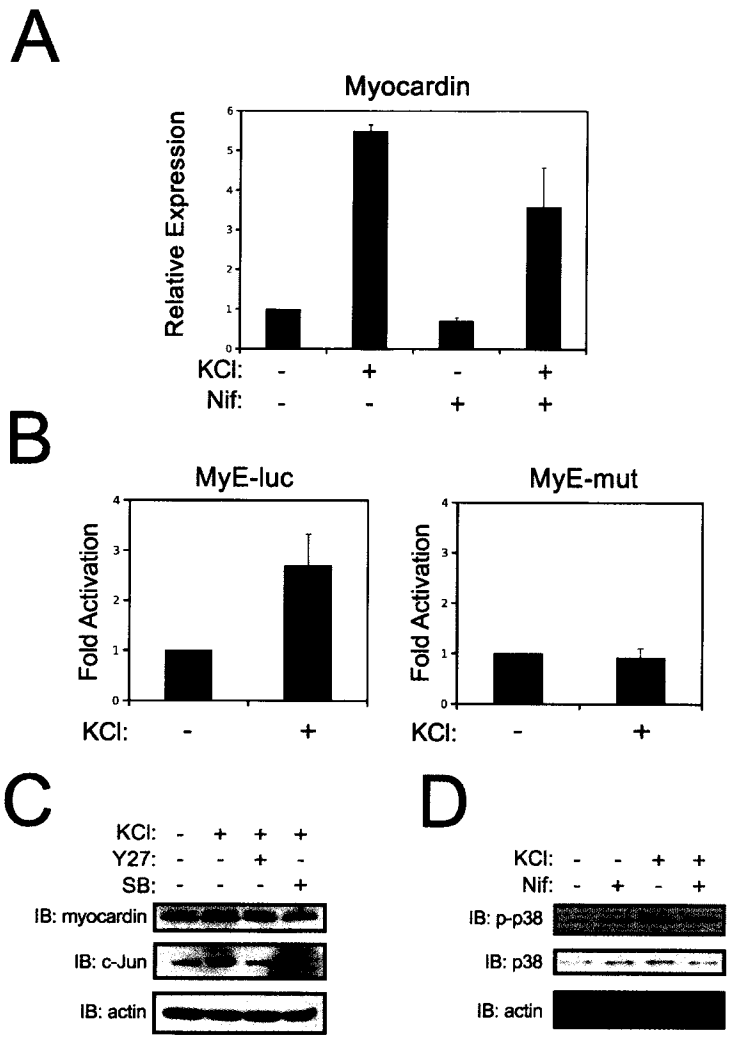


Figure 1. Depolarization-induced Expression of Myocardin Through ROCK and p38.

(A) Primary VSMCs were depolarized with 60 mM KCl, following pre-treatment with 1 μM of nifedipine (Nif), as indicated. Myocardin expression was evaluated by qPCR, corrected for GAPDH (n=2, ANOVA p=0.0764). (B) A10 cells were transfected with a myocardin-enhancer reporter gene (MyE-luc) or with a reporter gene with the MEF2 *cis* element mutated (MyE-mut). Following recovery, cell were depolarized with 60 mM KCl and subjected to luciferase assay. (C) A10 cells were pre-treated with either Y27632 (Y27, 5 μM) or SB203580 (SB, 5 μM) then depolarized. Extracts were subjected to immunobloting as indicated. (D) A10 cells were pre-treated with nifedipine, depolarized, and subjected to immunoblotting.

In addition, the induction of myocardin and c-Jun was found to be dependent on the MEF2 *cis* element found within the promoter region of these genes (Figure 1B and S1). Furthermore, the depolarization-induced expression of c-Jun was attenuated by the CaMK inhibitor, KN-62; whereas, myocardin expression was not attenuated by this inhibitor (Figure S1). We have previously demonstrated that c-Jun expression in VSMCs is regulated by a MEF2-HDAC4 repressor complex (120). Consistent with our previous results, the *c-jun* promoter was repressed by ectopic expression of HDAC4; however, we now demonstrate that this repression can be rescued by co-expression of an activated CaMKIIδ. Surprisingly, HDAC4 repression of *c-jun* could not be overcome by other CaMKs, such as CaMKI or CaMKIV (Figure S1). In addition, depolarization resulted in a reduced nuclear content of HDAC4, suggesting that CaMKII promotes nuclear export of HDAC4 to derepress *c-jun* expression (Figure S1).

Intriguingly, depolarization-induced expression of myocardin was attenuated by the p38 MAP kinase inhibitor, SB203580; whereas, the induction of c-Jun is unaffected by this inhibitor (Figure 1C). In addition, depolarization resulted in a nifedipine-sensitive increase in p38 activity, as indicated by an increase in phosphorylated p38 in response to KCl treatment (Figure 1D). These results indicate that distinct calcium-mediated signaling pathways regulate c-Jun and myocardin expression in VSMCs. Interestingly, the ROCK inhibitor, Y27632, could attenuate both myocardin and c-Jun expression induced by depolarization, which indicates some degree of cross-talk between these two pathways (see below and Figure 2E).

To further evaluate the role of RhoA/ROCK signaling in the regulation of myocardin expression, we utilized a myocardin-enhancer reporter gene that contains a MEF2 *cis* element (MyE). As shown in figure 2, Y27632 inhibited the myocardin enhancer, but not when the MEF2 *cis* element was mutated such that MEF2 can no longer bind.

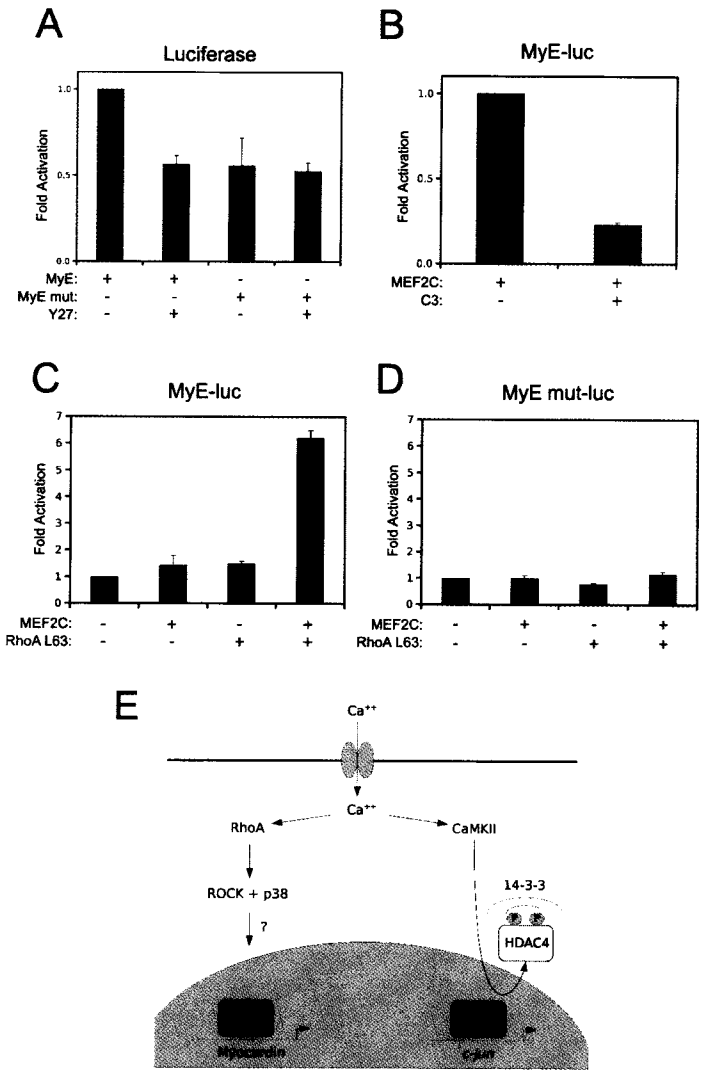


Figure 2. *RhoA Induces Myocardin Expression Through MEF2 Proteins.*

(A) A10 cells were transfected with the myocardin enhancer (MyE) or the enhancer with the MEF2 site mutated (MyE mut). Cells were treated with Y27632 (Y27, 5 μM) and harvested for luciferase assay. (B)(C)(D) Cells were transfected, as described in *(A)* along with a MEF2C, C3 transferase, and/or an active RhoA (RhoA L63) expression vectors. Extracts were subjected to luciferase assays. E) Model of the distinct signaling pathways that regulate MEF2-dependent myocardin and *c-jun* expression in VSMCs.

Additionally, the induction of this reporter-gene by MEF2C was completely prevented by co-expression of C3, a RhoA inhibitor (Figure 2B). Congruently, forced expression of MEF2C and an activated RhoA (RhoA L63) synergistically activate the myocardin enhancer, but again not when the MEF2 *cis* element is mutated (Figure 2C and D). Collectively, these data indicate that the RhoA/ROCK signaling pathway provides an important activating stimulus for MEF2C-induction of myocardin expression in VSMCs (Figure 2E).

p38 MAP kinase phosphorylation of MEF2C at Ser98 confers RhoA responsiveness.

Signals emanating from RhoA have been previously shown to activate p38 signaling and downstream targets in VSMCs (141). To further dissect the role of p38 MAP kinase in the regulation of myocardin expression, we utilized a Gal4 based one hybrid assay comprised of a MEF2C fusion containing the DNA binding domain of Gal4 fused to the C-terminal transcriptional activation domain of MEF2C. We expressed this fusion protein in A10 VSMCs with and without activated RhoA (Figure 3A). Surprisingly, RhoA failed to activate this fusion protein. In addition, neutralizing mutation of the classical p38 phospho-acceptor sites (Thr293, Thr300) had no effect on the transcriptional activity of this fusion protein (Figure 3A). We have previously utilized mass spectrometry to identify novel p38 phospho-residues within MEF2A and have identified several novel p38 phospho-acceptor sites on MEF2 , as well as three potential phospho-acceptor sites that could not originally be fully resolved due to the size of the tryptic phospho-peptide in which they were contained (45).

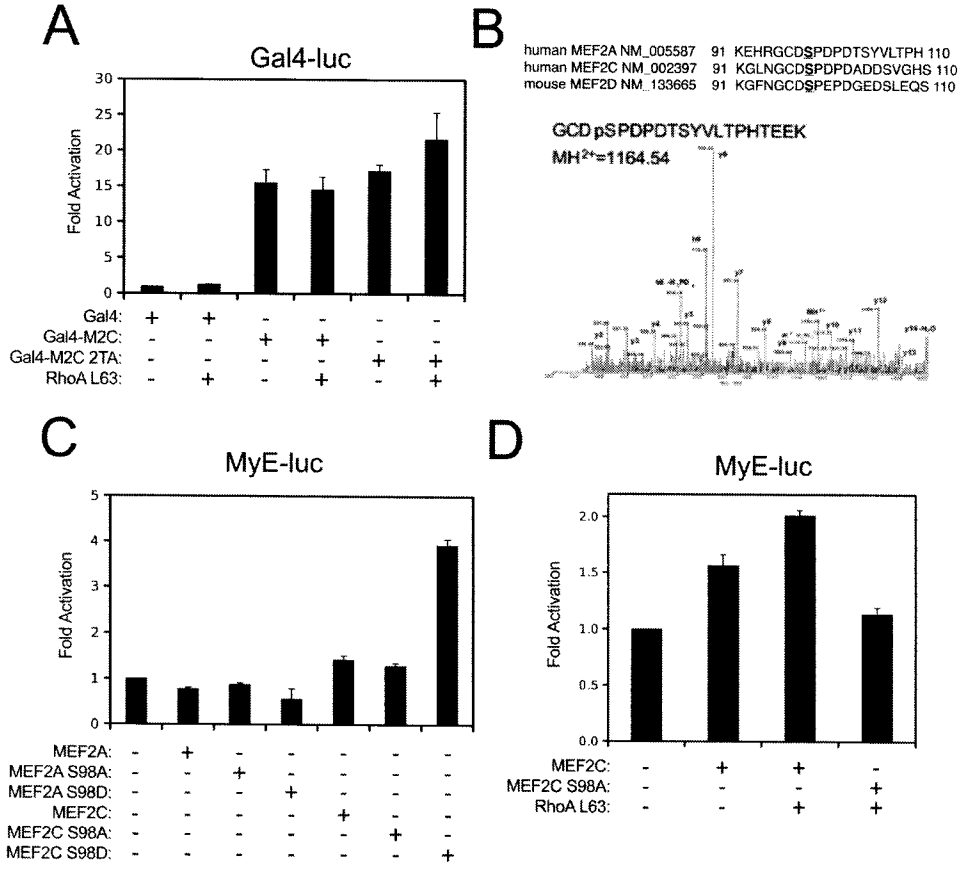


Figure 3. *Serine-98 of MEF2C Confers RhoA-Responsiveness.*

(A) A10 cells were transfected with a 5X-Gal4-luciferase reporter gene (Gal4-luc) and expression vectors for the Gal4 DNA binding domain (Gal4), a Gal4-MEF2C fusion protein lacking the N-terminal MADS/MEF2 domain (Gal4-M2C), a Gal4-MEF2C fusion protein with Thr293 and Thr300 mutated to alanine (Gal4-M2C 2TA), and/or an activated RhoA (RhoA L63), as indicated. (B) Alignment of amino acids 91-110 of MEF2A, -C, and –D. Confirmation of MEF2A-p38 phosphoacceptor sites by MS/MS. The phosphopeptides were identified by CID using precursor ion scanning. MEF2A 95-114 sequence (GCDpSPDPDTSYVLTPHTEEK) (MALDI-MS/MS). (C) COS cells were transfected with the myocardin enhancer (MyE) and either wild-type MEF2A or MEF2C, or MEF2s habouring alanine (S98A) or aspartic acid (S98D) mutations in serine-98. Extracts were sujected to luciferase assay. (D) COS cells were transfected with the myocardin enhancer (MyE), MEF2C, MEF2C S98A, or activated RhoA (RhoA L63), as indicated. Luceriferase assays were performed on cell extracts.

Using peptide mapping and collision-induced dissociation (CID) by nano-LC-MS/MS we have now resolved these phospho-peptides and identified Ser98 as a *bona fide* p38 phospho-acceptor site (Figure 3B). Next, we engineered neutralizing (S98A) and phospho-mimetic (S98D) mutations and evaluated their biological importance. As shown in figure 3C, S98D mutant of MEF2C has greater transcriptional activity on the myocardin enhancer than wild-type MEF2C. However, this was not the case for the S98D mutant of MEF2A, which had lower transcriptional activity than wild-type MEF2A. Finally, figure 3D shows that phosphorylation of Ser98 is required for the RhoA induction of the myocardin enhancer, in that the S98A mutant of MEF2C was completely resistant to RhoA stimulation. Collectively, these data support a model where RhoA activates downstream p38, which phosphorylates MEF2C on Ser98 to activate myocardin expression in VSMCs.

PP1α regulates MEF2-dependent gene expression in VSMCs.

We have recently identified PP1α as a dominant repressor of MEF2 transcriptional activity (61). In smooth muscle, PP1α is the catalytic subunit of the myosin light chain phosphatase complex (314). Shown in figure 4A, we demonstrate that forced expression of PP1α inhibits endogenous myocardin expression and powerfully attenuates the induction of myocardin expression by ectopic expression of MEF2C in cultured A10 cells. In addition, forced expression of PP1α completely prevented the induction of endogenous myocardin expression by activated RhoA (Figure 4B).

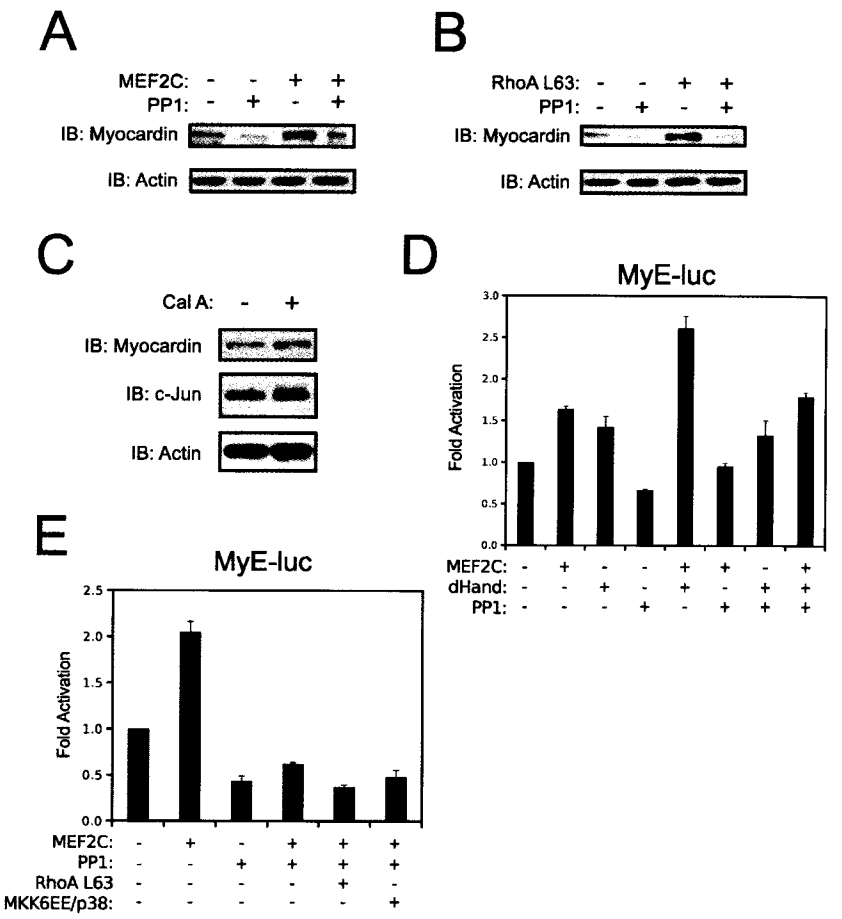


Figure 4. *Myocardin Expression is Opposed by PP1α.*

(A)(B) A10 cells were transfected with MEF2C, PP1, or activated RhoA (RhoA L63) using Lipofectamine reagent and puromycin-selected overnight. Protein extracts were subjected to immunoblotting as indicated. (C) A10 cells were treated with Calyculin A (0.5 ng/mL) for 2 hours. Protein extracts were immunoblotted as indicated. (D) COS7 cells were transfected with the myocardin enhancer, MEF2C, dHand, and/or PP1 as indicated. Luciferase assays were performed on the cells extracts. (E) COS cells were transfected with the myocardin enhancer (MyE), MEF2C, PP1α (PP1), activated RhoA (RhoA L63), or activated MKK6 and p38 (MKK6EE/p38), as indicated. Extracts were subjected to lucifierase assay.

Furthermore, we utilized the PP1α inhibitor, calyculin A, to address the role of PP1α in MEF2-dependent genes expression in VSMCs. As shown in figure 4C, calyculin A treatment resulted in a modest increase in the expression of both myocardin and c-Jun. Thus, ROCK regulation of PP1α might be an important mechanism for the attenuated expression of myocardin and c-Jun with Y27632 treatment (see below). MEF2C has been previously shown to interact with the bHLH transcription factors of the Hand (heart and neural crest derived) family (71). In addition, genetic ablation of MEF2C, dHand (Hand2), and myocardin all result in some degree of neural crest-derived vascular defect (9, 199, 231). Therefore, we chose to evaluate whether PP1α could inhibit a functional cooperation between MEF2C and dHand. As shown in figure 4D, MEF2C and dHand cooperatively activated the myocardin enhancer, yet forced expression of PP1α attenuated this effect. Next, we evaluated whether forced expression of activated p38 or RhoA might be able to overcome PP1α repression of MEF2C to induce myocardin expression. However, as shown in figure 4E, once repressed by PP1α, MEF2C is unresponsive to activated MKK6/p38 or RhoA signaling.

To further evaluate the role of PP1α in c-Jun expression, we performed a titration of calyculin A in A10 cells and observed first an increased c-Jun expression, followed by an increase in phosphorylated c-Jun at higher concentrations of calyculin A (Figure S2). Previous studies in lung epithelial cells have shown that calyculin A can activate JNK signaling to induce c-Jun phosphorylation (404). This appears to be consistent in VSMCs, as phosphorylation of c-Jun by calyculin A treatment is attenuated by pre-treatment with

the JNK inhibitor, SP600125 (Figure S2). In addition, our previous work has shown that PP1α helps recruit HDAC4 to MEF2 proteins, and that HDAC4 acts to repress c-Jun expression, but not myocardin expression, in VSMCs (61, 120). Thus, it appears that PP1α acts to repress c-Jun expression, in part, by inactivating JNK activity and recruiting HDAC4 to MEF2 proteins; however, myocardin expression appears to be regulated in a different manner.

CPI-17 rescues MEF2 repression by PP1α.

In VSMCs, the myosin light chain phosphatase is regulated by a smooth muscle-enriched phosphatase inhibitor, called PKC-potentiated protein phosphatase inhibitor of 17 kDa (CPI-17). CPI-17 physically interacts with PP1α to inhibit its activity (405). In addition to being potentiated by PKC, CPI-17 has also been shown to be activated by ROCK and PKN (361, 362). Therefore, we determined if CPI-17 could inhibit PP1α repression of MEF2 activity. As shown in figure 5B, CPI-17 can antagonize PP1α repression of the myocardin enhancer and restore the activation induced by MEF2C. In addition, figure 5A demonstrates that CPI-17 can compete away the physical interaction between MEF2C and PP1α, determined by co-immunoprecipitation. CPI-17 is activated by phosphorylation at Thr38, and stuctural analysis predicts that phospho-Thr38 serves to anchor the interaction with PP1α, resulting in maximal phosphatase inhibition (406, 407).

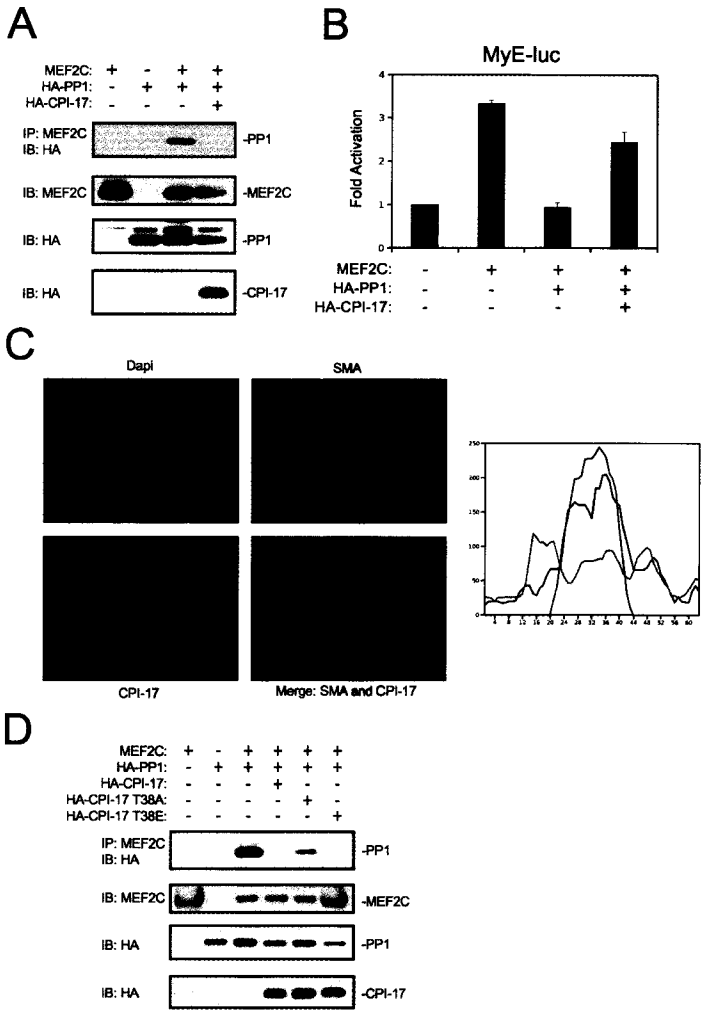


Figure 5. *PP1-Induced Repression of Myocardin is Attenuated by CPI-17.*

(A) COS7 cells were transfected with MEF2C, HA-PP1, or HA-CPI-17 as indicated. Extracts were immunoprecipitated with MEF2C antibody and immunoblotted for antibodies to HA. Proteins extracts were immunoblotted, as indicated, to demonstrate equal loading and efficiency. (B) 10T1/2 fibroblasts were transfected with the myocardin enhancer, and MEF2C, PP1, or CPI-17 as indicated, followed by luciferase assay. (C) Primary VSMCs were fixed, permeablized, and subjected to immunofluorescence for CPI-17, smooth muscle actin (SMA), and the Dapi nuclear stain. Cells were visualized by standard fluorescence techniques. Relative fluorescence of a representative cell was graphed with ImageJ.(D) COS7 cells were transfected, as described in *(A)* with the addition of Thr38 mutants of CPI-17. Protein extracts were immunoprecipitated and immunoblotted as described above.

We introduced both a neutralizing alanine mutation (T38A), and a phospho-mimetic glutamic acid mutation (T38E) at this residue to determine whether this site could regulate MEF2 activity. As shown in figure 5D, the T38E mutation was equally as effective as the wild-type CPI-17 at disrupting the MEF2C- PP1α interaction. However, the T38A mutation was less efficient at disrupting the PP1α interaction with MEF2C, indicating that phosphorylation of CPI-17 is necessary for MEF2 de-repression. To indicate whether CPI-17 could perform a nuclear role in transcriptional regulation, we investigated the cellular localization of CPI-17 by immunofluorescence microscopy in primary VSMCs (Figure 5C and S3). Given that previous studies have defined a role for CPI-17 in regulating calcium sensitivity, we anticipated an abundance of CPI-17 to co-localize with the actin cytoskeleton. Surprisingly, most of the cellular CPI-17 was confined to the nuclear compartment in VSMCs, suggesting a potentially important role for CPI-17 in the nucleus.

DISCUSSION

MEF2C plays an essential role in VSMC differentiation and is genetically upstream of the SRF-coactivator, myocardin (8, 145). MEF2 proteins are integrators of a number of cellular signaling pathways, and are also regulated by several interacting co-factors that either enhance or repress transcriptional activity. We document in this report that cellular signals emanating from RhoA serve to relieve MEF2C from the repressive effects of PP1α to increase myocardin expression in VSMCs (Figure 6).

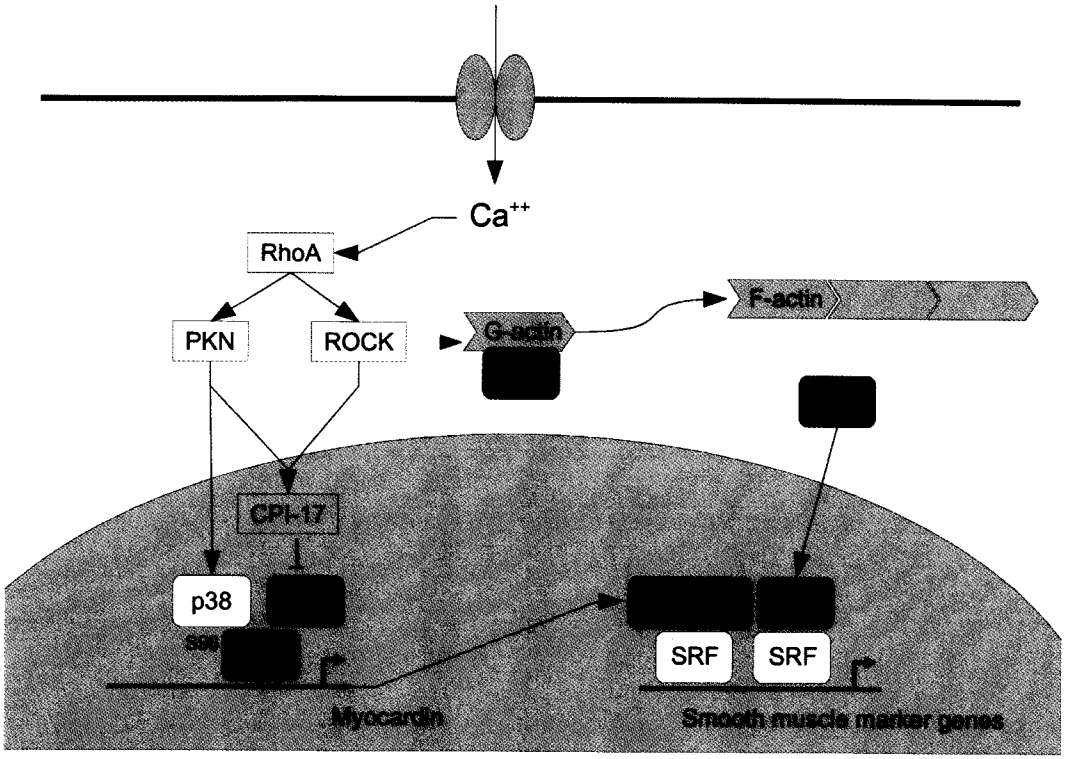


Figure 6. *Model of Calcium-Mediated Induction of Myocardin Expression in VSMCs.*

Based on the work presented in this manuscript, and previously published observations. MEF2-dependent myocardin expression is regulated by RhoA induced p38 phosphorylation of Ser98 and inhibition of PP1α repression by CPI-17. Myocardin activates SRF-dependent VSMC gene expression directly and by dimerizing with myocardin-related transcription factors (MRTFs) that translocate to the nucleus when G-actin polymerizes to form F-actin.

In addition, PP1α serves to modulate c-Jun expression through an entirely different mechanism involving recruitment of HDAC4 to MEF2 proteins and phosphatase-dependent regulation of JNK signaling.

Signal-Dependent Control of PP1α.

The cellular distribution and substrate specificity of PP1α isoforms is regulated by physical interaction with regulatory subunits, that typically contain a conserved RVXF domain (314). In VSMCs, PP1α is targeted to the myosin light chains by physical interaction with MYPT1; however, this RVXF domain is also conserved amongst MADS-box proteins, such as MEF2A-D and may serve to target PP1α to nuclear MEF2 proteins (61, 313). Interestingly, SRF also contains a conserved RVXF domain within its MADS-box, yet our data suggest that PP1α cannot overcome myocardin or TGF-β induction of SRF-target genes (Figure S2). In addition, the phosphatase activity of PP1α is regulated through interaction with specific inhibitor proteins like Inhibitor 1 and 2 (I1 and I2), and CPI-17. The potency of these inhibitor proteins is regulated by phosphorylation and dephosphorylation by cellular kinases/phosphatases, such as PKA, calcineurin, ROCK, and PKN (313, 314, 408). Our data demonstrates that phosphorylation of CPI-17 at Thr38 by ROCK and/or PKN regulates PP1α's ability to modulate gene expression; whereas, I1 and I2 have no effect on MEF2 transcriptional activity (61). The reason for this specificity is not known; however, it may be related to the proposed cytosolic distribution of I1 and I2 compared to the relatively nuclear distribution of CPI-17, or the ability of CPI-17 to compete with MEF2C for PP1α binding (see figure 5)(314).

PP1α Regulates MEF2-Target Genes.

We have previously shown that PP1α regulates the transcriptional activity of MEF2 proteins through a number of mechanisms: 1) PP1α physically interacts with both the N-terminus and C-terminus of MEF2A, -C, and –D to inhibit transcriptional activity directly; 2) PP1α dephosphorylates Ser408 of MEF2A; and 3) PP1α serves to recruit HDAC4 to MEF2 (61). We now document, within the cellular context of VSMC, that these previously identified mechanisms operate in a MEF2-target gene specific manner, where PP1α regulates myocardin expression through direct interaction with MEF2C, and regulates *c-jun* expression by recruiting HDAC4 to MEF2 proteins and dephosphorylation of JNK. Furthermore, we identify a nuclear role for CPI-17 in VSMCs, and document a previously unidentified p38 phosphorylation site within MEF2 proteins (Ser98) that specifically activates MEF2C in VSMCs. Given that the traditional p38-targeted residues (Thr293 and Thr300) proved to be unresponsive to RhoA stimulation, we propose that Ser98 is the primary residue responsible for RhoA-p38-MEF2C induction of myocardin in this cellular context.

In summary, we provide novel evidence that PP1α serves as critical regulator of MFE2-dependent gene expression in VSMC, and demonstrate for the first time that RhoA-mediated signaling plays a fundamental role in inducing myocardin expression through MEF2 proteins. These findings have important ramifications to the field of vascular smooth muscle development and in the progression of vascular stenotic diseases.

ACKNOWLEDGEMENTS

We wish to thank Drs. Michelle Bendeck and Guangpei Hou for technical assistance with the primary cultures. Seneca College Office of Research and Innovation is acknowledged for providing release-time support for JWG. This work was supported by a grant from the Canadian Institute of Health Research (CIHR) to JCM.

SUPPLEMENTAL FIGURES FOR MANUSCRIPT 2

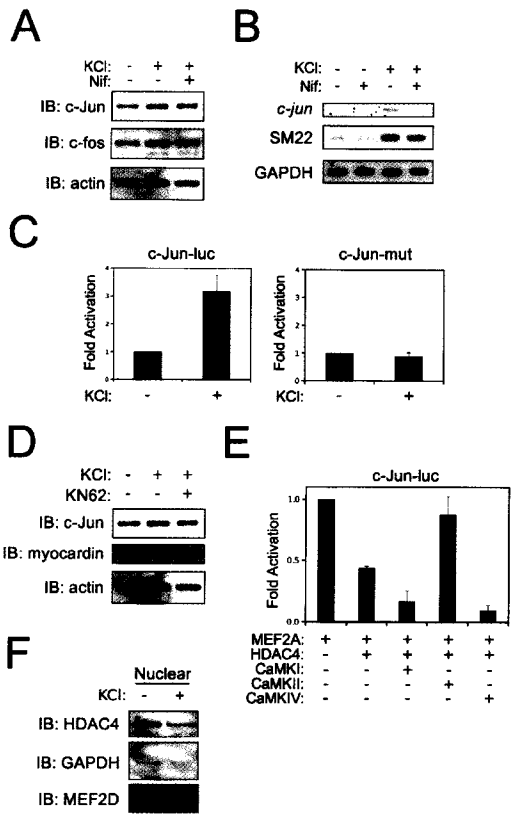


Figure S1. c-Jun Expression is Regulated by CaMKII, HDAC4, and MEF2.

(A)(B) Quiescent A10 cells were treated with 60mM KCl following a 15 minute treatment of 5mM Nifedipine (L-type calcium channel blocker). Immunoblotting was performed on protein extracts using c- Jun, c-fos and actin antibodies, and RT-PCR was performed on total RNA for SM22 and GAPDH. (C) A10 cells were transfected with the c-Jun promoter (c-Jun-luc) or with a reporter gene with the MEF2 *cis* element mutated (c-Jun mut). Following recovery, cell were depolarized with 60 mM KCl and subjected to luciferase assay. (D) A10 cells were treated with 60mM KCl for 2 hours following 15 minute pretreatment with 5 μM KN-62 (CaM kinase blocker). Protein extracts were immunoblotted with c-Jun, myocardin, and Actin antibodies. (E) A10 cells were transfected with a c-jun reporter-gene (c-Jun-luc), MEF2A, HDAC4, and activated CaMKI, CaMKII or CaMKIV, as indicated. (F) Nuclear extracts were probed for HDAC4, GAPDH, and MEF2 following depolarization.

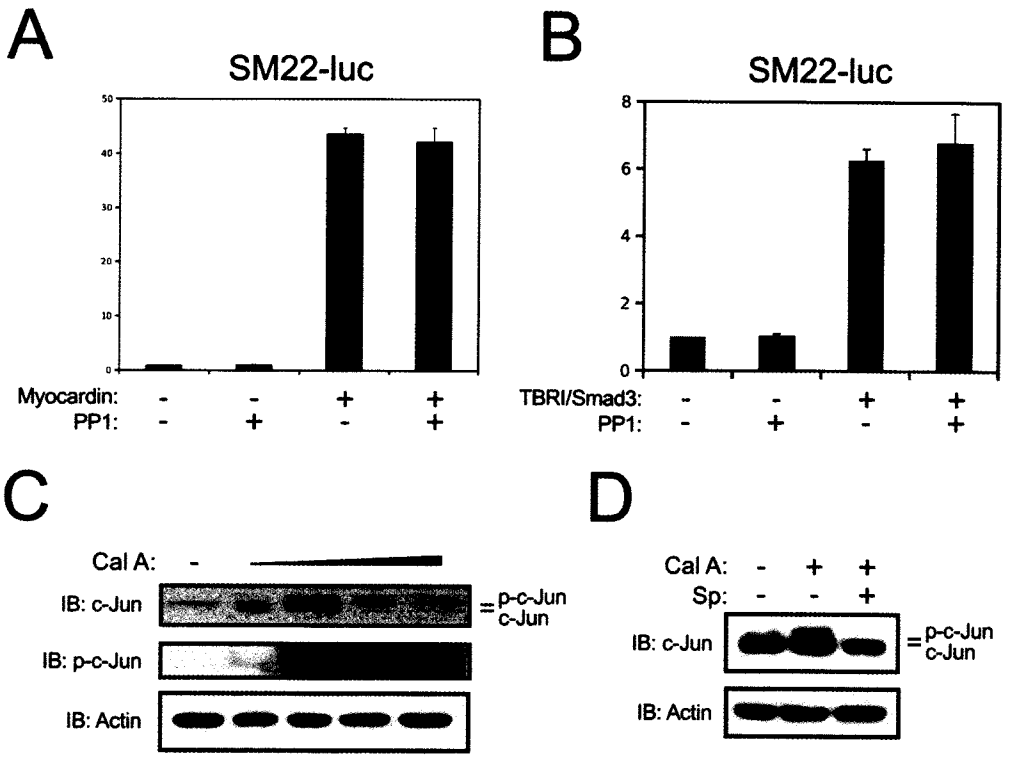


Figure S2. PP1α Cannot Overcome Myocardin, But Regulates JNK.

(A)(B) A10 cells were transfected with the SM22 promoter, myocardin, an activated type I TGF-β receptor (TBRI), Smad3, or PP1α, as indicated. Extracts were harvested for luciferase. (C) A10s were treated with increasing amounts of calyculin A (Cal A; 0.25 ng/mL, 0.5 ng/mL, 1 ng/mL, 2 ng/mL), and (D) A10s were treated with 0.5 ng/mL and SP600125 for 2 hour and harvested for immunoblotting.

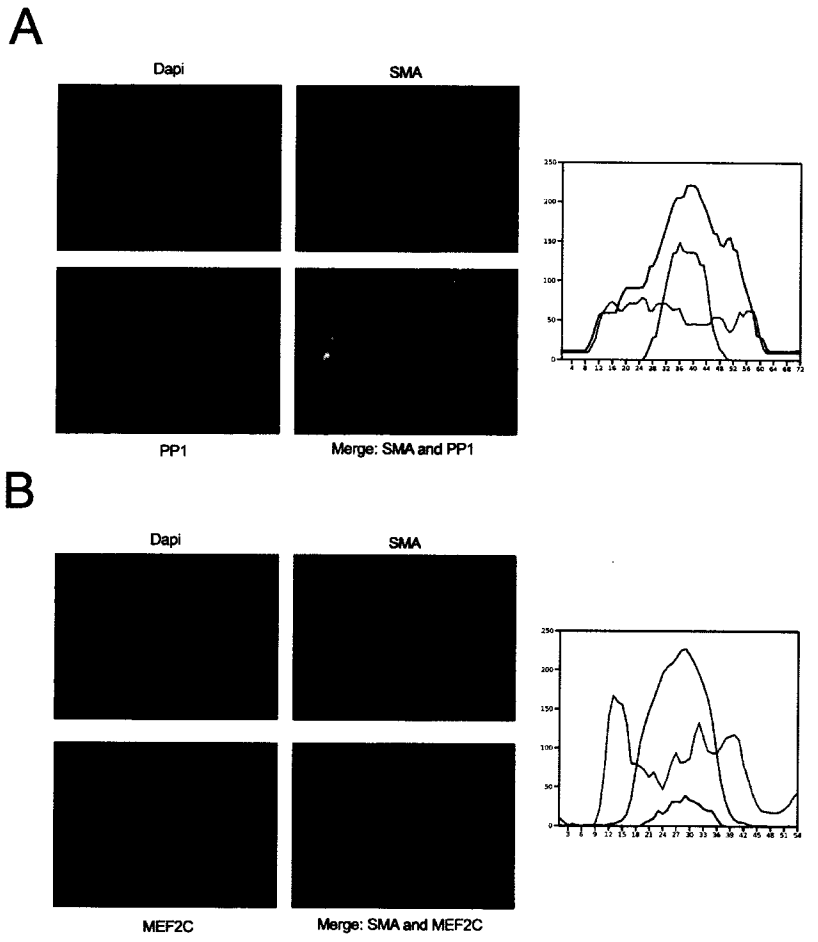


Figure S3. Immunofluorescence for CPI-17, PP1α, and MEF2C in Primary VSMCs.

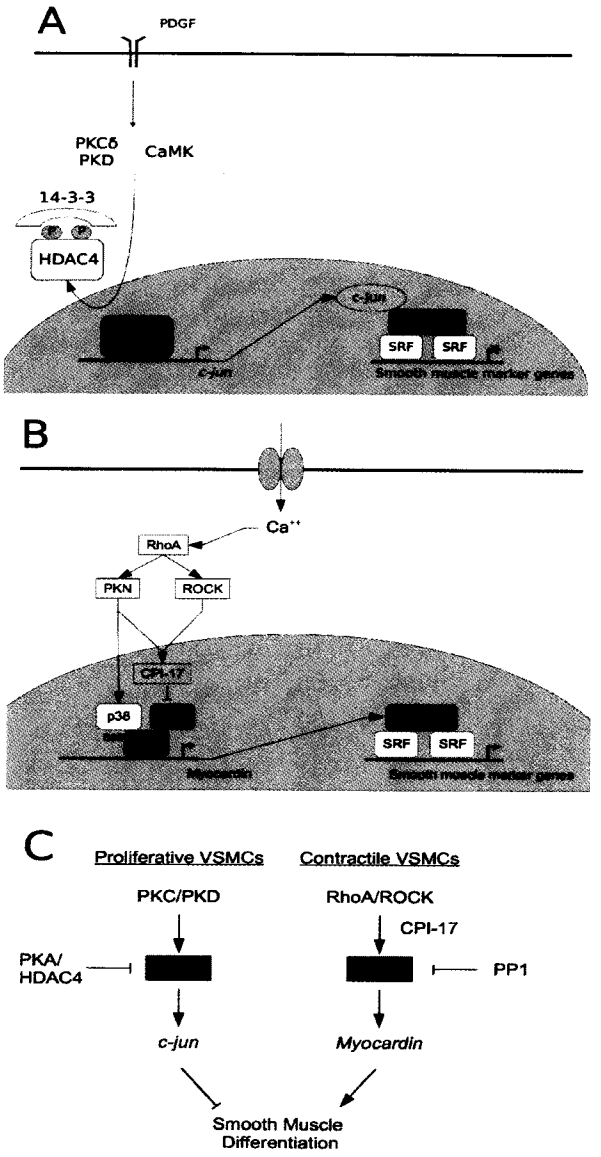
(A)(B) Primary rat aortic VSMCs were fixed in 4% paraformaldehyde, and permeabilized with methanol. Immunofluorescence was performed with primary antibodies to PP1α *(A)* and MEF2C *(C)*(red). Nuclear Dapi stain is shown in blue, and immunofluorescence for smooth muscle actin (SMA) in green. All images were acquired by standard fluorescence techniques and processed with ImageJ, including brightness/contrast and background subtract. Relative fluorescence of a representative cell was graphed with ImageJ.

DISSERTATION SUMMARY

Collectively, these studies further define the role of MEF2 proteins in VSMCs, an area that has been under-investigated compared to the role of MEF2 in the striated muscle types. In the first series of experiments, I investigated the role of MEF2 in the regulation of the immediate-early gene, *c-jun*. In VSMCs, MEF2 recruits HDAC4 to the *c-jun* promoter and acts as a repressor to promote cell quiescence. This repression is critical to the contractile phenotype of VSMCs, as c-Jun can physically interact with myocardin to inhibit activation of smooth muscle differentiation (see Summary Figure 1). Furthermore, this repression is promoted by PKA-induced inhibition of SIK1 to maintain HDAC4 in the nucleus. Upon growth factor stimulation, HDAC4 is exported from the nucleus through activation of CaMKII and PKCδ, thereby relieving MEF2 from repression.

In the second series of experiments, I evaluated MEF2-dependent myocardin expression and differentiation of VSMCs. Using a model of depolarization, I revealed that myocardin expression is sensitive to ROCK and p38 inhibition. Further investigation revealed a novel transcription regulating pathway involving components of a cellular calcium sensitivity mechanism. Regulation of MEF2-dependent myocardin expression involves RhoA-mediated activation of p38 and CPI-17, which relieve MEF2C from PP1α repression. Furthermore, p38 regulation of myocardin occurs through a novel phosphorylation site on MEF2C.

Thus, these data add significant knowledge to the field of vascular biology regarding the role of MEF2, and the signalling pathways that regulate its activity during the process of phenotype modulation of VSMCs. Furthermore, the results of this dissertation support a genetic interaction between MEF2 and SRF that underlies the phenotypic modulation of VSMCs (see Summary Figure 1). These findings have clinical implications to stenotic vascular disease states, vascular development, and congenital cardiac outflow tract defects.



Summary Figure 1. The Genetic Interaction Between MEF2 and SRF in VSMCs.

A) During proliferative conditions, growth factor stimulation relieves MEF2 from HDAC4 repression to promote *c-jun* expression. c-Jun physically interacts with myocardin to suppress SRF-dependent smooth muscle gene expression. B) During contractile conditions, MEF2 activates myocardin expression through RhoA signalling. Myocardin co-activates SRF-dependent smooth muscle genes, while *c-jun* expression is repressed through PKA-dependent inhibition of SIK1 (not shown). C) A schematic summary of the major findings of this dissertation.

APPENDICES

APPENDIX A: FUTURE WORK

RATIONALE

Several productive experimental avenues had to be set aside during the course of my PhD studies in order to complete of this dissertation. These findings have become the basis for my immediate future work. They are presented here under the following experimental hypotheses.

FUTURE OBJECTIVES

Hypotheses:

1) IGF-1 regulation of MEF2-dependent *c-jun* expression is biphasic, resulting in early induction through ERK5 and late repression through PKA.
2) The transcriptional activation of MEF2 proteins in VSMCs is regulated through an interaction with β-catenin.

EXPERIMENTAL PLAN

In this section the experimental approach to each stated hypotheses will be outlined:

1) IGF-1 regulation of MEF2-dependent *c-jun* expression is biphasic, resulting in early induction through ERK5 and late repression through PKA.

A recent study has highlighted the role of insulin-like growth factor 1 (IGF-1) and PI3' kinase/AKT signaling in the promotion of smooth muscle differentiation by activating the transcriptional activity of myocardin (306). Therefore, we utilized this model of VSMC differentiation to evaluate *c-jun* expression. We previously published that this IGF-1-mediated inhibition of c-Jun served as a critical modulator of VSMC differentiation (120). However, we now demonstrate that the effect of IGF-1 on c-Jun expression appears to be bi-phasic, in that 2 hours of treatment with IGF-1 results in an early induction of c-Jun followed by a late down-regulation (Appendix figure 1). Indeed, evaluation of the literature in this field yields conflicting reports regarding the role of IGF-1 in VSMC phenotype modulation. Therefore, I speculate the cellular response to IGF-1 is temporal and context specific. Consistent with this, our preliminary data suggests that IGF-1 induces c-Jun expression in A10 cells cultured at low density, and not when they are grown to confluence (not shown). In contrast, PDGF is capable of inducing c-Jun expression regardless of the cell density.

Congruent with the induction of c-Jun, IGF-1 treatment also resulted in phosphorylation of ERK5, a known MEF2-kinase and activator. Conversely, IGF-1 treatment had no effect on ERK1/2 phosphorylation. ERK5 has previously been shown to phosphorylate MEF2C at serine 387, and mutation of this residue to alanine eliminated most of MEF2C's activation in cultured A10 cells (Appendix figure 2). Although no specific inhibitor for ERK5 exists, ERK5 activity can be blocked by the general ERK inhibitor PD98059. Therefore, I am currently evaluating whether PD98059 can block the

early induction of c-Jun by IGF-1. The next series of experiments in our culture model will involve the generation of a siRNA targeted to ERK5. Utilizing this construct, we will be able to determine the necessity of ERK5 in the IGF-1 induction of c-Jun. To further these experiments *in vivo*, we could generate a cardiac and smooth muscle conditional deletion of the ERK5 gene, using the SM22-cre transgenic background. These mice can then be crossed with our MEF2 'sensor mouse' to determine the effect of ERK5 gene ablation on MEF2 transcriptional activity, as determined by X-gal staining, in VSMCs.

We have recently documented that PKA inhibits MEF2 transcriptional activity in skeletal muscle and VSMCs by promoting the nuclear accumulation of HDAC4 (120). Interestingly, ERK5 signaling has been previously shown to activate PKA signaling in cardiomyocytes by inhibiting phosphodiesterase 4 (PDE4), and we have documented that this PDE plays a functional role in regulating *c-jun* expression in VSMCs (120, 218). Therefore, I hypothesize that the late inhibition of c-Jun by IGF-1 is due to PKA-mediated repression of MEF2 activity. To evaluate this I will utilize PKA inhibitors, such as H89 and Rp-cAMPS, to rescue c-Jun from IGF-1-mediated repression. Indeed, my preliminary evidence, shown in appendix figure 3, demonstrates that SIK1 can overcome the IGF-1 repression of *c-jun*, as can an siRNA targeting HDAC4. This hypothesis is clinically relevant given the finding that extended use of cyclooxygenase II inhibitors (eg. Vioxx) in humans has been associated with a greater risk of vascular events due to loss of prostaglandin-induced PKA activity. To evaluate this idea *in vivo*, I could use our model of carotid injury in our MEF2-lacZ mouse.

We have previously shown that carotid injury in this mouse model results in increased MEF2 activity and c-Jun expression within the neointima, and I anticipate that mice treated with a cyclooxygenase II inhibitor will have enhanced neointima formation and MEF2 activity due to the loss of protective endothelium-derived prostaglandins that activate PKA (See Appendix figure 4 for diagram). Completion of these studies will expand our current knowledge regarding PKA-mediated MEF2 activity in VSMCs and identify potential pharmacological targets that may prove efficacious in preventing neointimal formation in vascular diseases caused by endothelial dysfunction.

2) The transcriptional activation of MEF2 proteins in VSMCs is regulated through an interaction with β-catenin.

We have previously screened a neonatal rat aortic cDNA library using the MADS-MEF2 domain of MEF2D (aa. 19-167) as bait (52). This screen identified several novel MEF2 interacting cofactors, which we have characterized, such as ERK5, Smad2, and PP1α. We are now characterizing another clone from this two-hybrid screen, which we have identified as β-catenin. β-catenin is a downstream effecter of the canonical Wnt signaling pathway and a regulator of cell proliferation and survival. Most cellular β-catenin is found at the cell periphery complexed within the cadherin complex that forms at cell-to-cell contact points, while cytosolic β-catenin is kept at low levels by proteosome-mediated degradation, which is regulated by phorphorylation by GSK3. Upon Wnt or IGF-1 stimulation, GSK3 is inhibited and β-catenin is allowed to accumulate and translocate to the nucleus, where it interacts with T-cell factor (TCF)

family members to active gene expression. As shown in Appendix figure 6, we have preliminary evidence that MEF2C and β-catenin synergistically active *c-jun* expression in cultured A10 cells. This synergy is eliminated when the MEF2 *cis* element within the *c-jun* promoter is mutated. We are continuing to characterize the interaction of MEF2C and β-catenin by co-immunoprecipitation (Appendix figure 6) and GST-pulldown assays, and speculate that this molecular interaction will stabilize during proliferative conditions and destabilize by cell-to-cell contact inhibition (see diagram in Appendix figure 5). In support of this, GSK3 inhibition and PI3' kinase inhibition had no effect on c-Jun expression in cultured VSMCs, which suggests that the canonical pathway regulating β-catenin activity does not regulate MEF2 in this model (not shown). However, we did observe that IGF-1 induction of c-Jun was highly dependent on cell contact. Interestingly, PDGF induction of c-Jun is not dependent on cell contact (not shown). This is consistent with previous reports that have demonstrated that PDGF can induce cleavage of the cadherin complex in VSMC and release β-catenin into the nucleus (409).

I will continue this line of research by designing a specific siRNA to β-catenin to evaluate whether the density-dependent induction of c-Jun requires β-catenin expression. I also plan to utilized this siRNA targeting β-catenin to evaluate the migratory phenotype of VSMCs, by using a wound-scratch technique, previously developed in our laboratory. In addition, we will ectopically express a constituently nuclear β-catenin and evaluate whether we can reinstitute c-Jun induction during contact inhibition. I am also in the process of cloning N-cadherin constructs, such as a mammalian expression vector to

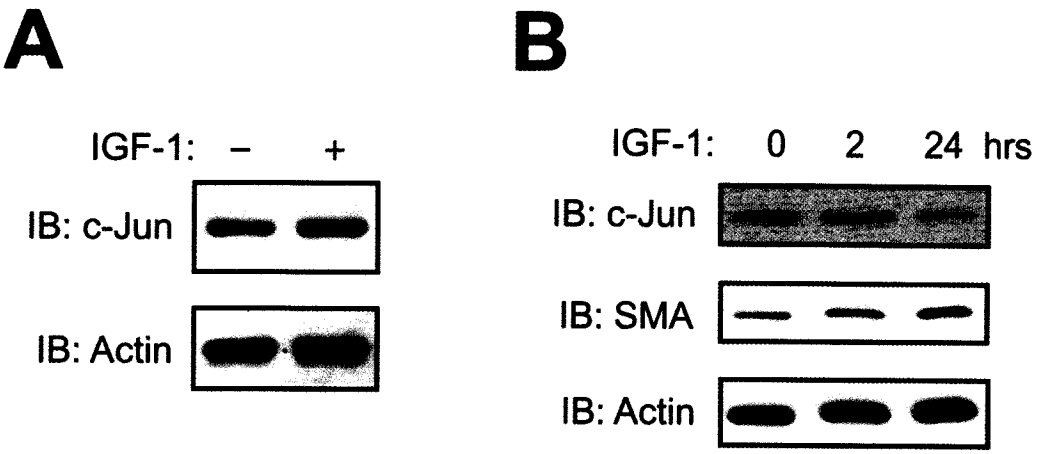
evaluate the hypothesis that forced expression of N-cadherin can sequester β-catenin and inhibit c-Jun expression; as well as, a dominant-negative construct that can release β-catenin and hypothetically increase MEF2-dependent gene expression. Given that ERK5 was also identified in the yeast two-hybrid screen with β-catenin, I wished to know if ERK5 signaling could cooperate with β-catenin to induce *c-jun* expression. As shown in appendix figure 7, activation of MEK5/ERK5 signaling by forced expression of activated constructs enhances the β-catenin induction of the *c-jun* promoter. Lastly, we have previously demonstrated that c-Jun expression in VSMCs is repressed by a MEF2/ HDAC4 complex. I speculate the MEF2C will interact either with HDAC4 or β-catenin in an exclusive manner, where interaction with HDAC4 results in transcriptional repression and interaction with β-catenin results in transcriptional activation (see Appendix figure 5 for diagram). To evaluate this hyposthesis we have performed competitive COIPs and functional reporter-assays with the *c-jun* promoter, and expression vectors for MEF2C, HDAC4, and β-catenin (Appendix Figure 8), which indicate that HDAC4 can compete for MEF2C binding with β-catenin. The next phase of this project is to establish the presence of β-catenin and HDAC4 on the *c-jun* promoter region within intact chromatin in VSMCs during opposing cellular conditions. This experimental phase will be evaluated in cultured A10 cells and primary aortic smooth muscle cells. Using a recently developed protocol in our laboratory, we will perform chromatin immunoprecipitation (ChIP) followed by quantitative PCR from extracts made from quiescent and PDGF-treated cells. Based on our evidence that PDGF-induced

activation of CaMKII promotes nuclear export of HDAC4, my hypothesis is that HDAC4 will be present on the *c-jun* promoter in quiescent conditions, but will be replaced by β-catenin following PDGF stimulation. Utilizing ChIP assays, I will be able to identify which MEF2 proteins (ie. MEF2A, -C, or –D) are bound to the *c-jun* and myocardin promoters in VSMCs, and identify whether distinct sets of regulatory cofactors are present at the *c-jun* and myocardin promoters that help dictate the distinct signaling pathways that regulate the expression of these genes. These data will be critical to determine the regulatory role of each MEF2 protein and their target-genes within a cellular context and intact chromatin. Following this, I would like to attempt quantitative ChIP analysis *in vivo*, to evaluate whether HDAC4 is exported from the *c-jun* promoter region following vascular injury, and replaced with β-catenin.

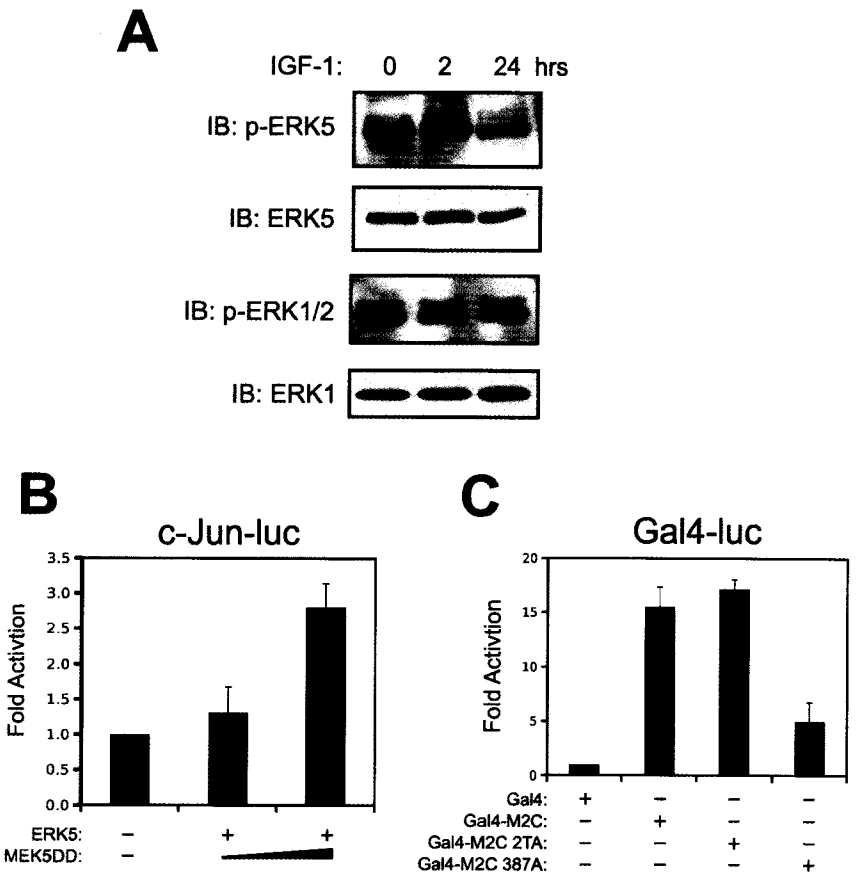
A recent study has identified the tumour-suppressor gene menin in the process of nuclear export of β-catenin. Furthermore, our group has previously identified menin as a critical regulator of mesenchymal cell differentiation. Therefore, I plan to evaluate the role of menin on the β-catenin-MEF2 interaction and the regulation of c-Jun expression in VSMCs.

Completion of these studies will begin to define critical levels of MEF2 regulation that help determine MEF2-dependent immediate-early gene and smooth muscle gene expression. We anticipate that these studies will further our understanding of the molecular mechanism of VSMC phenotypic modulation that underlies most human stenotic vascular lesions.

ADDITIONAL DATA

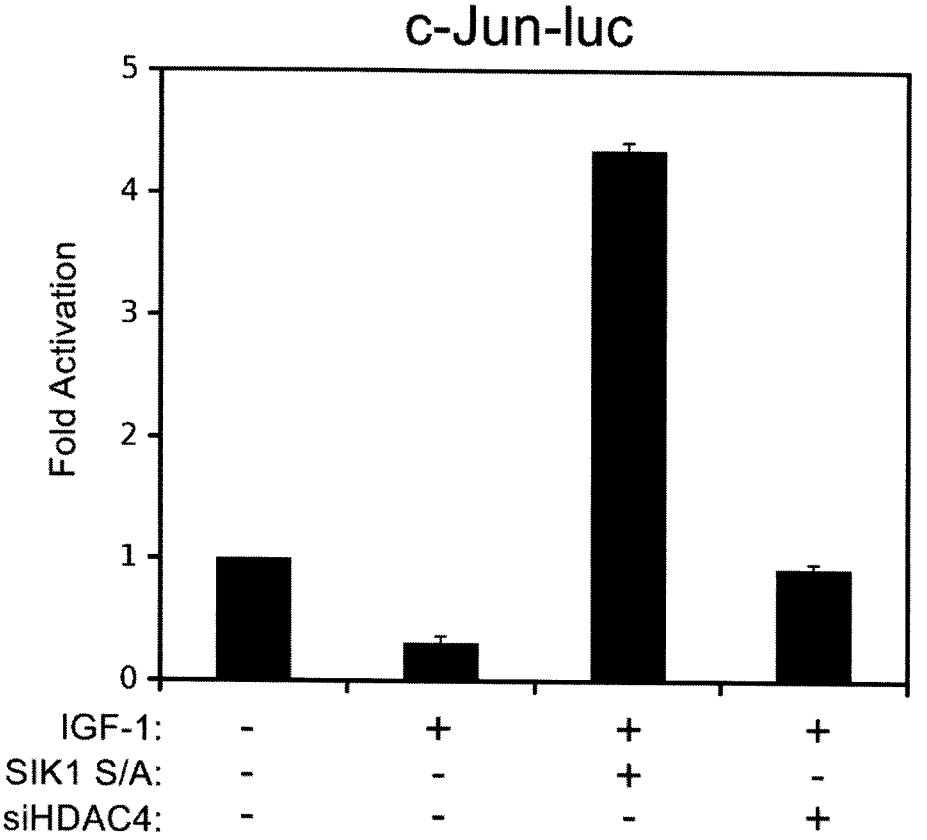


Appendix Figure 1. Biphasic regulation of c-Jun by IGF-1.
A) Quiescent cells were treated with 50 ng/mL of IGF-1 for 2 hours. Protein extracts were subjected to immunoblotting as indicated. B) A10 cells were treated with 50 ng/mL of IGF-1 for 2 hours and 24 hours and immunoblotted at indicated. SMA = smooth muscle alpha actin.



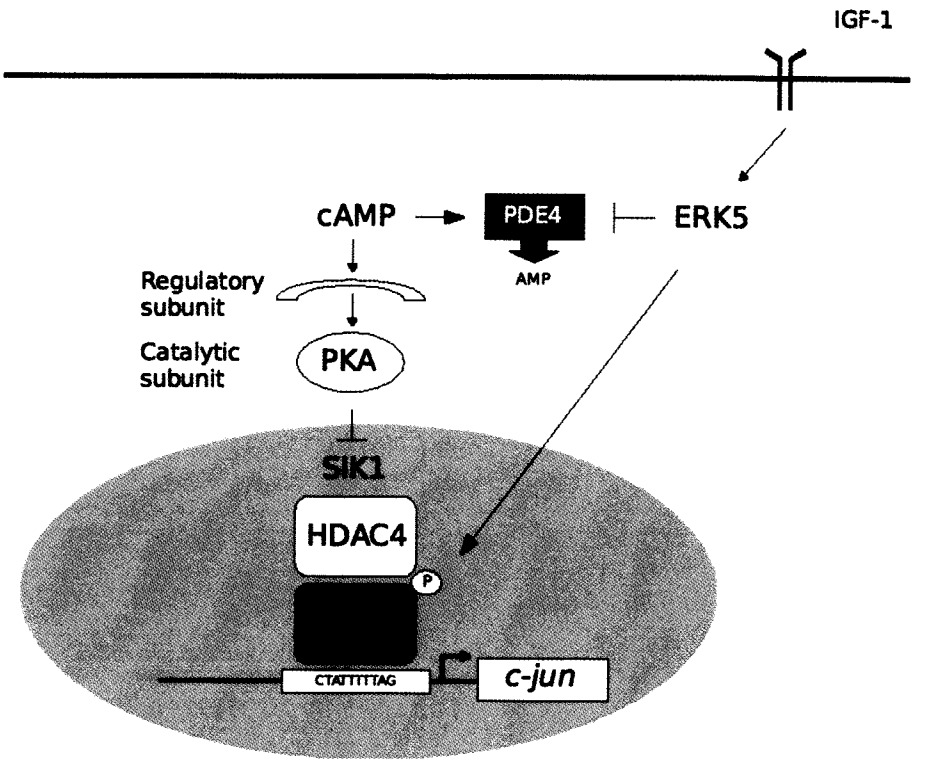
Appendix Figure 2. c-Jun expression is mediated through ERK5 activation of MEF2C.

A) Serum starved A10s were treated with IGF-1 (50 ng/mL) for 2 and 24 hours. Protein extracts were immunoblotted as indicated. B) A10 cells were transfected with c-Jun-luc, ERK, and increasing amounts of activated MEK5 (MEK5DD). Extracts were subjected to luciferase assay. C) A10 cells were transfected with a Gal4-luc reporter gene with expression vectors containing fusion proteins for Gal4, Gal4-MEF2C (Gal4-M2C), Gal4-MEF2C with the p38 phospho-residues T293 and T300 mutated to alanine (Gal4-M2C 2TA), or a Gal4-MEF2C with the ERK5 phospho-residue S387 mutated to alanine (Gal4-M2C 387A). Extracts were subjected to luciferase assay.

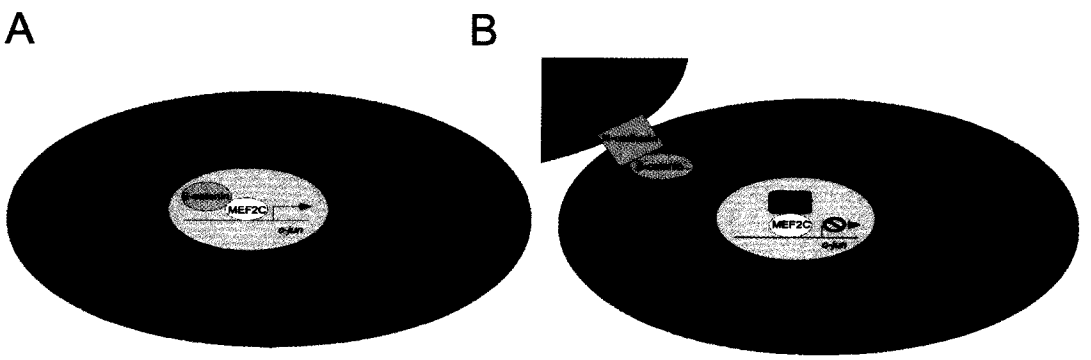


Appendix Figure 3. SIK1 and RNA Interference of HDAC4 Rescue IGF-1 Repression of c-Jun.

A10 cells were transfected with the *c-jun* promoter, SIK1 S577A (SIK1 S/A), and siHDAC4, as indicated. Following recovery, cells were treated with IGF-1 (50 ng/mL) overnight and harvested for luciferase.

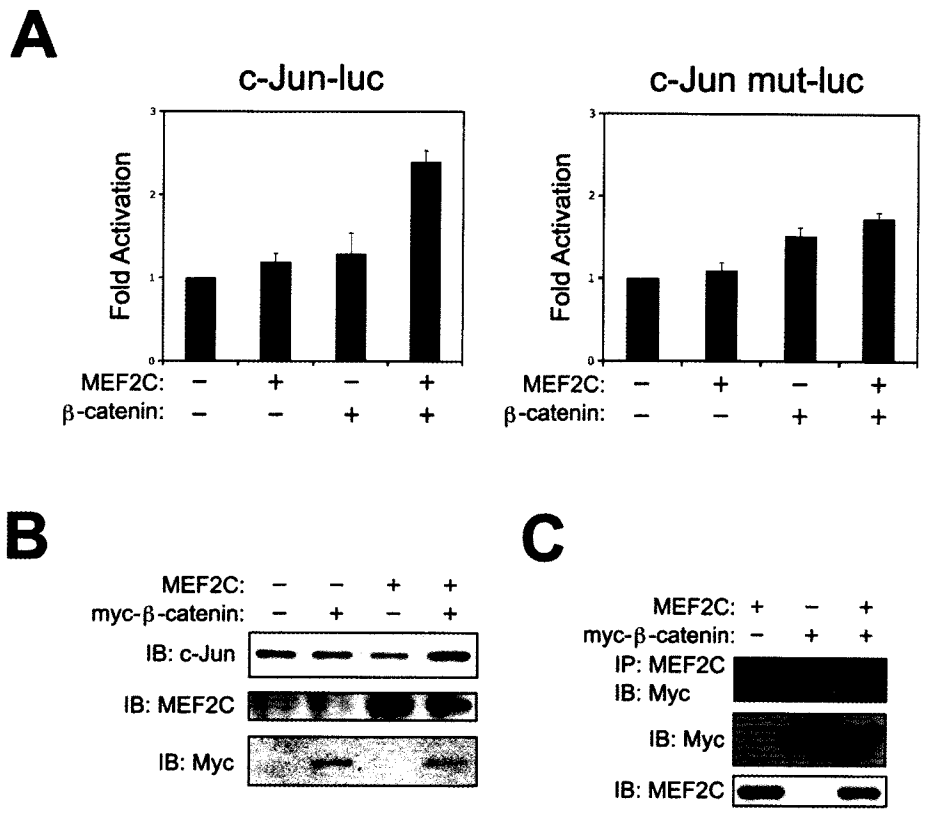


Appendix Figure 4. Proposed model of c-Jun regulation by IGF-1.
IGF-1 stimulation activates MEK5/ERK5 signalling, which results direct and early activation of MEF2-dependent c-Jun expression, followed by late PDE4-dependent c-Jun repression, through a HDAC4/MEF2 repressor complex.



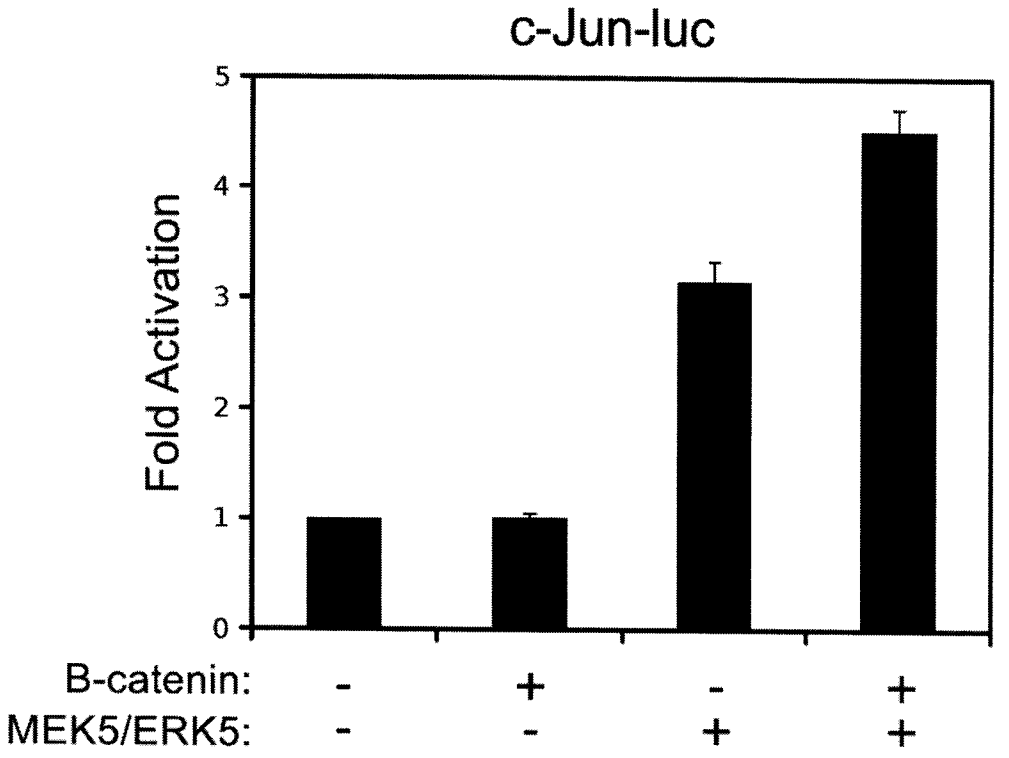
Appendix Figure 5. Proposed model of β-catenin regulation of MEF2-dependent c-jun expression.

A) In proliferative cells β-catenin and MEF2 are complexed in the nucleus to coactivate c-jun expression. B) In contact inhibited quiescent cells, β-catenin is sequestered to the cadherin complex, while HDAC4 represses MEF2-dependent c-jun expression.



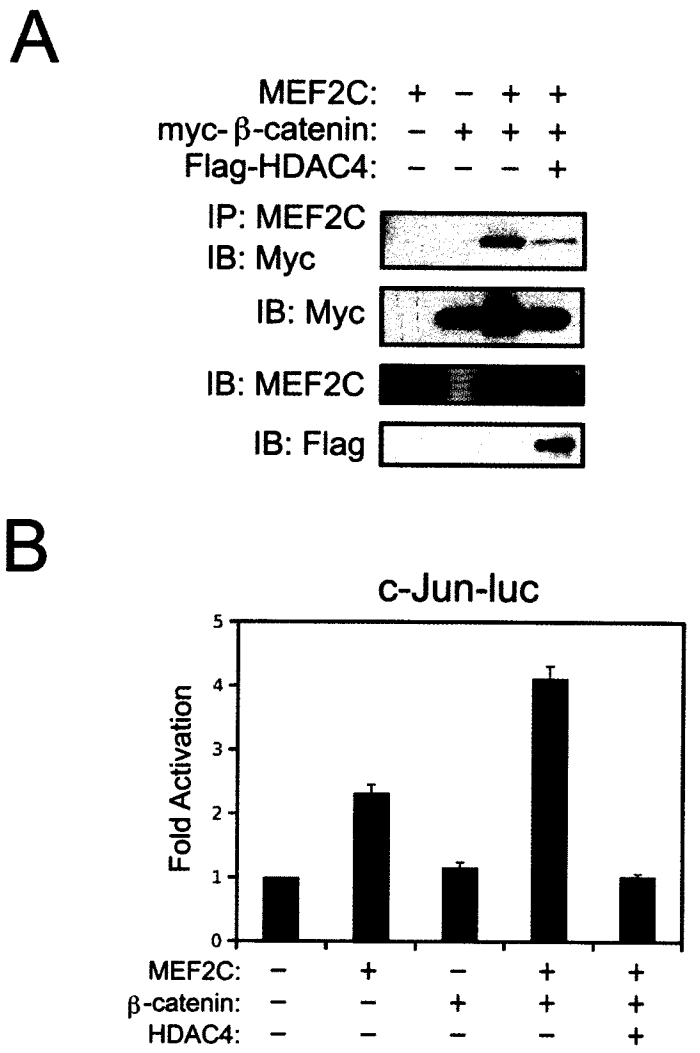
Appendix Figure 6. MEF2C and β-catenin synergistically activate c-jun expression.

A) A10 cell were transfected with the *c-jun* reporter-gene (c-Jun-luc) or a reporter with the MEF2 site mutated (c-Jun mut-luc) and MEF2C or β-catenin, as indicated. Extracts were subjected to luciferase assay. B) A10 VSMCs were transfected with MEF2C and β-catenin, as indicated. Positive cells were selected for by puromycin treatment for 3 days. Protein extracts were subjected to immunoblotting (IB)(Provided by Saviz Ehyai). C) COS7 cells were transfected with MEF2 or β-catenin, as indicated. Proteins extracts were immunoprecipitated (IP) with MEF2C antibody and immunoblotted (IB) as indicated (In collaboration with Saviz Ehyai).



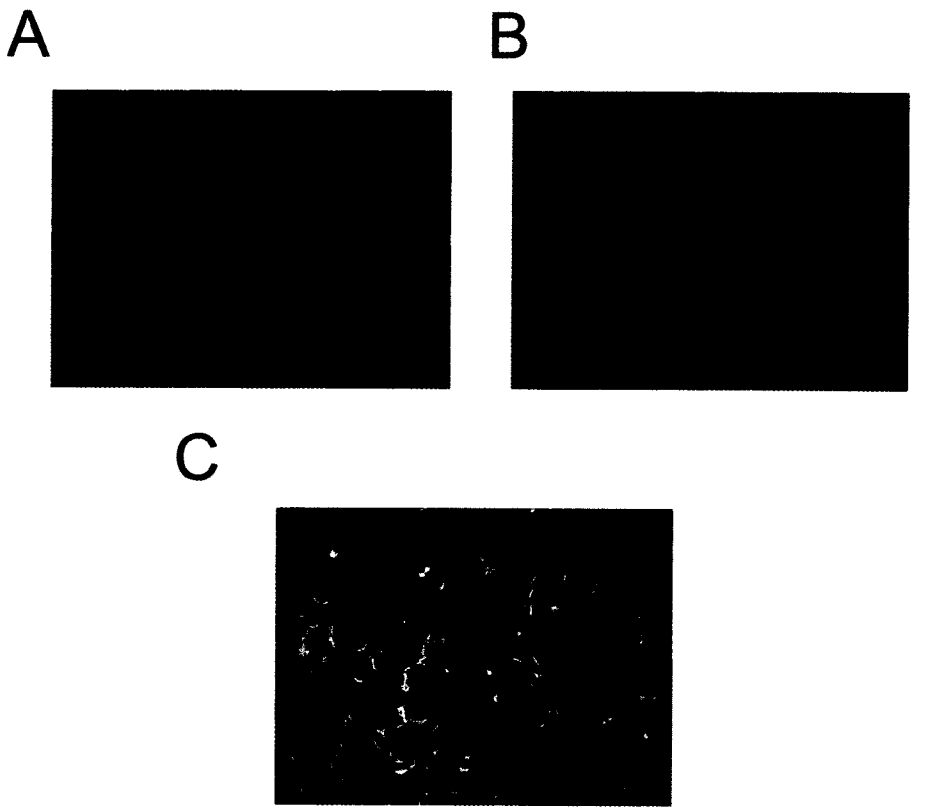
Appendix Figure 7. β-Catenin and ERK5 Cooperative to Induce c-Jun Expression.

A10 cells were transfected with the *c-jun* reporter gene, and β-catenin, or ERK5 and activated MEK5, as indicated. Luciferase assay was performed on cell extracts.



Appendix Figure 8. HDAC4 attenuates MEF2C and B-catenin induction of c-jun expression.

A) COS7 cells were transfected with MEF2C, β-catenin, or HDAC4, as indicated. Proteins extracts were immunoprecipitated (IP) with MEF2C antibody and immunoblotted (IB) as indicated (Provided by Saviz Ehyai). B) 10T1/2 cells were transfected with the *c-jun* reporter-gene (c-Jun-luc) and MEF2C, β-catenin, or HDAC4, as indicated. Extracts were subjected to luciferase assay.



Appendix Figure 9. Immunofluorescence in VSMC primaries.

Rat aortic VSMC primary cultures were obtained using the enzymatic dispersion method and subjected to immunofluorescence A) smooth muscle alpha-actin (green) and dapi (blue); B) smooth muscle myosin heavy chain (green) and dapi (blue); C) smooth muscle alpha-actin (green) and MEF2C (red).

EXPANDED MATERIALS AND METHODS

Cell Culture

The following cell lines were utilized in the aforementioned studies, A10, Cos7, C3H10T1/2, and VMSC rat primary cultures. What follows are general cell culture guidelines, recommendations by ATCC were also adhered to.

Reagents:

- 1x Dulbecco's PBS (without Ca^{++})

  NaCl 8g

  KCl 0.2g

  Na2HPO4 7H2O 1.44g

  KH2PO4 0.24g

  Add 3.75 g/L NaCO3 (1.5 g/L for A10s)

up to 800ml, pH to 7.4 with HCl, top to 1L

- 0.125% Trypsin-EDTA (Gibco) diluted in PBS
- DMEM supplemented with Penicillin-Streptomycin (Gibco) and L-glutamine (Gibco) added as required
- Freezing medium: Growth media in which the cells are normally cultured, supplemented with 10% DMSO (5% for A10s); sterilize the freezing medium by passing through a 0.2um filter.
- FBS, heat inactivated at 56°C for 30 min
- HS, heat inactivated at 56°C for 30 min

Cell Passaging

- Remove media from established stock cultures.
- Rinse the cell monolayer briefly with 4 ml of Versene. (this step is necessary to remove any traces of serum, which could inactivate the Trypsin)
- Add 1.0ml of 0.125% Trypsin-EDTA solution to 100mm dish or 0.5ml to 60mm dish, incubate at 37C for 1-4 min.
- Inactivate the Trypsin by adding 3.0 ml of DMEM.
- Pipette the cells up and down several times to ensure complete removal of the cells from the dish and to dissociate clumps of cells.
- Count the cells in a haemocytometer (optional) and seed a dilution of cells that allows for future cell growth but is not too low to retain viability of the culture by cross-feedings, incubate in new culture dishes.

Inducing Muscle Cell Differentiation

- Deplete cells at 60-80% confluence of growth factors by gently washing cells with PBS/DMEM and re-feeding with 5% HS in DMEM.
- Incubate cells for desired time at 37C with 5% CO2

Freezing Cells

- Prepare a cell suspension and pellet the cells by centrifugation at 1500 g.
- Resuspend the cells in freezing medium at a concentration of $1x10^6$ - $8x10^6$ cells/ml.

- Dispense 1 ml of cell suspension into each freezing vial.
- Place vials into polystyrene a box.
- Place box in -80 °C freezer and freeze overnight.
- For long term storage place vials in liquid nitrogen.

Thawing Frozen Cells

- Remove vial from the liquid-nitrogen freezer and thaw in 37 °C water bath.
- Dissociate clumps of cells using a Pasteur pipette.
- Transfer to a 15 ml conical tube containing 5 ml of media.
- Centrifuge for 10 min at 1500 g; aspirate the supernatant, and agitate tube vigorously to remove clumps of cells.
- Suspend cells in 5-10ml of growth medium.
- Count cells in haemocytometer
- Plate new stock plates at 10^6 cells/100 mm dish in 10 ml of growth medium.

Embryo Isolation Protocol

- Sacrifice a pregnant female (13.5 – 14.5 dpc) by cervical dislocation.
- Prepare three 100 mm dishes containing 15 ml PBS each.
- Place mouse on its back and wipe down with 70% ethanol.
- Make an incision down the midsection to expose uterine horns. Observe embryos on each side of the uterus and in the amniotic sacs.

- Pull embryos and uterine horns away from amniotic sac and place in a fresh dish of PBS.
- Detach embryos from amniotic sac and place in fresh dish of PBS.

β-galactosidase Staining of Adherent Cells, embryos, and sections

Reagents:

- 0.5 M Potassium ferric cyanide
- 0.5 M Potassium ferrous cyanide
- 40 mg/ml X-gal in DMSO
- 1 M MgCl2
- X-gal staining solution

  1 mg/ml X-gal

  5 mM Potassium ferric cyanide

  5 mM Potassium ferrous cyanide

  1 mM MgCl2

Methods:

- Wash cells/embryo/sections with PBS.
- Fix cells in 4 % paraformaldehyde for 15 min.
- Wash 3 x in PBS
- Stain in X-gal solution for 3 – 16 h.
- Wash with PBS and analyze.

Transfection of Mammalian Cells with DNA

Calcium-mediated transfections were performed in 35/100 mm cell culture dishes. What follows below are guidelines for transfection of 100 mm plates, reagents were scaled proportional to surface area for transfection of 35 mm plates.

Reagents:

- 2x HEBS (2.8 M NaCl, 15mM Na2HPO4, 50mM HEPES)

  8.18 g NaCl

  5.95 g HEPES

  0.1065 g Na2HPO4 (MW=142) or 0.201 g Na2HPO4-7H2O

  Add 400 ml ddH2O, pH to 7.15, bring volume up to 500 ml, filter sterilize, store at -20 °C.

- 2.5 M CaCl2

  2.78 g CaCl2 (MW=111)

  Add ddH2O up to 10 ml, filter sterilize, store at -20 °C.

Methods:

Transient transfection of adherent cells with Calcium

- Plate cells 24 h prior to transfection so that they are 30-50% confluent at time of transfection.
- Re-feed cell cultures with growth media 3 h prior to addition of DNA.
- Label sterile tubes and add 0.5 ml of 2x HEBS to each tube.

- Prepare DNA-CaCl2 solution as follows, add 25 μg DNA, bring up volume to 450 μl, mix, add 50 μl 2.5 M CaCl2, mix.
- While vortexing HEBS at low speed add DNA-CaCl2 solution dropwise.
- Add DNA mix dropwise to cell cultures.
- 16 h following addition of DNA, wash cells with PBS and re-feed with growth media for at least 8 h before experimentally treating cells.

Luciferase Assay

Luciferase assays were performed with commercially purchased substrate (Promega). The manufacturer's protocol was slightly modified and assays were performed as described below. All reporter assays were performed with cells grown in 35 mm dishes.

Reagents:

- Lysis buffer

  20 mM Tris, pH 7.4

  0.1% Triton-X 100
- Luciferase substrate (Promega)

Methods:

Harvesting

- Wash adherent cells with PBS.
- Add 200-300 μl of lysis buffer per dish.
- Incubate 5-10 min at room temperature.

- Scrape cells off with rubber policeman, collect into labelled tubes, vortex, spin, transfer supernatant to new tube.
- Freeze cell lysate until analysis.
- Thaw lysate when ready to analyze and transfer 100 [illegible]l to Luciferase assay tube.

Lambat (LB 958) Luminometer protocol for Luciferase assay

Washing (performed before and after using machine):

- Dry probe with Kim Wipe.
- Place probe in ddH20.
- Choose 'Others' option.
- Choose 'Operator Function'.
- Choose 'Reagent'.
- Choose 'Other'.
- Choose 'Wash'.
- Choose 'INJ 1'.
- Enter '5' for amount of cycles, press 'Enter'.
- Insert tube.
- Press 'Start'.
- Choose 'Repeat Washing'.
- Remove tube, discard water and replace tube back into machine.

- Choose 'Start'.
- Choose 'Exit'.

Luciferase Assay:

Priming (to ensure washing solution removed from injection tube)

- Dry probe with Kim Wipe.
- Place probe in Luciferase bottle.
- Choose 'Others'.
- Choose 'Operator Function'.
- Choose 'Reagent'.
- Choose 'Prime'.
- Choose 'INJ 1'.
- Insert tube.
- Press 'Start'.
- Choose 'Exit'.

Measurement (measurement of amount of Luciferase in samples based on reaction and light emitted)

- Choose 'Measure'.
- Choose 'Protocols'.
- Enter '5' for protocol number, press 'Enter'.
- Choose 'Yes'.
- Comment Press 'Enter'.

- Follow on screen instructions, and choose 'Exit' when finished.

Unloading (rids injection tube of Luciferase substrate)

- Choose 'Others'.
- Choose 'Operator Function'.
- Choose 'Reagent'.
- Choose 'Others'.
- Choose 'Manual Unload'
- Choose 'INJ 1', choose INJ 1 two more times.
- Put probe into the 1% bleach solution, and press 'exit'.

End Assay by repeating washing step with bleach.

β-Galactosidase Assay

Reagents:

- ONPG (4 mg/ml in ddH2O)
- Z buffer

  16.1 g Na2HPO4-7H2O (60 mM)

  5.5 g NaH2PO4-H2O (40 mM

  0.75 g KCl (10 mM)

  0.246 g MgSO4-7H2O (1 mM)

  Add 800 ml ddH2O, pH to 7, bring volume up to 1 L, filter sterilize, store at RT
- Reaction mix

500 µl Z buffer/sample

100 µl ONPG/sample

2.74 µl β-mercaptoethanol/sample

Prepare fresh and mix well

Prepare volume which is sufficient for all samples and blank to be read

- 1 M Na2CO3

Methods:

- Aliquot 50-100 µl of lysate prepared for Luciferase assay into new tube, add 600 µl β-Galactosidase reaction mix.
    - Incubate tubes at 37 °C until a color change is apparent (yellow).
    - Add 300 µl of 1M Na2CO3 to each tube to stop reaction.
    - Transfer samples to spectroscopy cuvette and measure absorbance of samples at 420 nm.

Protein Extracts

Keep protein samples cold at all times (unless otherwise directed). Nuclear and cytoplasmic extracts were made using the NE-PER nuclear and cytoplasmic extraction kit (Pierce). Whole cell extracts were prepared as follows:

Reagents:

- PBS (keep cold)

- Lysis buffer

  50 mM Tris, pH 8.0

  150 mM NaCl

  1 mM Sodium vanadate

  1 mM PMSF (add fresh)

  Protease inhibitor cocktail (add fresh, Sigma, P-8340)

- 2X SDS sample buffer (Biorad)

Add β-mercaptoethanol fresh as directed by manufacturer

Methods:

- Remove media from cells, wash with PBS, repeat.
- Add 700 µl PBS and gently scrape cells with rubber policeman, transfer to new tube.
- Centrifuge cells at 1500xg for 5 min.
- Remove PBS, approximate the cell pellet volume and dilute with five times that volume in lysis buffer.
- Vortex cells briefly every 10 min for 30 min.
- Centrifuge cell lysate at high speed (>10 000xg), transfer supernatant to new tube.
- Determine protein concentration by Bradford assay, and dilute protein samples with equal amounts 2 X SDS sample buffer added.

- Boil samples for 2-4 min, chill on ice for five minutes, store at - 80 °C.

Nuclear and Cytoplasmic Protein Extracts

(NE-PER kit, Pierce)

Before beginning protocol add protease inhibitors as described in protein extract section to solutions CER I, CER II, and NER.

- Gently scrape cells and pellet by centrifugation at 1 500xg for 5 min at 4 °C.
- Remove supernatant and add 200 µl of ice-cold CER I to the cell pellet.
- Vortex the tube for 15 sec and then incubate tube on ice for 10 min.
- Add 11 µl of ice-cold CER II to the tube.
- Vortex the tube for 5 sec on the highest setting and then incubate tube on ice for 1 min.
- Vortex the tube for 5 more sec and then centrifuge at 13 000xg for 5 min at 4 °C.
- Immediately transfer the supernatant (cytoplasmic extract) fraction to a clean pre-chilled tube. Place this tube on ice until use or storage.
- Resuspend the insoluble pellet fraction from step 7 in 100 µl of ice-cold NER.
- Vortex on the highest setting for 15 sec every 10 min for 40 min.

- Centrifuge the tube at 13 000xg for 10 min at 4 °C and then transfer supernatant to new tube.
- Determine protein concentration by Bradford assay and analyze samples by Western analysis.

SDS-PAGE

Reagents:

- PBS
- 10% Resolving gel (15ml)

  ddH20 5.9ml

  1.5M Tris pH 8.8 3.8ml

  30% acrylamide mix 5ml

  10% SDS 0.15ml

  10% APS 0.15ml

  TEMED .006ml
- Stacking gel (4ml)

  ddH20 2.7ml

  1.0 M Tris pH 6.8 0.5ml

  30% acrylamide mix 0.67ml

  10% SDS 0.04ml

  10% APS 0.04ml

TEMED 0.004ml

- 10X Laemmli (1L)

  ddH20 800 ml

  Tris 30.3g

  Glycine 144.2 g

  SDS 10g

  pH to 8.3

  bring volume up to 1L with ddH2O

Methods:

- Prepare resolving gel and then top with stacking gel with appropriate comb inserted in Hoefer mini-gel apparatus.
- Fill bottom and centre well of mini-gel apparatus with 1X Laemmli buffer.
- Load samples on gel.
- Run gel at 100 V through stacking and 150 V through running gel

Western Immunoblot

Reagents:

- Transfer buffer (100ml)

  Methanol 20ml

  1X Laemmli 80ml

- Prepare blocking buffer, washing solutions, ECL, and antibody diluent as per manufacturer's instruction.

Methods:

- After SDS PAGE, transfer protein to Immobilon-P (Millipore) membrane by wet transfer at 20 V for 16-20 h.
- Block membrane with 5 % (w/v) skim milk powder in PBS/TBS.
- Incubate membrane with primary antibody diluted 1:100-1:10 000 in blocking solution for 1-16 h at 4 °C.
- Wash membrane with PBS/TBST (3 X 5 min).
- Incubate membrane with secondary antibody 1:1000-1:100 000 in blocking solution for 1-16 h at 4 °C.
- Wash membrane with PBS/TBST (3 X 5 min).
- Develop blot with chemiluminescence reagent, expose blot to film, and develop.

Co-immunoprecipitation

- Prepare cell lysates as described in protein extracts section.
- Dilute protein extract 1:10 in lysis buffer.
- To 1 ml of cell lysate (250-1000μg total protein) add 1-5 μg of primary antibody and nutate at 4 °C for 1 h.

- Add 30-50 µl of Protein G-Agarose (commercially purchased) to IP reaction and nutate 4-16 h at 4 °C.
- Pellet IP reaction by centrifugation at 1000xg for 30 sec.
- Wash pellet with 1 ml of lysis buffer.
- Repeat steps 5 and 6 two more times.
- Resuspend pellet in 40 µl of 2 X SDS sample buffer and boil for 3 min, transfer supernatant to new tube.
- Analyze sample by Western analysis.

RNA Isolation

- Add 1 ml of Trizol to 100 – 35 mm dish, agitate for 5 min and then transfer solution to microfuge tube.
- Add 200 µl chloroform to cell suspension, vortex for 15 sec, and leave at RT for 2-3 minutes.
- Centrifuge samples at 12 000g for 15 min at 2-8 °C.
- Following centrifugation, there will be three phases visible within the tube. Transfer the aqueous phase (top) to a fresh tube
- Add 500µl of isopropanol to the aqueous phase and incubate atRT for 10 min.
- Centrifuge samples at 12 000g for 10 min at 2-8 °C.
- Following centrifugation, remove the supernatant and leave pellet.

- Wash RNA pellet with 70% ethanol.
- Centrifuge samples at 7 500g for 5 min at 2-8 °C.
- Remove supernatant and air dry for 5-10 min.
- Redissolve the pellet in 25-50 µl of DEPC-treated water by heating at 70 °C for 5 min.

Immunofluorescence

- Wash cells several times with cold PBS.
- Fix cells with 4 % paraformaldehyde in PBS for 10 min at RT.
- Wash cells several times with PBS.
- Permeabilize cells with 0.3 % Triton-X in PBS.
- Block cells with 10 % serum in PBS (use serum from species that secondary antibody is raised in).
- Incubate cells with primary antibody diluted anywhere from 1:50 – 1:1000.
- Wash cells several times with PBS.
- Incubate cells with secondary antibody (1:50 – 1:1000) directed against IgG from species the primary antibody was raised in.
- Wash cells several times with PBS, add a drop of appropriate mounting media, and cover slip before taking picture.

Lipofectamine in A10 cells

- Seed cells at 80% confluence in 10 cm plates the night before.
- Dilute 8 μg of DNA in 800 μl serum- and antibiotic-free media.
- Mix 20 μl of Lipofectamine reagent in 800 μl serum- and antibiotic-free media.
- Combine above, mix and incubate for 15 minutes (up to 45 minutes).
- Add 1.6 mls of serum- and antibiotic-free media to the mix.
- Re-feed cells in 3.2 mls of serum- and antibiotic-free media.
- Add the DNA/Lipo mix and gently rock.
- Incubate of 2 hours.
- Wash 2x in PBS and re-feed in growth media.

ABSTRACTS AND PRESENTATIONS

PKA promotes MEF2/HDAC repression of the c-Jun promoter in vascular smooth muscle cells

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Vascular smooth muscle cells (VSMCs) do not terminally differentiate, but can modulate their phenotype in response to extracellular stimuli. Although proliferative VSMCs are required during vascular repair, the activated phenotype also plays a role in vascular disease. To investigate the role of myocyte enhancer factor 2 (MEF2) in the induction of the growth-responsive gene, c-Jun, we utilized the A10 line. Mitogenic stimulation by platelet derived growth factor (PDGF) resulted in marked induction of c-Jun protein and promoter activity. This induction was attenuated by rottlerin and KN-62. Given that these signaling pathways have been shown to relieve the repressive effects of histone deacetylases (HDACs) on MEF2 proteins, we overexpressed HDAC4, which repressed the c-Jun promoter. Mutation of the MEF2 binding site in the c-Jun promoter resulted in activation during quiescent conditions, while treatment with trichostatin A, increased c-Jun protein. Interestingly, activation of protein kinase A (PKA) prevented PDGF induction of c-Jun, and repressed a MEF2-dependent reporter gene. PKA also caused nuclear accumulation of HDAC4 and stabilized the interaction of HDAC4 with MEF2D. Thus, it appears that MEF2 and HDAC4 act to repress c-Jun expression in quiescent

VSMCs, PKA enhances this repression, and PDGF derepresses through CaMKs and novel PKCs. Supported by CIHR and the Heart and Stroke Foundation of Canada.

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PKA REGULATED ASSEMBLY OF A MEF2/HDAC4 REPRESSOR COMPLEX CONTROLS C-JUN EXPRESSION IN VASCULAR SMOOTH MUSCLE CELLS

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Vascular smooth muscle cells (VSMCs) maintain the ability to modulate their phenotype in response to changing environmental stimuli. This phenotype modulation plays a critical role in development of most vascular disease states, including atherosclerosis and restenosis. The purpose of these investigations was to evaluate the role of MEF2 proteins in the induction of the immediate early gene, *c-jun*, a critical regulator of VSMC proliferation. In these studies, stimulation of cultured vascular smooth muscle cells with platelet derived growth factor (PDGF) resulted in marked induction of *c-jun* expression, which was attenuated by protein kinase C delta (PKCδ) and calcium/calmodulin-dependent protein kinase (CaMK) inhibition. Given that these signaling pathways have been shown to relieve the repressive effects of class II histone deacetylases (HDACs) on MEF2 proteins, we ectopically expressed HDAC4, and observed repression of *c-jun* expression. Congruently, suppression of HDAC4 by RNA interference resulted in

enhanced *c-jun* expression. Consistent with these findings, mutation of the MEF2 *cis* element in the *c-jun* promoter resulted in promoter activation during quiescent conditions, suggesting that the MEF2 *cis* element functions as a repressor in this context. Furthermore, we demonstrate that protein kinase A (PKA) attenuates c-Jun expression by promoting the formation of a MEF2/HDAC4 repressor complex by inhibiting salt-inducible kinase 1 (SIK1). Finally, we show that forced expression of c-Jun represses myocardin's ability to activate smooth muscle gene expression. Thus, it appears that MEF2 and HDAC4 act to repress c-Jun expression in quiescent VSMCs, PKA enhances this repression, and PDGF derepresses c-Jun expression through CaMKs and novel PKCs. Regulation of this molecular 'switch' on the *c-jun* promoter may thus prove critical for toggling between the activated and quiescent VSMC phenotypes.

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PKA REGULATED ASSEMBLY OF A MEF2/HDAC4 REPRESSOR COMPLEX CONTROLS C-JUN EXPRESSION IN VASCULAR SMOOTH MUSCLE CELLS

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ICRH Theme: Biomedical, Cardiovascular health

PURPOSE: Vascular smooth muscle cells (VSMCs) are specialized for regulating blood flow to tissues; however, theses cells also maintain the ability to increase their numbers in order to heal an injured vessel. This modulation of cell 'programming' also plays a critical role in development, and in most vascular diseases. Previous studies have shown that mice with a genetic mutation in the myocyte enhancer factor 2C (MEF2C) gene fail to form a proper vasculature and have decreased expression of smooth muscle proteins. Therefore, we hypothesized that MEF2 proteins would have a critical role regulating VSMC division and specialization.

METHODS: To evaluate the signals that regulate VSMC division and specialization, we utilized a cell culture model, and induced VSMC division by arterial injury in an animal model that makes a blue indicator when MEF2 proteins are activated.

RESULTS: Stimulation of cultured VSMCs with platelet-derived growth factor (PDGF), an agent know to induce cell division, resulted in an increased expression of the MEF2-target gene called c-Jun. This increased production of c-Jun was inhibited by pharmacological agents that block the protein kinase C delta (PKCδ), and calcium/ calmodulin kinase (CaMK) pathways. Given that these signaling pathways have been shown to relieve the repressive effects of histone deacetylases (HDACs) on MEF2 proteins, we performed gain and loss of function experiments on HDAC4 to reveal its involvement in c-Jun regulation. Using animal models of vascular injury we observed an increased MEF2 activation, and increased production of c-Jun. Furthermore, we demonstrate that protein kinase A (PKA) inhibits c-Jun production by promoting the

formation of a MEF2/HDAC4 repressor complex. We also demonstrate that c-Jun production is inhibited when VSMC specialize. Finally, we document a physical interaction between c-Jun and myocardin, and we demonstrate that c-Jun represses myocardin's ability to promote VMSC specialization.

CONCLUSION AND IMPACT: These studies demonstrate that MEF2 and HDAC4 act to repress c-Jun production in specialized VSMCs. PKA enhances this repression, and PDGF inhibits this repression. Regulation of this molecular 'switch' may prove critical for toggling between the cellular programs that regulate cell division and specialization during vascular disease.

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RhoA signalling regulates MEF2-dependent myocardin expression in vascular smooth muscle cells.

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ICRH Theme: Biomedical, Cardiovascular health

PURPOSE: Contraction of vascular smooth muscle cells (VSMC) is triggered by an increase in cellular calcium. Once elevated, VSMCs regulate their calcium sensitivity through the RhoA signaling pathway. Interestingly, this RhoA signaling cascade has also been shown to regulate the production of smooth muscle contractile proteins. In addition, mice that habour a genetic mutation in the myocyte enhancer factor 2C (MEF2C) gene

fail to form a proper vasculature and have decreased expression of smooth muscle contractile proteins. Therefore, we hypothesized that the RhoA pathway might regulate the activity of MEF2 proteins.

METHODS: To evaluate calcium-mediated signaling in VSMCs, we used a cell culture model treated with high levels of potassium chloride (KCl) to promote calcium entry, along with gene transfection techniques, and common pharmacological inhibitors that block specific signaling pathways.

RESULTS: KCl treatment of VSMCs increased the protein of level of the MEF2-target genes myocardin and c-Jun. Analysis of the gene regulatory regions for c-Jun and myocardin revealed that KCl induction requires the MEF2 binding site for increased expression. Interestingly, increased expression of myocardin was prevented by pharmacological inhibition of the RhoA and p38 signaling pathways; whereas, increased c-Jun expression was inhibited by blockade of the calcium/calmodulin pathway. We have previously identified protein phoshatase 1α (PP1) as a potent repressor of MEF2 activation that is regulated by p38 signaling to MEF2 proteins. In VSMCs, treatment with a PP1 inhibitor resulted in increased expression of myocardin. Consistent with our pharmacological findings, forced expression of PP1 could inhibit myocardin expression, and the RhoA-regulated PP1 inhibitor, CPI-17, could rescue PP1's repressive effects.

CONCLUSION AND IMPACT: These data provide evidence of a novel signaling pathway that links RhoA-mediated calcium sensitivity to MEF2-dependent myocardin expression in VSMC through a mechanism involving p38 and PP1α regulation of MEF2

proteins. This knowledge could have important implications for both vascular disease and birth defects associated with heart's outflow tract.

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