SIGNAL-DEPENDENT TRANSCRIPTIONAL REGULATION OF VASCULAR SMOOTH MUSCLE CELL DIFFERENTIATION

CHRISTINA PAGIATAKIS

A DISSERTATION SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

GRADUATE PROGRAM IN BIOLOGY
YORK UNIVERSITY
TORONTO, ONTARIO

FEBRUARY 2015

ABSTRACT

Vascular smooth muscle cells (VSMCs) play a key role in development as they are the major source of extracellular matrix components of vessel walls. During development, VSMCs will both proliferate and differentiate to form components of the vasculature. Differentiated VSMCs (contractile phenotype) line vessel walls to regulate blood flow. The proliferative phenotype of VSMCs (synthetic phenotype) refers to migration and proliferation of these cells to specific sites to contribute to the formation of the vasculature. Interestingly, VSMCs maintain the ability to proliferate post-natally in response to vascular injury. Therefore, the purpose of this body of work was to investigate the signalling pathways that regulate transcriptional control in VSMCs.

Calcium sensitivity in VSMCs is regulated by RhoA/ROCK-mediated inhibition of the myosin light chain phosphatase complex, and alterations in smooth muscle gene expression. We found that calcium signalling stimulates ROCK-mediated phosphorylation of the PP1α inhibitor CPI-17 at threonine 38, leading to derepression of MEF2C by PP1α and increased myocardin expression, which lies upstream of smooth muscle-specific structural genes.

Furthermore, TGF-β also potently induces VSMC marker genes at the transcriptional and protein levels in 10T1/2 mouse embryonic fibroblast cells. We found that the potent transcriptional regulator and nuclear retention factor, TAZ, is required for TGF-β induction of smooth muscle genes and is required in the maintenance of the differentiated VSMC phenotype. A synergistic interaction between TAZ and SRF in regulating smooth muscle gene activation and differentiation has also been observed, and TAZ expression enhances SRF binding to the smooth muscle α-actin promoter.

This work addresses several important aspects of signalling pathways involved in the regulation of the vascular smooth muscle phenotype and provides a further understanding of the role of SRF in vascular development and vascular disease.

DEDICATION

...for science

ACKNOWLEDGEMENTS

I would like to thank my parents, Spiros and Roula, who have always been my undying support, taught me everything I know, and who have always pushed me to excel. I owe everything to you; I wouldn't be where I am today without you. I would like to thank my little sister, Katerina, without whom would I be able to accomplish anything. Words are insufficient to fully express your role in my life; you are my rock, my support and my best friend. I love you.

I would like to thank my supervisor, Dr. McDermott, for his mentorship, guidance, advice and patience throughout my years as a PhD student. I owe my scientific and academic success to you. I would also like to acknowledge my committee members for their feedback and evaluation over the years, and for taking the time to read my work.

Thank you to my labmates, past and present, for making these past six years memorable, and for sharing in our scientific ups and downs. In particular, I would like to thank Joe, who mentored me throughout my studies, and who taught me more than he will ever know. I would also like to acknowledge Tetsuaki, Saviz, Dandan and Stephanie for their work and contributions over the past few years.

Finally, I would like to thank all my friends, who have been by my side throughout everything. To my best friend Louie; you have touched my life in ways you will never understand. Thank you for your unwavering love and support. You are my angel. To my girls Mary, Christina, Marianna, Eve, Maria, Frannie and Natalia, and my cousin Nicoletta; thank you for being my sisters and for being my home away from home.

TABLE OF CONTENTS

TABLE OF FIGURES

INTRODUCTION

MANUSCRIPT 1:

MANUSCRIPT 2:

Additional Work I: Book Chapter

Additional Work II: Manuscript 3

ABBREVIATIONS

CaMK	calcium/calmodulin-dependent kinase
CPI-17	PKC-potentiated protein phosphatase inhibitor of 17kDa
ERK	extracellular regulated kinase
HDAC	histone deacetylase
MADS	MCM1, Agamus, Deficiens, SRF
MAPK	mitogen activated protein kinase
MEF2	myocyte enhancer factor 2
MLC	myosin light chain
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
PDGF	platelet-derived growth factor
PKC	protein kinase C
PKN	protein kinase N
PP1α	protein phosphatase 1 alpha
ROCK	RhoA associated kinase
SMαA	smooth muscle alpha actin
SM-MHC	smooth muscle myosin heavy chain
SRF	serum response factor
TAZ	transcriptional co-activator with PDZ binding motif
TGFβ	transforming growth factor β1
VSMC	vascular smooth muscle cells

CHAPTER 1: REVIEW OF LITERATURE

INTRODUCTION

Vascular smooth muscle cells (VSMCs) are a key component of development, as they are the major source of extracellular matrix components of vessel walls (1). During development, VSMCs will both proliferate and differentiate to form components of the vasculature. In development, the proliferative phenotype of VSMCs (synthetic phenotype) refers to migration and proliferation of these cells to specific sites to form the vasculature, whereas differentiated VSMCs (contractile phenotype) line vessel walls to regulate blood flow.

Post-natally, VSMCs modulate their phenotype in response to various extracellular signals, and, unlike striated muscle, do not terminally differentiate. This phenotypic modulation and expression of either the synthetic or contractile phenotypes is not mutually exclusive. Differentiated VSMCs in mature vessels express matrix components and proliferate at low levels. However, following vascular injury, contractile VSMCs down-regulate muscle-specific differentiation genes and increase proliferation, to contribute to the vascular regenerative response and promote vessel healing (2). This phenotypic modulation, from contractile to proliferative, is key to maintaining the integrity of the vascular system, but also plays an important role in many vascular diseases, such as atherosclerosis and restenosis following angioplasty (3).

Presented below, is a detailed review of the literature surrounding the signalling pathways and proteins controlling vascular smooth muscle gene expression.

VASCULAR SMOOTH MUSCLE

The role of vascular smooth muscle cells (VSMCs) has been shown to be a key component of development, as they regulate blood flow through the vasculature, and are also a major source of extracellular matrix components of vessel walls (1). During development, VSMCs will both proliferate and differentiate to form components of the vasculature. In development, the proliferative phenotype of VSMCs (synthetic phenotype) refers to migration and proliferation of these cells to specific sites to contribute to the formation of the vasculature. On the other hand, differentiated VSMCs (contractile phenotype) line vessel walls to regulate blood flow. The earliest VSMC differentiation marker is smooth muscle α-actin (SMαA), whose expression can be detected as early as smooth muscle precursors are recruited into the vessel wall. Following SMαA induction, other smooth muscle-marker genes are sequentially induced: SM22, calponin, SM-MHC I, and finally SM-MHC II (1). Regulation of these genes by various transcription factors such as SRF, myocardin and MEF2 will be further discussed.

Post-natally, VSMCs modulate their phenotype in response to various extracellular signals, and unlike cardiac and skeletal muscles, do not terminally differentiate. This phenotypic modulation and expression of either the synthetic or contractile phenotypes is not mutually exclusive. Differentiated VSMCs in mature vessels express matrix components and proliferate at low levels. However, following vascular injury, contractile VSMCs down-regulate muscle-specific differentiation genes and increase proliferation, to contribute to the vascular regenerative response and promote vessel healing (2). This phenotypic modulation, from contractile to proliferative, is key to maintaining the integrity of the vascular system, but also plays an

important role in many vascular diseases, such as atherosclerosis and restenosis following angioplasty (3). Therefore, the phenotypic regulation of vascular smooth muscle cells and the decision to differentiate or proliferate is of scientific interest.

Smooth muscle cells are not only expressed in the vasculature, but also in other organs. The expression patterns of the structural and functional components of smooth muscle cells vary between cell types, and also within different structures of the vasculature. Quiescent vascular smooth muscle cells contribute significantly to the regulation of blood flow, blood pressure and vascular tone, and regulate the elasticity of the vascular wall. While the smooth muscle in blood vessels, bronchioles and sphincters contract tonically, the smooth muscle of the digestive tract contracts in a peristaltic manner to aid in digestion. In various smooth muscle tissue, specialized smooth muscle cells also perform non-contractile functions, in which they secrete factors in response to extracellular signals, pressure changes and osmotic changes (4). Although expression of RhoA/ROCK is ubiquitous, it has been noted that there are increased levels of mRNA in selected tissues, such as brain and muscle. Furthermore, it has been shown that altered expression of RhoA/ROCK in the vasculature accounts for incidence of vascular disease. Since ROCK activity is regarded as the major regulator of calcium sensitivity in vascular smooth muscle cells, its expression is higher in the pulmonary artery, mesenteric artery and portal veins, and regulates the tonic phase of muscle contraction (5).

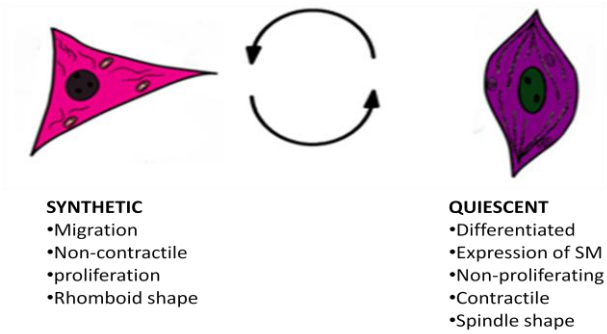

Figure 1: Schematic representation of vascular smooth muscle cell phenotypic switching between a quiescent/contractile and a proliferative/synthetic state. Adapted from (6).

CARDIOVASCULAR DEVELOPMENT

CARDIAC DEVELOPMENT

During development, the heart is the first organ to become fully functional in mice; during the late gastrulation stage, cardiac progenitor cells migrate medially from the lateral plate mesoderm. By E7.5, these cells form an arched structure called a cardiac crescent (3,7) The migration of these cells occurs in response to fibroblast growth factor 8 (Fgf8), while the induction of early cardiac marker genes (Nkx2.5 and Tbx5) occurs in response to bone morphogenetic proteins (BMPs), which are secreted from the underlying endoderm (3,8). At E8.5, the cardiac crescents fuse to form a beating primitive heart tube; the inflow region is located caudally (which will later become the atria), and the outflow is located cranially (which will later for the outflow tracts) (3,9). The formation of the heart tube occurs in response to an increase in Nkx2.5 and GATA4 and GATA 5 transcription factors. The linear heart tube is comprised of myocardial and endocardial layers. These layers are distinct and are separated by extracellular matrix (7,10).

Between E9.5 and 10.5, the linear heart tube undergoes a process called rightward looping, whereby the four chambers of the heart begin to form. The direction of looping is determined by Sonic hedgehog (shh) and Nodal signalling in the lateral mesoderm. The process of looping is essential for the proper formation and position of the atria and ventricles, and also proper alignment with the pulmonary and systemic outflow tracts (3,7). Although the exact mechanism by which looping occurs is unknown, it has been shown that MEF2C and Hand factors play a critical role; inhibition of these genes results in looping defects following heart tube formation (11,12).

During the looping process, at approximately E9.5, the pro-epicardium starts to form in the area of the sinoatrial junction. This structure will later give rise to the epicardium, the coronary vasculature and the cardiac conduction system (3,13). Epicardial cells migrate onto the myocardium to cover the heart's surface. The coronary vasculature forms as a result of epithelial to mesenchymal transition of the pro-epicardial cells. Following this, endothelial cells undergo vasculogenesis, and VSMCs are recruited (13).This process occurs in response to secretion of growth factors (VEGF, FGF and angiopoietin) from the myocardium, which promote vasculogenesis (13).

The process of vascular remodelling occurs to connect the coronary circulation with the developing systemic circulation. As the coronary arteries develop, they will secrete endothelin-1, which will cause the ventricular cardiomyocytes surrounding the coronary arteries to differentiate. This differentiation gives rise to cells that will form the purkinje fibres of the cardiac conduction system (14). The development of the ventricles, which occurs during and after cardiac looping, is regulated by the Hand (heart and neural crest derivatives) family of

bHLH transcription factors. Hand1/eHand has been implicated in left ventricle development and formation, whereas Hand2/dHand has been shown to be required in right ventricle formation (15).

The endocardial cushions, which will become cardiac cushions, begin to form by E10.5 in the primitive inflow and outflow tracts. These endocardial cushions will differentiate into the valves to allow blood flow through the heart, in a TGF-β-dependent manner. They are also involved in septation of both the atrioventricular canal and the conotruncus, which occurs as the outflow tract wedges on top of the inflow tract. This allows the inflow and outflow tracts to connect with the developing ventricular septum, which is growing from below (16). These processes, together, are what form the distinct left and right chambers of the heart. By E12.5, this, along with formation of aortic and pulmonary arteries is complete (3).

Heart growth occurs not only during the process of looping, but also afterward; both proliferation and differentiation occur to contribute to heart muscle growth, where the most rapidly dividing cardiomyocytes are located on the outer surface of the heart. This is thought to be in response to mitogen release by the epicardium. An important factor released from the epicardium is retinoic acid, which has been shown to be critical in not only the regulation of heart growth, but also specification. Post-natal cardiomyocytes are terminally differentiated, and do not proliferate (8). Various transcription and growth factors have been implicated in the regulation of cardiomyocyte proliferation, such as GSK3, which acts through β-catenin and Myc; GSK-3β-null mice display hyperproliferative cardiomyocytes (17). Another factor implicated in the regulation of cardiomyocyte proliferation is p38 MAPK; in adult cardiomyocytes inhibition of p38 induces proliferation (18). On the other hand, there are also various transcription factors

that are implicated in proliferation of cardiomyocytes, and therefore cardiomyocyte growth and hypertrophy. The MEF2 family, SRF, Myocardin and GATA factors have been studied in this context. SRF and Myocardin have both been shown to be required for cardiac gene expression, and MEF2A and MEF2C null mice have reduced expression of cardiac muscle genes, and also thin, dilated ventricles (19). Furthermore, GATA4 has been shown to be a co-factor of MEF2 and myocardin, which will induce gene expression of contractile proteins (20). Overall the heart and the developing vasculature need to be connected, a process which occurs by cell migration from the neural crest (originating from rhombomeres). These migrating cells have been shown to play a critical role in septation, and will form the vascular smooth muscle cells of the aortic and brachial arch arteries, which will later form the ductus arteriosus and the subclavian and carotid arteries (7).

The myocardial wall of the proximal outflow tract is formed from progenitors originating for the pharyngeal mesoderm. These progenitors will migrate to the heart after heart tube formation is complete; as this is the second migration of progenitor cells, they have been called the secondary heart field. The secondary heart field arises from the pharyngeal mesoderm, whereas the primary heart field (first instance of progenitor cell migration) is derived from the lateral plate mesoderm to give rise to the cardiac crescent (21). Studies have shown that the proximal outflow tract and the right ventricle are derived from the secondary heart field, which elongates the heart tube and aids in the growth of the right ventricle. There are many factors involved in the secondary heart field, including Isl-1, MEF2 and GATA. Isl-1 null mice display cardiovascular defects, and their hearts do not undergo the process of looping, and do not

develop a right ventricle or outflow tract (21). MEF2C null mice also display looping defects (11).

VASCULAR DEVELOPMENT

Vascular development occurs via two interconnected processes; vasculogenesis and angiogenesis. Vasculogenesis is the process of creating new vessels from undifferentiated mesodermal progenitors whereas angiogenesis is the process whereby the source of new cells is the endothelium of existing vessels (22). The process of vasculogenesis occurs cranially-to-caudially, and can be separated into four stages. Firstly, endothelial cells from mesodermal progenitors (angioblasts) will differentiate in response to VEGF and its receptor (VEGFR-2). Secondly, primordial vessels from endothelial cells will form in response to platelet/endothelial adhesion molecule, PECAM and CD34. Thirdly, formation of endothelial tubes occurs in response to VEGF and the receptor VEGFR-1. Finally, the formation of primary vascular networks will occur in response to the integrin family of receptors (22).

Once the primary vascular networks are formed, they are remodelled by angiogenesis, which involves branching, sprouting, migration and proliferation. These together will begin the formation of a more mature network, which will then recruit mural cells (pericytes and smooth muscle cells) in response to secreted factors (PDGF, TGF-β and angiopoietins) (23). VSMCs are recruited from diverse embryonic origins to developing vascular networks, largely in response to secreted PDGF, TGF-β1, and angiopoietins. Interestingly, recruitment of VSMCs to primitive vessels is concurrent with onset of blood flow and increasing pulse pressure (22).

The embryonic source of endothelial progenitors appears to be mesodermal; 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 at E8 (24). Studies have suggested that these lineages share a common progenitor, the hemangioblast; TAL1/SCL and T-box transcription factors have been shown to be expressed in both early and haematopoietic and angioblast lineages (25). Both cardiac heart fields (primary and secondary), as well as endothelial lineages, share a common mesodermal progenitor; interestingly, MEF2C has been shown to play a critical role in regulation of these (26,27).

The initial stages of vascular development are genetically regulated via a variety of factors. However, there are many environmental factors (haemodynamics, hypoxia, etc) that regulate vasculogenesis. For example, hypoxia will induce expression of a variety of genes (VEGF, Flk-1, Flt-1 and Tie2) to promote vascular remodelling. This phenomenon occurs via induction of HIF-1α (hypoxia inducible factor), which dimerizes with the constituitively expressed HIF-1β to form an active transcription factor complex and induce the above-mentioned genes (24). Disruption of VEGF results in embryonic lethality at E8.5 and absence of a dorsal aorta. Flk-1 null mice also result in embryonic lethality at E8.5 and defects in angiogenic and haematopoietic precursors. Disruption of Tie2 (angiopoietin receptor) results in embryonic lethality between E9.5 and E10.5, and endothelial tubes lack mural cells (22). As the vasculature is still forming, circulation begins. The onset of circulation inevitably results in various haemodynamic forces that will also contribute to the development of vascular networks. An example of this is the induction of eNOS (endothelial nitric oxide synthase) via forces created by flow of erythrocytes, which modulates vascular remodelling (28).

Recruitment of primitive vascular smooth muscle cells plays an important role in development of the vasculature, as they comprise a major part of the extracellular matrix that forms vessel walls. Primitive VSMCs have been shown to originate from a variety of developmental origins, even within the same vessel (29). The descending aorta has been used as a model for vessel wall morphogenesis studies; VSMC progenitor migration can thus be observed, ending up at the circumference of the vessel and creating the outer smooth muscle layer. Originally, VSMCs were thought to be derived from the splanchnic layer of the ventrolateral plate mesoderm, however recent studies have shown that these specifically derived VSMCs are restricted to the abdominal aorta and iliac arteries (22). Migrating neural crest cells contribute to pharyngeal arch development and VSMC differentiation of aortic arch arteries. Furthermore, a subset of migrating cells will mediate septation of the cardiac outflow tract to produce the aorta and pulmonary trunk (29). Furthermore, the role of VSMCs in extracellular matrix production is a key one, as the rate of ECM deposition is dependent on the dynamic changes in the vessel wall. Since matrix proteins bind to their associated integrin receptors to initiate intracellular signalling, these complexes are key in transducing mechanical forces into biological responses; these responses will activate signalling pathways important in regulating VSMC phenotype and vascular tone (30).

During the process of development, the aortic arch arteries are transient structures which will initially appear as six bilaterally paired vessels, which grow out of the cardiac outflow tract and common carotid arteries (16). Arches 1, 2, and 5 exist transiently, whereas arches 3, 4, and 6 undergo extensive remodelling to become the major embryonic outflow arteries. Arch 3 will become the internal carotid arteries while the right fourth arch remodels to become the

right subclavian artery, and the left fourth arch becomes the aortic arch. The right sixth branch regresses and the left sixth arch becomes the pulmonary arteries and the ductus arteriosus (16).

As previously mentioned, there are a variety of factors involved in the control of vascular development, and more specifically, the migration of VSMCs. One such factor is endothelin-1, which recruits neural crest-derived VSMCs through Hand and MEF2C (31). Furthermore, the myocardin family of transcription factors has been shown to play a role not only in induction of smooth muscle specific genes, but also the development of the aortic arch (32). Studies suggest the importance of GATA is in morphogenesis of VSMCs; inactivation of GATA6 results in patterning defects in the aortic arch arteries (33).

CALCIUM SIGNALLING PATHWAYS IN VSMCs

EXCITATION-CONTRACTION AND EXCITATION-TRANSCRIPTION COUPLING

In VSMCs, the rise and fall of intracellular calcium will initiate contraction and relaxation, respectively. The average intracellular concentration of free calcium is significantly lower than the extracellular free calcium (34). Thus contraction is activated by calcium binding to calmodulin (CaM), which directly activates myosin light-chain kinase (MLCK) (35). Activated MLCK will then phosphorylate myosin light-chain MLC_{20} at serine-19, in order for myosin ATPase to be activated by actin, resulting in activation of contraction (36). When intracellular levels of calcium fall, there is a resulting inactivation of MLCK; MLC_{20} will thus be dephosphorylated by myosin light-chain phosphatase (MLCP). This dephosphorylation results in deactivation of myosin ATPase and thus muscle relaxation (37). The process of activation of contraction by

elevated levels of calcium is termed excitation-contraction coupling (EC-coupling), and is a result of two processes: electromechanical coupling and pharmacomechanical coupling. The former involves changes in the cells' membrane potential, and the latter involves ligand activation of membrane-bound receptors. Both these processes result in the activation of contraction (35).

During electromechanical coupling, depolarization of the membrane will cause the opening of voltage-gated calcium channels (such as L-type calcium channel), calcium influx and muscle contraction. This process is thought to induce calcium release from the sarcoplasmic reticulum. In pharmacomechanical coupling, ligands (such as hormones or neurotransmitters) can modulate calcium sensitivity by regulating MLCP activity, release of calcium from the sarcoplasmic reticulum and also generation of second messenger molecules (36). Conversely, to allow for muscle relaxation, the concentration of intracellular calcium lowers by cell membrane hyperpolarization to prevent further calcium influx through the voltage-gated channels and calcium uptake by ATPases (38).

The process by which signalling pathways regulate EC-coupling is termed excitation-transcription coupling (ET-coupling). This process also regulates changes in gene expression. Since VSMCs can modulate their phenotype in response to extracellular signals, this process has been shown to be different than terminally differentiated muscle cells (cardiac and skeletal). Studies have shown that a key factor in the process in VSMCs is serum response factor (SRF), at which many calcium signalling pathways converge to regulate the expression of smooth muscle genes. Transcriptional regulation of SRF has been shown to modulate VSMC phenotype as a

result of its various cofactors, which determine to which promoters (smooth muscle specific or immediate-early) it is recruited to (39,40).

CALCIUM/CALMODULIN KINASE SIGNALLING

As mentioned above, EC-coupling and ET-coupling play a major role in the regulation of VSMC tone. An effector of this regulation is the calcium-calmodulin complex. This complex activates MLCK to phosphorylate MLC_{20} to activate contraction. Furthermore, this complex activates the calcium-calmodulin-dependent protein kinases (CaMKs), which have also been show to play a key role in the regulation of ET-coupling in VSMCs (41,42). CaMKII has been extensively studied in smooth muscle, and has been shown to play a role in the modulation of MLCK sensitivity to calcium, as well as regulating L-type calcium channels (38). Various studies have shown that this regulation is a result of its binding to subunits of L-type calcium channels (43). CaMKII has been shown to directly phosphorylate class II histone deacetylase 4 (HDAC4) to promote its nuclear export and therefore de-repress HDAC4-dependent genes. Furthermore, smooth muscle migration in response to platelet derived growth factor (PDGF) has been shown to be through CaMKII; activation of CaMKII by PDGF appears to be dependent on MEK1, integrin receptors and FGF signalling (44).

RHOA/ROCK SIGNALLING

An important signalling molecule implicated in smooth muscle cells and more specifically in EC- and ET- coupling, is the small GTPase, RhoA. RhoA, and its downstream target ROCK (Rho-kinase) have been implicated in calcium sensitivity via regulation of myosin light-

chain phosphatase (MLCP) (36). In smooth muscle, MLCP is composed of three subunits: the catalytic subunit (protein phosphatase 1, PP1), the regulatory subunit MYPT1 (M_{110}), and an accessory subunit (M_{21}) of unknown function (37). MYPT1 acts as a myosin binding subunit in order to target MLCP to myosin light chain. Activation of the RhoA/ROCK pathway results in MYPT1 phosphorylation and inactivation of phosphatase activity; this increases phosphorylation of myosin light chain resulting in enhanced calcium sensitivity and contraction (36,37).

Furthermore, RhoA has not only been shown to be activated in an agonist-dependent manner, but also by membrane depolarization (45). Membrane depolarization induces RhoA to translocate to the membrane, where the RhoA signalling cascade is activated (45). Interestingly, calcium influx increases the expression of SRF-dependent smooth muscle genes such as SMA, SM22 and SMMHC. This effect was found to be ROCK- and myocardin- dependent indicating that calcium signalling is important in activating differentiation genes in vascular smooth muscle cells through a RhoA/ROCK and myocardin/SRF pathways (45).

Another of the downstream targets of RhoA is protein kinase N (PKN), which in smooth muscle, has been shown to promote differentiation by increasing the expression of smooth muscle marker genes. Overexpression of active PKN also increases the activity of SM alpha actin, SM22 and SMMHC promoters. Furthermore, knockdown of PKN inhibits expression of these genes (46).

Along with regulating calcium sensitivity, RhoA also regulates serum response factor in a mechanism called “actin treadmilling.” The formation of actin filaments, termed F-actin, which are comprised of G-actin monomers, is induced by ROCK. SRF-dependent genes are then

activated upon an increase in F-actin and decrease in free G-actin (47). Furthermore, studies have shown that activation of RhoA leads to activation of smooth muscle-specific genes, such as SMA and SM22 (48). It has also been shown that this induction is sensitive to the ROCK inhibitor Y27632, indicating a RhoA/ROCK-specific mechanism. Interestingly, studies have shown that RhoA-mediated F-actin formation promotes nuclear localization of the SRF co-activator MRTF-A (myocardin related transcription factor A) resulting in its association with G-actin monomers to promote cytosolic retention. Once MRTF-A is localized in the nucleus, it will activate a variety of SRF-dependent genes (49).

PKC SIGNALLING

There are three classes of protein kinase C (PKC) isoenzymes; conventional PKCs (α, β and γ), novel PKCs (δ, ε, η and θ) and atypical PKCs (ζ, ι and λ). PKCs are activated through G-protein-coupled receptors, tyrosine kinase receptors and non-receptor tyrosine kinases (50). The conventional PKCs are implicated in EC-coupling, whereby they regulate calcium sensitivity via MLCP activity (36), and also ET-coupling via growth stimulus (51).

In smooth muscle ET-coupling, conventional PKCs function through growth stimuli, such as angiotensin II, a vasoconstrictor that induces *c-fos* expression through the serum response element in the promoter region. Interestingly, PKCα and PKCε activate the serum response element via Elk-1 activation (52). Conventional PKCs activate SRF-dependent immediate early genes (such as *c-fos*). The consensus PKC phosphorylation site (Ser-162) within the MADS box of SRF has been shown to specifically target SRF to growth-responsive genes, and sequestering them away from muscle-specific genes such as smooth muscle alpha actin (53).

Conversely, it has been shown that PKCs phosphorylate a protein called CPI-17 at threonine-38, which is a potent inhibitor of the MLCP catalytic subunit. This in turn inhibits MLCP to promote calcium sensitivity, and thus up-regulation of smooth muscle specific genes (54). Interestingly, there is cross-talk between PKC and RhoA/ROCK phosphorylation of CPI-17, as both play a major role in the phosphorylation of CPI-17 and therefore promoting calcium sensitivity (55,56). Therefore agonists such as angiotensin II play a role in both the activation of smooth muscle specific genes in quiescent cells via activation of PKC and CPI-17, and in the activation of proliferative genes via SRF.

CPI-17 FUNCTION AND REGULATION IN VSMC

ROLE OF PP1 IN VSMC CONTRACTION

Calcium sensitivity in smooth muscle cells is regulated by several factors, including activation the RhoA/ROCK and PKC pathways. More specifically, calcium sensitivity in VSMCs is regulated by RhoA/ROCK-mediated inhibition of the myosin light chain phosphatase complex. Depolarization has been shown to induce sustained contraction of smooth muscle cells, as well as increased activation of RhoA. In addition, removal of extracellular calcium attenuates RhoA activation (57). Interestingly, this signalling pathway also acts at the level of gene expression by increasing the expression of serum response factor (SRF)-dependent smooth muscle-marker genes through regulation of the myocardin-family of SRF co-activators (58).

Protein phosphatase 1 (PP1) has been implicated in the control of many cellular functions, including muscle contraction, cell division, transcription, glycogen metabolism, cell cycle control and smooth muscle contraction. The 38kDa catalytic subunit of PP1, along with its

non-catalytic subunits, is localized to a variety of substrates under different cellular conditions. There are a number of modulator proteins that regulate PP1 activity, including inhibitor-1, inhibitor-2, DARPP32 and NIPP-1. Interestingly, an unidentified inhibitor protein for PP1 was isolated from porcine aorta smooth muscle; upon phosphorylation, this protein could suppress not only the PP1 catalytic subunit, but also the PP1 holoenzyme activity. Cloning and characterization of this protein revealed a 17kDa protein potentiated by PKC phosphorylation, termed CPI-17 (PKC-potentiated PP1 inhibitory protein of 17kDa). Expression of CPI-17 was found to be specific to smooth muscle tissues (54). CPI-17 functions very differently from other PP1 inhibitors, such that phosphorylated CPI-17 rapidly inhibits both the catalytic subunit and the holoenzyme of MLCP with extremely high potency. Interestingly, PKC phosphorylates CPI-17 and PKA phosphorylates inhibitor-1 (59).

The catalytic subunit of PP1 contains a bimetal active centre, which can dephosphorylate multiple phospho-Ser/Thr substrates on various proteins. The consensus binding site of the regulatory subunit is RVXF. A KIQF consensus sequence produces a phosphorylation-dependent inhibition of the catalytic subunit of PP1, and has been shown to be the docking site for inhibitor-1 and DARPP-32. The PP1 catalytic subunit can bind to both regulatory subunits and inhibitor proteins, but not consecutively. CPI-17 can inhibit the myosin phosphatase enzyme complex, but RVXF is not required for this inhibition. Since phosphorylation of Threonine38 occurs in response to stimulation of intact smooth muscle by agonists (such as histamine and phenylepherine), and dephosphorylation occurs in response to nitric oxide production, the mode of catalytic subunit inhibition is of great interest. Using phosphomimetic Threonine38 mutants, it was found that the negative charge as a result of

glutamate substitution did not mimic the phosphate group, indicating that a phosphate or thiophosphate moiety on threonine38 is necessary for potent inhibition. It was also found that Tyr41 is crucial in preventing dephosphorylation of phospho-Thr38, by converting CPI-17 from an inhibitor to a substrate of MLCP. It has been shown that of all the inhibitors of PP1, CPI-17 is the only one to inhibit the MLCP holoenzyme (60).

In smooth muscle, PP1α is the catalytic subunit of the myosin light chain phosphatase complex, and determines calcium sensitivity and smooth muscle contraction. Contraction is triggered by the phosphorylation of myosin light chain (MLC), which is controlled via two signalling pathways; activation of MLC kinase (MLCK) and inhibition of MLC phosphatase (MLCP). MLCP is a heterotrimeric enzyme, which consists of the PP1 catalytic subunit (delta-isoform), a myosin targeting subunit (MYPT1) and an accessory M21 subunit. MYPT1 plays a critical role in tethering the PP1 catalytic subunit to myosin filaments (61). A rise in cytoplasmic calcium acts as the main trigger for phosphorylation of MLC through activation of MLCK (62). Inhibition of MLCP occurs upon G-protein activation in response to agonist stimuli; its activity is increased in response to nitric oxide production, which results in myosin dephosphorylation and smooth muscle relaxation (63). Increased calcium sensitization observed in muscle contraction is a result of inhibition of myosin phosphatase, increasing levels of myosin phosphorylation to reinforce contraction. Inhibition of myosin phosphatase is transmitted through G-protein-coupled mechanisms and the small GTPase RhoA plays a key role in this process. RhoA-induced inhibition of myosin phosphatase is a result of MYPT1 phosphorylation by Rho-associated kinase (ROCK) (61).

In addition, ROCK has been shown to regulate PP1α activity in VSMCs through the smooth muscle enriched phosphatase inhibitor CPI-17, which determines calcium sensitivity in smooth muscle contraction (63). CPI-17 is highly expressed in mature smooth muscle cells and is phosphorylated by PKC, PKN, and ROCK at threonine 38, a phosphorylation which enhances the affinity of CPI-17 for protein phosphatases, thus enhancing its inhibitory ability (64). The N-terminal region of myosin phosphatase contains an ankyrin repeat domain, which includes the PP1 binding site, which is the site for allosteric regulation of the catalytic subunit. The C-terminal domain is phosphorylated by multiple kinases, including ROCK, ZIPK and ILK, which results in inhibition of myosin phosphatase. In smooth muscle, the agonist-induced activation of G-proteins enhances the activity of calcium/calmodulin-dependent MLCK, which in turn suppresses the activity of myosin phosphatase through the activation of ROCK and PKC. Inhibition of myosin phosphatase results in sustained contraction of smooth muscle (64).

Studies have shown that protein kinase C (PKC) increases calcium sensitization, independent of RhoA/ROCK. PKC-dependent inhibition of myosin phosphate occurs through phorphorylation of CPI-17, which upon phosphorylation at threonine-38 has its inhibitory potency increased by over 1000-fold, and as a result, induces contraction in permeabilized smooth muscle. Thiophosphorylation of CPI-17 results in calcium sensitization, although even in its unphosphorylated form, CPI-17 is a potent inhibitor of PP1 (59).

STRUCTURE AND FUNCTION OF CPI-17

cDNA clones were isolated from a human aorta library; two isoforms of CPI-17 were identified: alpha and beta. The CPI-17α isoform contains 147 residues and is approximately

16.7kDa, whereas CPI-17β is 120 residues with a deletion of 27 residues from amino acids 68-94, and is 13.5kDa. The N-terminal domain of 67 residues is highly conserved among human, rat, mouse and pig. Full length CPI-17 contains 4 exons and 3 introns, whereby CPI-17β is an alternative splice variant as a result of deletion of the second exon of CPI-17α. CPI-17 is 5.3kb and is located on chromosome 19q13.1 (65). Zebrafish express a similar gene, however its functional relevance remains unclear. There have been no homologous genes detected in fruit fly, nematode and yeast genes, indicating that the CPI-17 family emerged at a later stage in evolution (66).

There has been no homology shown between CPI-17 and other PP1 inhibitors; CPI-17 contains three domains: N-terminal tail, C-terminal tail and a central 86-residue PHIN domain (residues 35-120). It has also been shown that Tyr41, Aps42 and Arg43 of CPI-17 are necessary for CPI-17's inhibitory activity (66). CPI-17 was mapped to determine regions required for inhibition of the PP1 holoenzyme. The central domain of CPI-17 (a.a 35-120), including Threonine38, is necessary for recognition by myosin phosphatase and interestingly, Tyr41 arrests dephosphorylation, resulting in inhibition (67).

CPI-17 forms a four-helix, V-shaped bundle comprised of a central anti-parallel helix pair containing B/C helices, flanked by two large spiral loops formed by the N- and C-termini. These are held together by an additional anti-parallel helix pair (A/D helices), which are stabilized by intercalated aromatic and aliphatic side chains. Phosphorylation at threonine38 induces a conformational change resulting in the displacement of Helix A (68). The CPI-17 inhibitory domain (a.a 22-120) is conserved amongst the CPI-17 family of PP1 inhibitor proteins (PHI-1, KEPI and GBPI). Multidimensional NMR experiments showed that the conformation of the

phosphorylated form of CPI-17 differs from that of the phosphomimetic mutants. Three-dimensional analysis of phosphorylated CPI-17 at Threonine38 revealed that there are 4 helices arranged in an anti-parallel orientation. Residues 22-31 in the N-terminal domain have a flexible conformation, and residues 32-40 (which contain Thr38) are termed the P-loop, which remains on the surface of the 4-helix bundle, keeping the P-Thr38 chain exposed to solvent. The phosphomimetic mutants of CPI-17 do not have an altered conformation in comparison to unphosphorylated CPI-17 (69).

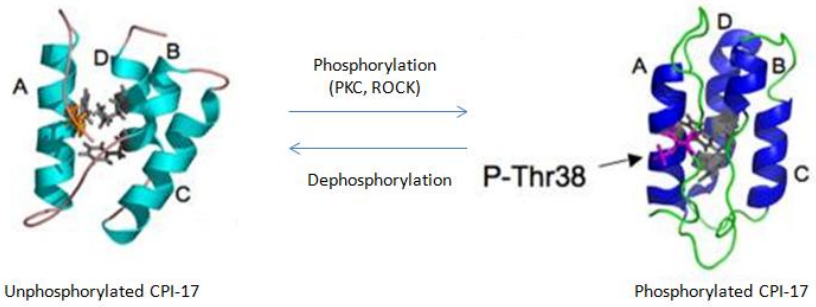

Figure 2: Structure of CPI-17 in phosphorylated and un-phosphorylated form. Adapted from (66)

ROLE OF CPI-17

CPI-17 is predominantly expressed in smooth muscle tissues and the brain, although there is higher expression in tonic muscles such as arteries, and lower expression in phasic muscles such is the ileum and urinary bladder. It is also expressed in embryonic epithelium. At E10, CPI-17 is exclusively expressed in the heart intercostal muscle and neuronal tissues and by E17, its expression is in smooth muscle tissues in the aorta, tongue, esophagus, intestine, lung and heart, and also in neuronal tissues. CPI-17 and SMA are co-expressed in arterial smooth

muscle and cardiac muscle cells, although the expression of CPI-17 is higher in arterial smooth muscle at E14 in comparison with E10. It is thought that the smooth muscle marker genes are expressed in embryonic cardiac muscle as a result of early myocardin activity; however, it is not yet known if the expression of CPI-17 is also regulated in this manner. From E10 to E14, the heart rate and blood flow of the embryo is significantly increased; this is because embryonic arterial smooth muscle cells increase their contractility through expression of CPI-17 in response to the gain of cardiac function and an increase in blood flow (63).

CPI-17 is down-regulated in the proliferative phenotype in smooth muscle cells and in the neointima (63). CPI-17 transcription has been recently studied; it is suppressed in response to proliferative stimuli such as PDGF, through the ERK1/2 pathway. CPI-17 transcription is elevated in response to inflammatory stress-induced and excitatory stimuli such as TGFβ, IL-1β, TNFα, sorbitol and serotonin. CPI-17 transcription is repressed with inhibition of JNK, p38, PKC and ROCK. The CPI-17 promoter is governed by proximal GC-boxes at the 5' flanking region. It is insensitive to knockdown of myocardin, but positively regulated by sorbitol-induced p38/JNK pathways and negatively regulated by PDGF-induced ERK1/2. CPI-17 expression is synchronized to smooth muscle specific genes, although the transcription factors that regulate these promoters are different (70).

Under de-differentiation conditions, CPI-17 expression declines. Studies have shown that this occurs concurrently with active import of CPI-17 into the nucleus to regulate histone phosphorylation and cell proliferation. Using Panc1 (pancreatic cancer cells) as a model system to study the proliferative role of CPI-17, it was shown that CPI-17 is nuclear when cells are in a proliferative state, and that the N-terminal tail of CPI-17 forms a basic residue cluster similar to

a nuclear import signal. Mutational analysis showed that phosphorylation of CPI-17 at Ser12 in the N-terminal tail is sufficient to attenuate NLS activity. Furthermore, nuclear CPI-17 was shown to inhibit a subset of PP1 that is responsible for dephosphorylation of histone H3, which upregulates cell proliferation (71).

CPI-17 is also expressed in the brain, and is phosphorylated *in vivo* on Ser128 by PKA and CamKII, and *in vitro* on Ser130 by CamKII (72). CPI-17 is thus involved in neuronal signalling, and deletion of CPI-17 by siRNA revealed elimination of cerebellar long-term synaptic depression of Purkinje cells mediated by PKC (73).

CPI-17 regulates the tumor suppressor protein, Merlin, which regulates proliferation, and is activated by dephosphorylation at Ser518. This phenomenon occurs upon serum withdrawl, cell-cell contact or cell-matrix contact. It has been shown that CPI-17 levels are increased in several human tumor cell lines, and downregulation of CPI-17 induces merlin dephosphorylation, which inhibits Ras activation (74).

The role of CPI-17 has also been studied in diabetic vascular pathology. In early diabetes, abnormalities in blood vessel constriction or dilatation can be detected. These abnormalities result in blood flow dysregulation and increased peripheral resistance resulting in diabetic retinopathy, hephropathy, neuropathy and hypertension. VSMCs from a type 2 diabetic mouse model show a significantly increased contractile response to stimuli, and studies show that several isoforms of PKC are activated in vascular tissue by hyperglycemia or diabetes, playing a role in diabetic cellular dysfunction. Studies revealed that both RhoA and CPI-17 are activated in the type 2 diabetic mouse in the mesenteric arteries and the aorta. Furthermore, the RhoA/ROCK pathway and CPI-17 phosphorylation appear to be upregulated in VSMCs under

high glucose conditions; RhoA is required for high glucose-induced CPI-17 phosphorylation, and enhanced ROCK activity contributes to the contractile hyperactivity of the mesenteric artery of the type 2 diabetic mouse model (75).

PP1, MYPT1 and M20 are involved in contraction and filament organization. Recent studies in barrier dysfunction in the pulmonary endothelium showed that these proteins are playing a role in the formation of extracellular gap formation. A two-hybrid study using a human lung cDNA library revealed five proteins involved in actin cytoskeleton reorganization and cell adhesion: plectin1, α-II-spectrin, OK/SW-CL16, gelsonin and junction plakoglobin (γ-catenin). CPI-17 was shown to interact with F-actin cytoskeleton scaffolding proteins and signalling molecules, and could potentially be an important modulator of the interaction between a cadherin/catenin complex on the plasma membrane or the actin cytoskeleton machinery (76).

MEF2 BIOLOGY

MEF2 FAMILY OF PROTEINS

MEF2 (myocyte enhancer factor-2) proteins belong to the MADS (MCM1, agamous, deficiens, SRF) family of transcription factors. In *S. cerevisiae, Drosophila* and *C. Elegans,* there is a single MEF2 gene; in vertebrates, the MEF2 family consists of four structurally conserved genes (A-D). The N-terminus of each MEF2 protein contains a conserved MADS/MEF2 domain, which mediates dimerization, DNA binding and cofactor interaction. The more divergent C-terminal domain functions as a transcriptional activation domain, and is the site of alternative splicing (77). The transcripts of these four genes are ubiquitous, but are more abundant in

skeletal muscle, heart, and brain. Interestingly, MEF2 expression alone is insufficient to induce a complete muscle phenotype (78). Where MEF2A and MEF2D are more ubiquitous in their expression, MEF2C is restricted to muscle, brain and spleen (79,80). MEF2D transcripts are also ubiquitously expressed (81). The MEF2 consensus binding site was identified as (C/T)TA(A/T)4TA(G/A), and MEF2A, C and D, but not B bind to this site with high affinity (82). The MEF2 consensus binding site has been identified in the regulatory regions of muscle contractile and structural genes, and also in the promoter regions of immediate-early genes, and in the promoter regions of genes regulating metabolism and apoptosis (83,84).

The N-terminal region of MEF2 proteins contains a highly conserved 57-amino acid DNA binding domain termed the MADS-box, which mediates homo- and heterodimerization between the different MEF2 proteins. Next to the MADS-box lies a 29-amino acid sequence termed the MEF2 domain. This domain is also highly conserved amongst MEF2 members, but interestingly is not found within other MADS-box proteins. This domain also serves to mediate DNA binding, dimerization, and recruitment of co-factors (58). The C-terminal region of MEF2 proteins is comprised of a transcription activation domain, which is the site of alternative splicing, resulting in less than 20% homology between the MEF2 factors. The C-terminal domain is also the region of phosphorylation by many kinases to regulate transcriptional activity. The C-termini of MEF2 A, C and D also contain a conserved nuclear localization signal (85,86).

SIGNALLING AND REGULATION

MEF2 proteins are regulated by various cofactors, and are also downstream of many signalling pathways, acting as central regulators for differentiation and organogenesis. Many interacting cofactors act to increase or decrease MEF2s transcriptional activity.

Interestingly, MEF2 proteins appear to be involved in both the proliferative smooth muscle phenotype, as well as in smooth muscle differentiation, depending on the extracellular signals received by the cell. MEF2 has been shown to be sensitive to a variety of calcium-mediated cellular signals, which positively modulate its transcriptional activity (58). For example, MEF2 proteins can be activated through calcium/calmodulin kinases (CaMKs), which phosphorylate class II histone deacetylases (HDACs) to relieve their repressive effects on MEF2 transcriptional activity (2). In addition to regulating the expression of VSMC contractile genes, MEF2 is also required for the serum-induction of the immediate-early *c-jun* gene, which is associated with cell-cycle progression (77). This phenotypic switching between contractile and proliferative states of VSMCs is mediated through interplay of MEF2 with HDAC4, and it appears that the two distinct phenotypes act in an opposing manner to eachother (2).

PP1α has been shown to inhibit MEF2 transcriptional activity by recruiting HDAC4 to MEF2A (60). Originally, PP1 proteins were thought to dephosphorylate class IIa HDACs to promote their nuclear import (87). It was later found that although PP1 physically interacts with MEF2 to promote nuclear retention of HDAC4, this phenomenon may occur in a phosphatase-dependent and –independent manner (60).

MEF2 IN VASCULAR SMOOTH MUSCLE DIFFERENTIATION

Of the four MEF2 isotypes, only the MEF2C-null mice display a vascular defect, indicating that only this family member has a non-reduntant role in VSMC differentiation. Consistent with this, the expression of MEF2C is enhanced in differentiating smooth muscle cells during development and is functionally important in cell-type-specific expression of smooth muscle genes (77,83,88). Genetic deletion of MEF2C in mice results in a lethal embryonic defect where VSMCs fail to differentiate (88); however, it is now apparent that MEF2C does not regulate the expression of smooth muscle contractile genes directly. Instead, MEF2C regulates smooth muscle gene expression through myocardin, a direct target gene of MEF2C in cardiac and VSMCs. Myocardin stimulates transcription from CArG-dependent muscle enhancers, but interestingly does not bind DNA directly; myocardin actually associates with SRF on CArG boxes to initiate transcription of smooth muscle specific genes (89).

Calcium sensitivity in VSMCs is regulated by RhoA/ROCK-mediated inhibition of the myosin light chain phosphatase complex. Depolarization has been shown to induce sustained contraction of smooth muscle cells, as well as increased activation of RhoA. In addition, removal of extracellular calcium attenuates RhoA activation (57). Interestingly, this signalling pathway also acts at the level of gene expression by increasing the expression of serum response factor (SRF)-dependent smooth muscle-marker genes through regulation of the myocardin-family of SRF co-activators (58).

Furthermore, protein phosphatase 1α (PP1α), the catalytic subunit of the myosin light chain phosphatase complex, has been shown to interact with MEF2, and has been identified to be a potent repressor of MEF2 transcriptional activation (60). In addition, ROCK has been

shown to regulate PP1α activity in VSMCs through CPI-17, which determines calcium sensitivity in smooth muscle contraction (63). CPI-17 is highly expressed in mature smooth muscle cells and is phosphorylated by PKC, PKN, and ROCK at threonine 38, a phosphorylation which enhances the affinity of CPI-17 for protein phosphatases, thus enhancing its inhibitory ability (64).

THE ROLE OF SRF AND MYOCARDIN

The serum response factor (SRF) is a founding member of the MADS domain of transcription factors (90,91). As previously discussed, these proteins, including the MEF2 family, all contain the MADS domain near their N-terminal region, which allows them to efficiently bind DNA, dimerize, and recruit regulatory co-factors that increase or decrease their transcriptional potency. The MADS domain of SRF lies within an extended region between amino acids 137 to 224, and SRF binds DNA as a homodimer. C-terminal to the MADS-box of SRF is a region thought to be important for the binding of regulatory cofactors (92). 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 (93). Interestingly, SRF is not a potent transcriptional activator on its own, as it requires binding of a co-activator within the extended MADS domain to achieve high levels of transcriptional activation (39,89).

SRF BINDING

An enhancer region was identified within the promoter of the immediate-early gene, *c-fos*, that was responsive to serum stimulation in cultured 3T3 cells (94). The first observed

protein to bind to this site was SRF, thus the enhancer region was termed the serum response element (SRE) (95,96). SRF binds to the core sequence of the SRE, CCATATTAGG, however, additional studies revealed the consensus SRF binding site as CC(A/T_6)GG, which has become known as the CArG box (97).

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 DNA (91). 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 (89,92). 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 (91). Such naturally occurring CArG degeneracy might provide a mechanism of SRF site-directed control in smooth muscle cells (98,99).

SRF AND SMOOTH MUSCLE GROWTH AND DIFFERENTIATION

Since smooth muscle cells do not terminally differentiate, the observation that SRF controls both immediate-early genes and smooth muscle specific structural genes was of great interest in the context of SRF-dependent gene expression (97,100). The activity of nearly all smooth muscle marker genes identified is dependent on one or more CArG elements found within the promoter region or the first intron (3). Therefore, the phenotypic regulation of VSMCs in response to various extracellular signals is largely dependent on SRF.

Functional analysis of SRF in muscle has shown that SRF-null mice die at gastrulation from a failure to form mesoderm (101). Inactivation of SRF in vascular smooth muscle results in decreased recruitment to the dorsal aorta with attenuation of smooth muscle marker gene expression (16). 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 (102).

The mechanism by which SRF distinguishes between growth or differentiation-specific genes is of great interest; SRF expression is higher in smooth muscle cells than in non-muscle cells, can be induced by smooth muscle differentiation factors, like TGF-β (103,104). At high levels of expression, SRF favours the activation of smooth muscle-specific genes (3). However, studies have also shown that CArG boxes within many smooth muscle promoters have a reduced binding affinity for SRF compared with the CArG box of *c-fos* (105). 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 (3). Therefore, it appears that SRF binding to DNA is regulated to target SRF to appropriate promoter regions; reduced SRF binding is critical for proper smooth muscle-specific gene expression during phenotype modulation (98). Interestingly, SRF activity appears to be regulated through RhoA-dependent actin treadmilling (47,106); RhoA stimulates transcription of CArG-dependent smooth muscle genes, while having no effect on the *c-fos* promoter (48,107).

Transcriptional control of SRF has been shown to be site-directed, and a result of interaction with specific coactivators. The *c-fos* CArG box is flanked by a binding site for ETS domain transcription factors, such as Elk-1. The majority of smooth muscle CArG boxes do not

lie adjacent to ETS binding sites (3,91) and growth factor-induced activation of mitogen activated protein (MAP) kinase signaling results in Elk-1 phosphorylation and recruitment of SRF to promote *c-fos* expression through the SRE (108). In the absence of mitogen stimulation, SRF is bound to smooth muscle-selective coactivators that promote the expression of smooth muscle marker genes (3,39,40,89,91,109).

MYOCARDIN

Myocardin was originally identified as an SRF cofactor with a SAP-domain, that is able to activate both cardiac and smooth muscle gene expression (89). Studies of myocardin revealed that there exist two isoforms; a cardiac-specific (myocardin-935) and a smooth muscle-specific (myocardin-856) isoform. The cardiac-specific isoform has been shown to physically interact with both SRF and MEF2 proteins, and contains an N-terminal extension that interacts with the MADS/MEF2 domain of MEF2C. This extension is not present in the shorter, smooth muscle-specific isoform (110).

Myocardin was discovered in a bioinformatics screen designed to identify unknown cardiac-specific genes, however, myocardin is also highly expressed in smooth muscle (111). Myocardin interacts with the MADS domain of SRF and forms a ternary complex on CArG-boxes but only in the presence of SRF. Mutation of the CArG-boxes in the SM22 promoter abolishes myocardin's potent activation of this reporter gene (111). SRF interacts with myocardin through an N-terminal region of basic amino acids and a glutamine-rich region. Disruption of either of these regions prevents the formation of a ternary complex with SRF (111). Myocardin contains

a 35 amino acid SAP (SAF-A/B, Acinus, and PIAS) domain, which has the potential to bind DNA, and also has a leucine zipper-like domain that is required for myocardin homodimerization (58).

Myocardin is able to activate smooth muscle marker genes in different cells types including L6 myoblasts and embryonic stems (ES) cells. Interestingly, it can also convert 10T1/2 fibroblasts into smooth muscle cells, but its overexpression cannot activate cardiac-specific genes. Myocardin deletion results in reduced smooth muscle gene expression in various smooth muscle cell lines and primary cultures (112-114). Mice homozygous for a myocardin loss-of-function mutation die at E10.5 and a lack of vascular smooth muscle differentiation in the dorsal aorta (91). Myocardin-null mice have no apparent decrease in cardiac gene expression, an observation which could be attributed to MRTFs ability to compensate for the loss of myocardin in the heart, but not in the developing aorta (84,115).

Myocardin activates smooth muscle genes by interacting with SRF. In quiescent smooth muscle cells, myocardin binds with SRF, however under PDGF stimulation, Elk-1 becomes phosphorylated and displaces myocardin from SRF in favour of SRE activation (116). Thus smooth muscle gene activation is regulated by opposing SRF cofactor interaction (15).

The ability of myocardin to activate SRF-dependent smooth muscle genes has also been shown to be regulated by physical association with histone acetyltransferases (HATs) and deacetylases (HDACs) (88). Myocardin recruits p300 (HAT) 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 (88).

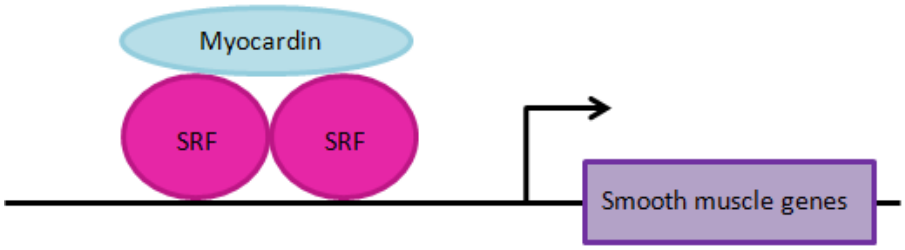

Figure 3: SRF control of smooth muscle-specific gene expression.

ROLE OF TGF-β IN VSMCs

Transforming growth factor-β (TGF-β) signalling is important in controlling developmental processes, regulation of cell growth, differentiation, adhesion, migration and cell death, and has also been implicated in a variety of diseases ranging from autoimmune diseases to cardiovascular diseases and cancer. TGF-β has also been shown to play a role in the regulation of vascular smooth muscle cell function. The canonical TGF-β pathway is transduced by phosphorylation of receptor-associated Smad proteins (R-Smads), which upon phosphorylation associate with a common Smad (Smad4), to allow for translocation into the nucleus (117,118). Nuclear localization of Smads results in transcriptional regulation of various target genes where the Smads, in association with other factors, act as transcriptional regulators various target genes (119). Furthermore, nuclear retention of heteromeric Smad complexes has been shown to be regulated through an association with TAZ, which is recruited to sites of Smad-mediated transcription (120).

The TGF-β superfamily of cytokines includes activins, bone morphogenetic proteins (BMPs) and growth and differentiation factors. Members of the TGF-β family signal via a

canonical pathway that involves a heterotetrameric complex of two type I and two type II Ser/Thr kinase receptors on the plasma membrane and downstream cytoplasmic effector proteins (Smads). TGF-β signalling is initiated by ligand binding to the transmembrane receptor TβR-I and TβR-II. Five type II and seven type I receptors exist in humans and other mammals. TGF-β promotes receptor oligomerization which leads to the phosphorylation of its type 1 receptor (TβRI) by the constituitively active type II receptor (TβRII). Ligand binding links the active type II receptor receptor kinases to the dormant type I receptor kinases, allowing the type II receptor to phosphorylate the type I receptor turning on its receptor kinase activity. The activation of TβRI results in phosphorylation of receptor-associated Smads (R-Smads) (Smads 2, 3) at their C-terminal SSXS motif, which upon phosphorylation oligomerize with the common Smad4 to translocate into the nucleus, where they bind to many promoters of various target genes and function as transcription factors to regulate gene expression. TGFβ target genes function in cell-cycle regulation, apoptotic regulation, extracellular matrix production, cytokine signalling, transcriptional regulation and differentiation. Conversely, Smads 6 and 7 function as repressor Smads by binding to the receptor and preventing phosphorylation of receptor Smads (R-Smads). Smad 7 functions through negative feedback by blocking Smad phosphorylation by TβRI and directs receptor ubiquitination and degradation via the ubiquiting ligases Smurf1 and Smurf2, thus attenuating the TGF-β signal and shutting down the pathway (121). Another factor that attenuates TGF-β signalling is the Ski/SnoN family of oncoporteins, which directly associate with Smad proteins to block the ability of the Smads to activate the expression of the majority of TGF-β-responsive genes (122).

The Smad family regulates transcription by being phosphorylated at their C-terminal region by activated type I TGF-β receptors. Smad proteins are comprised of 3 domains. The N-terminal region has a Mad-homology 1 (MH1) domain which interacts with other proteins and also carries a nuclear localization signal and a DNA-binding domain. The C-terminal region has a MH2 domain which binds to type I receptors and can also interact with other proteins to mediate Smad homo- and hetero-oligomerization to mediate transactivation potential of nuclear smad complexes. These two regions are connected by a middle linker domain that interacts with prolyl-isomerases and ubiquitin ligases. Induction by TGF-β (Alk 5, 2 and 2), Nodal (Alk 4 and 7) or Activin (Alk 4 and 2) ligands leads to phosphorylation of Smad2 and Smad3, whereas BMP (Alk 1, 2, 3 and 6) and GDF (Alk 4, 5 and 6) ligands induce phosphorylation of Smads 1, 5 and 8 (119).

TGF-β has also been implicated in inducing VSMC differentiation through the RhoA/ROCK signalling pathway (123). TGF-β induces a contractile VSMC phenotype in neural crest stem cells, by up-regulating smooth muscle marker genes such as smooth muscle α-actin, SM-22 and calponin, which is attenuated by the addition of the ROCK inhibitor, Y27632 (124,125). MEF2 proteins have also been shown to be down-stream targets of TGF-β signalling in other cell types through a physical interaction between MEF2 and Smad2 (126). TGF-β has been shown to have a role in the upregulation of smooth muscle specific genes such as SM22 and smooth muscle alpha actin. In VSMCs, TGF-β signalling has been shown to be RhoA-dependent. TGF-β induces smooth muscle cell differentiation, and the ROCK inhibitor Y27632 blocks the expression of SMC markers (SMA and SM22) in a TGF-β-dependent manner (126). Activation of the Smad pathway has been shown to be necessary in smooth muscle

differentiation, inducing a contractile phenotype, for which Smad2 and Smad3 have been found to be essential for this differentiation. TGF-β signalling is necessary for proper smooth muscle development during embryogenesis and is sufficient to induce certain smooth muscle markers from embryonic fibroblasts and primary neural crest cultures. Expression of Smad 2 and Smad 3 are necessary for induction of the smooth muscle phenotype, and have been found to be phosphorylated upon TGF-β and activin stimulation (125). As previously mentioned, SRF plays a central role in controlling smooth muscle-specific gene expression. Expression of SRF in mesenchymal cells is sufficient to induce SMC gene expression, and the dominant-negative mutant of SRF blocks TGF-β-induced SMC genes. TGF-β transiently induces the expression of SRF and various smooth muscle cell markers, a process which increases the binding of SRF to the SM22 promoter. Furthermore, Smad3 mediates TGFβ1-induced transactivation of the SM22 promoter, an effect which is repressed by Smads 6 and 7 (127); this was demonstrated by the silencing of Smad7 stimulating SRF-dependent promoter function. SRF was further shown to interact with Smad7 and SM22 promoter function was inhibited by Smad7 (128).

TAZ FUNCTION AND REGULATION

TAZ (transcriptional coactivator with PDZ-binding motif) (also termed WWTR1; WW domain containing transcription regulator 1), is a transcriptional regulator containing a WW domain which binds to consensus PPXY motifs. Its C-terminal domain contains a coiled-coil motif which is important in recruiting core components of transcriptional machinery to various target genes. The C-terminal transcriptional regulatory domain contains multiple phosphorylation sites and also a PDZ (post synaptic density protein PDS95, drosophila disc large

tumor suppressor Dlg1, zonula occludens-1 protein zo-1) binding motif, which aids in anchoring transmembrane proteins to the cytoskeleton to hold together signalling complexes (129,130). TAZ was originally identified in a proteomic screen for 14-3-3 interacting proteins and was found to function as a transcriptional modulator of mesenchymal stem cell differentiation by sitting at the convergence point of multiple signalling pathways regulating bone and fat development. Osteoblasts and adipocytes originate from the same mesenchymal stem cells; alternate activation of reciprocal transcriptional programs has been shown to be regulated by TAZ. TAZ directly inhibits the ability of PPARγ to stimulate gene expression and stimulates adipocyte differentiation. TAZ knockout mice display pronounced adipogenesis, and developmental abnormalities as a result of lack of bone formation. Interaction of TAZ and RunX2 promotes osteogenesis from mesenchymal stem cells (129,131).

TAZ was found to be downstream of TGF-β signalling, and binds to heteromeric Smad2/3-4 complexes and recruited to TGF-β response elements. In human embryonic stem cells, TAZ is required for maintenance of self-renewal markers, and a loss of TAZ shows inhibition of TGF-β signalling leading to differentiation into a neuroectoderm lineage. Furthermore, loss of TAZ results in failure of Smad2/3-4 complexes to accumulate in the nucleus to activate transcription of target genes. TGF-β signalling regulates embryonic stem cell pluripotency, and TAZ is shown to be required for hESC self-renewal. Inhibition of TAZ expression results in the loss of the pluripotency markers *Oct4* and *Nanog* (120). TAZ promotes cell proliferation and induces epithelial-to-mesenchymal transition (EMT) through binding to TEAD proteins, which are major downstream factors mediating function of TAZ, since TAZ lacks a DNA binding domain (132).

Interestingly, TAZ expression has been detected in the paraxial mesoderm, limb buds, and neural tube, which are all sources of myogenic cells. TAZ increases myogenin expression through direct interaction with MyoD, whereas its reduction delays myogenic differentiation (133). Recent work has also shown that TAZ up-regulates the smooth muscle α-actin promoter and represses the Smad3 promoter in injured epithelium, and affects wound healing and fibrogenesis, a mechanism which currently remains unknown (134).

Figure 4: Structure of TAZ. TB: TEAD binding domain. WW: WW domain. TA: Transactivation domain, containing glutamine-rich region and coiled-coil region. PDZ: PDZ-binding domain.

SUMMARY

The review of literature presented above illustrates the complexity of the networks involved in the regulation of vascular smooth muscle cell phenotypic control. The central role of SRF is evident in the transcriptional control of contractile smooth muscle genes, an effect which is modulated by several intracellular signalling pathways mediated either directly or indirectly through interacting co-factors. Identification of these signalling pathways will allow understanding of the molecular mechanisms involved in the contribution of cardiovascular diseases and can allow for development of therapeutic targets.

STATEMENT OF PURPOSE

The signalling pathways involved in the regulation of smooth muscle phenotypic modulation have been widely identified; however, the exact mechanisms by which these pathways function remain unknown.

Therefore, the purpose of this work was to identify and dissect the signalling pathways regulating vascular smooth muscle cell differentiation. This will ultimately contribute to the field of vascular biology through understanding the mechanisms underlying smooth muscle phenotypic modulation contributing to vascular diseases, such as atherosclerosis and restenosis. This purpose will be addressed experimentally through specific hypotheses:

1) RhoA/ROCK-MEF2C-CPI-17 signalling regulates vascular smooth muscle cell gene expression

2) TGF-β signalling promotes vascular smooth muscle cell gene expression through TAZ and SRF

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

CHAPTER 2: MANUSCRIPT 1

RATIONALE:

This manuscript focuses on dissecting the pathway by which MEF2 regulates myocardin expression in vascular smooth muscle cells. Previous work from our group showed that myocardin is a transcriptional target of MEF2 (2,135), possibly linking the regulation of MEF2 and SRF-dependent smooth muscle marker genes. This manuscript defines the mechanism of MEF2-dependent myocardin expression implicating RhoA signalling and the PP1 inhibitor, CPI-17.

AUTHOR CONTRIBUTIONS:

A NOVEL RhoA/ROCK- CPI-17 -MEF2C SIGNALING PATHWAY REGULATES VASCULAR SMOOTH MUSCLE CELL GENE EXPRESSION

Pagiatakis, C.[1§], Gordon, J.W.[1,3§#], Ehyai, S.[1], and McDermott, J.C.[1,2,3,4]

Published in *Journal of Biological Chemistry,* (2012) 287(11): 8361-8370.

Experimental design:

Christina Pagiatakis and Joe Gordon

Drafting manuscript:

Joe Gordon and Dr. John C McDermott

Conducting experiments:

Christina Pagiatakis: Figure 1, Figure 2A, B, C, D, E, G, H, Figure 3 A, B, C, D, E, F, H, I, Figure 4, Figure 5

Joe Gordon: Figure 2F, 3G, Figure 6

Saviz Ehyai: Figure 2I

MANUSCRIPT:

A NOVEL RhoA/ROCK- CPI-17 -MEF2C SIGNALING PATHWAY REGULATES VASCULAR SMOOTH MUSCLE CELL GENE EXPRESSION

Pagiatakis, C.[1§], Gordon, J.W.[1,3§#], Ehyai, S.[1], and McDermott, J.C.[1,2,3,4]

[1] Department of Biology, [2] Centre for Research in Mass Spectrometry (CRMS), [3] Muscle Health Research Centre (MHRC), [4] Centre for Research in Biomolecular Interactions (CRBI), York University.

§ These authors contributed equally to this work.

RhoA activates MEF2C through CPI-17.

Key words: MEF2C, muscle, myocardin, vascular smooth muscle, CPI-17, RhoA.

Background: MEF2C is essential for vascular smooth muscle development, yet the signaling pathways that regulate its function in this cell type remain largely unknown.

Results: We identify a novel regulator of MEF2C in vascular smooth muscle, called CPI-17.

Conclusion: Our data identify a genetic pathway involving CPI-17, MEF2C and myocardin.

Significance: These findings have important ramifications during vascular development and for stem cell programming.

INTRODUCTION

Differentiation of vascular smooth muscle cells (VSMC) is a fundamental aspect of normal development and vascular disease. During contraction, VSMCs modulate calcium sensitivity through RhoA/ROCK-mediated inhibition of the myosin light chain phosphatase complex (MLCP). Previous studies have demonstrated that this signaling pathway functions in parallel to increase the expression of smooth muscle genes through the myocardin-family of co-activators. MEF2C fulfills a critical role in VSMC differentiation and regulates myocardin expression, leading us to investigate whether the RhoA/ROCK signaling cascade might regulate MEF2 activity. Depolarization-induced calcium signaling increased the expression of myocardin, which was sensitive to ROCK and p38 MAPK inhibition. We previously identified protein phosphatase 1α (PP1α), a known catalytic subunit of the MLCP in VSMCs, as a potent repressor of MEF2 activity. PP1α inhibition resulted in increased expression of myocardin, while ectopic expression of PP1α inhibited the induction of myocardin by MEF2C. Consistent with these data, shRNA-mediated suppression of a PP1α inhibitor, CPI-17, reduced myocardin expression and inhibited VSMC differentiation, suggesting a pivotal role for CPI-17 in regulating MEF2 activity. These data constitute evidence of a novel signaling cascade that links RhoA-mediated calcium

sensitivity to MEF2-dependent myocardin expression in VSMCs through a mechanism involving p38 MAPK, PP1α, and CPI-17.

During development, vascular smooth muscle cells (VSMCs) migrate to primitive endothelial tubes while simultaneously executing a program of differentiation in order to contribute to the vascular architecture (136). Upon incorporation into the vasculature, VSMCs become quiescent and primarily regulate vascular tone (137). However, unlike terminally differentiated striated muscle cell types, VSMCs retain a capacity, referred to as the activated or synthetic 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 (138). The MADS-box (MCM-1, Agamous, Deficiens, Serum Response Factor) transcriptional regulators, serum response factor (SRF) and myocyte enhancer factor 2 (MEF2) 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, somewhat paradoxically, smooth muscle marker genes involved in the contractile phenotype (139-141). The cellular signals that direct SRF to these distinct sets of genes have been intensively studied, where SRF physically interacts with the myocardin family of co-activators in contractile VSMCs to induce smooth muscle marker gene expression (142). 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* (143). 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 (144). 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 (144). 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 (141). However, MEF2C has also been shown to be genetically upstream of myocardin and of critical importance to VSMC differentiation (140,145). Yet, the signaling pathways that regulate MEF2-dependent myocardin expression in VSMCs remain unknown; however, recent studies suggest that RhoA signaling may be involved (146,147). We recently identified protein phosphatase 1α (PP1α) as a potent *trans*-dominant repressor of MEF2 activity (148). Interestingly, in VSMCs PP1α serves as the catalytic subunit of the myosin light chain phosphatase complex (MLCP) and is regulated by RhoA signaling to control calcium sensitivity during contraction (149). In addition, signals emanating from RhoA in VSMCs have been previously shown to activate p38 MAP kinase (MAPK) signaling (150), a known activator of MEF2 transcriptional activity in multiple cell types (151-153). In this report we document for the first time, a novel signaling pathway in VSMCs that links RhoA-mediated regulation of calcium sensitivity to MEF2-dependent expression of myocardin. This pathway involves the de-repression of MEF2 from PP1α inhibition by a two-step mechanism involving p38 MAPK and ROCK-mediated activation of the PP1α inhibitor, CPI-17 (PKC-potentiated protein phosphatase inhibitor of 17 kDa). 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.

EXPERIMENTAL PROCEDURES

Plasmids. MEF2, PP1α, p38 MAPK (p38 MAPK), MKK6EE, and luciferase constructs were described previously (141,148). 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. The activated ROCK and PKN constructs were generous gifts from M. Scheid and Y. Ono, respectively. Thr38 mutations in CPI-17 were generated by site-directly mutagenesis. The shRNAs targeting MEF2C and CPI-17 were generated by ligating annealed oligonucleotides into the pSilencer 3.0 H1 vector, as per the manufacturer's instructions, where the target sequence for the MEF2C 3' UTR was 5'-AACAGAAATGCTGAGATACGC-3' and the target sequence for CPI-17 was 5'-AAAGCCCAGATTGTTTCTAAG-3'.

Primary VSMC and Immortalized Cell Cultures. Primary rat aortic smooth muscle cells were isolated by enzymatic cell dispersion, as described in Hou et al (154). Rat A10 myoblasts (ATCC; CRL-1476) were maintained in growth media consisting of 10% fetal bovine serum (FBS). Quiescence was obtained by re-feeding the cells with serum-free DMEM overnight. C3H10T1/2 mouse embryonic fibroblasts (ATCC; CCL-226) and COS7 cells were maintained in standard DMEM with 10% FBS.

shRNA Transfections. The shRNAs targeting MEF2C, CPI-17, 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.

Luciferase and β-Galactosidase Assays. Transient transfections of A10, C3H10T1/2 and COS7 cells were performed by a modified calcium phosphate-DNA precipitation with pCMV-β-galactosidase serving as an internal control for transfection efficiency. Luciferase and β-galactosidase activityies were measured as described previously (141).

Immunoblot Analysis. Protein extractions were achieved using an NP-40 lysis buffer described previously (148). Protein concentrations were determined by Bradford assay, and 15 µg were resolved using SDS-PAGE and transferred 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. Primary antibodies included, rabbit myocardin (Santa Cruz), c-Jun and c-fos (Santa Cruz), p38 and p-p38 MAPK (NEB), and CPI-17 and p-CPI-17 (Santa Cruz), and smooth muscle α-actin (Sigma).

Co-immunoprecipitation. COS7 cells were transfected using calcium phosphate method and protein extracts were harvested, as described above. Immunoprecipitation was performed using the ExactaCruz kit (Santa Cruz), as per manufacturer's instructions. Elusions were analyzed by immunoblot, as described above.

Immunofluorescence. Primary VSMCs were fixed in 4% paraformaldehyde, permeabilized in ice-cold methanol, and incubated with a primary smooth muscle α-actin antibody (Sigama), CPI-17 antibody (Santa Cruz) with FITC- and TRITC-conjugated secondary antibodies. Cells were visualized using fluorescence microscopy.

Quantitative RT-PCR. Total RNA was isolated from primary VSMCs using a Cell-to-cDNA kit (Ambion), and quantitative PCR was performed using SYBR green (Applied Biosystems), and analyzed using the $\Delta\Delta$CT method, as described previously (141).

RESULTS

Depolarization enhances MEF2-dependent gene expression through distinct calcium-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) and nifedipine, an L-type calcium channel blocker. Depolarization, in both primary cultures and the A10 VSMC 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 1). In addition, the induction of myocardin and c-Jun was found to be dependent on the MEF2 *cis* elements found within the proximal promoter regions of these genes (Figure 1B and 1D). Endothelin-1 (ET-1) has also been implicated in regulating calcium sensitivity in VSMCs during contraction through RhoA-dependent signaling, and our evidence suggests that ET-1 induces myocardin expression through the MEF2 *cis* element in a manner similar to depolarization (Figure 1E)(155). Finally, to evaluate whether MEF2C could activate endogenous myocardin expression, we transfected A10 cells with MEF2C and observed an increase in myocardin expression determined by qPCR (Figure 1F).

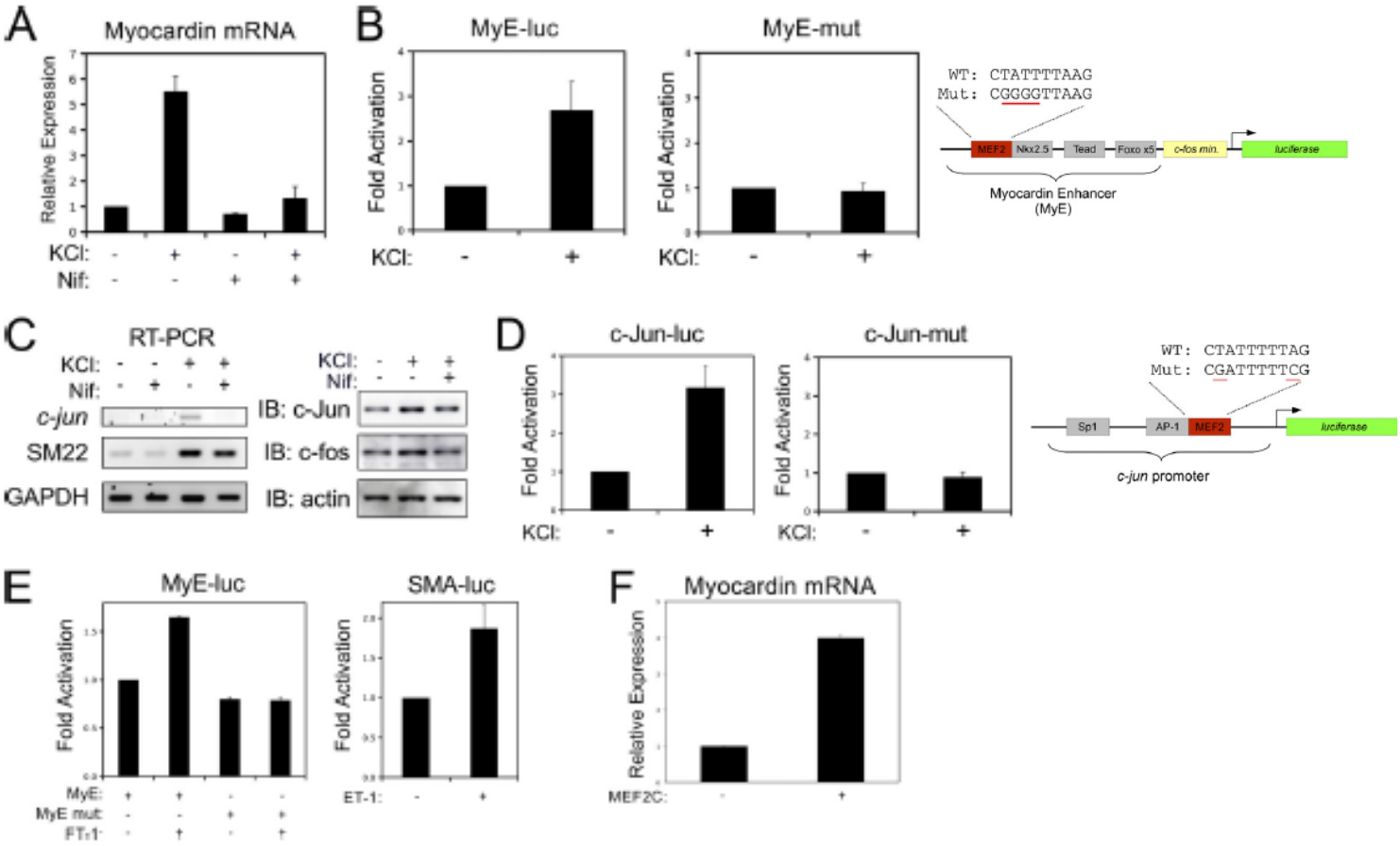

Figure 1: Depolarization-induced expression of MEF2-target genes in VSMCs. (A) Primary VSMCs were depolarized with 60 mM KCl, following pre-treatment with 1 µM of nifedipine (Nif), as indicated. Myocardin expression was evaluated by qPCR, corrected for GAPDH using the ΔΔCT method. (B) A10 cells were 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) Quiescent A10 cells were treated with 60mM KCl following a 15 minutes treatment of 5µM 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 c-jun and SM22 and GAPDH. (D) 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, cells were depolarized with 60 mM KCl and subjected to luciferase assay. (E) A10 cells were transfected with the myocardin enhancer and smooth muscle α-actin promoter, as indicated. Cells were treated overnight with endothelin-1 (10nM) and extracts were subjected to luciferase assay. (F) A10 cells were transfected with MEF2C (as indicated). Myocardin expression was evaluated by qPCR and corrected for GAPDH using the ΔΔCT method. Error bars indicate SEM.

In order to dissect the calcium-dependent signaling pathways responsible for the induction of MEF2-dependent smooth muscle genes at the protein level, we utilized well-characterized pharmacological inhibitors in our culture model, after testing the specificity of myocardin antibodies to detect exogenous myocardin in COS7 cells (Figure 2A). Interestingly, 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 2B). We have previously demonstrated that c-Jun expression in VSMCs is regulated by a MEF2-HDAC4 repressor complex (141). 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 counteracted by co-expression of an activated CaMKIIδ. Surprisingly, the HDAC4 repression of *c-jun* could not be overcome by other CaMKs, such as CaMKI or CaMKIV (Figure 2C). In addition, depolarization resulted in a reduced nuclear content of HDAC4, suggesting that CaMKII promotes nuclear export of HDAC4 to de-repress *c-jun* expression (not shown).

Intriguingly, depolarization-induced expression of myocardin was attenuated by the p38 MAPK inhibitor, SB203580; whereas, the induction of c-Jun is unaffected by this inhibitor (Figure 2D). In addition, depolarization resulted in a nifedipine-sensitive increase in p38 MAPK activity, as indicated by an increase in phosphorylated p38 MAPK in response to KCl treatment (Figure 2E). These results, and the work of others, indicate that distinct calcium-mediated signaling pathways regulate c-Jun and myocardin expression in VSMCs (Figure 2F). 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.

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 2H, the ROCK inhibitor, Y27632, inhibited the myocardin enhancer, but not when the MEF2 *cis* element was mutated such that MEF2 can no longer bind (140). Additionally, the induction of this reporter-gene by MEF2C was prevented by co-expression of C3, a RhoA inhibitor (not shown). Congruently, forced expression of MEF2C and an activated RhoA (RhoA L63) cooperatively activate the myocardin enhancer, but again not when the MEF2 *cis* element is mutated (Figure 2G).

To evaluate the necessity of MEF2C for myocardin expression and VSMC differentiation, we engineered a short-hairpin RNA to reduce MEF2C expression (shMEF2C). As shown in Figure 2I, the shMEF2C reduced endogenous MEF2C expression in cultured A10 cells, which resulted in a corresponding reduction in the expression of myocardin and its down-stream VSMC target-gene, smooth muscle α-actin (SMA). Furthermore, the shMEF2C mediated reduction in myocardin expression and VSMC differentiation could be overcome by ectopic expression of human MEF2C, which is not suppressed by the shRNA, validating the specificity of the shMEF2C effect. Finally, the reduction in SMA induced by the shMEF2C could be by-passed by exogenous expression of myocardin (Figure 2I). These results are consistent with our hypothesis that MEF2C regulates VSMC differentiation through myocardin (140), yet to our knowledge, this is the first report to evaluate this notion through both gain- and loss-of-function experiments. Collectively, these data indicate that the RhoA/ROCK signaling pathway provides an important activating stimulus for MEF2C-mediated induction of myocardin expression in VSMCs.

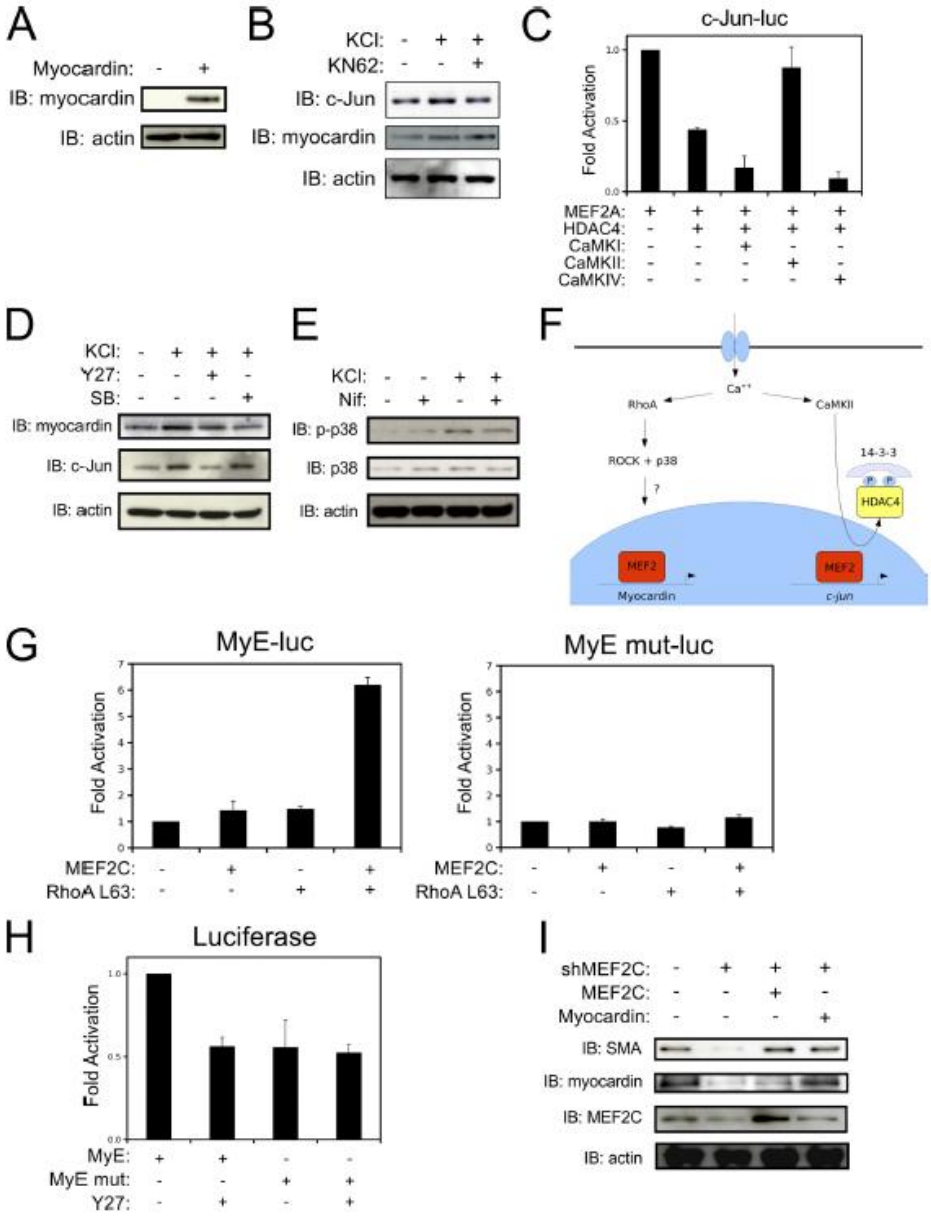

Figure 2: Distinct calcium-mediated signaling pathways regulate myocardin and c-Jun expression in VSMCs. (A) COS7 cells were transfected with Myocardin-856 and subjected to immunoblotting with Myocardin antibody (SC-33766, Santa Cruz). (B) A10 cells were treated with 60mM KCl for 2 hours following 15 minute pretreatment with 5 μM KN-62 (CaM kinase inhibitor) or DMSO as a vehicle control. Protein extracts were immunoblotted with c-Jun, myocardin, and Actin antibodies. (C) A10 cells were transfected with a *c-jun* reporter-gene (c-Jun-luc), MEF2A, HDAC4, and activated CaMKI, CaMKII or CaMKIV, as indicated. (D) A10 cells were pre-treated with either Y27632 (Y27, 5 μM) or SB203580 (SB, 5 μM), or DMSO as a vehicle control for 15 minutes, then depolarized for two hours. Extracts were subjected to immunobloting as indicated. (E) A10 cells were pre-treated with nifedipine, depolarized, and subjected to immunoblotting. (F) Model of the distinct signaling pathways that regulate MEF2-dependent myocardin and *c-jun* expression in VSMCs. (G) A10 cells were transfected with MyE or the enhancer with the MEF2 site mutated (MyE mut) along with a MEF2C, and/or an active RhoA (RhoA L63) expression vectors. Extracts were subjected to luciferase assays. (H) A10 cells were transfected as described in (G), treated with Y27632 (Y27, 5 μM) and harvested for luciferase assay. (I) Cells were transfected with a plasmid encoding a short-hairpin RNA targeting MEF2C (shMEF2C), and expression vectors for human MEF2C or myocardin, as indicated. Cultures were enriched for expression of the shRNA by puromycin selection and extracts were subjected to immunoblotting. Error bars indicate SEM.

PP1α regulates MEF2-dependent gene expression in VSMCs.

We have recently identified PP1α as a dominant repressor of MEF2 transcriptional activity (148). In smooth muscle, PP1α is the catalytic subunit of the myosin light chain phosphatase complex (MLCP) (156). Exogenous expression of PP1α inhibits endogenous myocardin expression and powerfully attenuates the induction of myocardin by ectopic expression of activated RhoA in cultured A10 cells (Figure 3A and 3B). In addition, exogenous expression of PP1α completely prevented the induction of endogenous myocardin expression by MEF2C (Figure 3D). Furthermore, we utilized the PP1α inhibitor, calyculin A, to address the role of PP1α in MEF2-dependent gene expression in VSMCs. Calyculin A treatment increased the expression of both myocardin and c-Jun (Figure 3C). Thus, ROCK regulation of PP1α might be an important mechanism for the attenuated expression of myocardin and c-Jun with Y27632 treatment (Figure 2D). MEF2C has previously been shown to interact with the bHLH transcription factors of the Hand (heart and neural crest derived) family (157). In addition, genetic ablation of MEF2C, dHand (Hand2), and myocardin all result in some degree of neural crest-derived vascular defect (158-160). Therefore, we chose to evaluate whether PP1α could inhibit a functional cooperation between MEF2C and dHand. As shown in figure 3E, MEF2C and dHand cooperatively activated the myocardin enhancer, yet exogenous expression of PP1α attenuated this effect. To evaluate whether PP1α could block smooth muscle gene expression directly (ie. Downstream of MEF2C), we utilized a SM22-dependent reporter gene. As shown in figure 3F and 3G, forced expression of PP1α could not overcome the induction of this promoter by myocardin or Smad3. These results indicate that PP1α regulates smooth muscle gene expression through MEF2C and not through genetically downstream transcription factors, such as SRF.

To further evaluate the role of PP1α in c-Jun expression, we performed a titration of calyculin A in A10 cells and observed an increase in phosphorylated c-Jun at higher concentrations of calyculin A (Figure 3H). Previous studies in lung epithelial cells have shown that calyculin A can activate JNK

signaling to induce c-Jun phosphorylation (161). 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 3I). 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 (141,148). 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.

Next, we evaluated whether exogenous expression of activated p38 MAPK or RhoA might be able to overcome PP1α repression of MEF2C to induce myocardin expression. However, as shown in figure 4A, once repressed by PP1α, MEF2C is unresponsive to activated MKK6/p38 MAPK or RhoA signaling in COS7 cells. In VSMCs, the myosin light chain phosphatase complex (MLCP) is regulated by a smooth muscle-enriched phosphatase inhibitor called PKC-potentiated protein phosphatase inhibitor of 17 kDa (CPI-17), which is not expressed in COS7 cells (not shown). Consistent with previously published structural data, figure 4C demonstrates that CPI-17 physically interacts with PP1α, evaluated by co-immunoprecipitation, which leads to inhibition of phosphatase activity (162). In addition to being potentiated by PKC, CPI-17 has also been shown to be activated by ROCK and PKN (163,164). Therefore, we determined if exogenous expression of CPI-17 in COS7 cells could inhibit PP1α repression of MEF2 activity. As shown in figure 4B, CPI-17 can antagonize PP1α repression of the myocardin enhancer and restore the activation induced by MEF2C. In addition, figure 4D demonstrates that CPI-17 can compete away the physical interaction between MEF2C and PP1α, determined by co-immunoprecipitation. 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 4E). 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, much of the cellular CPI-17 was confined to the nuclear compartment in VSMCs, suggesting a potentially important role for CPI-17 in the nucleus. These results were confirmed biochemically using nuclear and cytosolic fractionation, which demonstrated that CPI-17 is expressed in both the nuclear and cytosolic compartments (Figure 4F).

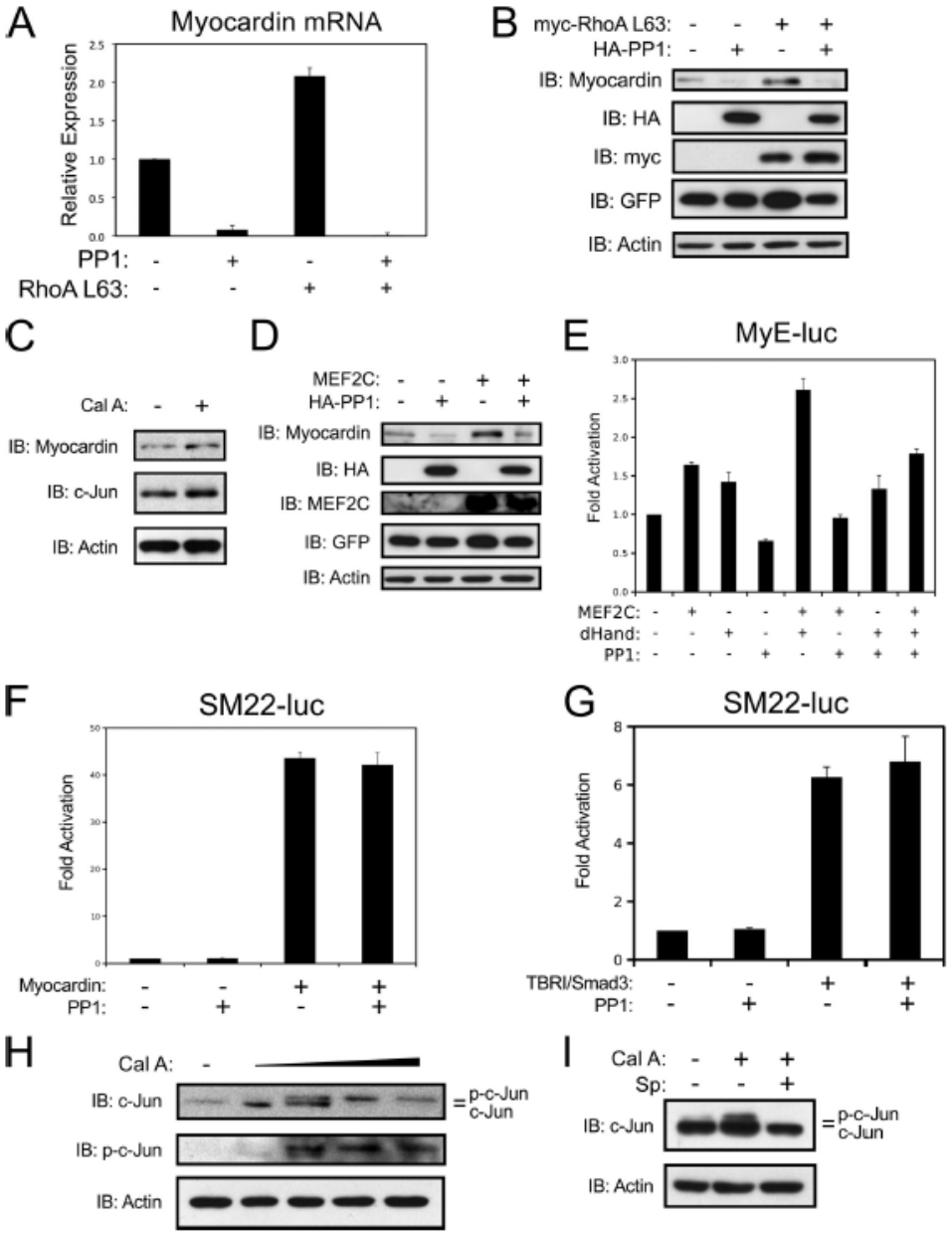

Figure 3: Myocardin expression is opposed by PP1α. (A) A10 cells were transfected with HA-PP1α (PP1) and activated myc-RhoA (myc-RhoA L63) using Lipofectamine reagent and puromycin-selected overnight. Myocardin expression was evaluated by qPCR and corrected for GAPDH using the ΔΔCT method. (B) A10 cells were transfected as described in (A). Protein extracts were subjected to immunoblotting as indicated. (C) A10 cells were treated with Calyculin A (0.5 ng/mL) or DMSO as a vehicle control for 2 hours. Protein extracts were immunoblotted as indicated. (D) A10 cells were transfected with HA-PP1α and MEF2C as described in (A). Protein extracts were subjected to immunoblotting as indicated. (E) COS7 cells were transfected with the myocardin enhancer, MEF2C, dHand, and/or PP1α (PP1) as indicated. Luciferase assays were performed on the cells extracts. (F)(G) 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. (H) 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 (I) A10 cells were treated with 0.5 ng/mL of calyculin A and 5 μM of SP600125 for 2 hour and harvested for immunoblotting. Error bars indicate SEM.

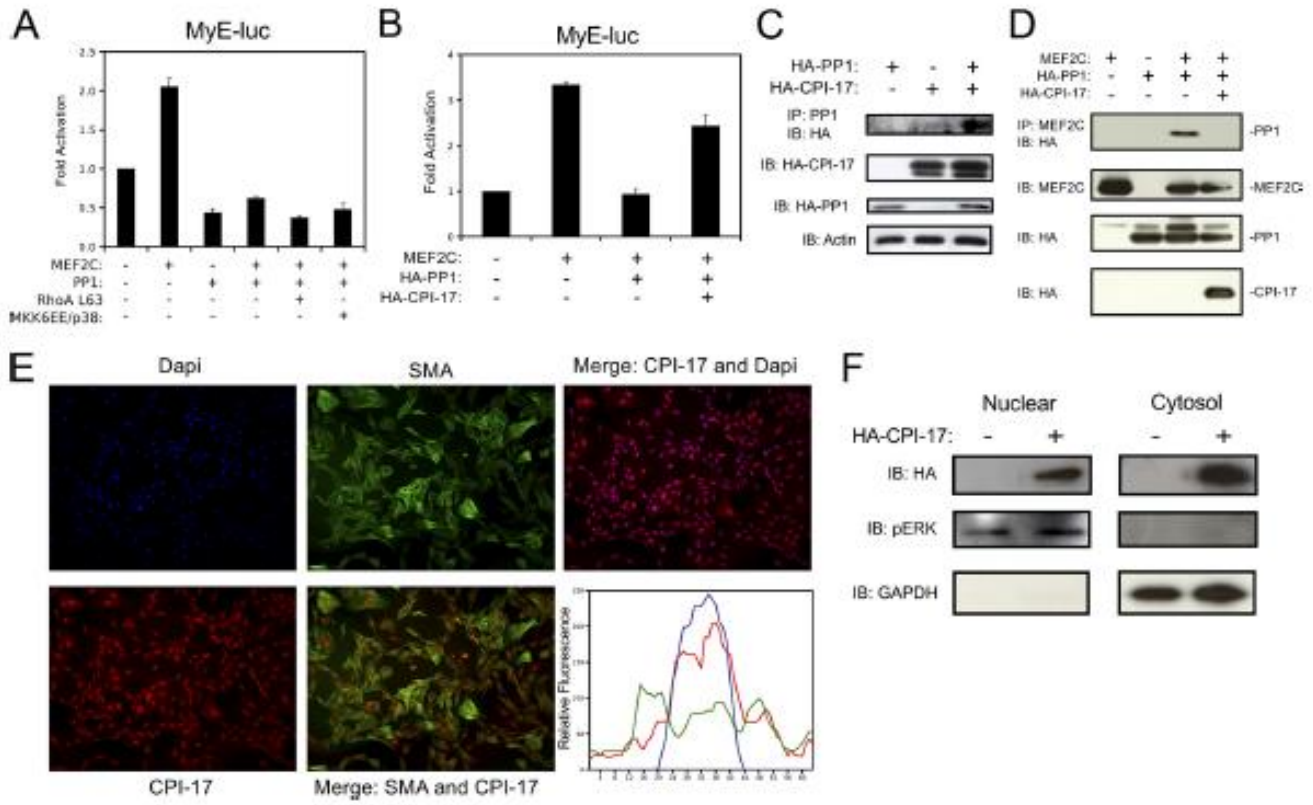

Figure 4: PP1α-induced repression of myocardin is attenuated by CPI-17. (A) COS7 cells were transfected with the myocardin enhancer (MyE), MEF2C, PP1α (PP1), activated RhoA (RhoA L63), or activated MKK6 and p38 (MKK6EE/p38), as indicated. Extracts were subjected to lucifierase assay. (B) Cells were transfected with the myocardin enhancer, and MEF2C, PP1, or CPI-17 as indicated, followed by luciferase assay. (C) COS7 cells were transfected with HA-CPI-17 and HA-PP1α. Protein extracts were immunoprecipitated with PP1α antibody and immunoblotted, as indicated. (D) 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. Protein extracts were immunoblotted, as indicated, to demonstrate equal loading and transfection efficiency. (E) 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. (F) A10 cells were transfected with HA-CPI-17 and subjected to nuclear/cytosolic fractionation. Lysates were immunoblotted as indicated. Error bars indicate SEM.

CPI-17 is activated by phosphorylation at Thr38, and structural analysis predicts that phospho-Thr38 serves to anchor the interaction with PP1α, resulting in maximal phosphatase inhibition (165,166). Therefore, we utilized a Thr38 phospho-specific antibody to evaluate the role of RhoA-dependent signaling on CPI-17 phosphorylation. As shown in figure 5A, expression of activated RhoA, ROCKII, and PKN, increased Thr38 phosphorylation of CPI-17 in A10 cells. Furthermore, these kinases also increased the expression of total CPI-17, which is consistent with a recent report that demonstrated CPI-17 expression was regulated in a manner similar to other smooth muscle marker genes (167). Next, we introduced both a neutralizing alanine mutation (T38A), and a phospho-mimetic glutamate mutation (T38E) at the Thr38 residue to determine whether this site could regulate MEF2 activity. As shown in figure 5B, the T38E mutation was equally as effective as the wild-type CPI-17 at disrupting the MEF2C-PP1α interaction. However, as predicted, 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.

Finally, to evaluate the endogenous role of CPI-17 in myocardin expression, we engineered a short-hairpin RNA to reduce CPI-17 expression (shCPI-17). Shown in figure 5C, shCPI-17 attenuated the induction of myocardin by activated RhoA, determined by qPCR. Furthermore, shown in figure 5D, the shCPI-17 reduced endogenous CPI-17 expression and attenuated its induction following exogenous expression of RhoA. Interestingly, reduced CPI-17 expression was accompanied by a corresponding decrease in myocardin and SMA protein expression, implicating CPI-17 as a critical regulator of VSMC differentiation. Titration of the shCPI-17 on the myocardin enhancer (MyE-luc) resulted in a dose-dependent decrease in myocardin expression that could be rescued with forced expression of human CPI-17, which is resistant to the shCPI-17 and validates the specificity of the shRNA. Finally, we determined if MEF2C and CPI-17 could convert a pluripotent cell-line towards a

VSMC phenotype. Utilizing C3H10T1/2 mouse embryonic fibroblasts, we exogenously expressed CPI-17 and MEF2C, alone and in combination and determined the effect on SMA expression. As shown in figure 5F, neither CPI-17 nor MEF2C expression had an impact on basal levels of SMA expression in this cell-line. However, when combined, CPI-17 and MEF2C markedly induced SMA expression, indicating that MEF2C requires derepression by CPI-17 to activate a VSMC phenotype.

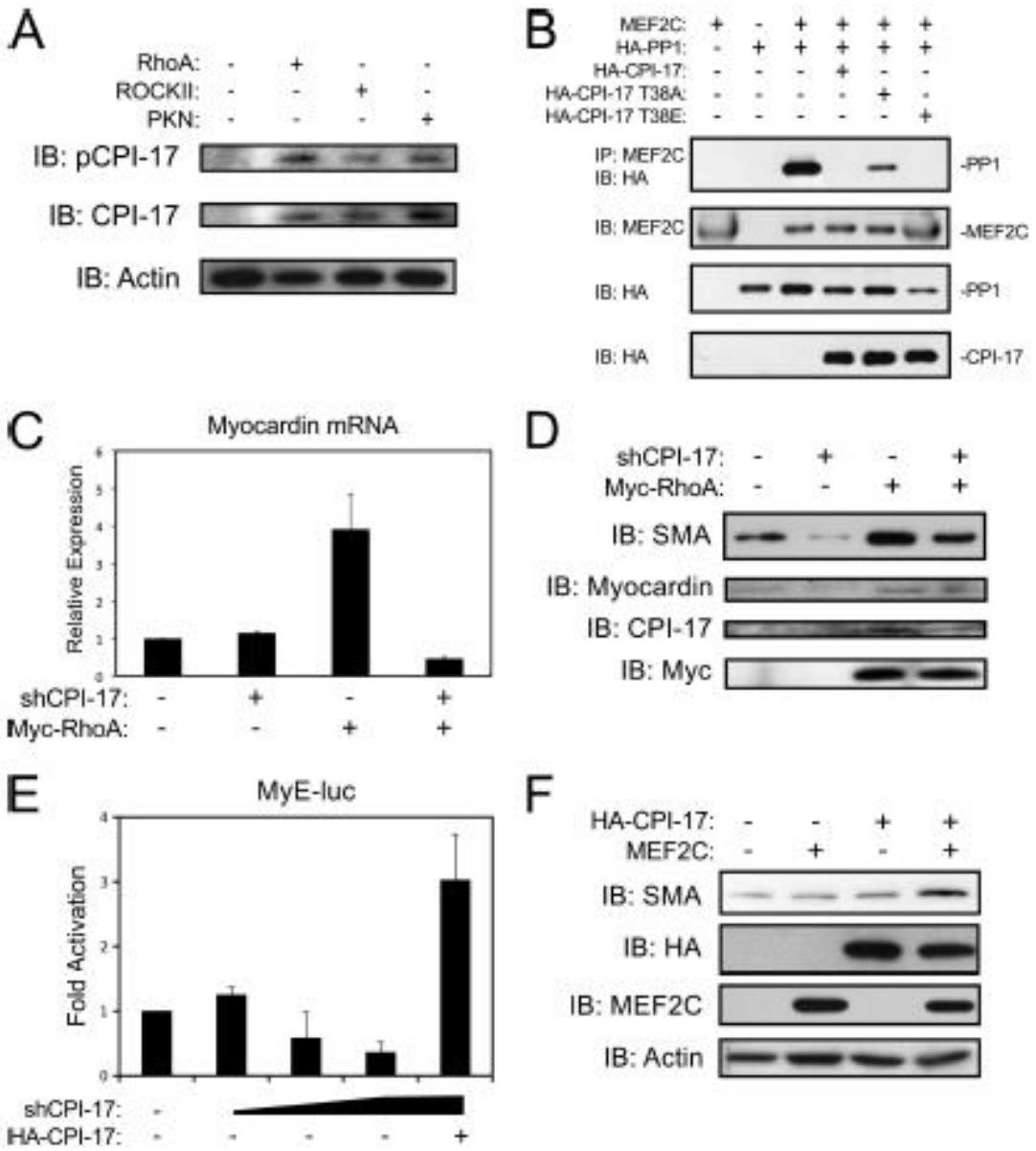

Figure 5: Phosphorylation of CPI-17 at threonine-38 regulates MEF2-dependent VSMC differentiation. (A) A10 cells were transfected with activated RhoA, ROCKII, or PKN using Lipofectamine reagent and puromycin-selected overnight. Protein extracts were subjected to immunoblotting as indicated. (B) COS7 cells were transfected, as described in (4D) with the addition of Thr38 mutants of CPI-17. Protein extracts were immunoprecipitated and immunoblotted as previously described. (C) A10 cells were transfected as described in (A) with a plasmid encoding a short hairpin RNA targeting CPI-17 (shCPI-17) and an expression vector for active RhoA. Following puromycin selection, myocardin expression was evaluated by qPCR, corrected for GAPDH using the ΔΔCT method. (D) A10 were transfected as described in (C). Following puromycin selection, extracts were subjected to immunoblotting. (E) Rat primary VSMCs were transfected by Lipofectamine reagent with the myocardin reporter gene (MyE-luc), increasing amounts of shCPI-17, and an expression plasmid for human CPI-17. Extracts were subjected to luciferase assay. (F) 10T1/2 mouse embryonic fibroblast cells were transfected with MEF2C or HA-CPI-17 as indicate. Cells were placed in low serum conditions (5% horse serum) for 96 hours and subjected to immunoblotting as indicated. Error bars indicate SEM.

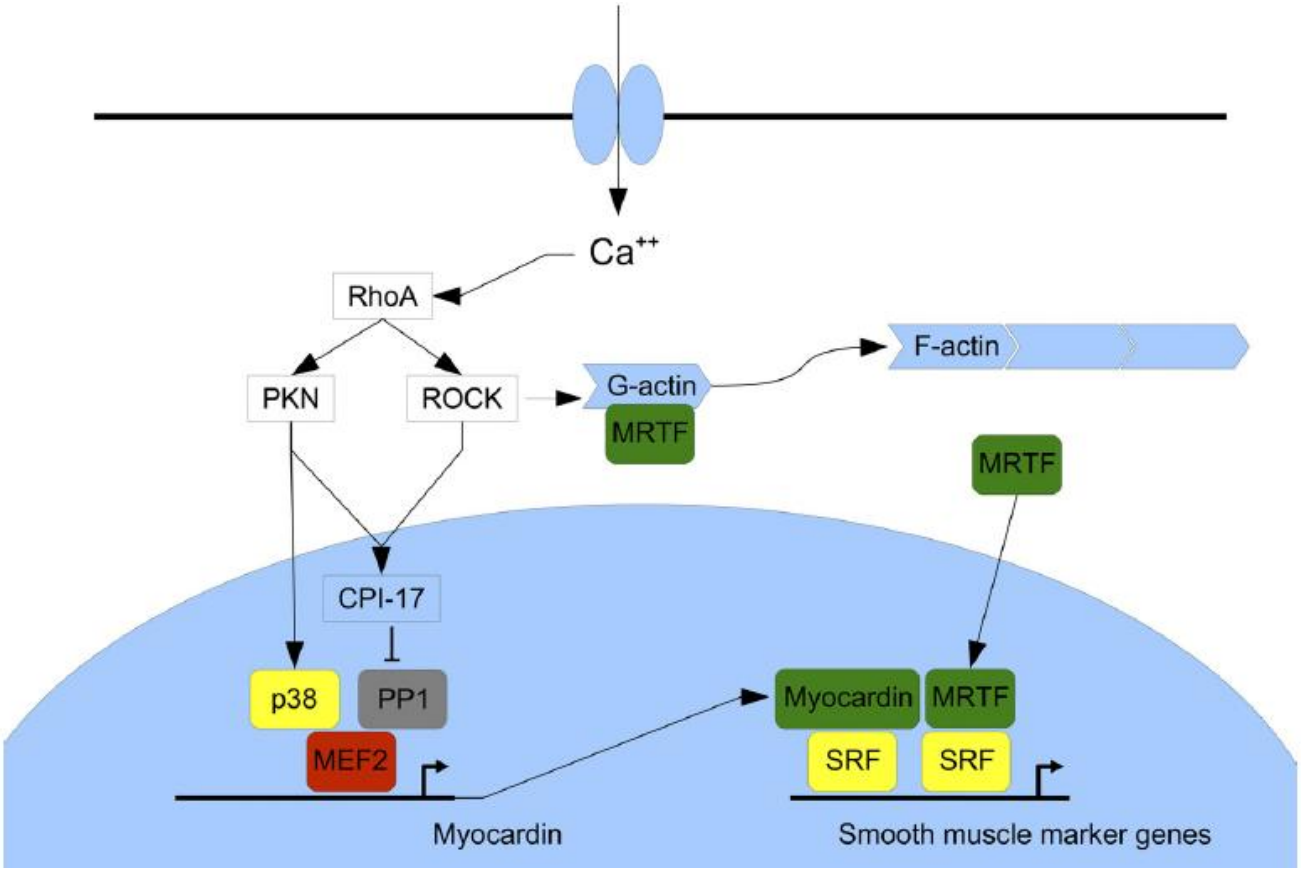

Figure 6: Model of calcium-mediated induction of myocardin expression in VSMCs. Based on the work presented in this manuscript, and previously published observations. MEF2-dependent myocardin expression is regulated by p38 MAPK and RhoA-induced derepression of PP1α by CPI-17. Myocardin activates SRF-dependent 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.

DISCUSSION

MEF2C plays an essential role in VSMC differentiation and is genetically upstream of the SRF-coactivator, myocardin (140,145). 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). Furthermore, we demonstrate, for the first time, that this genetic pathway connecting CPI-17, MEF2C, and myocardin is critical for VSMC differentiation (Figures 1 and 5). 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 is regulated by physical interaction with regulatory subunits, that typically contain a conserved RVXF domain (156). 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 (148,149). 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 3F). In addition, the phosphatase activity of PP1α is regulated through interaction with specific inhibitor proteins like Inhibitor 1 and 2 (I1 and I2, PP1 inhibitors), and CPI-17. The potency of these inhibitor proteins is regulated by phosphorylation and dephosphorylation by cellular kinases and phosphatases, such as PKA, calcineurin, ROCK, and PKN (66,149,156). 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 (148). 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, and/or the ability of CPI-17 to compete with MEF2C for PP1α binding (156).

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 (148). We now document, within the cellular context of VSMCs, 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 regulating VSMC gene expression. Recent analysis of CPI-17 expression in mouse embryos has revealed that in addition to its restricted expression pattern in smooth muscle post-natally, CPI-17 is also expressed transiently in the developing heart and intercostal muscles (167). Interestingly, myocardin and several smooth-muscle marker genes, such as SM22, all display at least transient expression in striated and smooth muscle types during development (168-170). In light of our evidence demonstrating the critical role of CPI-17 in the regulation of myocardin expression, these data suggest a potentially larger role for CPI-17 regulating the development of all three muscle types.

In summary, we provide novel evidence that PP1α serves as critical regulator of MEF2-dependent gene expression in VSMCs, 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, and uncover potentially new therapeutic targets for manipulation of VSMC differentiation in stem cell programming.

FOOTNOTES

This work was supported by a grant from the Heart and Stroke foundation of Canada (HSFC) to JCM.

We wish to thank Drs. Michelle Bendeck, Guangpei Hou, and Olga Ornatsky for technical assistance with the primary cultures, and Nezeka Alli and Dr. Tetsuaki Miyake for technical assistance with cloning, mutagenesis and RNAi experiments.

CHAPTER 3: MANUSCRIPT 2

RATIONALE

Many signalling pathways regulate the fate of vascular smooth muscle cells into a quiescent or proliferative phenotype. We showed in our previous study that the regulation of *myocardin*, which lies upstream of smooth muscle-specific genes, is a result of RhoA/ROCK signalling to CPI-17, to derepress MEF2 from PP1 inhibition. Myocardin is a potent co-activator of SRF; their interaction up-regulates many SRF-dependent smooth muscle specific genes. The signalling pathways regulating SRF are of great interest in the context of cardiovascular disease therapeutic targets. This manuscript focuses on dissecting the pathway by which TGF-β signalling induces and maintains vascular smooth muscle phenotype through co-operativity of SRF and TAZ, a potent transcriptional co-activator and nuclear retention factor.

AUTHOR CONTRIBUTIONS:

TGFβ-TAZ/SRF SIGNALING REGULATES VASCULAR SMOOTH MUSCLE CELL DIFFERENTIATION

Pagiatakis, C.[1,3,4], Sun, D.[1,3,4], Wales, S.[1,3,4], Miyake, T.[1] and McDermott, J.C.[1,2,3,4]

Submitted for publication, *Journal of Biological Chemistry*

Experimental design:

Christina Pagiatakis

Drafting manuscript:

Christina Pagiatakis and Dr. John C McDermott

Conducting experiments:

Christina Pagiatakis: Figure 1, Figure 2C,D, Figure 3, Figure 4B, Figure 5

Dandan Sun: Figure 2A, B

Stephanie Wales: Figure 4A, C

Tetsuaki Miyake: Figure 1B, Supplementary Figures 1, 2, 3 and 4

MANUSCRIPT:

TGFβ-TAZ/SRF SIGNALING REGULATES VASCULAR SMOOTH MUSCLE CELL DIFFERENTIATION

Pagiatakis, C.[1,3,4], Sun, D.[1,3,4], Wales, S.[1,3,4], Miyake, T.[1] and McDermott, J.C.[1,2,3,4]

TGF-β activates VSMC differentiation through TAZ and SRF

[1] Department of Biology, [2] Centre for Research in Mass Spectrometry (CRMS), [3] Muscle Health Research Centre (MHRC), [4] Centre for Research in Biomolecular Interactions (CRBI), York University.

Key words: TGFβ, vascular smooth muscle cells, differentiation, Smad, cardiovascular disease, SRF, TAZ

Abstract:

Rationale: Vascular smooth muscle cells (VSMCs) do not terminally differentiate; they modulate their phenotype between proliferative and differentiated states, which is a major factor contributing to vascular diseases. TGF-β signaling has been implicated in inducing VSMC differentiation, although details of the exact mechanism remain largely unknown. Our goal was to assess the network of transcription factors involved in the induction of VSMC differentiation.

Objective: To determine the role of TAZ and SRF in promoting the quiescent VSMC phenotype.

Methods and Results: TGF-β potently induces VSMC marker genes in 10T1/2 mouse embryonic fibroblast cells. Smad proteins function downstream of TGF-β signalling to regulate various target genes. The potent transcriptional regulator TAZ has been shown to retain Smad complexes on DNA. Thus, the role of TAZ in regulation of VSMC differentiation genes was investigated. Using siRNA-mediated gene silencing, our studies reveal that TAZ is required for TGF-β induction of smooth muscle genes and is required in the maintenance of the differentiated VSMC phenotype; synergy between TAZ and SRF in regulating smooth muscle gene activation has also been observed. Furthermore, TAZ expression enhances SRF recruitment to the smooth muscle α-actin promoter.

Conclusions: These data provide evidence of a novel signalling pathway that links TGF-β signalling to induction of smooth muscle genes in embryonic fibroblasts through a mechanism involving regulation of TAZ and SRF proteins. These observations elucidate a novel level of control of VSMC induction which may have implications for vascular diseases and congenital vascular malformations.

Introduction

Vascular smooth muscle cells (VSMCs), unlike other muscle types, do not terminally differentiate; they toggle between a proliferative and differentiated state in response to various extracellular signals (171). The regulation of this phenotypic modulation plays a key role in vascular injury, whereby smooth muscle-specific differentiation genes are down-regulated, contributing to the vascular regenerative response through up-regulation of proliferative genes (2). The fluctuation between contractile and proliferative phenotypes is a major factor contributing to many vascular diseases, such as atherosclerosis and restenosis following angioplasty (3). Therefore, the regulation of vascular smooth muscle phenotype is of important clinical relevance for cardio-vascular disease.

Serum response factor (SRF) is a member of the MADS (Mcm1, Agamous, Deficiens, SRF) domain superfamily of transcription factors, containing a MADS domain near its N-terminal region, which allows efficient DNA binding and homo-dimerization. C-terminal to the MADS-box of SRF is a region thought to be important for the binding of regulatory co-factors (90). The C-terminus of SRF contains a transcriptional activation domain, which is regulated by phosphorylation upon serum stimulation(93). Together these properties allow efficient growth factor regulated transcriptional activation through the consensus SRF binding site, CC $(AT)_6$ GG, which is termed the serum response element (SRE), or CArG box (172).

However, SRF is not a potent transcriptional activator on its own, as it requires binding of a co-activator within its MADS domain to achieve high levels of activation (39). An interesting property of SRF is that it can activate both growth-dependent proto-oncogenes (such as *c-fos*), and also cell type-specific promoters such as α-actin (100). In smooth muscle, these characteristics have physiological relevance since smooth muscle cells do not terminally differentiate. Interestingly, nearly all smooth muscle marker genes identified are dependent on one or more CArG elements found

within their promoter or first intron (3). Thus, it appears that smooth muscle phenotypic modulation is dependent on SRF recruitment to muscle-specific promoters. Cardiac ablation of SRF results in embryonic lethality at E11.5 with thin dilated myocardium, (16,173) and inactivation of SRF in vascular smooth muscle results in their decreased recruitment to the dorsal aorta with attenuation of smooth muscle marker gene expression (16). SRF expression is higher in smooth muscle cells than in non-muscle cell types, and SRF expression is induced by smooth muscle differentiation factors such as TGF-β (104).

The canonical transforming growth factor β (TGF-β) pathway is propagated by phosphorylation of receptor-associated Smad proteins (R-Smads) following TGF-β stimulation. Phosphorylated R-Smads associate with the common Smad (Smad4) which is required for the complex to be translocated into the nucleus (117,118). Nuclear localization of Smads results in transcriptional regulation of various target genes where the Smads, in concordance with other factors, act as transcriptional regulators of various genes (119). Furthermore, nuclear retention of Smad complexes has been shown to be regulated through an association with TAZ, which is recruited to sites of Smad-mediated transcription (120). TGF-β has also been implicated in inducing VSMC differentiation through the RhoA/ROCK signalling pathway (123). TGF-β induces a contractile VSMC phenotype in neural crest stem cells, by up-regulating smooth muscle marker genes such as smooth muscle α-actin, SM-22 and calponin (124,125), however, details of the transcriptional mechanism for up-regulation of these genes is incomplete. Moreover, it is likely that TGF-β can also induce smooth muscle gene expression in fibroblasts which may have important clinical ramifications.

Here, we report a novel role for TAZ in smooth muscle gene expression and differentiation that is mediated by a TGF-β pathway dependent, synergistic association between TAZ and SRF. Our

findings highlight the importance of TAZ in the maintenance of the vascular smooth muscle phenotype.

Methods

Cell culture

Primary mouse aortic smooth muscle cells were isolated by enzymatic cell dispersion, modified from (174) and maintained in growth medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) with High Glucose and L-Glutamine (Hyclone) supplemented with 10% fetal bovine serum (FBS; HyClone) and 1% Penicillin/Streptomycin (Invitrogen). For pharmacological treatments, cells were serum-starved for 24 hours prior to drug administration. C3H10T1/2 mouse embryonic fibroblasts (ATCC; CCL-226) were maintained as described above, and re-fed in 5% horse serum to achieve quiescence. For conversion assays, C3H10T1/2 were grown to confluence and made quiescent for 4 days prior to harvesting. C3H10T1/2 cells treated with recombinant human TGF-β1 (R&D Systems 240-B) were treated in 5% horse serum at 10ng/mL for 24 hours. Primary VSMCs treated with the TGF-β inhibitor SB431542 (Sigma S4317) were treated in serum free medium at 5μM for 24 hours.

Transfections

C3H10T1/2 were transfected using the calcium phosphate precipitation method. Cells were re-fed 16 hours post-transfection, and harvested 24 hours later. For siRNA experiments, C3H10T1/2 and primary mouse aortic smooth muscle cells were transfected with Lipofectamine (Invitrogen), as per manufacturer's instructions. Cells were supplemented with 20% FBS 5 hours post-transfection, re-fed after 16 hours and harvested 24 hours later or placed in quiescent conditions for pharmacological treatment.

Plasmids

Expression plasmids for FLAG-TAZ and FLAG-TAZS89A were purchased from Addgene (Dr. Jeff Wrana, 24809 and 24185, respectively). The HA-TAZ expression vector was cloned by Dr. Tetsuaki Miyake (fwd primer: 5' ACCTCGAAGCCCTCTTCAACTCT3'. Tagged with HA, EcoRI/XhoI). The Smad3 construct was a generous gift from J. Wrana, and the SRF expression plasmid was obtained from P. Shaw. The SM-MHC promoter was a gift from S. White, the smooth muscle α-actin and calponin reporter genes were generously provided by J. Miano, and the SM22 reporter gene was provided by E. Olson. The pRL-Renilla (Promega) reporter construct was used as an internal transfection control.

siRNA

Knockdown of target genes was done using siRNA obtained from Sigma-Aldrich. siTAZ #1 (SASI Mm01 00107363), siTAZ#2 (SASI Mm01 00107364) and siTAZ#3 (SASI Mm01 00107368) were used at 50nM concentrations, and siSRF#1 (SASI Mm01 00170496), siSRF#2 (SASI Mm02 00325543) and siSRF#3 (SASI Mm01 00170499) were used at 100nM concentrations.

Immunoblots

Cells were washed with 1× phosphate buffered saline (PBS) and lysed in NP-40 lysis buffer (50 mM Tris, 150 mM NaCl, 0.5% NP-40, 2 mM ethylenediaminetetraacetic acid (EDTA), 100 mM NaF and 10 mM Na pyrophosphate) containing protease inhibitor cocktail (Sigma-Aldrich), 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich) and 1 mM sodium orthovanadate (Bioshop). Protein concentrations were determined by Bradford assay (Bio-Rad) and 1-20 µg of total protein were resolved on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto Immobilon-FL Polyvinylidene Difluoride (PVDF) membrane (Millipore) for 1 h or overnight. Non-specific binding sites were blocked using 5% milk in TBS. Membranes were incubated with primary antibodies overnight at 4°C in 5% Bovine Serum Albumin (BSA) in TBS. Primary

antibodies included TAZ (1:1000, Cell Signaling 8418), SRF (1:1000, Cell Signaling 5147), FLAG (1:1000, Sigma F1804), SMαA (1:20000, Sigma A5228), Actin (1:2000, Santa Cruz 1616), HA (Developmental Studies Hybridoma Bank) and Smad3 (1:1000, Cell Signaling 9513). Appropriate HRP-conjugated secondary antibody (Goat anti-rabbit IgG-HRP (170–6515) and goat anti-mouse IgG-HRP (170–6516), BioRad, (1:2000) were added for 2 hours at room temperature. Protein was detected with Enhanced Chemiluminescence (ECL) western blotting substrate (Pierce).

Luciferase analysis

Cells were washed with 1× PBS and then lysed in Luciferase Lysis Buffer (20 mM Tris pH 7.4 and 0.1% Triton X-100). Lysate was briefly vortexed, and enzymatic activity was measured in each sample on a luminometer using Luciferase assay substrate (E1501, Promega) or Renilla assay substrate (E2820, Promega) (internal transfection efficiency control). Corresponding immunoblots of luciferase extracts contained equal volumes from each triplicate.

Immunofluorescence

Primary VSMCs were fixed in 4% paraformaldehyde, permeabilized in ice-cold methanol, and incubated with a primary smooth muscle α-actin antibody (1:500, Sigma) or TAZ antibody (1:500, Cell Signaling), and with FITC- and TRITC-conjugated secondary antibodies (1:200). Cells were visualized using confocal fluorescence microscopy. Mouse thoracic aorta was serially sectioned into 5µm sections using a -20°C cryotome and mounted on OCT mounting medium. Immunofluorescence was carried out on sections as indicated above.

ChIP

Methods were carried out as previously described (175); however, a third Immunoprecipitation (IP) Wash Buffer was added (IP Wash Buffer III; 20 mM Tris pH 8.1, 250 mM LiCl, 1% NP-40, 1%

deoxycholate and 1 mM EDTA). Antibodies were used at the following amounts: SRF (Cell Signaling): 5µL, TAZ (Cell Signaling): 10µL, and Rabbit IgG (Millipore): 1µg.

Quantitative polymerase chain reaction (qPCR)

Note that 2.5 µl gDNA or cDNA was combined with iTaq universal SYBR Green supermix (Bio-Rad) and 500 nM primers in a final volume of 20 µl. cDNA was diluted 1:10 in Nuclease-free water (Ambion) prior to use. Each sample was prepared in triplicate and analyzed using Rotor-Gene Q (Qiagen). Parameters for quantitative reverse transcriptase-PCR (qRT-PCR): 30 s 95°C, [5 s 95°C, 30 s 60°C] × 40 cycles. Parameters for ChIP-qPCR: 5 min 95°C, [5 s 95°C, 15 s 60°C] × 40 cycles. Fold enrichment (ChIP-qPCR) and fold change (qRT-PCR) was quantified using the ΔΔCt method. Primers used are as described in (176).

Results

TGF-β induces expression of smooth muscle gene expression

Using the pluripotent 10T1/2 mouse embryonic fibroblast cell line, cells were treated with TGF-β in a conversion assay, to determine whether TGF-β was sufficient to induce smooth muscle gene expression and phenotype switching; cells were placed in low serum conditions, and were allowed to differentiate for four days post TGF-β treatment. TGF-β significantly increases the expression of smooth muscle α-actin (SMαA) and SM22, indicating a conversion of the fibroblasts to smooth muscle (Figure 1A). Interestingly, the endogenous expression of TAZ and SRF are also increased upon TGF-β stimulation (Figure 1A). These data suggest that TGF-β induction of VSMC differentiation could be a result of its up-regulation of downstream effectors such as TAZ and/or SRF along with the more conventional activation of Smad family members (Smad 2 or 3). Under TGF-β conditions, 10T1/2 embryonic fibroblast cells also undergo phenotypic changes, whereby the cell

morphology appears to have a more elongated, smooth-muscle-like phenotype which is consistent with the gene expression changes observed (SMαA staining), and SM22 protein expression is induced (Figure 1B, S1, S2, S3, S4). In addition, treatment of primary mouse aortic vascular smooth muscle cells with the TGF-β inhibitor SB431542, which inhibits the TGF-β-mediated activation of Smad proteins, shows a decrease in endogenous smooth muscle alpha actin and SRF expression, indicating that TGF-β signalling through the Smad pathway also plays an important role in the maintenance of the quiescent vascular smooth muscle phenotype (Figure 1C).

In concert with the proteomic changes alluded to above, promoter level analysis using a luciferase reporter system of several smooth muscle-specific promoters (*SMαA-luc, SMMHC-luc, Calponin-luc* and *SM22-luc*) revealed that treatment with TGF-β also results in activation of these promoters (Figure 1D). Under identical conditions, and using the same cell lysate as in the promoter analysis, we also observed a corresponding increase in SMαA, SM22 and TAZ protein levels, confirming that TGF-β is playing a potent role in the activation of the VSCM differentiation program, indicating that TGF-β is crucial in the commitment of fibroblast conversion to the smooth muscle phenotype (Figure 1D).

TAZ is expressed in primary VSMCs and plays an important role in the quiescent phenotype

TAZ protein has been implicated in the modulation of canonical TGF-β signalling, and a key factor in Smad biology, whereby TAZ retains Smad complexes in the promoter regions of various genes to regulate gene expression controlling proliferation, differentiation, apoptosis and epithelial-mesenchymal transition (EMT) (133). However, TAZ has not been previously implicated in the control of vascular smooth muscle cell gene expression and differentiation. Immunofluorescence analysis using antibodies specific to TAZ and smooth muscle α-actin, reveals that TAZ is expressed in primary

aortic vascular smooth muscle cells, and is localized primarily in the nucleus, with lower expression levels in the cytosol (Figure 2A). Furthermore, immunofluorescence staining of mouse aortic sections with antibodies specific to TAZ and SMαA reveals that TAZ is also expressed in the tunica media concurrently with smooth muscle α–actin protein, indicating its expression in the vascular smooth muscle cells lining the arterial wall (Figure 2B).

Promoter analysis of the SMαA-luc promoter was performed using a luciferase-based reporter system to determine the effects of TAZ on the regulation of this smooth muscle promoter. Exogenous expression of TAZ showed a significant increase in SMA promoter activity (Figure 2C), indicating that TAZ can potently activate this promoter and may play a functional role in the regulation of smooth muscle phenotype. Although TAZ has been implicated in the regulation of SMαA during fibrogenesis, epithelial to myofibroblast transition (EMyT) and epithelial to mesenchymal transition (EMT) (134), the involvement of TAZ in smooth muscle specific gene expression and maintenance of the quiescent phenotype is a novel observation and we sought to further define the mechanism by which TAZ regulates smooth muscle differentiation. Interestingly, TAZ depletion by siRNA-mediated gene silencing in primary smooth muscle cells, results in a decrease of endogenous SMA and SM22 expression, confirming that TAZ is playing a crucial role in the induction of vascular smooth muscle cell gene expression and the quiescent phenotype (Figure 2D).

TAZ, Smad3 and SRF cooperate to activate smooth muscle-specific gene expression

We observed both a significant increase in SMA promoter activity with exogenous TAZ expression and a corresponding decrease in VSMC gene expression when TAZ was depleted, suggesting that TAZ may play an important, if not critical role in promoting VSMC differentiation. We therefore hypothesized that TAZ may co-operate with one or more of the known transcription factors

involved in activation of the SMαA promoter. Since siRNA mediated TAZ depletion reduced the effect of TGF-β on smooth muscle gene expression we also predicted that TAZ may co-operate with a factor downstream of TGFβ signalling. Since it has been reported that TAZ and Smad3 can interact in HEK293T cells, we analyzed the effect of co-expressing TAZ and Smad3 on the SMαA-luc promoter. Figure 3A indeed shows that nuclear TAZ (TAZS89A) and Smad3 have a potent synergistic effect on the SMαA promoter, activating it over 120-fold. Since the SMαA promoter contains CaRG boxes, and it has already been identified that SRF and Smad3 up-regulate VSMC gene expression leading to differentiation, we next pursued the idea that TAZ may function with SRF and Smad3 on the SMαA promoter. Promoter analysis using a luciferase reporter reveals that exogenous expression of nuclear TAZ, SRF and Smad3 together, co-operate to increase the activity of the SMαA promoter. Interestingly, we observe a synergistic effect under only SRF and nuclear TAZ conditions (Figure 3B). Since SRF is known to play a role in the up-regulation of promoter activity, siRNA to SRF was used to determine whether TGF-β can activate differentiation in the absence of SRF. Depletion of SRF by siRNA mediated gene silencing reveals that TGF-β cannot induce the SMαA-luc promoter in the absence of SRF, (Figure 3C) and furthermore, SMA protein cannot be induced to the same level when SRF is depleted (Figure 3D). Collectively, these data indicate an unequivocal requirement for SRF in TGFβ-mediated VSMC differentiation.

TAZ and SRF synergistically activate smooth muscle differentiation

Chromatin immunoprecipitation revealed that under TGF-β conditions, TAZ is enriched on the smooth muscle α-actin promoter at the first CaRG box (Figure 4A). Interestingly, there is also enrichment of SRF on the smooth muscle actin promoter under TGFβ conditions (Figure 4A). The induction of fibroblast cells to the smooth muscle phenotype, both *in vitro* and *in vivo,* results from

canonical TGF-β signalling pathway and its downstream effectors' recruitment to the SRF binding region of the smooth muscle alpha actin promoter. A fibroblast conversion assay utilizing exogenous expression of either wild-type TAZ or nuclear TAZ (TAZS89A) and SRF indeed reveals a marked induction of SMαA protein expression when SRF is co-expressed with nuclear localized TAZ, indicating a co-operation between these two transcription factors in the commitment of the vascular smooth muscle cell phenotype (Figure 4B). Since we revealed the role of SRF in the TGF-β/TAZ mediated regulation of smooth muscle-specific gene expression, we hypothesized that the enrichment of these two factors on the SMαA promoter are dependent on each other. Co-immunoprecipitation analysis showed that the binding of SRF to the most proximal CaRG box of the SMαA promoter is decreased under siRNA-mediated TAZ depletion (Figure 4C). This suggests a novel role for TAZ in the retention of SRF on the SMαA promoter to regulate smooth muscle cell differentiation.

Collectively, these data suggest that the canonical TGF-β pathway is a requirement for full activation of smooth muscle genes, and that the combination of downstream effectors comprising TAZ and SRF are required for full scale (Figure 5), robust activation of smooth muscle gene expression. TGF-β and TAZ are also required for the commitment phase of the fibroblasts to the vascular smooth muscle cell lineage.

Discussion

Vascular diseases including atherosclerosis and restenosis are a result of aberrant vascular smooth muscle cell activation, proliferation and subsequent migration to the site of injury, whereas differentiated or quiescent VSMCs express matrix components and proliferate at low levels. The reversible nature of the phenotypic modulation of synthetic versus contractile phenotypes is largely a

function of regulation of either proliferative or smooth muscle -specific genes, respectively (177). Therefore, an understanding of the molecular mechanisms toggling smooth muscle cells between these dynamic cellular states is fundamental to our understanding of vascular development and disease processes. Moreover, the possibility of therapeutically manipulating the smooth muscle phenotype in a variety of vascular diseases also relies on our knowledge of the underlying biology of the system.

The MADS box transcription factor SRF plays a critical role in the modulation of the VSMC phenotype. Interestingly, it plays a paradoxical role in regulating both contractile and synthetic phenotypes, by binding to CArG boxes on the promoter regions of different genes, through differential recruitment of co-activators (91). Smooth muscle structural genes contain multiple copies of the CArG box, and SRF regulates the quiescent phenotype by recruiting co-activators such as myocardin. It appears that smooth muscle phenotype modulation is dependent largely on SRF site-directed control elements (3,109). However, SRF can also activate proliferation by activating immediate-early genes such as *c-fos* through the Elk-1 co-activator (178). Clearly, the involvement of SRF in both the proliferative and quiescent states requires further dissection in order to fully understand how specificity is wired into the binary cellular state.

Inactivation of SRF in VSMCs results in attenuation of smooth muscle marker gene expression, and in cultured VSMCs, mimics proliferative effects of growth factor-induced activation of immediate-early genes, concurrently with down-regulation of smooth muscle structural genes(172). These findings indicate that SRF-dependent target genes contribute to but are dispensable for proliferation in smooth muscle, but are indispensable for differentiation (179,180). SRF expression is higher in smooth muscle cells than in most non-muscle cell lineages, and its expression is induced by various smooth muscle differentiation factors including TGF-β; at high levels of expression, SRF expression

favours activation of smooth muscle-specific genes (3,103,104). TGF-β plays an important role in the differentiation of smooth muscle cells from neural crest cells (181), and its downstream effectors, Smads 2 and 3, have been shown to be essential for VSMC differentiation (125). Thus, there is likely an important intersection between TGF-β signalling and SRF dependent gene expression in the specification of the smooth muscle differentiation program.

In this study, we provide the first evidence that TAZ, induced by TGF-β signaling, and SRF cooperate to potently regulate vascular smooth muscle differentiation. In particular, we show that TAZ is expressed in vascular smooth muscle cells, and in the tunica media of murine thoracic aorta. Furthermore, we report that under TGF-β stimulation in mouse embryonic fibroblasts, TAZ expression is induced concurrently with SMαA. These data are in agreement with a growing body of evidence showing that TGF-β signalling plays a critical role in up-regulating smooth muscle structural genes and inducing differentiation in fibroblasts (182).

Furthermore, our data show that TGF-β-dependent SMαA activation is SRF-dependent, and that together, TAZ and SRF robustly activate smooth muscle structural genes promoting the quiescent phenotype. In agreement with this, our data indicate that nuclear localized TAZ functions with SRF to promote induction of the VSMC phenotype from fibroblast cells. These data correlate with recent findings indicating that TAZ stimulates the SMαA promoter and TAZ silencing prevents wound-restricted expression of SMαA in wound healing of injured epithelium (134). We provide evidence that TAZ expression modulates binding of SRF to smooth muscle-specific genes in a chromatin context, an effect which is TGF-β dependent. In summary, we present evidence implicating TAZ in the maintenance of the VSMC quiescent phenotype, and also in the conversion of fibroblasts to smooth muscle cells. These findings have important ramifications in uncovering potentially new therapeutic targets in the manipulation of VSMC differentiation and stem cell reprogramming.

***Acknowledgments*:**

We would like to thank Joydeep Chaudhuri for assistance with aorta sectioning and Joe Gordon for assistance and support.

Sources of Funding:

This work was supported by a grant from the Heart and Stroke Foundation of Canada to JCM.

Disclosures:

None.

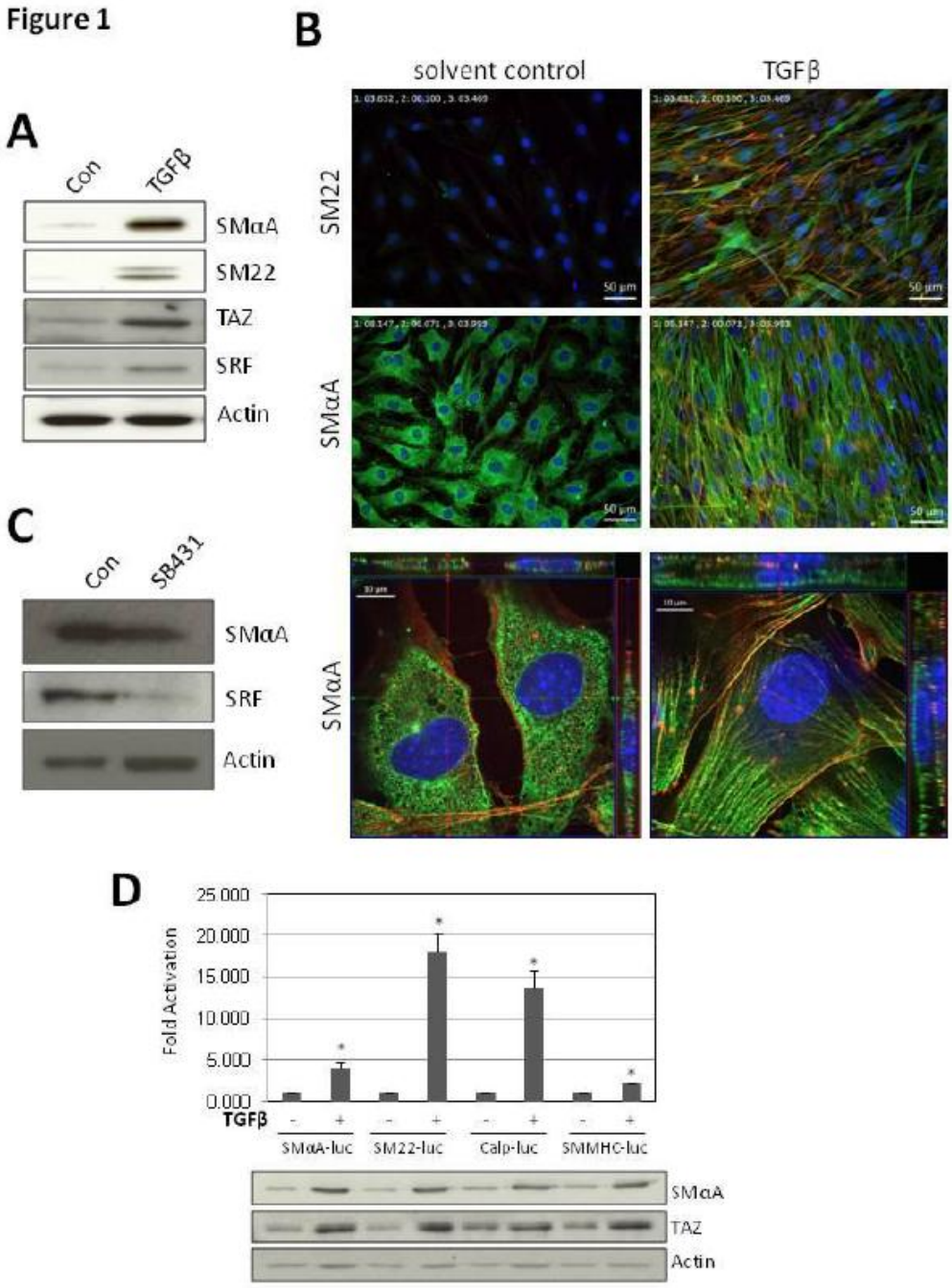

Figure 1: TGFβ induces expression of smooth muscle gene expression A) 10T1/2 mouse embryonic fibroblasts were treated with 10ng/ml TGFβ in low serum conditions for 24 hours. Media was changed to low serum after 24 hours and cells were left quiescent for 4 days and harvested for protein and blotted as indicated. **B)** 10T1/2 cells were treated as in A. Cells were stained with either anti-SMαA (1:500) (Sigma) or anti-SM22 (1:500) (Abcam) primary antibodies (green) and DAPI (blue) and F-actin (red). Images were obtained using spinning-disc microscopy. **C)** Primary mouse aortic vascular smooth muscle cells were treated in serum free conditions with 10μM SB431542 (Sigma) TGFβ inhibitor for 24 hours and subsequently harvested for protein and immunoblotted as indicated. **D)** 10T1/2 cells were transfected with either *SMαA-luc, SM22-luc, Calponin-luc* or *SMMHC-luc* and allowed to recover for 24 hours. Cells were switched to low serum conditions and treated with 10ng/ml TGFβ for 24 hours and harvested for both luciferase and protein. * indicates $P<0.05$ using student T-test.

Figure 2

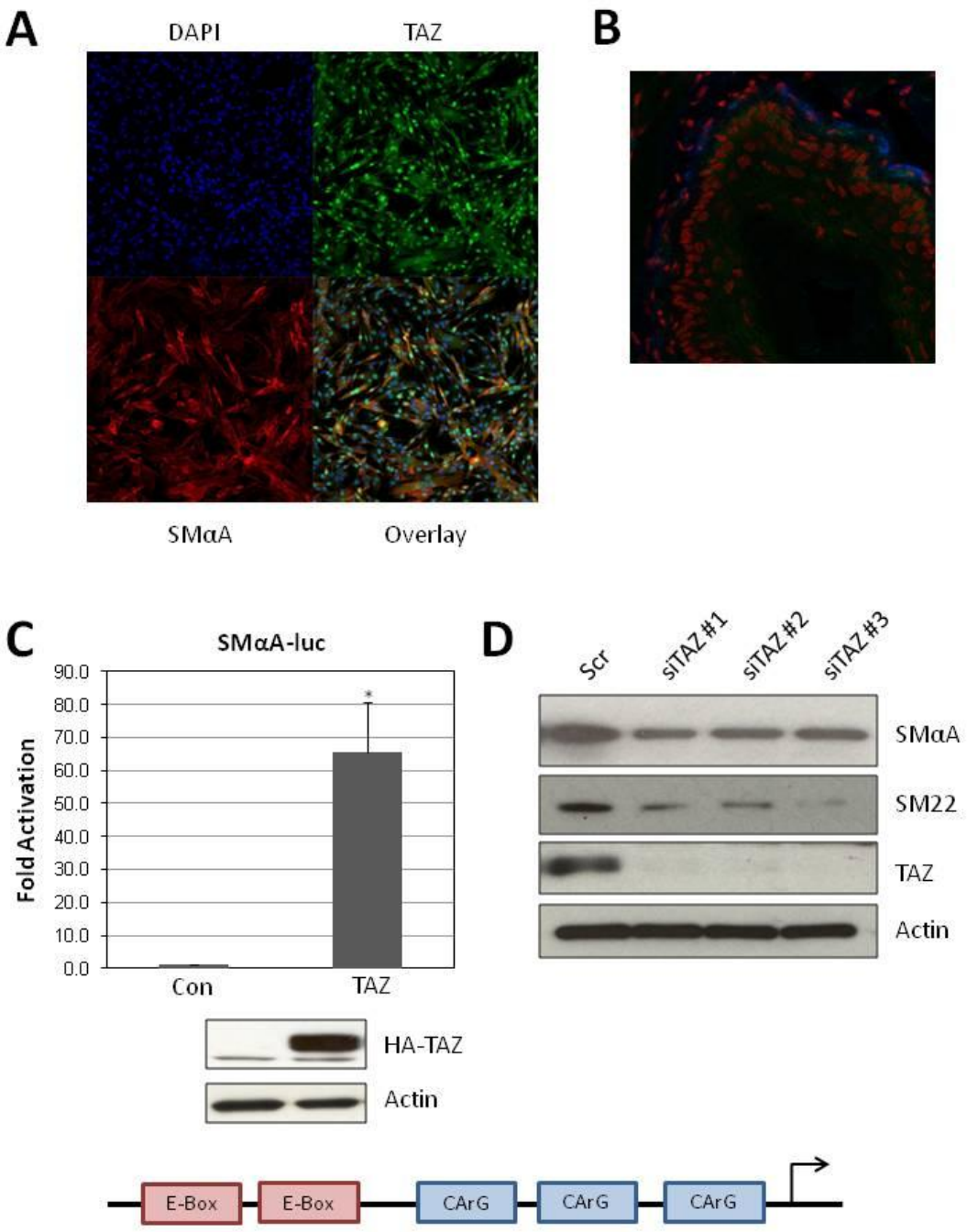

Figure 2: TAZ is expressed in primary VSMCs and is important in the quiescent phenotype A) Primary mouse aortic vascular smooth muscle cells were isolated and seeded for immunofluorescence. Cells were stained for anti-TAZ (1:200) (Cell signalling) and anti-SMαA (1:500) (Sigma) primary antibodies and subjected to confocal microscopy under 20x magnification as indicated. DAPI was used as a nuclear marker. **B)** Aortas were isolated from 4-week old mice, sectioned transversally and stained as in A). **C)** 10T1/2 mouse embryonic fibroblast cells were transiently transfected with *SMαA-luc* and HA-TAZ constructs for 16 hours and allowed to recover for 24 hours. Cells were subsequently harvested for both luciferase and protein and immunoblotted as shown. **D)** Primary mouse aorta smooth muscle cells were transfected with 50nmol of three different siRNAs targeting TAZ, using lipofectamine, as indicated above, for 5 hours. Cells were left to recover for 24 hours and harvested for protein and blotted as indicated above. * indicates $P<0.05$ using student T-test.

Figure 3

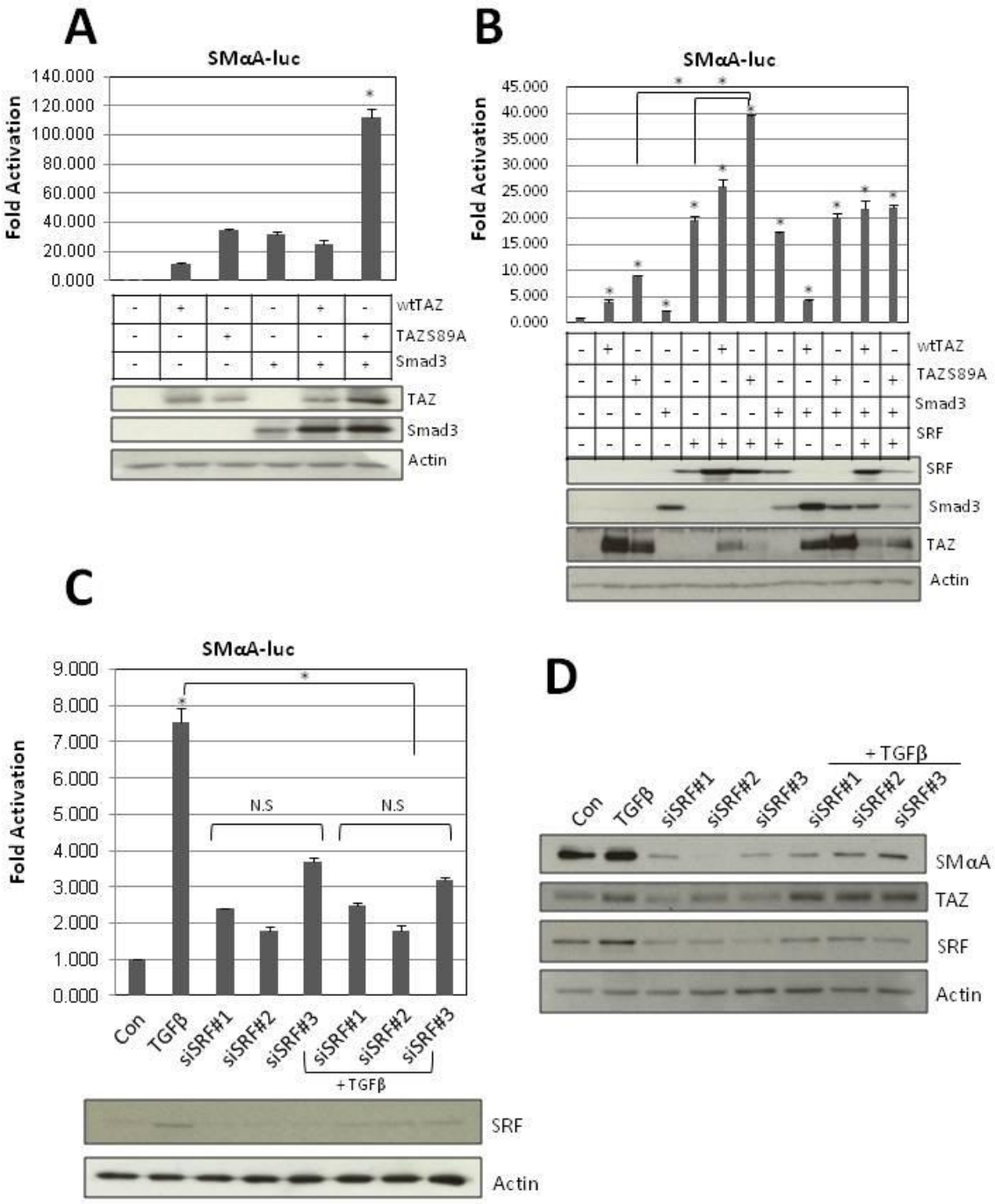

Figure 3: ***TAZ, Smad3 and SRF cooperate to activate smooth muscle-specific gene expression*****. A)** 10T1/2 cells were transfected with *SMαA-luc*, TAZ, TAZS89A and Smad3 constructs for 16 hours and allowed to recover. Cells were harvested for both luciferase and protein and blotted as indicated above. **B)** 10T1/2 cells were transfected as in A, with TAZ, TAZS89A, Smad3 and SRF, and processed as in A. **C)** 10T1/2 cells were transfected with both *SMA-luc* and 50nmol of siSRF for 5 hours. Cells were left to recover for 24 hours and subsequently treated with 10ng/ml of TGFβ in low serum conditions for 24 hours. Cells were harvested for both protein and luciferase and blotted as indicated above. **D)** 10T1/2 cells were transfected with 50nmol of siSRF for 5 hours. Cells were allowed to recover for 24 and treated with 10ng/ml TGFβ in low serum conditions for 24 hours and harvested for protein and immunoblotted as shown. **P*<0.05, NS=no significance; using one-way ANOVA.

Figure 4

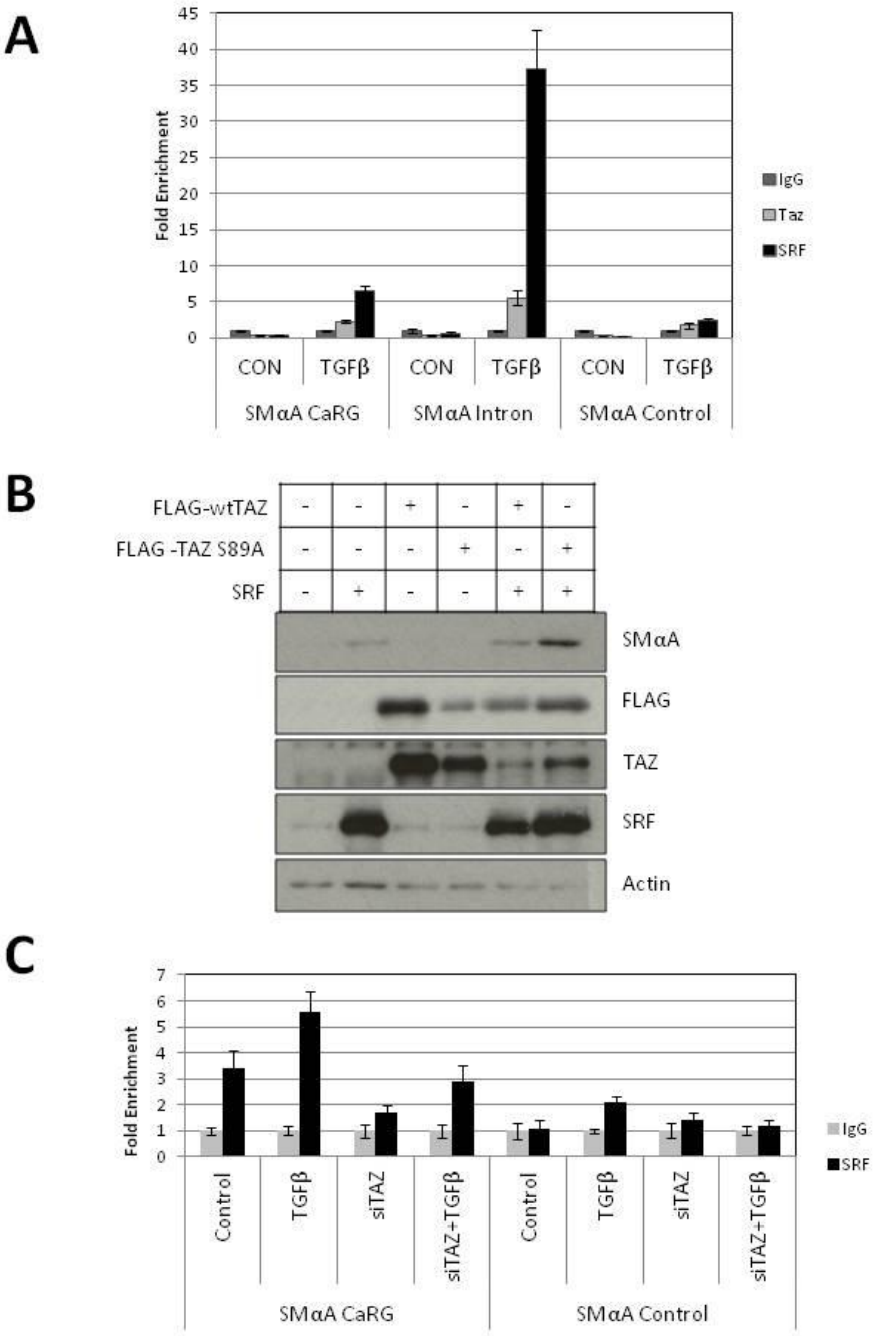

Figure 4: TAZ and SRF synergistically activate smooth muscle differentiation. A) 10T1/2 cells were treated with 10ng/ml of TGFβ for 24 hours in low serum conditions and harvested for chromatin immunoprecipitation and qPCR, as shown above. Error bars represent standard error of the mean. Primers for "SMαA CaRG" flank the first CaRG box in the promoter region, primers for "SMαA intron" flank the first CaRG box in the first intron of *SMαA,* and primers for "SMαA control" flank a random sequence +6kB of the transcription start site. **B)** 10T1/2 cells were transfected with wild type TAZ or TAZS89A and SRF as indicated above. Cells were allowed to recover for 24 hours in 10%FBS and left in low serum conditions for 48 hours, harvested for protein and immunoblotted as indicated above. **C)** 10T1/2 cells were transfected with 50nm of siTAZ for 5 hours, supplemented with 20% FBS, and subsequently switched to 10%FBS conditions to recover for 24hours. Cells were switched to low serum conditions and treated with 10ng/ml of TGFβ for 24, and subsequently harvested for chromatin immunoprecipitation and qPCR as indicated.

Figure 5

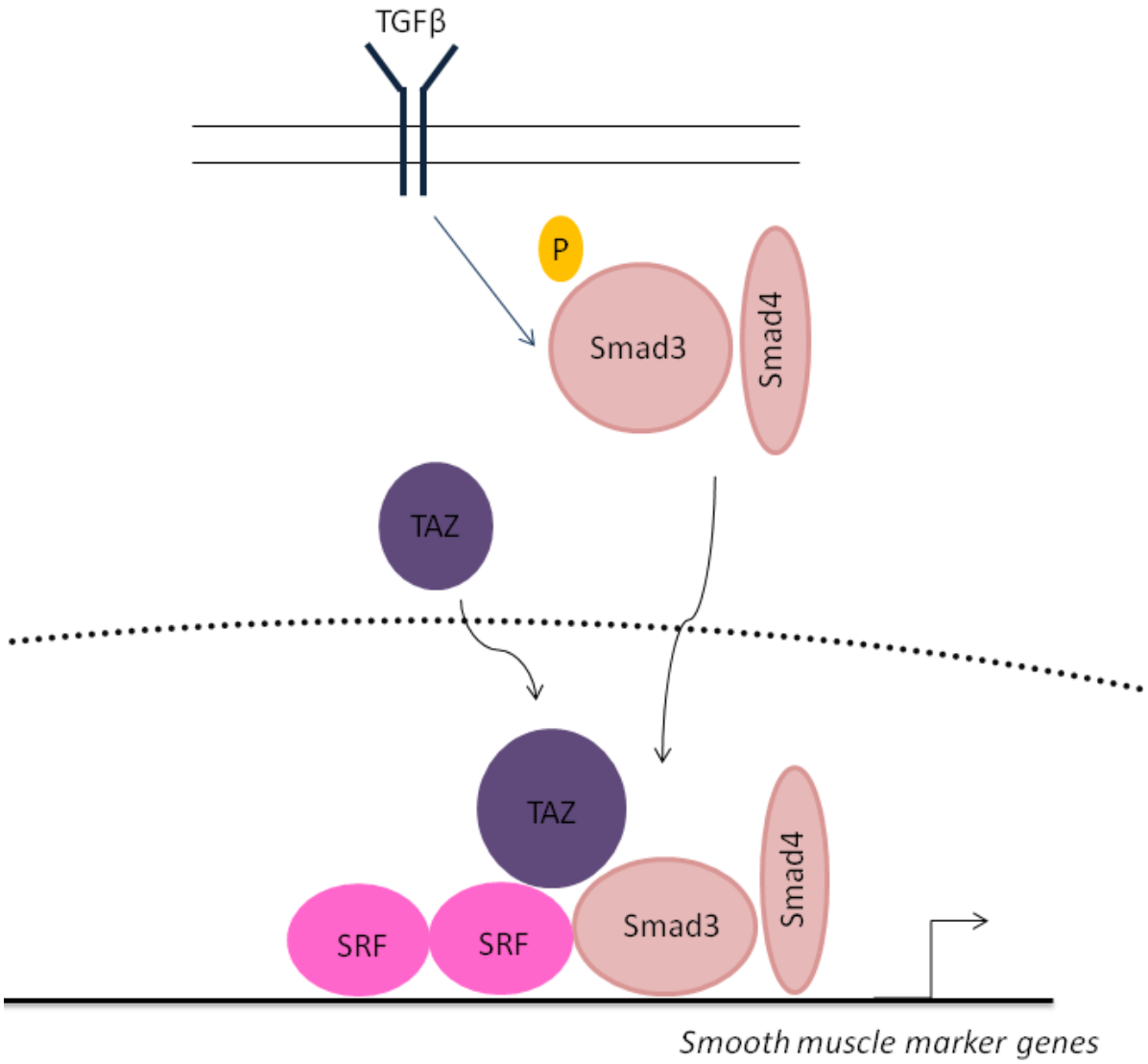

Figure 5: Summary of TAZ and SRF regulation of the *Smooth muscle α-actin* promoter

Supplemental Figures:

Figure S1:

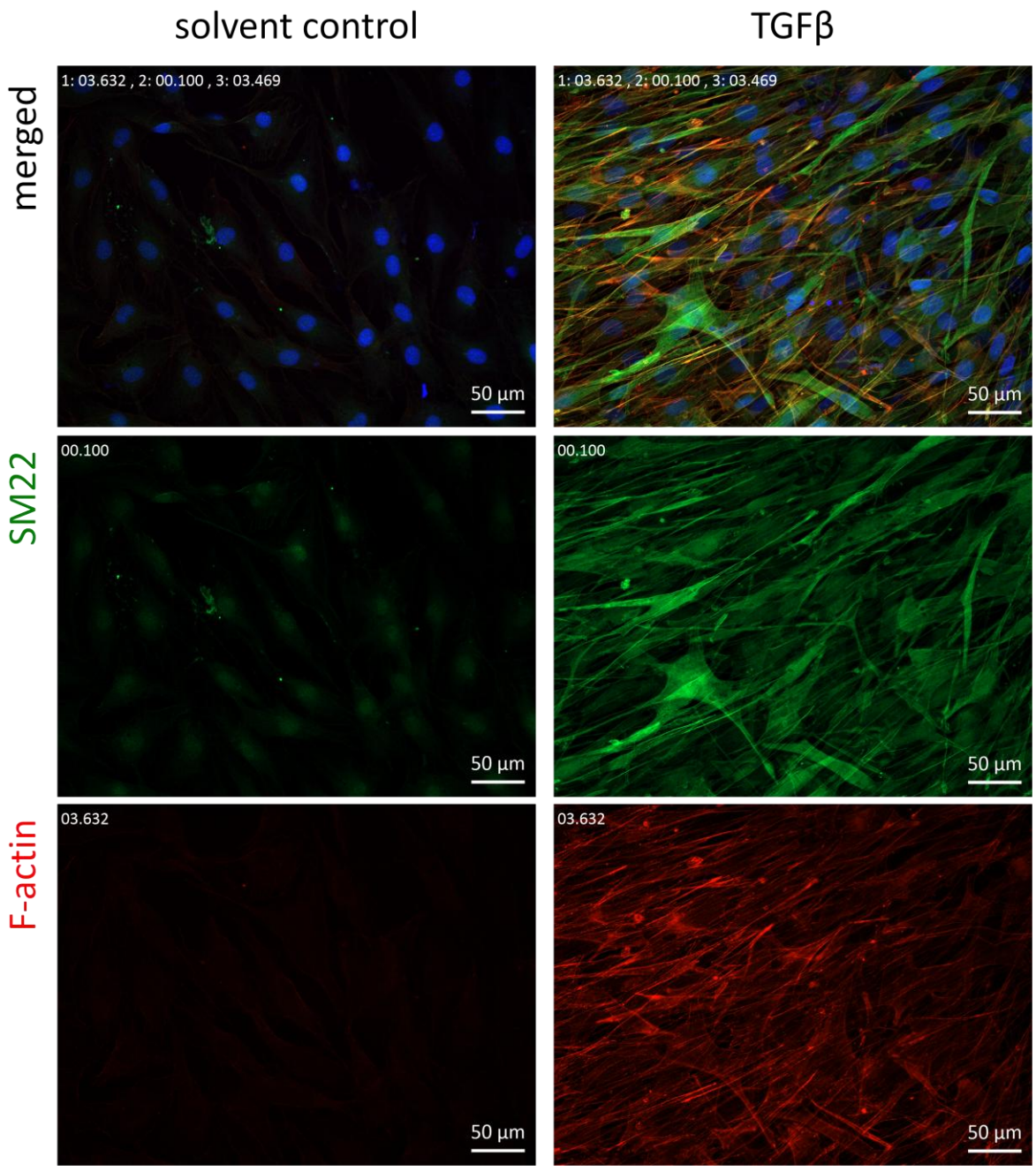

Figure S1: 10T1/2 mouse embryonic fibroblasts were treated with 10ng/ml TGFβ in low serum conditions for 24 hours. Media was changed to low serum after 24 hours and cells were left quiescent for 4 days and harvested for protein and blotted as indicated. Cells were stained with anti-SM22 (1:500) (Abcam) primary antibody (green), Hoechst (blue) and F-actin (red). Images were obtained using spinning-disc microscopy. Exposure time and actual cell size is as indicated above.

Figure S2:

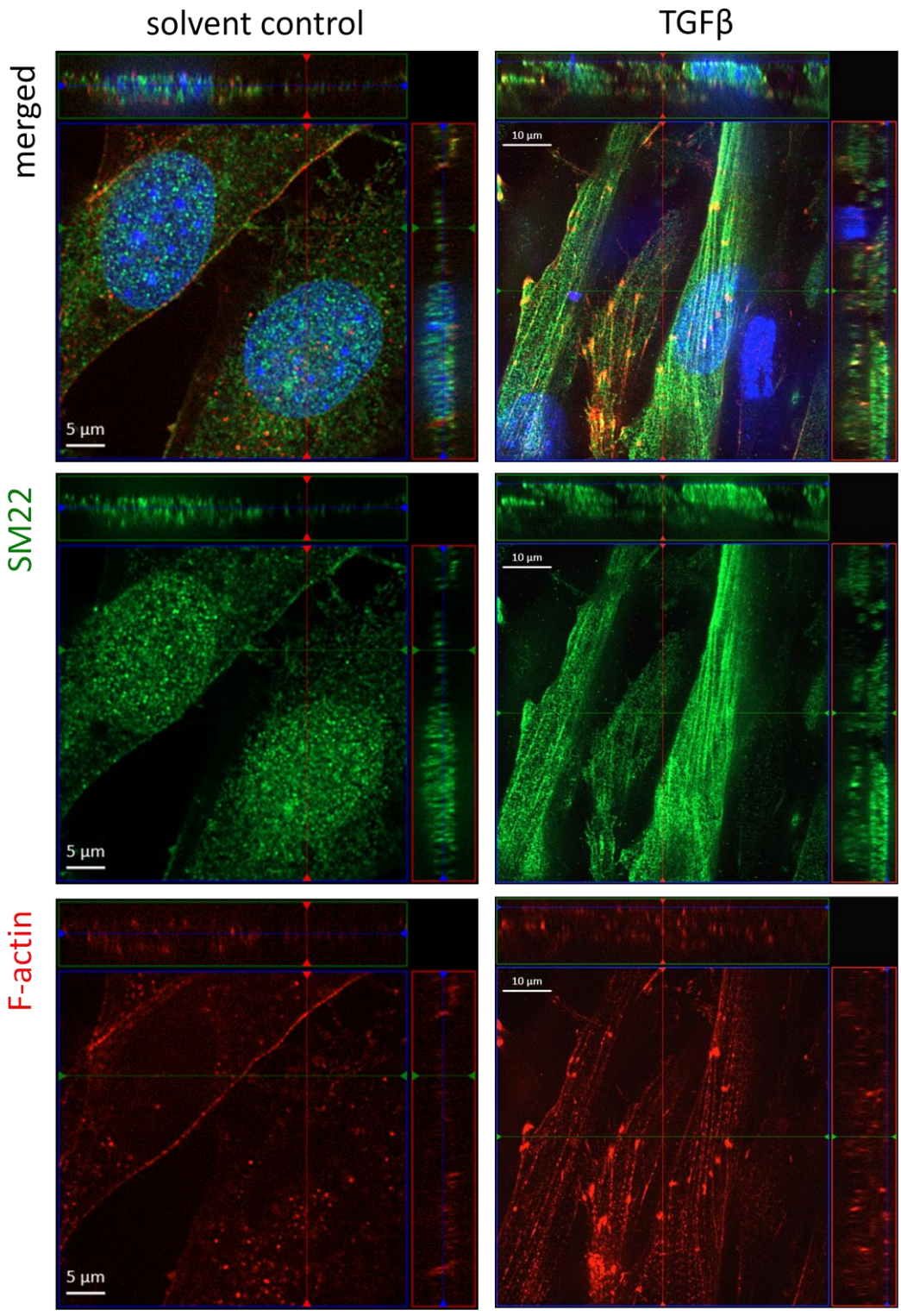

Figure S2: 10T1/2 mouse embryonic fibroblasts from Figure S1 are visualized at higher magnification. Actual cell size is as indicated above.

Figure S3:

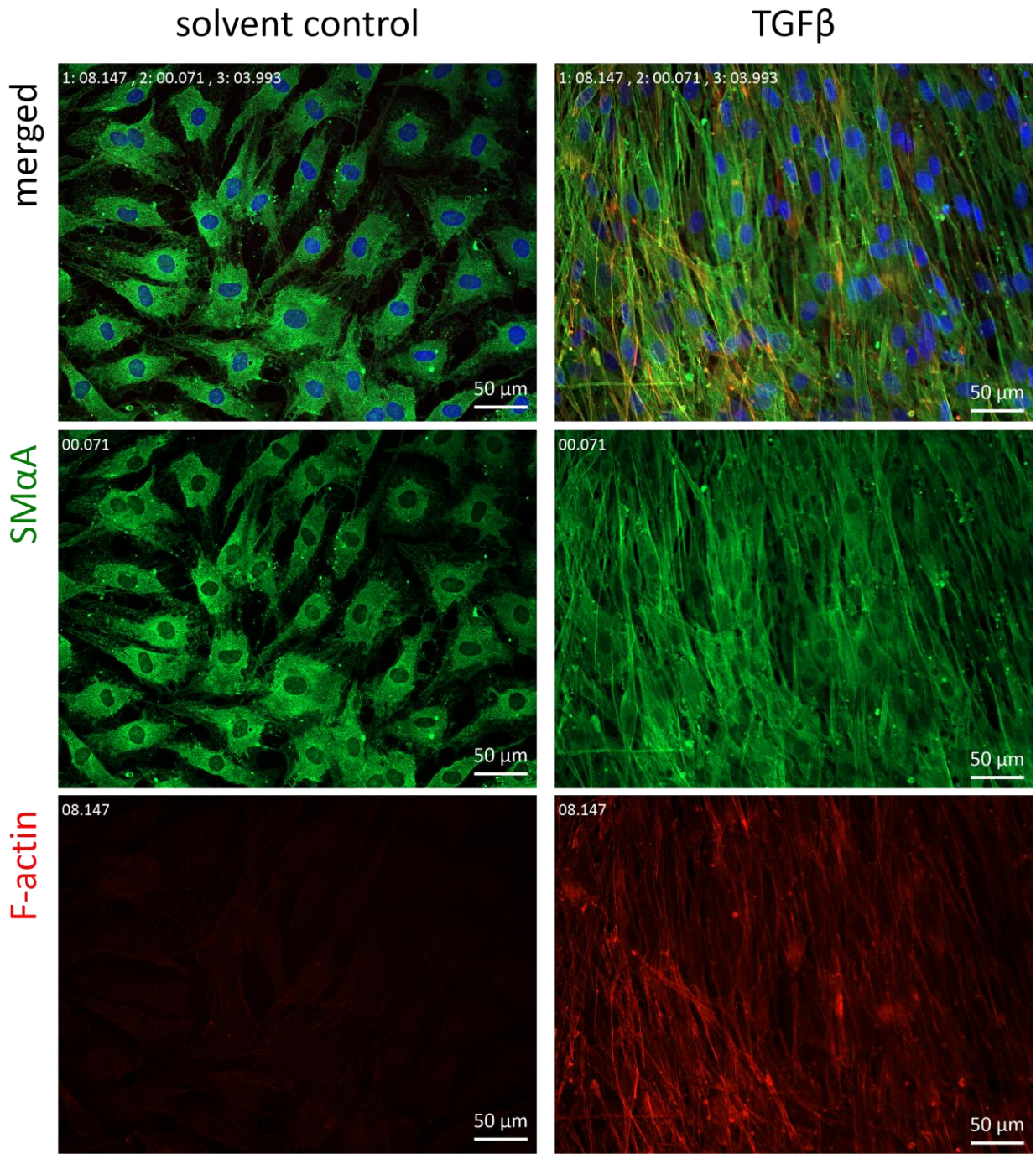

Figure S3: 10T1/2 mouse embryonic fibroblasts were treated with 10ng/ml TGFβ in low serum conditions for 24 hours. Media was changed to low serum after 24 hours and cells were left quiescent for 4 days and harvested for protein and blotted as indicated. Cells were stained with anti-SMαA (1:500) (Sigma) primary antibody (green), Hoechst (blue) and F-actin (red). Images were obtained using spinning-disc microscopy. Exposure time and actual cell size is as indicated above.

Figure S4:

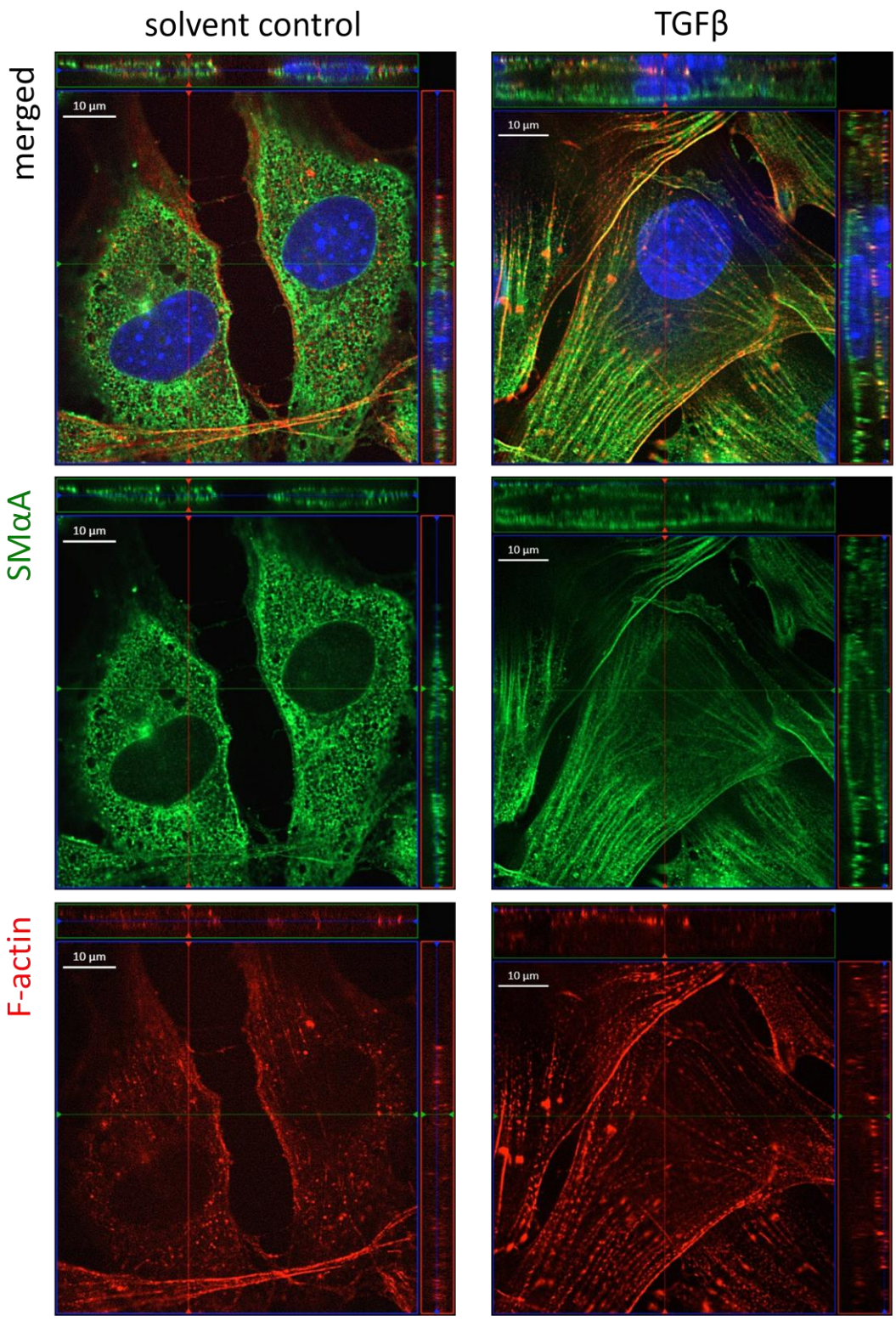

Figure S4: 10T1/2 mouse embryonic fibroblasts from Figure S3 are visualized at higher magnification. Actual cell size is as indicated above.

CHAPTER 4: DISSERTATION SUMMARY

The desicion of vascular smooth muscle cells to differentiate or proliferate is controlled by many signalling pathways, and is of great importance in the understanding of cardiovascular diseases affecting the integrity of blood vessels. As terminal differentiation of vascular smooth muscle cells does not occur, the mechanisms regulating the fluctuation between the synthetic or quiescent phenotype are critical as potential targets for potential therapeutic intervention.

Collectively, these studies evaluate the role of the signalling pathways involved in promoting vascular smooth muscle cell differentiation, centering on the control of SRF. I have further defined the role of several signalling molecules converging on the control of smooth-muscle specific gene expression in vascular smooth muscle cells, thus promoting the quiescent phenotype.

Calcium signalling to RhoA/ROCK promotes de-repression of MEF2C by PP1 through its phosphorylation of CPI-17, a potent inhibitor of PP1 activity. The de-repression of MEF2C results in activation of myocardin gene expression, which lies upstream of the smooth muscle α-actin promoter, whereby myocardin binds to SRF homodimers to activate its expression, and thus promoting the differentiated vascular smooth muscle phenotype. Furthermore, I showed that SRF is central in the regulation of *smooth muscle α-actin* through the TGF-β signalling pathways, whereby TGF-β promotes co-operativity between TAZ and SRF to activate expression of smooth muscle-specific genes and the quiescent phenotype.

The mechanisms underlying the control of vascular smooth muscle cell differentiation are still being characterized. This work provides further understanding of some of these pathways and describes a novel regulator of SRF-dependent smooth muscle gene expression. The identification of TAZ involvement in the smooth muscle quiescent phenotype allows for further investigation of smooth muscle-specific promoter control. The exact mechanism by which TGF-β signalling regulates

TAZ/SRF-dependent gene expression remains largely unknown. It is important to investigate the downstream effectors of TGF-β signalling to TAZ and SRF, and the factors controlling TAZ shuttling between the cytosol and nucleus.

Implication of TGF-β in the phenotypic transition of smooth muscle cells (from neural crest cells and fibroblasts) indicates the importance in identifying the mechanisms regulating the fibroblast to myofibroblast transition so as to control aberrant activation and cardiac pathologies associated with excessive myofibroblast activity. TGF-β has been shown to be a potent driver of fibroblast to myofibroblast phenoconversion, EMT and ECM deposition. Our work shows that TGF-β signalling promotes conversion of fibroblasts to smooth muscle cells. It is important to further distinguish between this phenoconversion (determined by expression of SMαA) and actual differentiation of smooth muscle cells (determined by expression of SM-MHC). The transition of fibroblasts to supermature myofibroblasts, and finally smooth muscle cells are characterized by distinct expression of marker proteins, and not only a change in phenotype. Smooth muscle α-actin is expressed as early as the fibroblast stage; supermature fibroblasts can be characterized by expression of ED-A fibronectin; smooth muscle cells, however are the only that express smooth muscle – myosin heavy chain (183,184).

These data add to our understanding of the signalling pathways involved in the control and modulation of the vascular smooth muscle phenotype in response to various signals. The ability to control vascular smooth muscle cell proliferation is critical for prevention or formation of atherosclerotic plaques, arterial stenosis and restenosis following angioplasty. These findings will play a key role in the development of novel therapeutic approaches, aimed specifically at these molecular pathways targeting vascular development, congenital heart defects and vascular stenotic diseases.

CHAPTER 5: FUTURE DIRECTIONS

This work identified a key role of calcium and TGF-β signalling in regulating vascular smooth muscle differentiation. To further illucidate the role of TGF-β and TAZ in vascular smooth muscle differentiation, it is important to look at the downstream effectors of TGF-β and the regulation of TAZ expression.

A potential downstream target of TGF-β is the RhoA/ROCK signalling pathway, which is known to be important in the regulation of PP1 and CPI-17. Since it has also been shown that TAZ nuclear/cytosolic shuttling is regulated by LATS kinase and PP1, it is important to investigate whether RhoA/ROCK signalling controls TAZ shuttling through CPI-17 inhibition of PP1. Since several studies have shown that PP1 controls the dephosphorylation of TAZ at serine-89, the 14-3-3 binding site, PP1 and CPI-17 could be playing a key role in promoting TAZ nuclear localization, and cooperativity with SRF. Since our work has shown that TAZ is important in maintaining SRF on the smooth muscle alpha-actin promoter, the interaction between TAZ and SRF needs to be investigated, and whether Smad3 (which has been shown to interact with both TAZ and SRF independently) mediates this interaction. Elucidating this pathway could provide a novel approach in therapeutically targeting the control of smooth muscle differentiation, and linking calcium signalling with TGF-β-dependent transcriptional control of VSMCs.

Our work has been centred around 10T1/2 mouse embryonic fibroblasts, and primary mouse aortic vascular smooth muscle cells. Using cultured primary VSMCs has several advantages over immortalized cell lines, such as expression of several structural proteins, however, removal of VSMCs from the *in vivo* environment may also alter phenotypic and differentiation properties as a result of isolation from the surrounding cells and structures.

It is therefore important for this work to progress to an *in vivo* setting to further investigate the role of TAZ in VSMC differentiation. Firstly, it will be valuable to stain an atherosclerotic artery to determine TAZ expression compared to a healthy artery (Manuscript #2). Using a clinical stent model, inhibition of TAZ or RhoA/ROCK can show if these factors are important in preventing re-stenosis of the artery. Using conditional knockout of TAZ with either the Cre-Lox or the Crispr system in smooth muscle cells, VSMCs can be cultured to determine the effect of TAZ in this tissue. Furthermore, aortic banding, and staining of aortic sections of the TAZ knockout mice can illustrate the importance of TAZ on expression of several smooth muscle-specific proteins.

These experiments will further dissect the signalling pathways involved in vascular smooth muscle differentiation, and the understanding of the role of TGF-β and TAZ in this context.

ADDITIONAL WORK I: BOOK CHAPTER

Molecular mechanisms of smooth muscle and fibroblast phenotype conversions in the failing heart

Authors: Christina Pagiatakis and John C. McDermott

Chapter Published in: Cardiac Fibrosis and Heart Failure: Cause or effect?
Editors: Ian M.C. Dixon and Jeffery T. Wiggle
Springer International Publishing

Abstract:

The mechanisms of gene regulation in cardiac hypertrophy and fibrosis are important in understanding the regulation of pathological gene expression in the heart. Cardiac hypertrophy is characterized by enlargement of the heart as a result of an increase in cardiomyocyte size and also enhanced fibrosis due primarily to phenotypic conversion of fibroblasts to myofibroblasts. Also, atherosclerosis, a disease characterized by formation of plaque within the arterial wall, and restenosis, which is the process of arterial wall healing in response to vascular injury, are highly affected by vascular remodelling. Vascular smooth muscle cells thus play a key role in vascular remodelling, as they modulate their phenotype in response to vascular injury and are a significant source of extracellular matrix components of the vessel wall. In view of the profound effects of both the fibroblast to myofibroblast conversion and also the role of vascular smooth muscle cells in vascular remodelling, we review the activation of the smooth muscle □□ actin gene in these contexts to examine the common and non-overlapping molecular circuitry underlying these cellular processes in the cardiovascular system.

Cardiac hypertrophy is characterized by enlargement of the heart as a result of an increase in cardiomyocyte size and enhanced fibrosis due primarily to phenotypic conversion of fibroblasts to myofibroblasts. This is typically a result of increased biomechanical stress. There are two types of cardiac hypertrophy: physiological hypertrophy and pathological hypertrophy (185). The former occurs during normal growth and development, and also in response to exercise and pregnancy, whereas the latter occurs typically due to loss of cardiomyocytes following myocardial infarction or as a result of arterial hypertension. Physiological hypertrophy is not associated with adverse cardiac function, fibrosis or heart failure, whereas pathological hypertrophy results in congestive heart failure, arrhythmia and mortality (186). It should be noted that cardiac hypertrophy can also have a genetic basis due to mutations in contractile proteins of the cardiac muscle sarcomere, such as β-myosin heavy chain, myosin light chain, troponin, actin, myosin binding protein C and α-tropomyosin (187). However, most of the studies reviewed here are based on hypertrophic adaptations to hypertension or experimental model systems that mimic it.

During pathological hypertrophy in the adult, there is an up-regulation of genes normally associated with embryonic and fetal development concurrently with a down-regulation of adult myocardial genes. There are various alterations in cardiac gene expression which result in both apoptosis and fibrosis (188). This phenomenon is termed ‘fetal gene activation’ and involves increased expression of β-MHC, atrial natriuretic factor, SM22, smooth muscle and skeletal muscle α-actin.. Furthermore, cardiomyocytes decrease their overall oxidative capacity and rely on anaerobic glucose metabolism; it appears that physiological hypertrophy plays an adaptive role to increased cardiac wall stress. Conversely, pathological hypertrophy will ultimately result in congestive heart failure (189).

The mechanisms of gene regulation in cardiac hypertrophy are important in understanding the regulation of pathological gene expression in the heart. For example, forced expression of activated calcineurin induces hypertrophy, fetal gene activation and heart failure in the transgenic mouse (185). CaMKs phosphorylate class IIa HDACS to relieve their repressive effects on transcription, and forced expression of CaMKIV in the heart also induces hypertrophy, concomitanly with increased expression of ANF and down-regulation of α-MHC (190). CAMKII is also an important factor in cardiac excitation-contraction coupling in response to β-adrenergic signalling (43), but also plays an important role in pathological cardiac remodelling in response to endothelin-1 (191). Interestingly, CaMKII targets HDAC4 specifically, to promote fetal gene activation following α-adrenergic agonist treatment (192). Mice deficient in CaMKIIδ are protected from pathological hypertrophy and fetal gene activation (193), however targeted deletion of HDAC5 and HDAC9 results in cardiac hypertrophy and increased pressure overload resulting in cardiac remodelling and increased fetal gene activation (194).

Cardiac remodelling, a phenomenon which occurs in response to heamodynamic load and/or injury, is characterized by a physical alteration in the hearts' dimension, mass or shape, and there are several molecular pathways which regulate cardiac remodelling. There are several agonists of cardiac remodelling, including Angiotensin II (AngII), Endothelin-1 (Et-1) and α-adrenergic stimulation (195). Many studies have shown that these agonists that activate Gq-coupled receptors target specific downstream targets such as PKC and PKD1, which are important for the nuclear export of HDAC5 in cardiomyocytes (196). Reduced expression of PKD1 prevents agonist-induced hypertrophy in cardiomyocytes, whereas conditional deletion of PKD1 in mice shows improved cardiac function and reduced hypertrophy (197).

Atherosclerosis and Restenosis

Atherosclerosis is a disease that is characterized by the formation of a plaque (also termed atheroma), within the arterial intima and media. Following the formation of the atheroma, the lumen will eventually narrow to cause ischemia. Disruption of the atheroma results in thrombus formation, which is the leading cause of angina, myocardial infarction and even cerebral infarction in the brain (198).

Atherogenesis is a process that occurs in response to chronic injury such as shear stress or oxidative stress. Examples of sources of endothelial injury are free radicals produced from cigarette smoking, hypertension, diabetes mellitus, oxidized LDL and elevated homocysteine (198). Free radicals and reactive oxygen species not only contribute to intimal injury, but also neutralize the protective effects of nitric oxide produced by the endothelium on the vasculature (199). Injury to the intima results in changes to the endothelium's capacity to regulate its adhesiveness and permeability to various circulating factors. Importantly, upon injury, the endothelium will increase its production of vasoconstrictors (such as Ang-II and Et-1), which result in activation of cytokines and growth factors and subsequent internalization of oxidized LDL within the vessel wall. Internalization of LDL within the vessel wall stimulates conversion of macrophages into foam cells, which form the initial lesion during atherogenesis. Secretion of cytokines, chemokines and growth factors at the arterial lesion result in an inflammatory response which promotes proliferation and migration of vascular smooth muscle cells from the media to the lesion within the vessel wall (198).

As the VSMCs are being activated and migrate towards the site of lesion formation, the formation of the atherosclerotic plaque continues, while the VSMCs form a fibrous cap over the lesion. At this stage, the lesion is considered an advanced plaque, which will continue to develop as a result of increase proliferation of VSMCs, macrophages and T-cells. As the plaque grows larger,

degradation of the fibrous cap is promoted by secretion of MMPs from the activated macrophages, resulting in instability of the plaque, and subsequent hemorrhage and rupture of the plaque (198) (Figure 1).

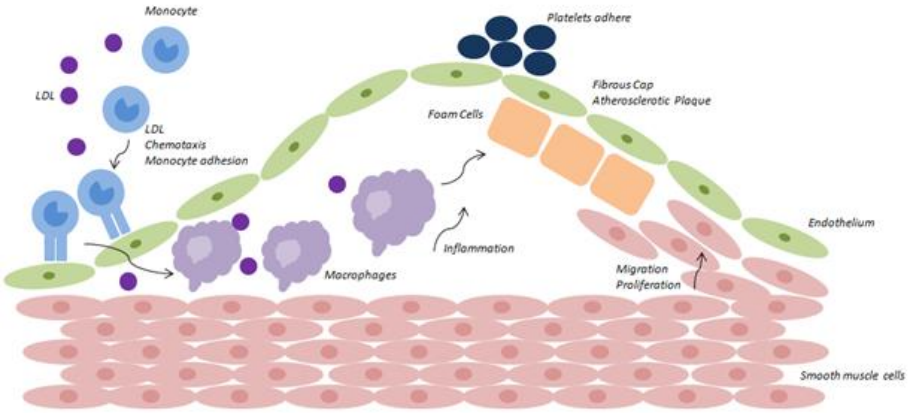

Figure 1 Summary of atherogenesis: macrophages are recruited to consume oxidezed LDL and become foam cells within the arterial wall. Platelets adhere to the dysfunctional endothelium to release growth factors, causing VSMCs to alter their phenotype and proliferate and migrate to the site of plaque formation.

Angiotensin II is a factor that plays a major role in not only formation of the plaque, but also its instability and rupture. Under oxidative stress conditions, Ang-II promotes vasoconstriction, inflammation and vessel remodelling. Vessel constriction is a result of angiotensin stimulating the release of endothelin-1 and noradrenaline, and also stimulates expression of monocyte chemoattractant protein 1 (MCP-1) and tumor necrosis factor (TNFα). Angiotensin also activates NADH, which in turn promotes vascular oxidative stress, and induces expression of various growth factors, including PDGF, bFGF and IFG-1, which contribute to the vascular inflammatory response (199).

Restenosis is the process of the arterial wall healing in response to mechanical injury. It is comprised of two stages: neointimal hyperplasia and vessel remodelling. Neointimal hyperplasia is a result of platelet aggregation and inflammatory cell infiltration as a result of release of cytokines and

growth factors which stimulate recruitment of activated VSMCs to the site of injury. The resulting neointima is comprised of synthetic VSMCs, extracellular matrix components and macrophages. As a result of vessel remodelling, production of extracellular matrix components increases, and VSMCs at the site of neointimal formation exhibit down-regulated expression of SM-MHC isoforms post-injury, but unaltered expression of SMα-actin. Interestingly, six months post arterial injury, expression of SM-MHC is recovered (200).

Role of VSMCs in atherosclerosis

The role of vascular smooth muscle cells (VSMCs) has been shown to be a key component of development, as they are the major source of extracellular matrix components of vessel walls (1). During development, VSMCs will both proliferate and differentiate to form components of the vasculature. In development, the proliferative phenotype of VSMCs (synthetic phenotype) refers to migration and proliferation of these cells to specific sites to form the vasculature. On the other hand, differentiated VSMCs (contractile phenotype) line vessel walls to regulate blood flow. The earliest VSMC differentiation marker is smooth muscle α-actin (SMαA), whose expression can be detected as early as smooth muscle precursors are recruited into the vessel wall. Following SMαA induction, other smooth muscle-marker genes are sequentially induced: SM22, calponin, SM-MHC I, and finally SM-MHC II (1).

Post-natally, VSMCs modulate their phenotype in response to various extracellular signals, and, unlike striated muscle, do not terminally differentiate. This phenotypic modulation and expression of either the synthetic or contractile phenotypes is not mutually exclusive. Differentiated VSMCs in mature vessels express matrix components and proliferate at low levels. However, following vascular injury, contractile VSMCs down-regulate muscle-specific differentiation genes and increase

proliferation, to contribute to the vascular regenerative response and promote vessel healing (2). This phenotypic modulation, from contractile to proliferative, is key to maintaining the integrity of the vascular system, but also plays an important role in many vascular diseases, such as atherosclerosis and restenosis following angioplasty (3).

VSMCs lie in the media of vessels, and are considered mature contractile cells that regulate the integrity of the vasculature, and also blood flow. In areas of turbulent flow, as well as arterial bifurcations, where differences in pressure exist, there is a higher chance of developing atherosclerotic lesions (201). Under shear stress or mechanical strain, VSMCs in these areas modify their phenotype as a result of atherogenic stimuli (fibronectin, collagen, PDGF and reactive oxygen species), to a synthetic one. VSMCs residing in the media will migrate into the developing lesion. The synthetic phenotype is characterized by increased DNA synthesis and expression of cell-cycle markers and a decreased expression of smooth muscle marker genes such as SM-MHC and SMα-actin. There is also a morphological change to the cells, whereby myofilaments are replaced with rough endoplasmic reticulum and golgi, as well as a change in the cell shape from a more elongated shape to a rounder one (3) (Figure 2).

Activated VSMCs contribute to plaque formation and size, not only by migrating to the site of injury, but also by affecting lipid uptake through LDL receptors, by contributing to inflammatory cytokine production and by altering the production of extracellular matrix components (201).

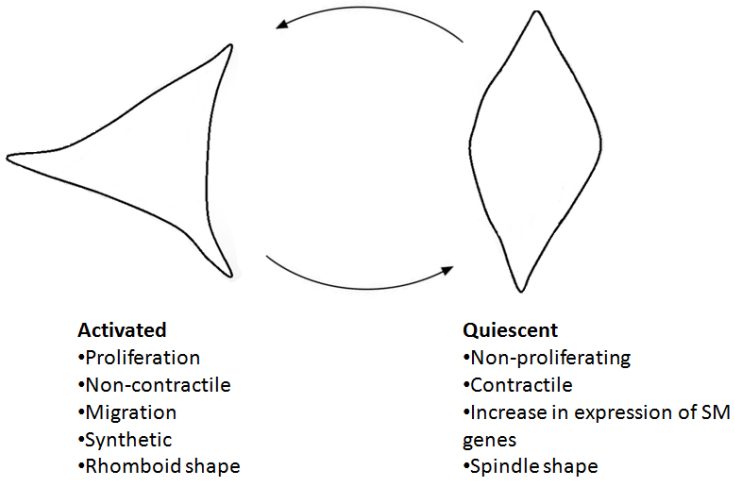

Figure 2 Schematic representation of the phenotypic switch of vascular smooth muscle cells between a quiescent/contractile and a proliferative/synthetic state (6).

Transcriptional Regulation of smooth muscle phenotype conversions

There are several transcription factors as well as transcriptional co-regulators that have been shown to regulate smooth muscle phenotype. GATA-6, a transcription factor of zinc finger motif DNA-binding domain proteins, has been shown to play a key role in regulating SMC-specific promoters. In quiescent (or contractile) smooth muscle cells, GATA-6 is expressed, however in response to injury, it is down-regulated (202). GATA-6 has been shown to be a key player in the induction of the differentiated smooth muscle phenotype; upregulation promotes withdrawal from the cell cycle. In a vascular injury model, phenotypic switch of smooth muscles into the de-differentiated or synthetic phenotype is associated with a downregulation of GATA6 (203).

The smooth muscle myosin heavy chain promoter has been shown to be regulated by GATA6 through specific regulatory elements (204). Apart from GATA6, there are various transcription factors that function in a combinatorial manner to regulate transcription of smooth muscle specific genes, and thus the quiescent, differentiated phenotype. It has been shown that GATA6, SRF and TAAT binding sites in the promoter regions of smooth muscle-specific genes act in concert to promote

transcription (205). For example, myocardin, a potent co-activator of SRF in both cardiac and smooth muscle cells functions through its interaction with SRF, which binds to *cis* elements termed CArG boxes, which are found in the promoters of muscle specific genes. Interestingly, CArG boxes are also found in the promoters of serum inducible genes, which regulate proliferation. Thus it is evident how smooth muscle cells can fluctuate between a proliferated and differentiated state, as a result of which co-activators are regulating SRF on the CArG boxes of various genes. Therefore, binding of myocardin to SRF on muscle-specific promoters induces transcriptional activation and a differentiated phenotype (89).

Fibrosis

A key factor in the majority of heart disease is the presence of fibrosis, an excess production of extracellular matrix proteins which alters the structure, shape of the heart. These changes to the heart, brought on by cardiovascular injury, have severe effects on ventricular contractility, valvular function and electrical conduction of the heart (206).

One of the key factors that has been implicated in fibrosis is TGFβ1. Although TGFβ1 is known to promote collagen production, little is known about the mechanism by which it induces fibrosis. Studies have shown that TGFβ1 inhibition can attenuate fibrosis in the heart. Interestingly, genetic studies on TGFβ1 gene polymorphism and dysregulation have been shown to be factors in having predisposition to heart disease. Therefore pharmacologic or targeted gene therapies are potentially important therapeutic approaches to treating fibrosis (207).

To understand fibrosis, it is important to understand the structure and composition of the heart. The heart is not an organ that is comprised solely of muscle cells. In fact, in the heart, the number of fibroblast cells actually outnumbers that of cardiac myocytes. It is primarily the fibroblasts

in the heart that give rise to the extracellular matrix and allows for fibrosis in the myocardium (207). The connective and elastic tissue in the heart is important for the maintenance of structure and architecture of various components of the heart. However, fibrotic tissue in excess will give rise to cardiac pathologies. Increased levels of collagen within the myocardium have an effect on ventricular elasticity (208). Stiffening of the ventricle will then have an effect on myocyte contraction and relaxation, resulting in aberrant ventricular filling and thus increased pressure (209). Presence of fibrotic tissues also has a detrimental effect on systolic function, due to increased collagen concentration in the myocardium. Although extracellular matrix proteins usually function as a repair mechanism, in fibrotic conditions where there is a reduction of muscle tissue, the outcome is poor ventricular contraction and reduced cardiac output. The fibrotic heart is not able to produce adequate pressures for systemic perfusion, as a result of increased collagen concentration and changes in ventricular geometry (210). Overall, the presence and upregulation of fibrotic proteins results in a change in ventricular size and shape, which negatively influences heart function.

Fibroblast to myofibroblast transition

Fibroblasts are spindle shaped cells that reside in the majority of tissues and organs of the body that are associated with extracellular matrix molecules. They are characterized by expression of vimentin and absence of expression of desmin and SMα-actin. Activated fibroblasts are associated with synthesis and secretion of ECM molecules such as collagens, proteoglycans and fibronectin. Fibroblast cells originate from the mesenchyme and portray a diverse phenotypic variability such as non-contractile fibroblast, protomyofibroblast and contractile myofibroblast. Myofibroblasts are distinguished from fibroblasts by their expression of SMα-actin in stress fibres and various ECM proteins. Although myofibroblasts express SMα-actin, they can be distinguished from actual smooth

muscle cells by their lack of desmin and smooth muscle myosin expression (Figure 3). The origin of myofibroblasts is uncertain. They may arise from transdifferentiation of fibroblasts and smooth muscle cells, however, whether the populations of myofibroblasts derived from fibroblasts or smooth muscle cells form distinct or similar populations is unknown. Whether or not fibroblasts can differentiate into smooth muscle cells or *vice versa* also remains unclear, however it is possible that fibroblasts can differentiate into myofibroblast-like cells, whose protein expression pattern resembles that of smooth muscle cells (211,212).

Recent studies have shown that following fibrosis or injury, the recruited fibroblasts or myofibroblasts may arise from different sources. Such sources could be de-differentiated epithelial cells by epithelial-mesenchymal transition (EMT), bone marrow derived mesenchymal stem cells or tissue derived mesenchymal stem cells (213).

Following tissue injury, such as myocardial infarction, the balance between collagen synthesis and degradation is regulated by myofibroblasts. The origin of these cells is mainly from cardiac fibroblasts and have the ability to respond to various mechanical, paracrine and autocrine factors. In response to mechanical stretch or pro-inflammatory cytokines, myofibroblasts increase synthesis and deposition of ECM proteins to replace necrotic myocardial tissue; this process results in scar formation (214). Myofibroblasts play a key role in the formation of stress fibres, expression of smooth muscle genes and collagen synthesis and deposition. Normal myofibroblast function involves stabilizing the infarcted area and promotes scar tussue formation and contraction. However, abnormal amounts of myofibroblasts as a result of persistent signal elevation can result in abnormal myocardial stiffness and impairment of ventricular function due to the excessive fibrotic deposition (215).

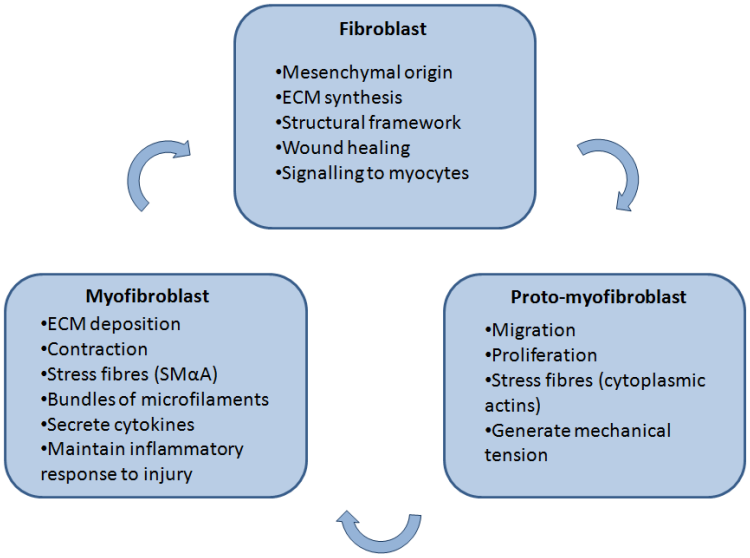

Figure 3 Schematic representation of the basic characteristics of fibroblast to protomyofibroblast to myofibroblast transition (216).

Molecular regulation of the SMA gene in phenotype conversion

In normal arteries, VSMCs regulate vascular tone, and are quiescent, expressing high levels of contractile, smooth muscle-specific genes. Upon arterial injury, VSMCs lose expression of the contractile genes and proliferate. This is termed phenotypic modulation (176). Fully differentiated smooth muscle cells upregulate genes encoding proteins that are involved in smooth muscle contraction, such as α-actin, myosin heavy chain, myosin light chain, caldesmon, vinculin, calponin, SM22 and metavinculin. However, many of these genes are also expressed in other cell types; for example, myosin light chain, caldesmon, vinculin and metavinculin smooth muscle isoforms are products of alternatively spliced genes that are expressed in a variety of cell types. The most abundant of the smooth muscle-specific genes is smooth muscle α-actin, which is exclusively expressed in smooth muscle, and smooth muscle-related cells in normal adults. Although it is expressed transiently in cardiac and skeletal muscle during development, and also in myofibroblasts

in tumors, wounds, and in proliferating smooth muscle cells in atherosclerotic lesions. Thus, because of its essential nature in VSMCs, the transcriptional regulation of the smooth muscle α-actin gene locus has become a paradigm for understanding the molecular regulation of differentiation and phenotypic conversions in smooth muscle cells (217).

The smooth muscle α-actin gene proximal promoter region contains several conserved regulatory elements that are essential in its regulation. One such element is the CArG box ($CC[A/T_6]GG$), which was first identified as the serum response element (SRE) in the promoter region of the immediate early gene *c-fos*. It was first identified as playing a role in inducing promoter activity in response to growth factor stimulation. The CArG box is a consensus binding site for the serum response factor (SRF), which binds to DNA as a homodimer to promote transcription of a variety of genes, including smooth muscle-specific genes. Although it is ubiquitously expressed, SRF is essential in the control of the smooth muscle α-actin promoter. Interestingly, it has been shown to regulate two opposing events: activation of muscle-specific genes to promote differentiation, and activation of immediate early genes to promote proliferation (98).

The key to SRF-dependent regulation of smooth muscle-specific gene expression was found to be through the co-factor myocardin, which has been shown to be essential for smooth muscle cell differentiation. Myocardin transactivates multiple smooth muscle genes in a CArG dependent manner, but interestingly fails to activate *c-fos,* in the same manner, indicating that its role in differentiation is dependent on SRF. Unlike SRF, Myocardin is not ubiquitously expressed, and its expression is restricted to cardiac and smooth muscle tissue, it is evident that it plays a key role in the regulation of smooth muscle phenotypic regulation. Furthermore, studies have shown that activation of smooth muscle specific genes through myocardin occurs as a result of the interaction of myocardin and SRF, and not due to direct binding of myocardin to the promoter region of these genes (98).

Furthermore, the transcription factor Myocyte Enhancer Factor 2 (MEF2), has been shown to play a critical role in the phenotypic modulation of smooth muscle cells, and like SRF, regulates both immediate early genes and smooth muscle marker genes. Calcium signalling has been implicated in the control of this phenotypic switching by controlling two distinct signalling pathways. It has been shown that induction of immediate-early genes occurs via de-repression of MEF2 from HDAC4 in a calcium/calmodulin-dependent manner (2), whereas the induction of smooth muscle specific genes occurs via a MEF2-dependent RhoA/ROCK dependent signalling pathway. MEF2 has been shown to be genetically upstream of myocardin, and recent studies have documented that the RhoA/ROCK pathway is functioning through MEF2 and myocardin to regulate calcium sensitivity in smooth muscle cells. This pathway involves the de-repression of MEF2 from PP1, the catalytic subunit of myosin light chain phosphatase which regulates contraction in smooth muscle cells, by the PP1 inhibitor CPI-17 (PKC-potentiated protein phosphatase inhibitor of 17kDa). Activation of the RhoA/ROCK pathway induces phosphorylation of CPI-17, which physically interacts with PP1 to relieve its repressive effects on MEF2, thus inducing expression of myocardin, resulting in its interaction with SRF and concomitant upregulation of smooth muscle marker genes, including smooth muscle α-actin (Figure 4) (171).

Recent studies have also implicated transforming growth factor β (TGFβ) in the phenotypic transition of smooth muscle cells. Little is known about the exact mechanism by which TGFβ functions to potently up-regulate smooth muscle specific genes, however studies have shown that in neural crest cells and fibroblasts, TGFβ induces smooth muscle α-actin, as well as other smooth-muscle specific genes, potentially through canonical Smad signalling, and the RhoA/ROCK pathway (123,218).

Therefore, it is important to identify the mechanisms regulating the fibroblast to myofibroblast transition so as to control aberrant activation and cardiac pathologies associated with excessive myofibroblast activity. Studies have demonstrated the hormone relaxin is produced in the

heart to stimulate mouse neonatal cardiomyocyte growth. Interestingly relaxin has been shown to inhibit TGFβ1-induced fibroblast to myofibroblast transition; this was indicated by a downregulation of smooth muscle α-actin and type I collagen expression. It was found that the Notch-1 signalling pathway is involved in this pathway, and inhibition of Notch-1 potentiated TGFβ1 induced myofibroblast differentiation and abrogated the inhibitory effects of relaxin. Thus Notch appears to also play an important role by downregulating TGFβ-dependent fibroblast to myofibroblast transitions, providing another potential therapeutic target (219,220).

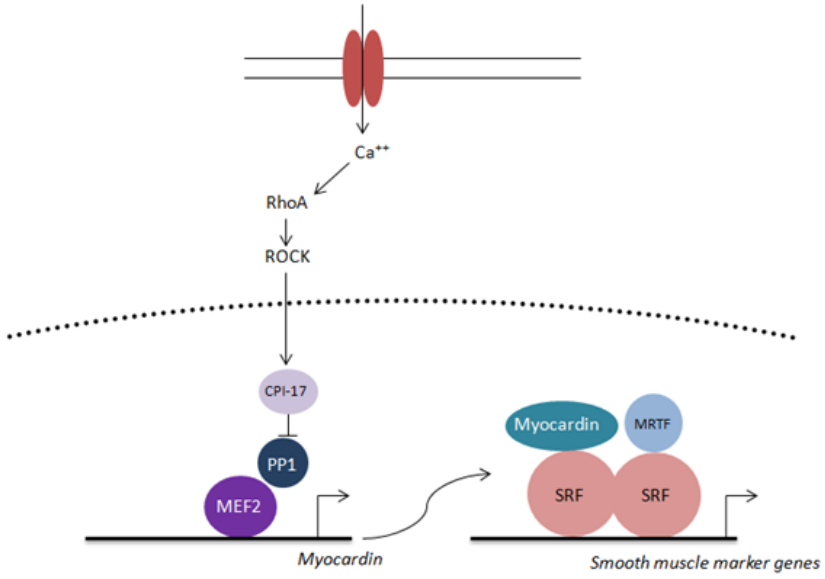

Figure 4 Summary of the regulation of myocardin/SRF smooth muscle marker gene expression through a RhoA/ROCK-MEF2-CPI-17-dependent mechanism (171).

Concluding remarks

Cardiovascular disease is one of the leading causes of death worldwide. A characteristic of patients presenting with hypertension and heart failure is cardiac fibrosis, due to constant activation of the tissue repair program and persistent activation of fibroblast migration to the site of injury.

Initially, this mechanism serves to synthesize new extracellular matrix, however prolonged activation results in excess scar tissue formation leading to fibrosis. Although the mechanisms underlying fibrosis are being characterized, there is still much to learn concerning the underlying molecular etiology of fibrosis in order to allow advances in therapeutic interventions. Basic studies have implicated a complex milieu of hormones and signalling pathways that contribute to the control of gene expression and ultimately the fibrotic phenotype. One important challenge for cardiovascular disease will be to develop novel therapeutic approaches aimed at these molecular pathways.

ADDITIONAL WORK II: MANUSCRIPT 3

AUTHOR CONTRIBUTIONS:

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

Joseph W. Gordon[1], Christina Pagiatakis[1], Jahan Salma[1], Min Du[1], John J. Andreucci[1], Jianzhong Zhao[1], Guangpei Hou[2], Robert L. Perry[1], Qinghong Dan[2], David Courtman[2], Michelle Bendeck[2], and John C. McDermott[1]

Published in *Journal of Biological Chemistry* (2009) 284(28): 19027-19042

Experimental design:

Dr. Joe Gordon

Drafting manuscript:

Dr. Joe Gordon and Dr. John C McDermott

Contribution:

Christina Pagiatakis: Figure 2E, Figure 3A, Figure 4B,C,D, Figure 5D, Figure 6B, C, Figure 8 B, C, D

MANUSCRIPT:

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

Joseph W. Gordon[1], Christina Pagiatakis[1], Jahan Salma[1], Min Du[1], John J. Andreucci[1], Jianzhong Zhao[1], Guangpei Hou[2], Robert L. Perry[1], Qinghong Dan[2], David Courtman[2], Michelle Bendeck[2], and John C. McDermott[1]

Running Head: MEF2/HDAC4 repress c-Jun in VSMC

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 show that forced expression of c-Jun represses myocardin's ability to activate smooth muscle gene expression, and this repression appears to be the result of physical interaction. 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.

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

Vascular smooth muscle cells (VSMCs), unlike their skeletal and cardiac counterparts, do not terminally differentiate, but can modulate their phenotype under conditions of growth or differentiation (1). 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 (1). 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 (1). 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 DNA sequence known as a CArG box, which can be found in multiple copies in many smooth muscle genes analyzed to date (2). 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 (2). This dual role for SRF is largely regulated by recruiting coactivators, such as myocardin (3), to activate smooth muscle genes, or ternary complex factors (TCFs), such as Elk-1, to activate immediate-early genes (4).

A second 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 (5), and the histidine-rich calcium binding protein (HRC), a sarcoplasmic reticulum protein expressed in skeletal, cardiac, and smooth muscle (6). In addition, gene-targeting studies have revealed that MEF2C is required for proper vascular patterning and vascular smooth muscle differentiaton (7). 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 (8).

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) (9)(10). 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* (11). 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 (12)(13)(14), and we have previously shown that the novel PKC isoforms, PKC δ and ε, can activate MEF2 proteins in HeLa and COS cells (15). 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 (16). 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 (17). One mechanism by which PKA has been shown to inhibit smooth muscle proliferation is to inhibit the expression of *c-jun* (18). 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 (19)(20)(21). 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 addtion, gain and loss of function manipulation of HDAC4 levels reveal its involvement in regulation of *c-jun* expression in VSMCs, making this is the 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.

Experimental Procedures

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 (15)(21)(22). 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 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. (5), 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 (22), 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 quiescense. 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 (23). Luciferase and β-galactosidase activityies were measured as described previously (24).

Immunoblot Analysis. Protein extractions were acheived using an NP-40 lysis buffer described previously (23). 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 (Sigama), 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'-GATCCACTGGTGCTTAACATTTGATTCAAGAGATCAAATGTTAAGCACCAGTTTTTTTGGAAA-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. Wire injury of mouse carotid arteries was described previously (25). Immunofluorescence and X-gal staining of mice harbouring three tandem MEF2 consensus DNA binding sites driving a LacZ reporter-gene which was described previously (26).

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 (NA) 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 entire AAA sections was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) followed by further purification with RNeasy mini RNA isolation system (Qiagen, Chatsworth, CA). 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 (8). 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 (26)(27). As shown in figure 1A, carotid injury elicited a widespread increase in c-Jun and MEF2A expression, consistent with previous reports (8). 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-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 (not shown). 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 (28)(29). 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). Lastly, ectopic expression of activated CaMKs and activated PKD 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, A10 cells within the confluent regions 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 C57BL/6 mouse that habours the MEF2-LacZ reporter gene (30)(31). Figure 3B shows HDAC4 staining is increased within the neointima of injured rat carotid arteries, where the HDAC4 immunofluorescence is much more diffuse than the nuclear stain. Lastly, previous evidence has suggested a causal link between the JNK-c-Jun pathway and the development of aneurysms (32). 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. 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 on an aneurysm (33). 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. 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 (not shown). 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 and 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 (21). 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 (18). 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, like Rp-cAMPS, and H89 (Figure S3); however, these pharmacological inhibitors were highly toxic in this cell line, similar to previously published work in A7r5 VSMC treated with the β-adrenergic receptor antagonist, propanolol (34). 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 (not shown).

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. In addition, figure 6A and 6B demonstrates 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-immunoprecipitation in COS7 cells (21). 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 (20)(35). 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 (35). 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. Athough 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 transcriptonal activity of myocardin (36). 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). 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 (37). 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 (38)(39). 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. 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 much greater immunoprecipitation of myocardin that a conrol rabbit IgG (not shown). To validate this interaction between c-Jun and myocardin, performed a mammalian two-hybrid assay in 10T1/2 cells using Gal4-c-Jun and myocardin-VP16 fusion proteins. As shown in figure 8E, myocardin-VP16 could activate 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 (40). 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) (41). Previous studies shown that MMP-9 is involved in both proliferative VSMC disease and degenerating disease, such as aneuysm (32)(42)(43). As shown in figure 9A, 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 (44)(45). Interestingly, c-Jun expression can be induced by cardiac wall stress and hypertrophy *in vitro* and *in vivo* (46)(47). 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 9B, the cardiac isoform of myocardin (myocardin 935) potently activated the promoters for alpha-cardiac actin and alpha-myosin heavy

chain. 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 (48). Interestingly, the PGC-1 promoter was induction 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 (17). 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 (16)(49). 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 (50). The role of MEF2 in vascular smooth muscle cells is less well characterized although a role for MEF2C in VSMC

differentiation and vascluar ontogeny has been invoked (7). 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 (38)(51).

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 (52)(53). In addition, we demonstrate that PKA can enhance the repression of *c-jun* by increasing the nuclear localizaion 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 (16). In addition, PKA has been implicated in promoting VSMC differentiation and increasing the expression of smooth muscle marker genes, such as SM-MHC (54). 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 (49). 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 (49). 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 (55). 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 (56). 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 phosphorlyate MEF2 proteins *in vivo* to inhibit skeletal muscle differentiaion (21). 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). 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

Salary support for RLP was in part provided by a postdoctoral fellowship from the Muscular Dystrophy Association of Canada (MDAC) and CIHR. Seneca College Office of Research and Innovation and Southlake Regional Health Centre are acknowledged for providing release-time support for JWG and JJA. This work was supported by a grant from the Canadian Institute of Health Research (CIHR) to JCM.

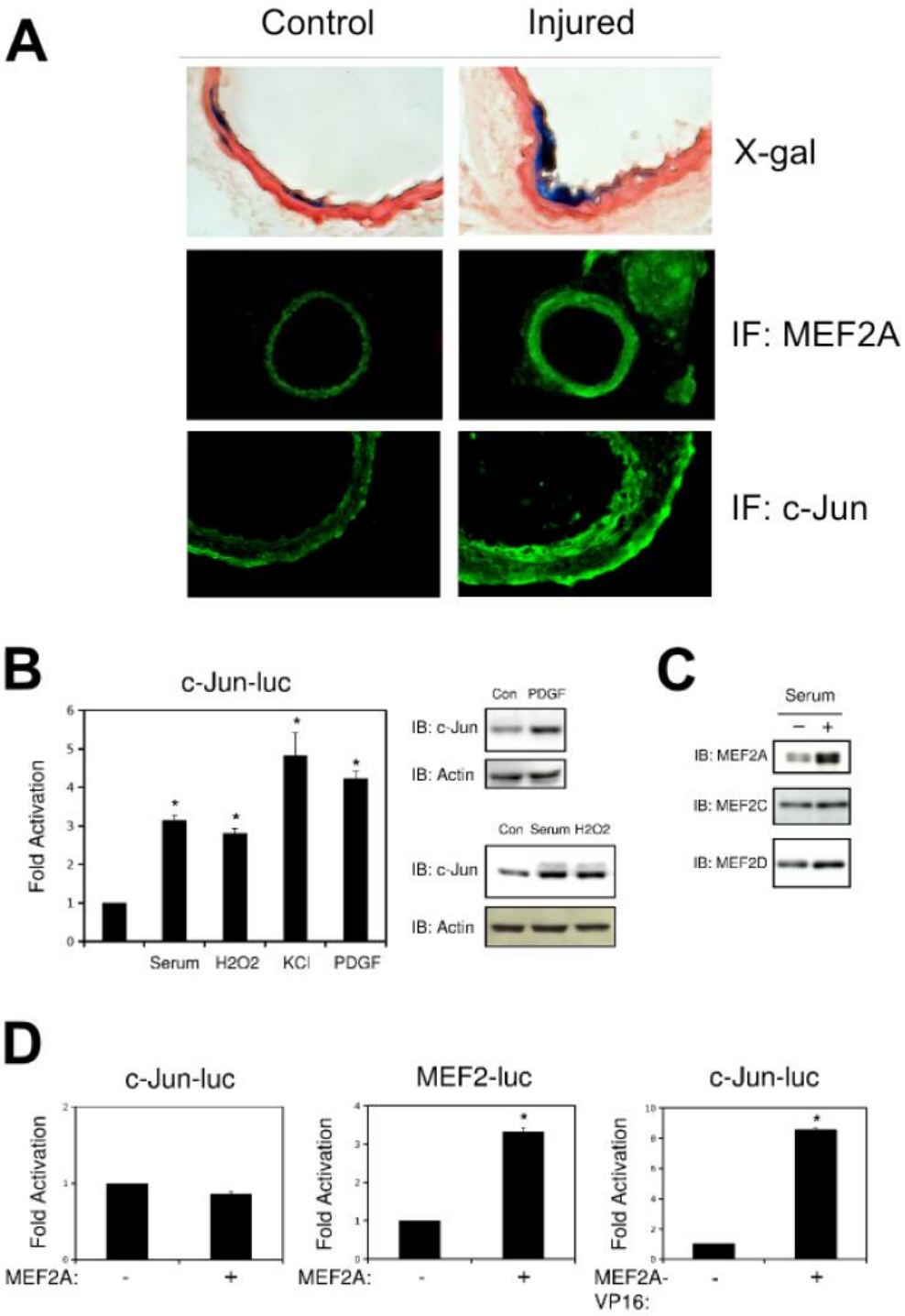

Figure 1: MEF2 activity and expression in VSMCs *in vivo* and *in vitro*. Common carotid arteries of MEF2-LacZ mice were injured by inserting a 2mm wire into the external carotid. Contralateral arteries were used as the control. A) X-gal staining and immunofluorescence for MEF2A 14 days following injury. 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 or 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.

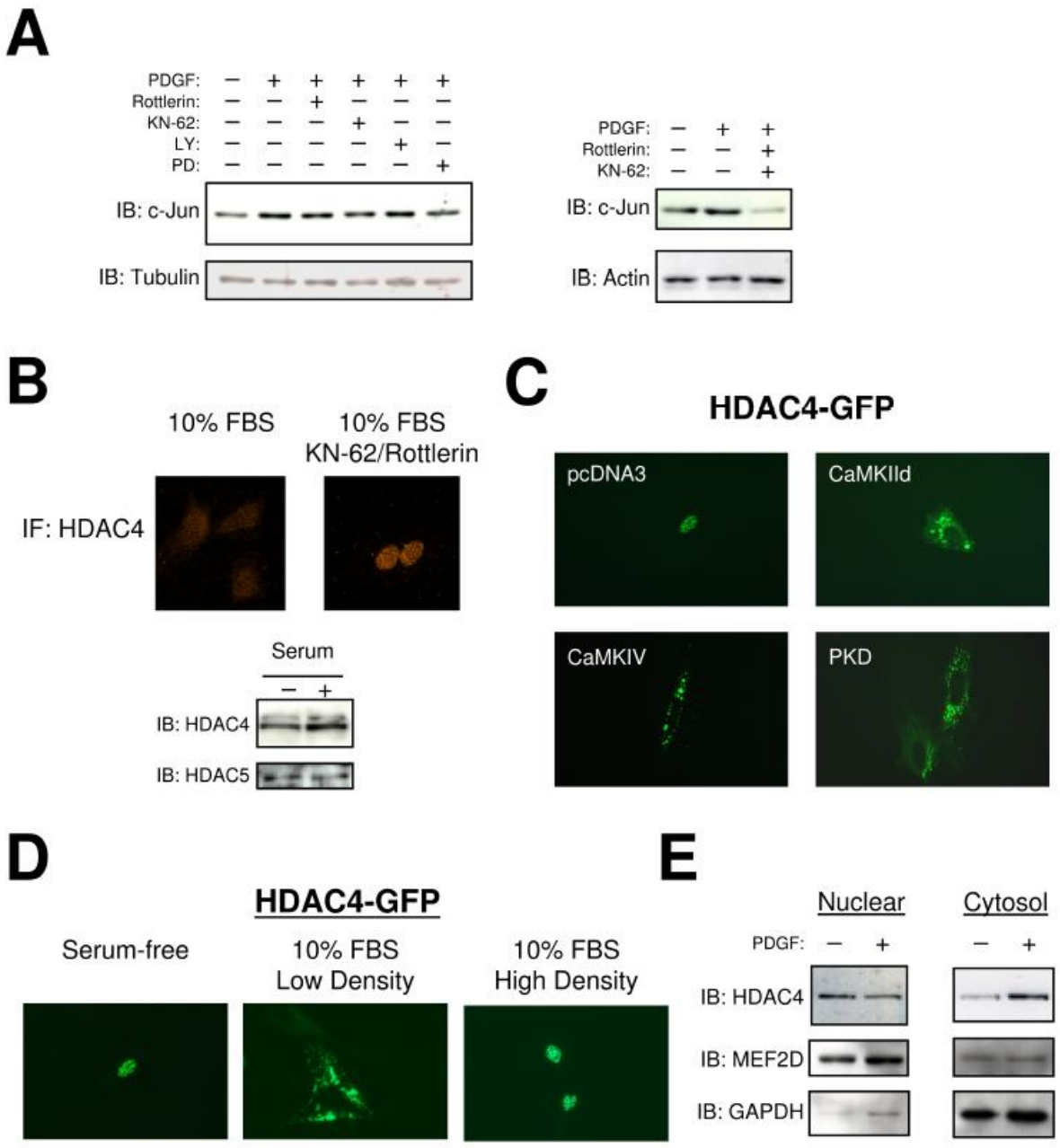

Figure 2: PDGF induction of c-Jun is mediated by CaMK, PKCδ, and MEK. A) Serum starved A10 cells were treated with PDGF (10 ng/mL) for 2 hours following 15 minute pretreatment with rottlerin (5 µM), KN-62 (5 µM), LY294002 (10 µM) or PD98059 (10 µM). Protein extracts were immunoblotted with a c-Jun antibody (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.

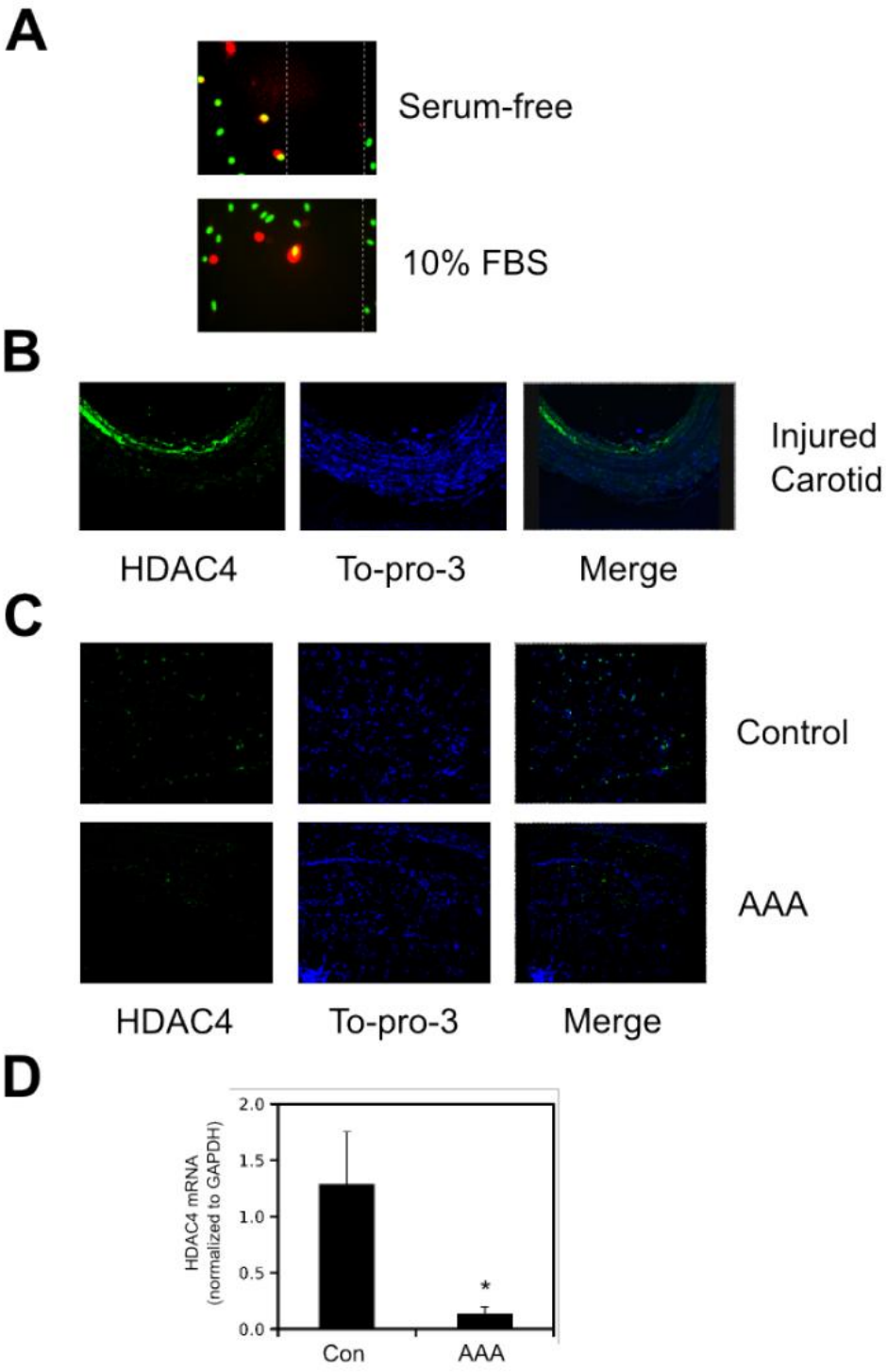

Figure 3: HDAC4 expression in models of vascular disease. *A*, A10 cells were grown to confluence and scraped with a standard 200-µl pipette tip. The cells were re-fed either serum-free medium or medium containing 10% FBS overnight and then fixed for immunofluorescence. *Red*, HDAC4; *green*, 4',6-diamidino-2-phenylindole (*i.e.* nuclear). *B*, Sprague-Dawley rats were subjected to balloon-injury of the carotid artery. Following 14 days of recovery, the arteries were fixed and harvested for immunofluorescence. *Green*, HDAC4; *blue*, To-pro-3 (*i.e.* nuclear). *C*, human aortic aneurysms or a nondiseased control specimen were harvested during elective surgical reconstruction and fixed for immunofluorescence. *Green*, HDAC4; *blue*, To-pro-3 (*i.e.* nuclear). *D*, human control (*Con*) and aortic aneurysm sections were immunostained for smooth muscle α-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).

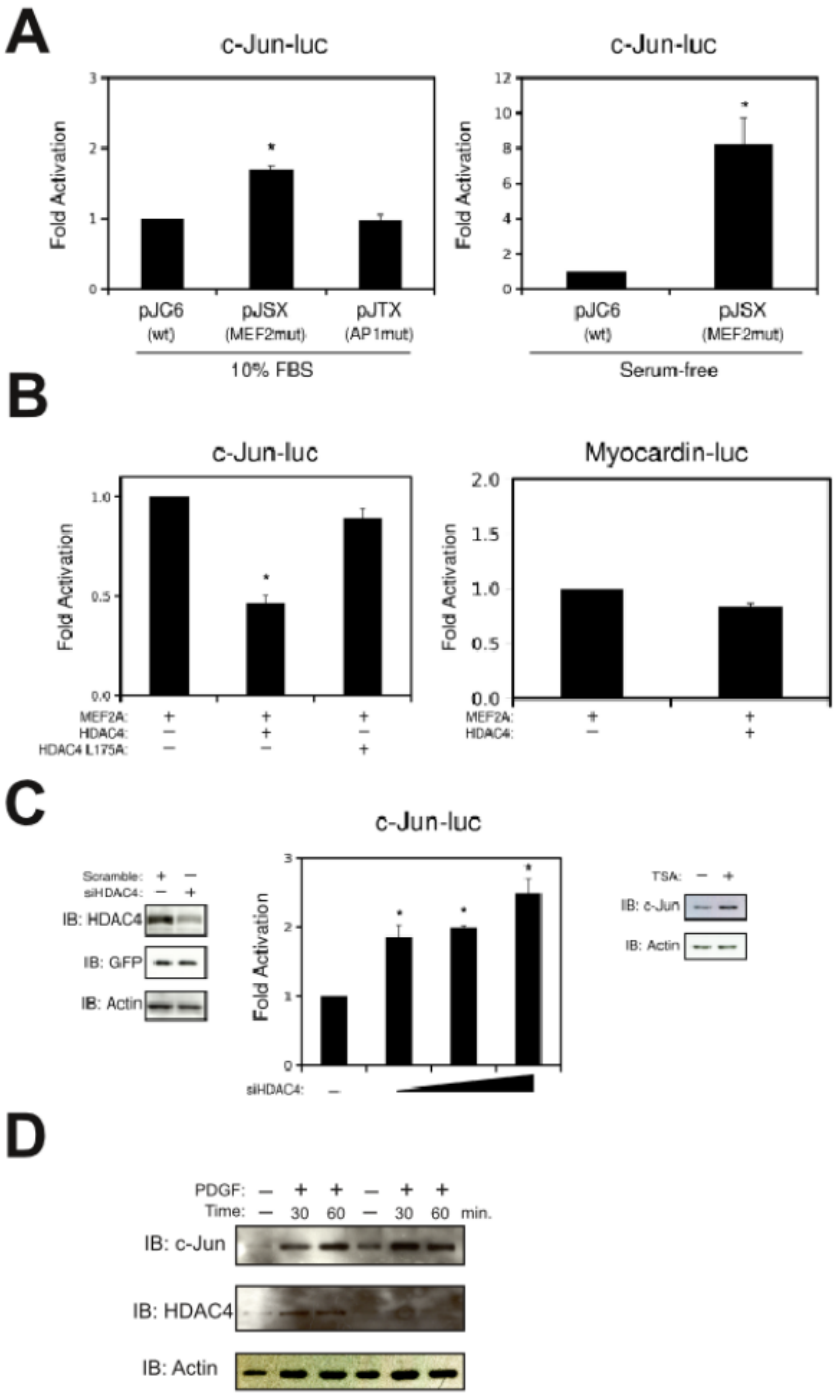

Figure 4: The MEF2 *cis* element in the *c-jun* promoter acts as a repressor element in quiescent VSMCs. A) A10 cells were transfected with a wild-type *c-jun* promoter (pJC6), a *c-jun* reporter with the MEF2 binding site mutated (pJSX), or a *c-jun* reporter gene with the AP1 site mutated (pJTX). Cells 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 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.

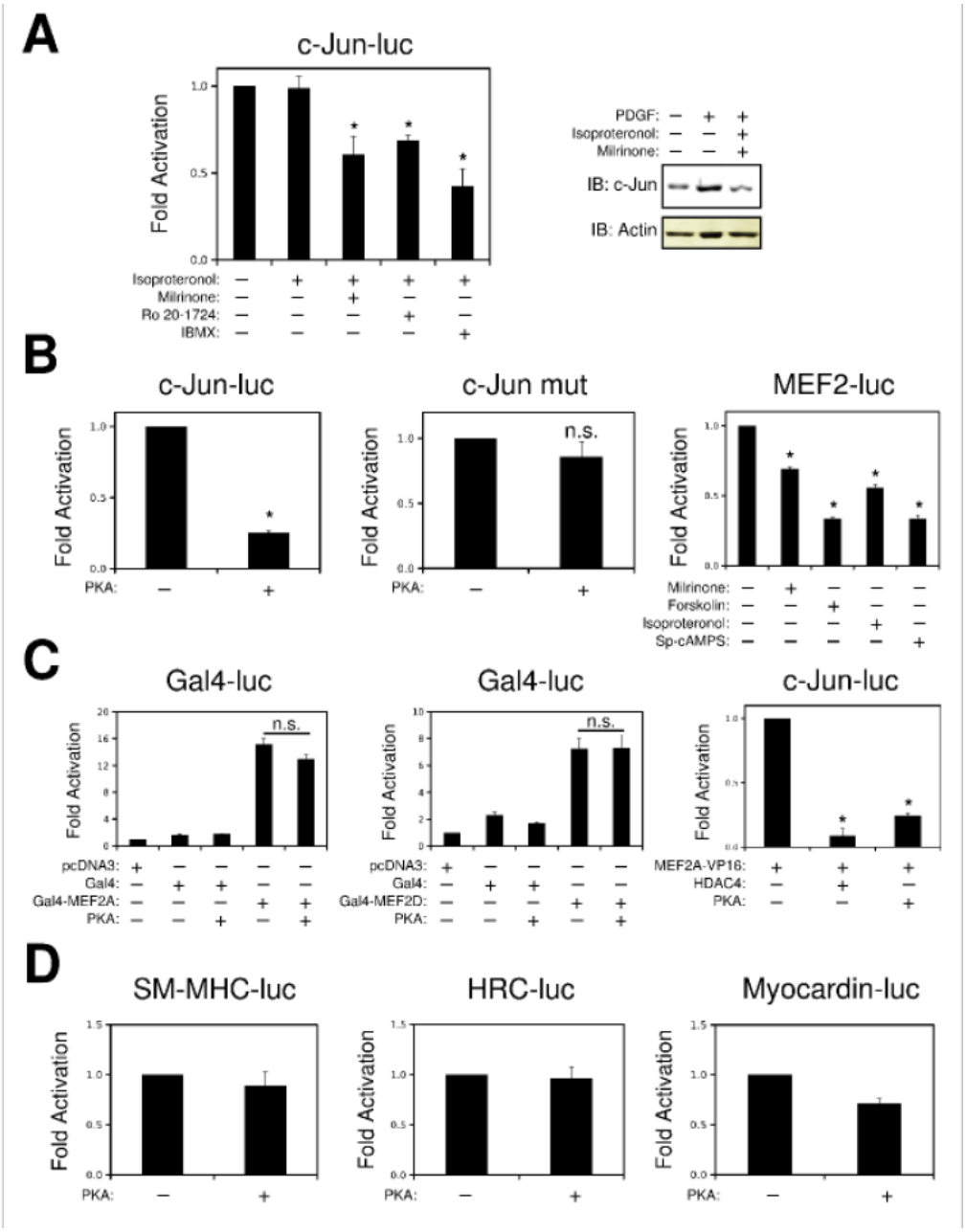

Figure 5: PKA inhibits induction of the *c-jun* promoter through a MEF2-dependent mechanism. A) A10 cells transfected with c-Jun-luc were treated with isoproteronol (1 µM), milrinone (10 µM), Ro 20-1724 (10 µM), or IBMX (500 µM) as indicated overnight. Serum-starved A10s were pre-incubated with milrinone (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 constuct 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. (n.s., not significant) 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.

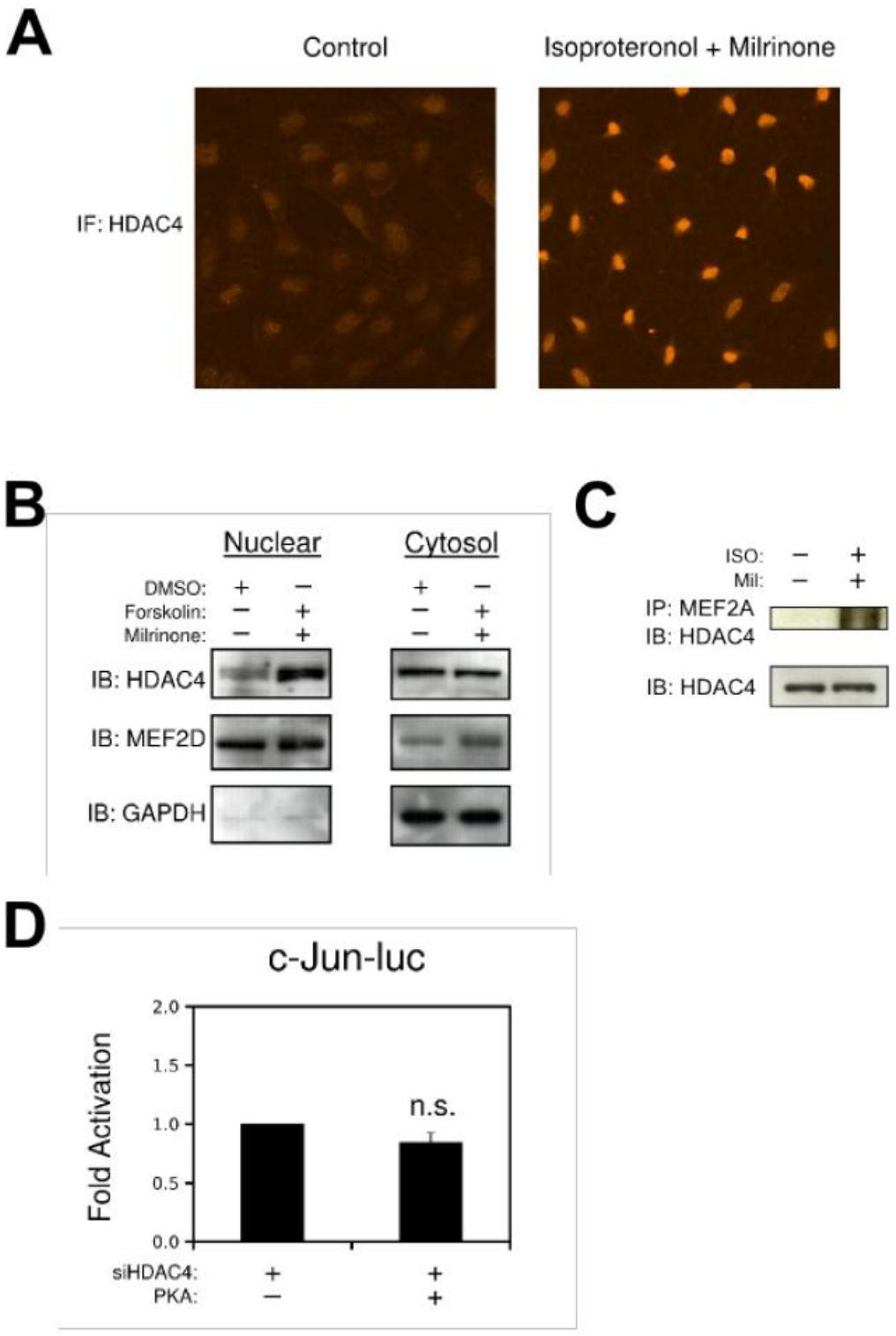

Figure 6: PKA inhibits *c-jun* expression through HDAC4. A) Growth phase VSMCs were treated with milrinone (10 µM, Sigma) and isoproteronol (1 µM) for 2 hours followed by fixation with 4% paraformaldahyde. Fixed cells were then subjected to immunofluorescence with an HDAC4 primary antibody (Sigma). B) 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) VSMCs were transfected with c-Jun-luc, siHDAC4, or PKA, as indicated. (n.s., not significant)

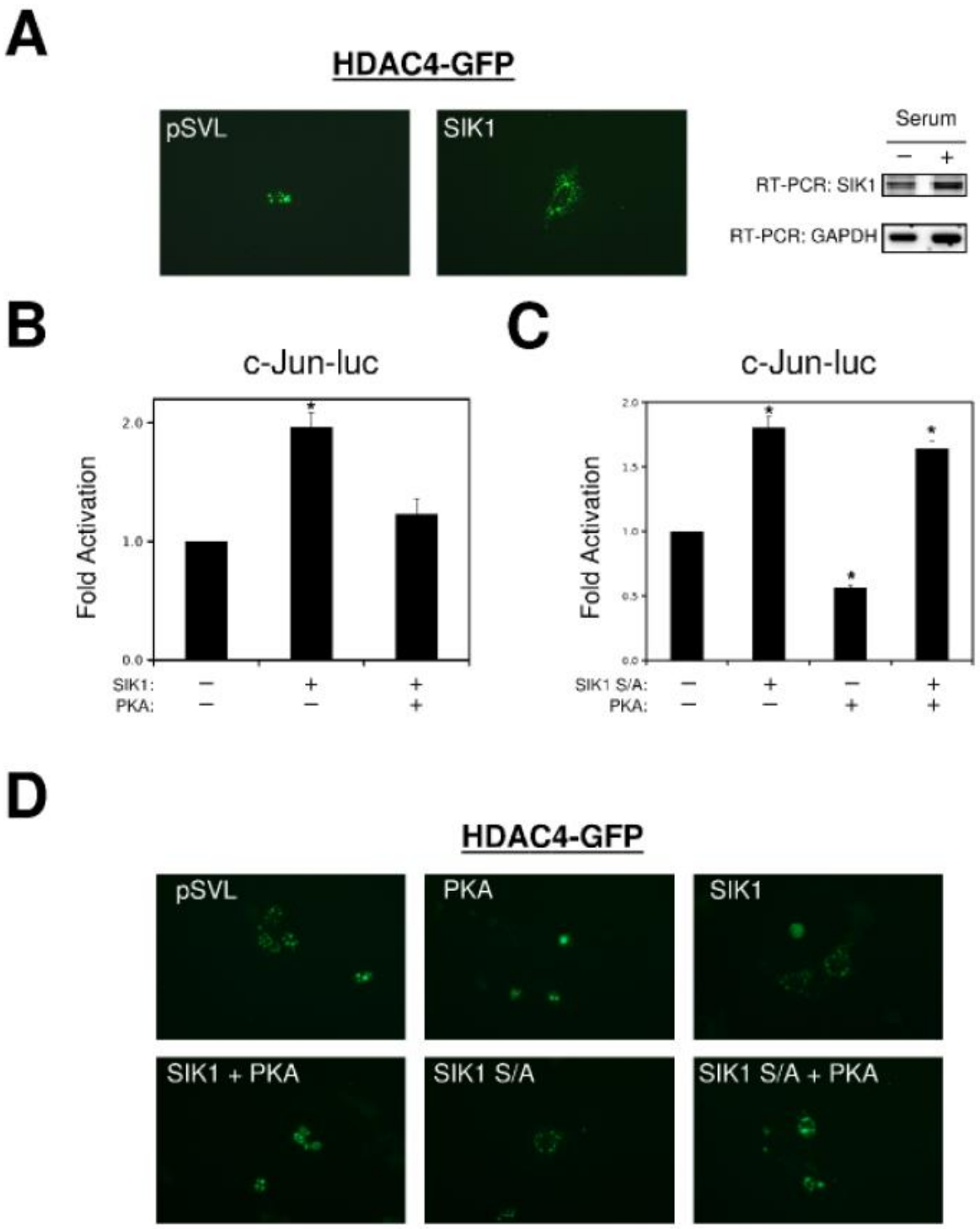

Figure 7: PKA inhibits HDAC4 nuclear export through SIK1. A) VSMCs were transfected with HDAC4-GFP and SIK1 or empty pSVL. Following serum starvation micrographs were obtained by standard fluorescent techniques. Growth phase A10s in 10% FBS (+) or serum-free media (-) were harvested for total RNA and 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.

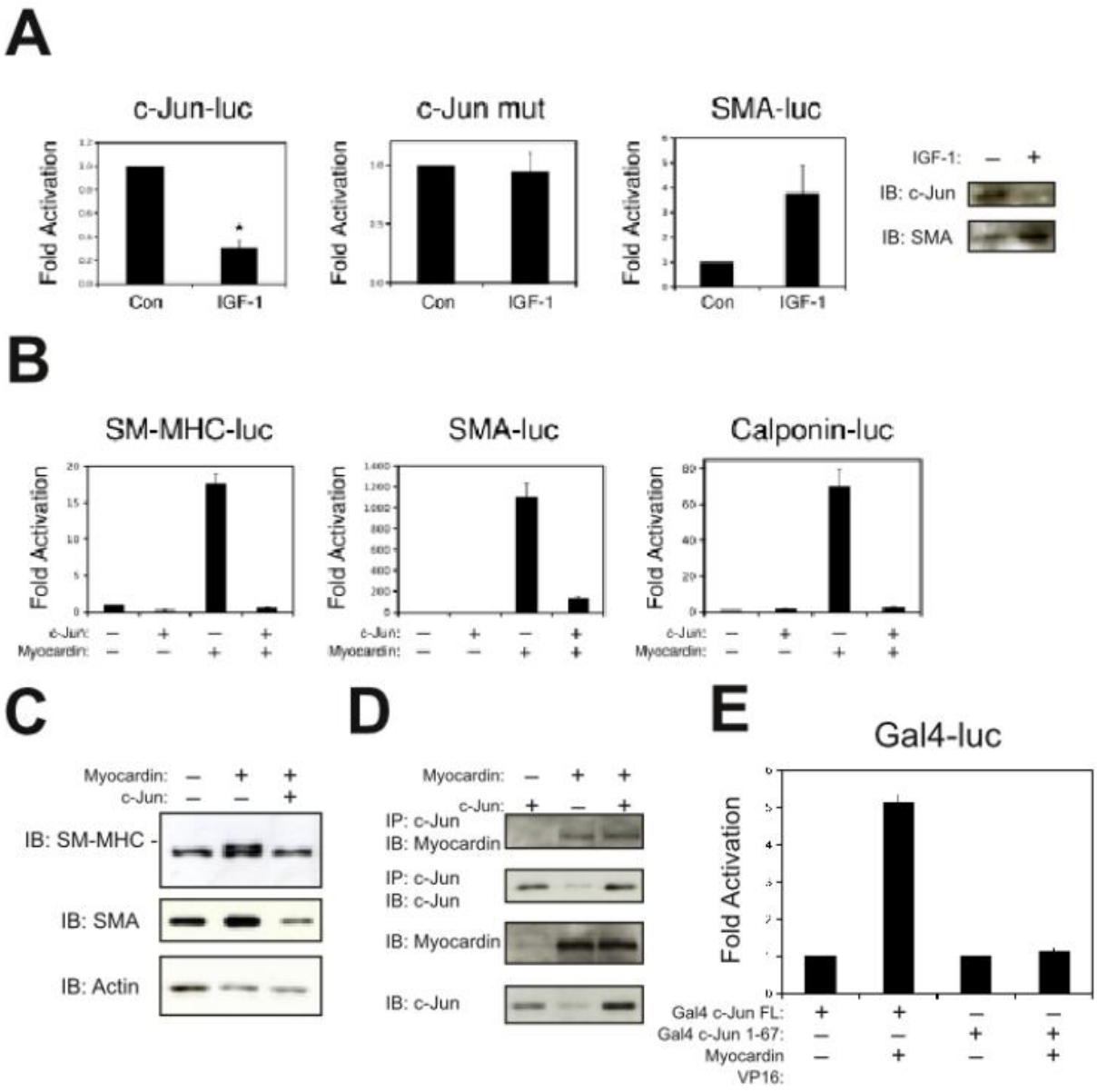

Figure 8: Down-regulation of c-Jun is critical for VSMC differentiation. *A*, VSMCs were transfected with wild-type c-Jun-luc, c-Jun-luc containing a mutation in the MEF2 binding site (*c-Jun mut*), or a smooth muscle α-actin reporter gene (*SMA-luc*). Quiescent cells were treated with 50 ng/ml of IGF-1 overnight and harvested for luciferase extracts. Protein extracts from overnight treated IGF-1 A10 cells were subjected to immunoblotting for c-Jun or SMA (Sigma). *B*, 10T1/2 fibroblasts were transfected with smooth myosin heavy chain (*SM-MHC-luc*), smooth muscle α-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. The cells were harvested for luciferase 24 h after recovery. *C*, 10T1/2 cells were transfected with myocardin 856 and c-Jun, as indicated. After a 24 h recovery, the 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. The extracts were subject to luciferase assay. *Con*, control.

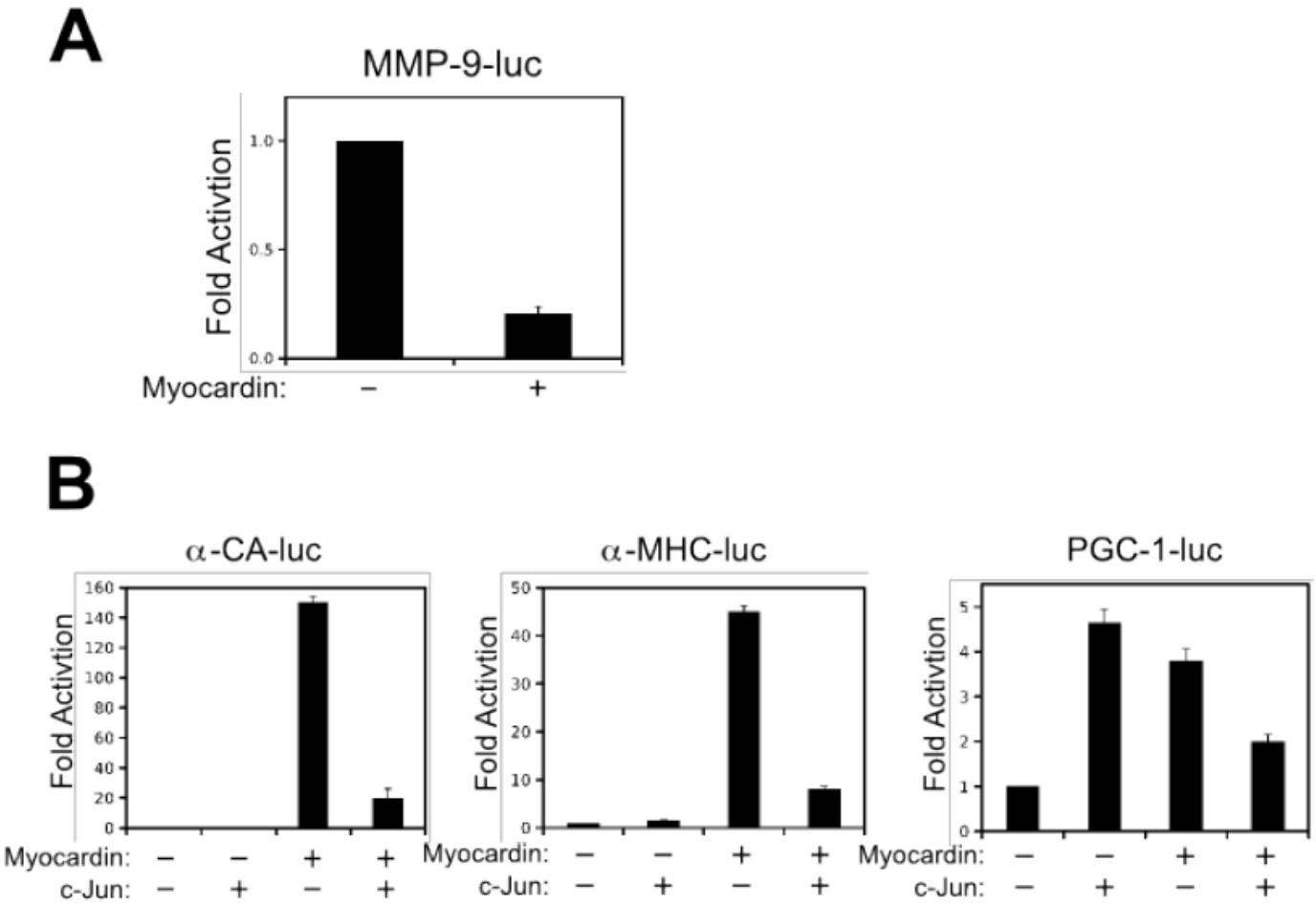

Figure 9: Downregulation of c-Jun is critical for VSMC differentiation. A) VSMCs were transfected with wild-type c-Jun-luc, c-Jun-luc containing a mutation in the MEF2 binding site (c-Jun mut), or a smooth muscle alpha-actin reporter gene (SMA-luc). Quiescent cells were treated with 50 ng/mL of IGF-1 overnight and havested for luciferase extracts. 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, as indicated. Cells were havested for luciferase 24 hours after recovery. C) 10T1/2 cells were transfected with the smooth muscle isoform of myocardin 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 SM-MHC (Biomedical Science).

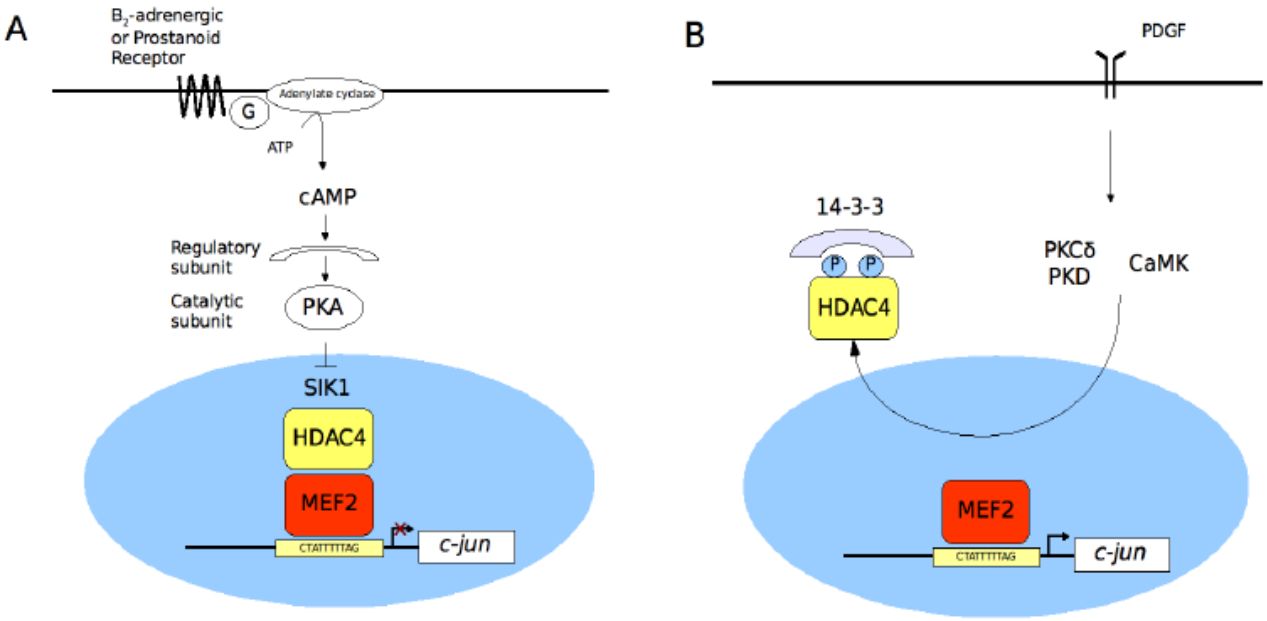

Figure 10: Model of *c-jun* regulation in VSMCs. In quiescent conditions, *c-jun* expression is repressed by a MEF2/HDAC4 complex, which is promoted by PKA-induced inhibition of SIK1. Growth factor (ie. PDGF) stimulation of VSMCs results in PKCδ/PKD- and CaMK-induced derepression through HDAC4 nuclear export, and MEK/ERK-dependent activation of *c-jun*.

REFERENCES

1. Moretti, A., Caron, L., Nakano, A., Lam, J. T., Bernshausen, A., Chen, Y., Qyang, Y., Bu, L., Sasaki, M., Martin-Puig, S., Sun, Y., Evans, S. M., Laugwitz, K. L., and Chien, K. R. (2006) Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. *Cell* **127**, 1151-1165
2. Gordon, J. W., Pagiatakis, C., Salma, J., Du, M., Andreucci, J. J., Zhao, J., Hou, G., Perry, R. L., Dan, Q., Courtman, D., Bendeck, M. P., and McDermott, J. C. (2009) Protein kinase A-regulated assembly of a MEF2{middle dot}HDAC4 repressor complex controls c-Jun expression in vascular smooth muscle cells. *The Journal of biological chemistry* **284**, 19027-19042
3. Owens, G. K., Kumar, M. S., and Wamhoff, B. R. (2004) Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiological reviews* **84**, 767-801
4. Aguilar, H. N., and Mitchell, B. F. (2010) Physiological pathways and molecular mechanisms regulating uterine contractility. *Human reproduction update* **16**, 725-744
5. Loirand, G., Guerin, P., and Pacaud, P. (2006) Rho kinases in cardiovascular physiology and pathophysiology. *Circulation research* **98**, 322-334
6. Davis-Dusenbery, B. N., Wu, C., and Hata, A. (2011) Micromanaging vascular smooth muscle cell differentiation and phenotypic modulation. *Arteriosclerosis, thrombosis, and vascular biology* **31**, 2370-2377
7. Kim, M. S., Fielitz, J., McAnally, J., Shelton, J. M., Lemon, D. D., McKinsey, T. A., Richardson, J. A., Bassel-Duby, R., and Olson, E. N. (2008) Protein kinase D1 stimulates MEF2 activity in skeletal muscle and enhances muscle performance. *Molecular and cellular biology* **28**, 3600-3609
8. Akkila, W. M., Chambers, R. L., Ornatsky, O. I., and McDermott, J. C. (1997) Molecular cloning of up-regulated cytoskeletal genes from regenerating skeletal muscle: potential role of myocyte enhancer factor 2 proteins in the activation of muscle-regeneration-associated genes. *The Biochemical journal* **325 (Pt 1)**, 87-93
9. Srivastava, D., and Olson, E. N. (2000) A genetic blueprint for cardiac development. *Nature* **407**, 221-226
10. Olson, E. N., and Schneider, M. D. (2003) Sizing up the heart: development redux in disease. *Genes & development* **17**, 1937-1956
11. Nguyen, H. T., Bodmer, R., Abmayr, S. M., McDermott, J. C., and Spoerel, N. A. (1994) D-mef2: a Drosophila mesoderm-specific MADS box-containing gene with a biphasic expression profile during embryogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 7520-7524
12. Olson, E. N. (2006) Gene regulatory networks in the evolution and development of the heart. *Science* **313**, 1922-1927
13. Molkentin, J. D., Lin, Q., Duncan, S. A., and Olson, E. N. (1997) Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes & development* **11**, 1061-1072

14. Srivastava, D., Cserjesi, P., and Olson, E. N. (1995) A subclass of bHLH proteins required for cardiac morphogenesis. *Science* **270**, 1995-1999
15. Morin, S., Pozzulo, G., Robitaille, L., Cross, J., and Nemer, M. (2005) MEF2-dependent recruitment of the HAND1 transcription factor results in synergistic activation of target promoters. *The Journal of biological chemistry* **280**, 32272-32278
16. Miano, J. M., Ramanan, N., Georger, M. A., de Mesy Bentley, K. L., Emerson, R. L., Balza, R. O., Jr., Xiao, Q., Weiler, H., Ginty, D. D., and Misra, R. P. (2004) Restricted inactivation of serum response factor to the cardiovascular system. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 17132-17137
17. Firulli, A. B., McFadden, D. G., Lin, Q., Srivastava, D., and Olson, E. N. (1998) Heart and extra-embryonic mesodermal defects in mouse embryos lacking the bHLH transcription factor Hand1. *Nature genetics* **18**, 266-270
18. Srivastava, D., Thomas, T., Lin, Q., Kirby, M. L., Brown, D., and Olson, E. N. (1997) Regulation of cardiac mesodermal and neural crest development by the bHLH transcription factor, dHAND. *Nature genetics* **16**, 154-160
19. Verzi, M. P., Agarwal, P., Brown, C., McCulley, D. J., Schwarz, J. J., and Black, B. L. (2007) The transcription factor MEF2C is required for craniofacial development. *Developmental cell* **12**, 645-652
20. Puigserver, P., Rhee, J., Lin, J., Wu, Z., Yoon, J. C., Zhang, C. Y., Krauss, S., Mootha, V. K., Lowell, B. B., and Spiegelman, B. M. (2001) Cytokine stimulation of energy expenditure through p38 MAP kinase activation of PPARgamma coactivator-1. *Molecular cell* **8**, 971-982
21. Mjaatvedt, C. H., Nakaoka, T., Moreno-Rodriguez, R., Norris, R. A., Kern, M. J., Eisenberg, C. A., Turner, D., and Markwald, R. R. (2001) The outflow tract of the heart is recruited from a novel heart-forming field. *Developmental biology* **238**, 97-109
22. Srivastava, D. (2006) Making or breaking the heart: from lineage determination to morphogenesis. *Cell* **126**, 1037-1048
23. Drake, C. J., Hungerford, J. E., and Little, C. D. (1998) Morphogenesis of the first blood vessels. *Annals of the New York Academy of Sciences* **857**, 155-179
24. Covello, K. L., and Simon, M. C. (2004) HIFs, hypoxia, and vascular development. *Current topics in developmental biology* **62**, 37-54
25. Gaengel, K., Genove, G., Armulik, A., and Betsholtz, C. (2009) Endothelial-mural cell signaling in vascular development and angiogenesis. *Arteriosclerosis, thrombosis, and vascular biology* **29**, 630-638
26. Kasler, H. G., Victoria, J., Duramad, O., and Winoto, A. (2000) ERK5 is a novel type of mitogen-activated protein kinase containing a transcriptional activation domain. *Molecular and cellular biology* **20**, 8382-8389
27. Garry, D. J., and Olson, E. N. (2006) A common progenitor at the heart of development. *Cell* **127**, 1101-1104
28. Kattman, S. J., Huber, T. L., and Keller, G. M. (2006) Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. *Developmental cell* **11**, 723-732

29. Lucitti, J. L., Jones, E. A., Huang, C., Chen, J., Fraser, S. E., and Dickinson, M. E. (2007) Vascular remodeling of the mouse yolk sac requires hemodynamic force. *Development* **134**, 3317-3326
30. O'Callaghan, C. J., and Williams, B. (2000) Mechanical strain-induced extracellular matrix production by human vascular smooth muscle cells: role of TGF-beta(1). *Hypertension* **36**, 319-324
31. Lin, Q., Schwarz, J., Bucana, C., and Olson, E. N. (1997) Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. *Science* **276**, 1404-1407
32. Huang, J., Cheng, L., Li, J., Chen, M., Zhou, D., Lu, M. M., Proweller, A., Epstein, J. A., and Parmacek, M. S. (2008) Myocardin regulates expression of contractile genes in smooth muscle cells and is required for closure of the ductus arteriosus in mice. *The Journal of clinical investigation* **118**, 515-525
33. Li, J., Zhu, X., Chen, M., Cheng, L., Zhou, D., Lu, M. M., Du, K., Epstein, J. A., and Parmacek, M. S. (2005) Myocardin-related transcription factor B is required in cardiac neural crest for smooth muscle differentiation and cardiovascular development. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 8916-8921
34. Knot, H. J., and Nelson, M. T. (1998) Regulation of arterial diameter and wall [Ca2+] in cerebral arteries of rat by membrane potential and intravascular pressure. *The Journal of physiology* **508 (Pt 1)**, 199-209
35. Somlyo, A. P., and Somlyo, A. V. (1994) Signal transduction and regulation in smooth muscle. *Nature* **372**, 231-236
36. Somlyo, A. P., and Somlyo, A. V. (2003) Ca2+ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiological reviews* **83**, 1325-1358
37. Cohen, P. T. (2002) Protein phosphatase 1--targeted in many directions. *Journal of cell science* **115**, 241-256
38. Wamhoff, B. R., Bowles, D. K., and Owens, G. K. (2006) Excitation-transcription coupling in arterial smooth muscle. *Circulation research* **98**, 868-878
39. Kumar, M. S., and Owens, G. K. (2003) Combinatorial control of smooth muscle-specific gene expression. *Arteriosclerosis, thrombosis, and vascular biology* **23**, 737-747
40. Liu, N., and Olson, E. N. (2006) Coactivator control of cardiovascular growth and remodeling. *Current opinion in cell biology* **18**, 715-722
41. Najwer, I., and Lilly, B. (2005) Ca2+/calmodulin-dependent protein kinase IV activates cysteine-rich protein 1 through adjacent CRE and CArG elements. *American journal of physiology. Cell physiology* **289**, C785-793
42. Stevenson, A. S., Cartin, L., Wellman, T. L., Dick, M. H., Nelson, M. T., and Lounsbury, K. M. (2001) Membrane depolarization mediates phosphorylation and nuclear translocation of CREB in vascular smooth muscle cells. *Experimental cell research* **263**, 118-130
43. Maier, L. S., and Bers, D. M. (2007) Role of Ca2+/calmodulin-dependent protein kinase (CaMK) in excitation-contraction coupling in the heart. *Cardiovascular research* **73**, 631-640

44. Lundberg, M. S., Curto, K. A., Bilato, C., Monticone, R. E., and Crow, M. T. (1998) Regulation of vascular smooth muscle migration by mitogen-activated protein kinase and calcium/calmodulin-dependent protein kinase II signaling pathways. *Journal of molecular and cellular cardiology* **30**, 2377-2389
45. Wamhoff, B. R., Bowles, D. K., McDonald, O. G., Sinha, S., Somlyo, A. P., Somlyo, A. V., and Owens, G. K. (2004) L-type voltage-gated Ca2+ channels modulate expression of smooth muscle differentiation marker genes via a rho kinase/myocardin/SRF-dependent mechanism. *Circulation research* **95**, 406-414
46. Deaton, R. A., Su, C., Valencia, T. G., and Grant, S. R. (2005) Transforming growth factor-beta1-induced expression of smooth muscle marker genes involves activation of PKN and p38 MAPK. *The Journal of biological chemistry* **280**, 31172-31181
47. Hill, C. S., Wynne, J., and Treisman, R. (1995) The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. *Cell* **81**, 1159-1170
48. Mack, C. P., Somlyo, A. V., Hautmann, M., Somlyo, A. P., and Owens, G. K. (2001) Smooth muscle differentiation marker gene expression is regulated by RhoA-mediated actin polymerization. *The Journal of biological chemistry* **276**, 341-347
49. Posern, G., Miralles, F., Guettler, S., and Treisman, R. (2004) Mutant actins that stabilise F-actin use distinct mechanisms to activate the SRF coactivator MAL. *The EMBO journal* **23**, 3973-3983
50. Newton, A. C. (1997) Regulation of protein kinase C. *Current opinion in cell biology* **9**, 161-167
51. Taubman, M. B., Berk, B. C., Izumo, S., Tsuda, T., Alexander, R. W., and Nadal-Ginard, B. (1989) Angiotensin II induces c-fos mRNA in aortic smooth muscle. Role of Ca2+ mobilization and protein kinase C activation. *The Journal of biological chemistry* **264**, 526-530
52. Soh, J. W., Lee, E. H., Prywes, R., and Weinstein, I. B. (1999) Novel roles of specific isoforms of protein kinase C in activation of the c-fos serum response element. *Molecular and cellular biology* **19**, 1313-1324
53. Iyer, D., Chang, D., Marx, J., Wei, L., Olson, E. N., Parmacek, M. S., Balasubramanyam, A., and Schwartz, R. J. (2006) Serum response factor MADS box serine-162 phosphorylation switches proliferation and myogenic gene programs. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 4516-4521
54. Eto, M., Senba, S., Morita, F., and Yazawa, M. (1997) Molecular cloning of a novel phosphorylation-dependent inhibitory protein of protein phosphatase-1 (CPI17) in smooth muscle: its specific localization in smooth muscle. *FEBS letters* **410**, 356-360
55. Dimopoulos, G. J., Semba, S., Kitazawa, K., Eto, M., and Kitazawa, T. (2007) Ca2+-dependent rapid Ca2+ sensitization of contraction in arterial smooth muscle. *Circulation research* **100**, 121-129
56. Kitazawa, T., Eto, M., Woodsome, T. P., and Khalequzzaman, M. (2003) Phosphorylation of the myosin phosphatase targeting subunit and CPI-17 during Ca2+ sensitization in rabbit smooth muscle. *The Journal of physiology* **546**, 879-889
57. Sakurada, S., Takuwa, N., Sugimoto, N., Wang, Y., Seto, M., Sasaki, Y., and Takuwa, Y. (2003) Ca2+-dependent activation of Rho and Rho kinase in membrane depolarization-

induced and receptor stimulation-induced vascular smooth muscle contraction. *Circulation research* **93**, 548-556

58. McKinsey, T. A., Zhang, C. L., and Olson, E. N. (2002) MEF2: a calcium-dependent regulator of cell division, differentiation and death. *Trends in biochemical sciences* **27**, 40-47
59. Li, L., Eto, M., Lee, M. R., Morita, F., Yazawa, M., and Kitazawa, T. (1998) Possible involvement of the novel CPI-17 protein in protein kinase C signal transduction of rabbit arterial smooth muscle. *The Journal of physiology* **508 (Pt 3)**, 871-881
60. Perry, R. L., Yang, C., Soora, N., Salma, J., Marback, M., Naghibi, L., Ilyas, H., Chan, J., Gordon, J. W., and McDermott, J. C. (2009) Direct interaction between myocyte enhancer factor 2 (MEF2) and protein phosphatase 1alpha represses MEF2-dependent gene expression. *Molecular and cellular biology* **29**, 3355-3366
61. Koyama, M., Ito, M., Feng, J., Seko, T., Shiraki, K., Takase, K., Hartshorne, D. J., and Nakano, T. (2000) Phosphorylation of CPI-17, an inhibitory phosphoprotein of smooth muscle myosin phosphatase, by Rho-kinase. *FEBS letters* **475**, 197-200
62. Sakai, H., Chiba, Y., Hirano, T., and Misawa, M. (2005) Possible involvement of CPI-17 in augmented bronchial smooth muscle contraction in antigen-induced airway hyper-responsive rats. *Molecular pharmacology* **68**, 145-151
63. Kim, J. I., Young, G. D., Jin, L., Somlyo, A. V., and Eto, M. (2009) Expression of CPI-17 in smooth muscle during embryonic development and in neointimal lesion formation. *Histochemistry and cell biology* **132**, 191-198
64. Hamaguchi, T., Ito, M., Feng, J., Seko, T., Koyama, M., Machida, H., Takase, K., Amano, M., Kaibuchi, K., Hartshorne, D. J., and Nakano, T. (2000) Phosphorylation of CPI-17, an inhibitor of myosin phosphatase, by protein kinase N. *Biochemical and biophysical research communications* **274**, 825-830
65. Yamawaki, K., Ito, M., Machida, H., Moriki, N., Okamoto, R., Isaka, N., Shimpo, H., Kohda, A., Okumura, K., Hartshorne, D. J., and Nakano, T. (2001) Identification of human CPI-17, an inhibitory phosphoprotein for myosin phosphatase. *Biochemical and biophysical research communications* **285**, 1040-1045
66. Eto, M. (2009) Regulation of cellular protein phosphatase-1 (PP1) by phosphorylation of the CPI-17 family, C-kinase-activated PP1 inhibitors. *The Journal of biological chemistry* **284**, 35273-35277
67. Hayashi, Y., Senba, S., Yazawa, M., Brautigan, D. L., and Eto, M. (2001) Defining the structural determinants and a potential mechanism for inhibition of myosin phosphatase by the protein kinase C-potentiated inhibitor protein of 17 kDa. *The Journal of biological chemistry* **276**, 39858-39863
68. Ohki, S., Eto, M., Kariya, E., Hayano, T., Hayashi, Y., Yazawa, M., Brautigan, D., and Kainosho, M. (2001) Solution NMR structure of the myosin phosphatase inhibitor protein CPI-17 shows phosphorylation-induced conformational changes responsible for activation. *Journal of molecular biology* **314**, 839-849
69. Eto, M., Kitazawa, T., Matsuzawa, F., Aikawa, S., Kirkbride, J. A., Isozumi, N., Nishimura, Y., Brautigan, D. L., and Ohki, S. Y. (2007) Phosphorylation-induced conformational switching of CPI-17 produces a potent myosin phosphatase inhibitor. *Structure* **15**, 1591-1602

70. Kim, J. I., Urban, M., Young, G. D., and Eto, M. (2012) Reciprocal regulation controlling the expression of CPI-17, a specific inhibitor protein for the myosin light chain phosphatase in vascular smooth muscle cells. *American journal of physiology. Cell physiology* **303**, C58-68
71. Eto, M., Kirkbride, J. A., Chugh, R., Karikari, N. K., and Kim, J. I. (2013) Nuclear localization of CPI-17, a protein phosphatase-1 inhibitor protein, affects histone H3 phosphorylation and corresponds to proliferation of cancer and smooth muscle cells. *Biochemical and biophysical research communications* **434**, 137-142
72. Dubois, T., Howell, S., Zemlickova, E., Learmonth, M., Cronshaw, A., and Aitken, A. (2003) Novel in vitro and in vivo phosphorylation sites on protein phosphatase 1 inhibitor CPI-17. *Biochemical and biophysical research communications* **302**, 186-192
73. Eto, M., Kitazawa, T., and Brautigan, D. L. (2004) Phosphoprotein inhibitor CPI-17 specificity depends on allosteric regulation of protein phosphatase-1 by regulatory subunits. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 8888-8893
74. Jin, H., Sperka, T., Herrlich, P., and Morrison, H. (2006) Tumorigenic transformation by CPI-17 through inhibition of a merlin phosphatase. *Nature* **442**, 576-579
75. Xie, Z., Su, W., Guo, Z., Pang, H., Post, S. R., and Gong, M. C. (2006) Up-regulation of CPI-17 phosphorylation in diabetic vasculature and high glucose cultured vascular smooth muscle cells. *Cardiovascular research* **69**, 491-501
76. Kim, K. M., Adyshev, D. M., Kasa, A., Zemskov, E. A., Kolosova, I. A., Csortos, C., and Verin, A. D. (2013) Putative protein partners for the human CPI-17 protein revealed by bacterial two-hybrid screening. *Microvascular research* **88**, 19-24
77. Potthoff, M. J., and Olson, E. N. (2007) MEF2: a central regulator of diverse developmental programs. *Development* **134**, 4131-4140
78. Yu, Y. T., Breitbart, R. E., Smoot, L. B., Lee, Y., Mahdavi, V., and Nadal-Ginard, B. (1992) Human myocyte-specific enhancer factor 2 comprises a group of tissue-restricted MADS box transcription factors. *Genes & development* **6**, 1783-1798
79. McDermott, J. C., Cardoso, M. C., Yu, Y. T., Andres, V., Leifer, D., Krainc, D., Lipton, S. A., and Nadal-Ginard, B. (1993) hMEF2C gene encodes skeletal muscle- and brain-specific transcription factors. *Molecular and cellular biology* **13**, 2564-2577
80. Martin, J. F., Schwarz, J. J., and Olson, E. N. (1993) Myocyte enhancer factor (MEF) 2C: a tissue-restricted member of the MEF-2 family of transcription factors. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 5282-5286
81. Breitbart, R. E., Liang, C. S., Smoot, L. B., Laheru, D. A., Mahdavi, V., and Nadal-Ginard, B. (1993) A fourth human MEF2 transcription factor, hMEF2D, is an early marker of the myogenic lineage. *Development* **118**, 1095-1106
82. Olson, E. N., Perry, M., and Schulz, R. A. (1995) Regulation of muscle differentiation by the MEF2 family of MADS box transcription factors. *Developmental biology* **172**, 2-14
83. Black, B. L., and Olson, E. N. (1998) Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. *Annual review of cell and developmental biology* **14**, 167-196
84. Han, T. H., and Prywes, R. (1995) Regulatory role of MEF2D in serum induction of the c-jun promoter. *Molecular and cellular biology* **15**, 2907-2915

85. Yu, Y. T. (1996) Distinct domains of myocyte enhancer binding factor-2A determining nuclear localization and cell type-specific transcriptional activity. *The Journal of biological chemistry* **271**, 24675-24683
86. Zhao, M., New, L., Kravchenko, V. V., Kato, Y., Gram, H., di Padova, F., Olson, E. N., Ulevitch, R. J., and Han, J. (1999) Regulation of the MEF2 family of transcription factors by p38. *Molecular and cellular biology* **19**, 21-30
87. Grozinger, C. M., and Schreiber, S. L. (2000) Regulation of histone deacetylase 4 and 5 and transcriptional activity by 14-3-3-dependent cellular localization. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 7835-7840
88. Lin, Q., Lu, J., Yanagisawa, H., Webb, R., Lyons, G. E., Richardson, J. A., and Olson, E. N. (1998) Requirement of the MADS-box transcription factor MEF2C for vascular development. *Development* **125**, 4565-4574
89. Pipes, G. C., Creemers, E. E., and Olson, E. N. (2006) The myocardin family of transcriptional coactivators: versatile regulators of cell growth, migration, and myogenesis. *Genes & development* **20**, 1545-1556
90. Messenguy, F., and Dubois, E. (2003) Role of MADS box proteins and their cofactors in combinatorial control of gene expression and cell development. *Gene* **316**, 1-21
91. Miano, J. M. (2003) Serum response factor: toggling between disparate programs of gene expression. *Journal of molecular and cellular cardiology* **35**, 577-593
92. Pellegrini, L., Tan, S., and Richmond, T. J. (1995) Structure of serum response factor core bound to DNA. *Nature* **376**, 490-498
93. Misra, R. P., Rivera, V. M., Wang, J. M., Fan, P. D., and Greenberg, M. E. (1991) The serum response factor is extensively modified by phosphorylation following its synthesis in serum-stimulated fibroblasts. *Molecular and cellular biology* **11**, 4545-4554
94. Treisman, R. (1985) Transient accumulation of c-fos RNA following serum stimulation requires a conserved 5' element and c-fos 3' sequences. *Cell* **42**, 889-902
95. Prywes, R., and Roeder, R. G. (1986) Inducible binding of a factor to the c-fos enhancer. *Cell* **47**, 777-784
96. Treisman, R. (1986) Identification of a protein-binding site that mediates transcriptional response of the c-fos gene to serum factors. *Cell* **46**, 567-574
97. Minty, A., and Kedes, L. (1986) Upstream regions of the human cardiac actin gene that modulate its transcription in muscle cells: presence of an evolutionarily conserved repeated motif. *Molecular and cellular biology* **6**, 2125-2136
98. Hendrix, J. A., Wamhoff, B. R., McDonald, O. G., Sinha, S., Yoshida, T., and Owens, G. K. (2005) 5' CArG degeneracy in smooth muscle alpha-actin is required for injury-induced gene suppression in vivo. *The Journal of clinical investigation* **115**, 418-427
99. Miano, J. M., Long, X., and Fujiwara, K. (2007) Serum response factor: master regulator of the actin cytoskeleton and contractile apparatus. *American journal of physiology. Cell physiology* **292**, C70-81
100. Treisman, R. (1987) Identification and purification of a polypeptide that binds to the c-fos serum response element. *The EMBO journal* **6**, 2711-2717
101. Arsenian, S., Weinhold, B., Oelgeschlager, M., Ruther, U., and Nordheim, A. (1998) Serum response factor is essential for mesoderm formation during mouse embryogenesis. *The EMBO journal* **17**, 6289-6299

102. Kaplan-Albuquerque, N., Van Putten, V., Weiser-Evans, M. C., and Nemenoff, R. A. (2005) Depletion of serum response factor by RNA interference mimics the mitogenic effects of platelet derived growth factor-BB in vascular smooth muscle cells. *Circulation research* **97**, 427-433
103. Croissant, J. D., Kim, J. H., Eichele, G., Goering, L., Lough, J., Prywes, R., and Schwartz, R. J. (1996) Avian serum response factor expression restricted primarily to muscle cell lineages is required for alpha-actin gene transcription. *Developmental biology* **177**, 250-264
104. Hautmann, M. B., Madsen, C. S., and Owens, G. K. (1997) A transforming growth factor beta (TGFbeta) control element drives TGFbeta-induced stimulation of smooth muscle alpha-actin gene expression in concert with two CArG elements. *The Journal of biological chemistry* **272**, 10948-10956
105. Hautmann, M. B., Madsen, C. S., Mack, C. P., and Owens, G. K. (1998) Substitution of the degenerate smooth muscle (SM) alpha-actin CC(A/T-rich)6GG elements with c-fos serum response elements results in increased basal expression but relaxed SM cell specificity and reduced angiotensin II inducibility. *The Journal of biological chemistry* **273**, 8398-8406
106. Sotiropoulos, A., Gineitis, D., Copeland, J., and Treisman, R. (1999) Signal-regulated activation of serum response factor is mediated by changes in actin dynamics. *Cell* **98**, 159-169
107. Camoretti-Mercado, B., Liu, H. W., Halayko, A. J., Forsythe, S. M., Kyle, J. W., Li, B., Fu, Y., McConville, J., Kogut, P., Vieira, J. E., Patel, N. M., Hershenson, M. B., Fuchs, E., Sinha, S., Miano, J. M., Parmacek, M. S., Burkhardt, J. K., and Solway, J. (2000) Physiological control of smooth muscle-specific gene expression through regulated nuclear translocation of serum response factor. *The Journal of biological chemistry* **275**, 30387-30393
108. Marais, R., Wynne, J., and Treisman, R. (1993) The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell* **73**, 381-393
109. Wang, D. Z., and Olson, E. N. (2004) Control of smooth muscle development by the myocardin family of transcriptional coactivators. *Current opinion in genetics & development* **14**, 558-566
110. Creemers, E. E., Sutherland, L. B., Oh, J., Barbosa, A. C., and Olson, E. N. (2006) Coactivation of MEF2 by the SAP domain proteins myocardin and MASTR. *Molecular cell* **23**, 83-96
111. Santalucia, T., Moreno, H., Palacin, M., Yacoub, M. H., Brand, N. J., and Zorzano, A. (2001) A novel functional co-operation between MyoD, MEF2 and TRalpha1 is sufficient for the induction of GLUT4 gene transcription. *Journal of molecular biology* **314**, 195-204
112. Naya, F. J., Black, B. L., Wu, H., Bassel-Duby, R., Richardson, J. A., Hill, J. A., and Olson, E. N. (2002) Mitochondrial deficiency and cardiac sudden death in mice lacking the MEF2A transcription factor. *Nature medicine* **8**, 1303-1309
113. Montessuit, C., Rosenblatt-Velin, N., Papageorgiou, I., Campos, L., Pellieux, C., Palma, T., and Lerch, R. (2004) Regulation of glucose transporter expression in cardiac myocytes: p38 MAPK is a strong inducer of GLUT4. *Cardiovascular research* **64**, 94-104

114. Morin, S., Charron, F., Robitaille, L., and Nemer, M. (2000) GATA-dependent recruitment of MEF2 proteins to target promoters. *The EMBO journal* **19**, 2046-2055
115. Kayahara, M., Wang, X., and Tournier, C. (2005) Selective regulation of c-jun gene expression by mitogen-activated protein kinases via the 12-o-tetradecanoylphorbol-13-acetate- responsive element and myocyte enhancer factor 2 binding sites. *Molecular and cellular biology* **25**, 3784-3792
116. Handschin, C., Rhee, J., Lin, J., Tarr, P. T., and Spiegelman, B. M. (2003) An autoregulatory loop controls peroxisome proliferator-activated receptor gamma coactivator 1alpha expression in muscle. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 7111-7116
117. Moustakas, A., and Heldin, C. H. (2009) The regulation of TGFbeta signal transduction. *Development* **136**, 3699-3714
118. Kardassis, D., Murphy, C., Fotsis, T., Moustakas, A., and Stournaras, C. (2009) Control of transforming growth factor beta signal transduction by small GTPases. *The FEBS journal* **276**, 2947-2965
119. Ross, S., and Hill, C. S. (2008) How the Smads regulate transcription. *The international journal of biochemistry & cell biology* **40**, 383-408
120. Varelas, X., Sakuma, R., Samavarchi-Tehrani, P., Peerani, R., Rao, B. M., Dembowy, J., Yaffe, M. B., Zandstra, P. W., and Wrana, J. L. (2008) TAZ controls Smad nucleocytoplasmic shuttling and regulates human embryonic stem-cell self-renewal. *Nature cell biology* **10**, 837-848
121. Beyer, T. A., Narimatsu, M., Weiss, A., David, L., and Wrana, J. L. (2013) The TGFbeta superfamily in stem cell biology and early mammalian embryonic development. *Biochimica et biophysica acta* **1830**, 2268-2279
122. Liu, X., Sun, Y., Weinberg, R. A., and Lodish, H. F. (2001) Ski/Sno and TGF-beta signaling. *Cytokine & growth factor reviews* **12**, 1-8
123. Chen, S., Crawford, M., Day, R. M., Briones, V. R., Leader, J. E., Jose, P. A., and Lechleider, R. J. (2006) RhoA modulates Smad signaling during transforming growth factor-beta-induced smooth muscle differentiation. *The Journal of biological chemistry* **281**, 1765-1770
124. Chen, S., Kulik, M., and Lechleider, R. J. (2003) Smad proteins regulate transcriptional induction of the SM22alpha gene by TGF-beta. *Nucleic acids research* **31**, 1302-1310
125. Chen, S., and Lechleider, R. J. (2004) Transforming growth factor-beta-induced differentiation of smooth muscle from a neural crest stem cell line. *Circulation research* **94**, 1195-1202
126. Quinn, Z. A., Yang, C. C., Wrana, J. L., and McDermott, J. C. (2001) Smad proteins function as co-modulators for MEF2 transcriptional regulatory proteins. *Nucleic acids research* **29**, 732-742
127. Qiu, P., Feng, X. H., and Li, L. (2003) Interaction of Smad3 and SRF-associated complex mediates TGF-beta1 signals to regulate SM22 transcription during myofibroblast differentiation. *Journal of molecular and cellular cardiology* **35**, 1407-1420
128. Camoretti-Mercado, B., Fernandes, D. J., Dewundara, S., Churchill, J., Ma, L., Kogut, P. C., McConville, J. F., Parmacek, M. S., and Solway, J. (2006) Inhibition of transforming

growth factor beta-enhanced serum response factor-dependent transcription by SMAD7. *The Journal of biological chemistry* **281**, 20383-20392

129. Hong, J. H., Hwang, E. S., McManus, M. T., Amsterdam, A., Tian, Y., Kalmukova, R., Mueller, E., Benjamin, T., Spiegelman, B. M., Sharp, P. A., Hopkins, N., and Yaffe, M. B. (2005) TAZ, a transcriptional modulator of mesenchymal stem cell differentiation. *Science* **309**, 1074-1078
130. Mahoney, W. M., Jr., Hong, J. H., Yaffe, M. B., and Farrance, I. K. (2005) The transcriptional co-activator TAZ interacts differentially with transcriptional enhancer factor-1 (TEF-1) family members. *The Biochemical journal* **388**, 217-225
131. Hong, J. H., and Yaffe, M. B. (2006) TAZ: a beta-catenin-like molecule that regulates mesenchymal stem cell differentiation. *Cell cycle* **5**, 176-179
132. Zhang, H., Liu, C. Y., Zha, Z. Y., Zhao, B., Yao, J., Zhao, S., Xiong, Y., Lei, Q. Y., and Guan, K. L. (2009) TEAD transcription factors mediate the function of TAZ in cell growth and epithelial-mesenchymal transition. *The Journal of biological chemistry* **284**, 13355-13362
133. Jeong, H., Bae, S., An, S. Y., Byun, M. R., Hwang, J. H., Yaffe, M. B., Hong, J. H., and Hwang, E. S. (2010) TAZ as a novel enhancer of MyoD-mediated myogenic differentiation. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **24**, 3310-3320
134. Speight, P., Nakano, H., Kelley, T. J., Hinz, B., and Kapus, A. (2013) Differential topical susceptibility to TGFbeta in intact and injured regions of the epithelium: key role in myofibroblast transition. *Molecular biology of the cell* **24**, 3326-3336
135. Creemers, E. E., Sutherland, L. B., McAnally, J., Richardson, J. A., and Olson, E. N. (2006) Myocardin is a direct transcriptional target of Mef2, Tead and Foxo proteins during cardiovascular development. *Development* **133**, 4245-4256
136. Drake, C. J., Hungerford, J. E., and Little, C. D. (1998) Morphogenesis of the first blood vessels. *Annals of the New York Academy of Sciences* **857**, 155-179
137. Owens, G. K. (1995) Regulation of differentiation of vascular smooth muscle cells. *Physiological reviews* **75**, 487-517
138. Owens, G. K., Kumar, M. S., and Wamhoff, B. R. (2004) Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiological reviews* **84**, 767-801
139. Miano, J. M. (2003) Serum response factor: toggling between disparate programs of gene expression. *Journal of molecular and cellular cardiology* **35**, 577-593
140. Creemers, E. E., Sutherland, L. B., McAnally, J., Richardson, J. A., and Olson, E. N. (2006) Myocardin is a direct transcriptional target of Mef2, Tead and Foxo proteins during cardiovascular development. *Development (Cambridge, England)* **133**, 4245-4256
141. Gordon, J. W., Pagiatakis, C., Salma, J., Du, M., Andreucci, J. J., Zhao, J., Hou, G., Perry, R. L., Dan, Q., Courtman, D., Bendeck, M. P., and McDermott, J. C. (2009) Protein Kinase A-regulated Assembly of a MEF2{middle dot}HDAC4 Repressor Complex Controls c-Jun Expression in Vascular Smooth Muscle Cells. *The Journal of Biological Chemistry* **284**, 19027-19042
142. Wang, D.-Z., and Olson, E. N. (2004) Control of smooth muscle development by the myocardin family of transcriptional coactivators. *Current opinion in genetics & development* **14**, 558-566

143. Wang, Z., Wang, D.-Z., Hockemeyer, D., McAnally, J., Nordheim, A., and Olson, E. N. (2004) Myocardin and ternary complex factors compete for SRF to control smooth muscle gene expression. *Nature* **428**, 185-189
144. Wamhoff, B. R., Bowles, D. K., McDonald, O. G., Sinha, S., Somlyo, A. P., Somlyo, A. V., and Owens, G. K. (2004) L-type voltage-gated Ca2+ channels modulate expression of smooth muscle differentiation marker genes via a rho kinase/myocardin/SRF-dependent mechanism. *Circulation research* **95**, 406-414
145. Lin, Q., Lu, J., Yanagisawa, H., Webb, R., Lyons, G. E., Richardson, J. A., and Olson, E. N. (1998) Requirement of the MADS-box transcription factor MEF2C for vascular development. *Development (Cambridge, England)* **125**, 4565-4574
146. Ren, J., Albinsson, S., and Hellstrand, P. (2010) Distinct effects of voltage- and store-dependent calcium influx on stretch-induced differentiation and growth in vascular smooth muscle. *J Biol Chem* **285**, 31829-31839
147. Martin, K., Weiss, S., Metharom, P., Schmeckpeper, J., Hynes, B., O'Sullivan, J., and Caplice, N. (2009) Thrombin stimulates smooth muscle cell differentiation from peripheral blood mononuclear cells via protease-activated receptor-1, RhoA, and myocardin. *Circ Res* **105**, 214-218
148. Perry, R. L. S., Yang, C., Soora, N., Salma, J., Marback, M., Naghibi, L., Ilyas, H., Chan, J., Gordon, J. W., and McDermott, J. C. (2009) Direct interaction between myocyte enhancer factor 2 (MEF2) and protein phosphatase 1alpha represses MEF2-dependent gene expression. *Molecular and Cellular Biology* **29**, 3355-3366
149. Somlyo, A. P., and Somlyo, A. V. (2003) Ca2+ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiological reviews* **83**, 1325-1358
150. Deaton, R. A., Su, C., Valencia, T. G., and Grant, S. R. (2005) Transforming growth factor-beta1-induced expression of smooth muscle marker genes involves activation of PKN and p38 MAPK. *The Journal of biological chemistry* **280**, 31172-31181
151. Han, J., Jiang, Y., Li, Z., Kravchenko, V. V., and Ulevitch, R. J. (1997) Activation of the transcription factor MEF2C by the MAP kinase p38 in inflammation. *Nature* **386**, 296-299
152. Ornatsky, O. I., Cox, D. M., Tangirala, P., Andreucci, J. J., Quinn, Z. A., Wrana, J. L., Prywes, R., Yu, Y. T., and McDermott, J. C. (1999) Post-translational control of the MEF2A transcriptional regulatory protein. *Nucleic acids research* **27**, 2646-2654
153. Zhao, M., New, L., Kravchenko, V. V., Kato, Y., Gram, H., di Padova, F., Olson, E. N., Ulevitch, R. J., and Han, J. (1999) Regulation of the MEF2 family of transcription factors by p38. *Molecular and cellular biology* **19**, 21-30
154. Hou, G., Vogel, W., and Bendeck, M. P. (2001) The discoidin domain receptor tyrosine kinase DDR1 in arterial wound repair. *The Journal of Clinical Investigation* **107**, 727-735
155. Gong, M. C., Iizuka, K., Nixon, G., Browne, J. P., Hall, A., Eccleston, J. F., Sugai, M., Kobayashi, S., Somlyo, A. V., and Somlyo, A. P. (1996) Role of guanine nucleotide-binding proteins--ras-family or trimeric proteins or both--in Ca2+ sensitization of smooth muscle. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 1340-1345

156. Cohen, P. T. W. (2002) Protein phosphatase 1--targeted in many directions. *Journal of cell science* **115**, 241-256
157. Morin, S., Pozzulo, G., Robitaille, L., Cross, J., and Nemer, M. (2005) MEF2-dependent recruitment of the HAND1 transcription factor results in synergistic activation of target promoters. *The Journal of biological chemistry* **280**, 32272-32278
158. Verzi, M. P., Agarwal, P., Brown, C., McCulley, D. J., Schwarz, J. J., and Black, B. L. (2007) The transcription factor MEF2C is required for craniofacial development. *Developmental Cell* **12**, 645-652
159. Yamagishi, H., Olson, E. N., and Srivastava, D. (2000) The basic helix-loop-helix transcription factor, dHAND, is required for vascular development. *The Journal of clinical investigation* **105**, 261-270
160. Huang, J., Cheng, L., Li, J., Chen, M., Zhou, D., Lu, M. M., Proweller, A., Epstein, J. A., and Parmacek, M. S. (2008) Myocardin regulates expression of contractile genes in smooth muscle cells and is required for closure of the ductus arteriosus in mice. *The Journal of clinical investigation* **118**, 515-525
161. Chu, S., and Ferro, T. J. (2006) Identification of a hydrogen peroxide-induced PP1-JNK1-Sp1 signaling pathway for gene regulation. *American Journal of Physiology. Lung Cellular and Molecular Physiology* **291**, L983-992-L983-992
162. Eto, M., Kitazawa, T., and Brautigan, D. L. (2004) Phosphoprotein inhibitor CPI-17 specificity depends on allosteric regulation of protein phosphatase-1 by regulatory subunits. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 8888-8893
163. Hamaguchi, T., Ito, M., Feng, J., Seko, T., Koyama, M., Machida, H., Takase, K., Amano, M., Kaibuchi, K., Hartshorne, D. J., and Nakano, T. (2000) Phosphorylation of CPI-17, an inhibitor of myosin phosphatase, by protein kinase N. *Biochemical and biophysical research communications* **274**, 825-830
164. Koyama, M., Ito, M., Feng, J., Seko, T., Shiraki, K., Takase, K., Hartshorne, D. J., and Nakano, T. (2000) Phosphorylation of CPI-17, an inhibitory phosphoprotein of smooth muscle myosin phosphatase, by Rho-kinase. *FEBS letters* **475**, 197-200
165. Eto, M., Ohmori, T., Suzuki, M., Furuya, K., and Morita, F. (1995) A novel protein phosphatase-1 inhibitory protein potentiated by protein kinase C. Isolation from porcine aorta media and characterization. *Journal of Biochemistry* **118**, 1104-1107
166. Eto, M., Kitazawa, T., Matsuzawa, F., Aikawa, S.-I., Kirkbride, J. A., Isozumi, N., Nishimura, Y., Brautigan, D. L., and Ohki, S.-Y. (2007) Phosphorylation-induced conformational switching of CPI-17 produces a potent myosin phosphatase inhibitor. *Structure (London, England: 1993)* **15**, 1591-1602
167. Kim, J., Young, G., Jin, L., Somlyo, A., and Eto, M. (2009) Expression of CPI-17 in smooth muscle during embryonic development and in neointimal lesion formation. *Histochemistry and Cell Biology*
168. Du, K. L., Ip, H. S., Li, J., Chen, M., Dandre, F., Yu, W., Lu, M. M., Owens, G. K., and Parmacek, M. S. (2003) Myocardin is a critical serum response factor cofactor in the transcriptional program regulating smooth muscle cell differentiation. *Molecular and cellular biology* **23**, 2425-2437

169. Wang, D., Chang, P. S., Wang, Z., Sutherland, L., Richardson, J. A., Small, E., Krieg, P. A., and Olson, E. N. (2001) Activation of cardiac gene expression by myocardin, a transcriptional cofactor for serum response factor. *Cell* **105**, 851-862
170. Long, X., Creemers, E. E., Wang, D.-Z., Olson, E. N., and Miano, J. M. (2007) Myocardin is a bifunctional switch for smooth versus skeletal muscle differentiation. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 16570-16575
171. Pagiatakis, C., Gordon, J. W., Ehyai, S., and McDermott, J. C. (2012) A novel RhoA/ROCK-CPI-17-MEF2C signaling pathway regulates vascular smooth muscle cell gene expression. *The Journal of biological chemistry* **287**, 8361-8370
172. Manabe, I., and Owens, G. K. (2001) CArG elements control smooth muscle subtype-specific expression of smooth muscle myosin in vivo. *The Journal of clinical investigation* **107**, 823-834
173. Parlakian, A., Tuil, D., Hamard, G., Tavernier, G., Hentzen, D., Concordet, J. P., Paulin, D., Li, Z., and Daegelen, D. (2004) Targeted inactivation of serum response factor in the developing heart results in myocardial defects and embryonic lethality. *Molecular and cellular biology* **24**, 5281-5289
174. Xu, S., Fu, J., Chen, J., Xiao, P., Lan, T., Le, K., Cheng, F., He, L., Shen, X., Huang, H., and Liu, P. (2009) Development of an optimized protocol for primary culture of smooth muscle cells from rat thoracic aortas. *Cytotechnology* **61**, 65-72
175. Dionyssiou, M. G., Salma, J., Bevzyuk, M., Wales, S., Zakharyan, L., and McDermott, J. C. (2013) Kruppel-like factor 6 (KLF6) promotes cell proliferation in skeletal myoblasts in response to TGFbeta/Smad3 signaling. *Skeletal muscle* **3**, 7
176. Grabski, A. D., Shimizu, T., Deou, J., Mahoney, W. M., Jr., Reidy, M. A., and Daum, G. (2009) Sphingosine-1-phosphate receptor-2 regulates expression of smooth muscle alpha-actin after arterial injury. *Arteriosclerosis, thrombosis, and vascular biology* **29**, 1644-1650
177. Grosser, T., Fries, S., and FitzGerald, G. A. (2006) Biological basis for the cardiovascular consequences of COX-2 inhibition: therapeutic challenges and opportunities. *The Journal of clinical investigation* **116**, 4-15
178. Wang, Z., Wang, D. Z., Hockemeyer, D., McAnally, J., Nordheim, A., and Olson, E. N. (2004) Myocardin and ternary complex factors compete for SRF to control smooth muscle gene expression. *Nature* **428**, 185-189
179. Liu, N., Bezprozvannaya, S., Williams, A. H., Qi, X., Richardson, J. A., Bassel-Duby, R., and Olson, E. N. (2008) microRNA-133a regulates cardiomyocyte proliferation and suppresses smooth muscle gene expression in the heart. *Genes & development* **22**, 3242-3254
180. Liu, N., Williams, A. H., Kim, Y., McAnally, J., Bezprozvannaya, S., Sutherland, L. B., Richardson, J. A., Bassel-Duby, R., and Olson, E. N. (2007) An intragenic MEF2-dependent enhancer directs muscle-specific expression of microRNAs 1 and 133. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 20844-20849
181. Huang, W. Y., Xie, W., Guo, X., Li, F., Jose, P. A., and Chen, S. Y. (2011) Smad2 and PEA3 cooperatively regulate transcription of response gene to complement 32 in TGF-beta-

induced smooth muscle cell differentiation of neural crest cells. *American journal of physiology. Cell physiology* **301**, C499-506

182. Guo, X., Stice, S. L., Boyd, N. L., and Chen, S. Y. (2013) A novel in vitro model system for smooth muscle differentiation from human embryonic stem cell-derived mesenchymal cells. *American journal of physiology. Cell physiology* **304**, C289-298
183. Santiago, J. J., Dangerfield, A. L., Rattan, S. G., Bathe, K. L., Cunnington, R. H., Raizman, J. E., Bedosky, K. M., Freed, D. H., Kardami, E., and Dixon, I. M. (2010) Cardiac fibroblast to myofibroblast differentiation in vivo and in vitro: expression of focal adhesion components in neonatal and adult rat ventricular myofibroblasts. *Developmental dynamics : an official publication of the American Association of Anatomists* **239**, 1573-1584
184. Gharaee-Kermani, M., Kasina, S., Moore, B. B., Thomas, D., Mehra, R., and Macoska, J. A. (2012) CXC-type chemokines promote myofibroblast phenoconversion and prostatic fibrosis. *PloS one* **7**, e49278
185. Frey, N., and Olson, E. N. (2003) Cardiac hypertrophy: the good, the bad, and the ugly. *Annual review of physiology* **65**, 45-79
186. Sadoshima, J., and Izumo, S. (1997) The cellular and molecular response of cardiac myocytes to mechanical stress. *Annual review of physiology* **59**, 551-571
187. Michele, D. E., and Metzger, J. M. (2000) Contractile dysfunction in hypertrophic cardiomyopathy: elucidating primary defects of mutant contractile proteins by gene transfer. *Trends in cardiovascular medicine* **10**, 177-182
188. Czubryt, M. P., and Olson, E. N. (2004) Balancing contractility and energy production: the role of myocyte enhancer factor 2 (MEF2) in cardiac hypertrophy. *Recent progress in hormone research* **59**, 105-124
189. Finck, B. N., and Kelly, D. P. (2007) Peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1) regulatory cascade in cardiac physiology and disease. *Circulation* **115**, 2540-2548
190. Passier, R., Zeng, H., Frey, N., Naya, F. J., Nicol, R. L., McKinsey, T. A., Overbeek, P., Richardson, J. A., Grant, S. R., and Olson, E. N. (2000) CaM kinase signaling induces cardiac hypertrophy and activates the MEF2 transcription factor in vivo. *The Journal of clinical investigation* **105**, 1395-1406
191. Wu, X., Zhang, T., Bossuyt, J., Li, X., McKinsey, T. A., Dedman, J. R., Olson, E. N., Chen, J., Brown, J. H., and Bers, D. M. (2006) Local InsP3-dependent perinuclear Ca2+ signaling in cardiac myocyte excitation-transcription coupling. *The Journal of clinical investigation* **116**, 675-682
192. Backs, J., Song, K., Bezprozvannaya, S., Chang, S., and Olson, E. N. (2006) CaM kinase II selectively signals to histone deacetylase 4 during cardiomyocyte hypertrophy. *The Journal of clinical investigation* **116**, 1853-1864
193. Ling, H., Zhang, T., Pereira, L., Means, C. K., Cheng, H., Gu, Y., Dalton, N. D., Peterson, K. L., Chen, J., Bers, D., and Brown, J. H. (2009) Requirement for Ca2+/calmodulin-dependent kinase II in the transition from pressure overload-induced cardiac hypertrophy to heart failure in mice. *The Journal of clinical investigation* **119**, 1230-1240
194. Chang, S., McKinsey, T. A., Zhang, C. L., Richardson, J. A., Hill, J. A., and Olson, E. N. (2004) Histone deacetylases 5 and 9 govern responsiveness of the heart to a subset of

stress signals and play redundant roles in heart development. *Molecular and cellular biology* **24**, 8467-8476
195. Yusuf, S., Sleight, P., Pogue, J., Bosch, J., Davies, R., and Dagenais, G. (2000) Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. *The New England journal of medicine* **342**, 145-153
196. Vega, R. B., Harrison, B. C., Meadows, E., Roberts, C. R., Papst, P. J., Olson, E. N., and McKinsey, T. A. (2004) Protein kinases C and D mediate agonist-dependent cardiac hypertrophy through nuclear export of histone deacetylase 5. *Molecular and cellular biology* **24**, 8374-8385
197. Harrison, B. C., Kim, M. S., van Rooij, E., Plato, C. F., Papst, P. J., Vega, R. B., McAnally, J. A., Richardson, J. A., Bassel-Duby, R., Olson, E. N., and McKinsey, T. A. (2006) Regulation of cardiac stress signaling by protein kinase d1. *Molecular and cellular biology* **26**, 3875-3888
198. Ross, R. (1999) Atherosclerosis--an inflammatory disease. *The New England journal of medicine* **340**, 115-126
199. Dzau, V. J. (2001) Theodore Cooper Lecture: Tissue angiotensin and pathobiology of vascular disease: a unifying hypothesis. *Hypertension* **37**, 1047-1052
200. Costa, M. A., and Simon, D. I. (2005) Molecular basis of restenosis and drug-eluting stents. *Circulation* **111**, 2257-2273
201. Doran, A. C., Meller, N., and McNamara, C. A. (2008) Role of smooth muscle cells in the initiation and early progression of atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology* **28**, 812-819
202. Mano, T., Luo, Z., Malendowicz, S. L., Evans, T., and Walsh, K. (1999) Reversal of GATA-6 downregulation promotes smooth muscle differentiation and inhibits intimal hyperplasia in balloon-injured rat carotid artery. *Circulation research* **84**, 647-654
203. Morrisey, E. E. (2000) GATA-6: the proliferation stops here: cell proliferation in glomerular mesangial and vascular smooth muscle cells. *Circulation research* **87**, 638-640
204. Kakita, T., Hasegawa, K., Morimoto, T., Kaburagi, S., Wada, H., and Sasayama, S. (1999) p300 protein as a coactivator of GATA-5 in the transcription of cardiac-restricted atrial natriuretic factor gene. *The Journal of biological chemistry* **274**, 34096-34102
205. Nishida, W., Nakamura, M., Mori, S., Takahashi, M., Ohkawa, Y., Tadokoro, S., Yoshida, K., Hiwada, K., Hayashi, K., and Sobue, K. (2002) A triad of serum response factor and the GATA and NK families governs the transcription of smooth and cardiac muscle genes. *The Journal of biological chemistry* **277**, 7308-7317
206. Helske, S., Lindstedt, K. A., Laine, M., Mayranpaa, M., Werkkala, K., Lommi, J., Turto, H., Kupari, M., and Kovanen, P. T. (2004) Induction of local angiotensin II-producing systems in stenotic aortic valves. *Journal of the American College of Cardiology* **44**, 1859-1866
207. Nag, A. C. (1980) Study of non-muscle cells of the adult mammalian heart: a fine structural analysis and distribution. *Cytobios* **28**, 41-61
208. MacKenna, D. A., Vaplon, S. M., and McCulloch, A. D. (1997) Microstructural model of perimysial collagen fibers for resting myocardial mechanics during ventricular filling. *The American journal of physiology* **273**, H1576-1586

209. Covell, J. W. (1990) Factors influencing diastolic function. Possible role of the extracellular matrix. *Circulation* **81**, III155-158
210. Deten, A., Holzl, A., Leicht, M., Barth, W., and Zimmer, H. G. (2001) Changes in extracellular matrix and in transforming growth factor beta isoforms after coronary artery ligation in rats. *Journal of molecular and cellular cardiology* **33**, 1191-1207
211. Desmouliere, A., Darby, I. A., and Gabbiani, G. (2003) Normal and pathologic soft tissue remodeling: role of the myofibroblast, with special emphasis on liver and kidney fibrosis. *Laboratory investigation; a journal of technical methods and pathology* **83**, 1689-1707
212. Eyden, B. (2007) The myofibroblast: a study of normal, reactive and neoplastic tissues, with an emphasis on ultrastructure. *Journal of submicroscopic cytology and pathology*, 7-166
213. Chambers, R. C., Leoni, P., Kaminski, N., Laurent, G. J., and Heller, R. A. (2003) Global expression profiling of fibroblast responses to transforming growth factor-beta1 reveals the induction of inhibitor of differentiation-1 and provides evidence of smooth muscle cell phenotypic switching. *The American journal of pathology* **162**, 533-546
214. Fan, D., Takawale, A., Lee, J., and Kassiri, Z. (2012) Cardiac fibroblasts, fibrosis and extracellular matrix remodeling in heart disease. *Fibrogenesis & tissue repair* **5**, 15
215. Kolonin, M. G., Evans, K. W., Mani, S. A., and Gomer, R. H. (2012) Alternative origins of stroma in normal organs and disease. *Stem cell research* **8**, 312-323
216. Micallef, L., Vedrenne, N., Billet, F., Coulomb, B., Darby, I. A., and Desmouliere, A. (2012) The myofibroblast, multiple origins for major roles in normal and pathological tissue repair. *Fibrogenesis & tissue repair* **5**, S5
217. Shimizu, R. T., Blank, R. S., Jervis, R., Lawrenz-Smith, S. C., and Owens, G. K. (1995) The smooth muscle alpha-actin gene promoter is differentially regulated in smooth muscle versus non-smooth muscle cells. *The Journal of biological chemistry* **270**, 7631-7643
218. Tang, Y., Yang, X., Friesel, R. E., Vary, C. P., and Liaw, L. (2011) Mechanisms of TGF-beta-induced differentiation in human vascular smooth muscle cells. *Journal of vascular research* **48**, 485-494
219. Sassoli, C., Chellini, F., Pini, A., Tani, A., Nistri, S., Nosi, D., Zecchi-Orlandini, S., Bani, D., and Formigli, L. (2013) Relaxin prevents cardiac fibroblast-myofibroblast transition via notch-1-mediated inhibition of TGF-beta/Smad3 signaling. *PloS one* **8**, e63896
220. Fahed, A. C., Gelb, B. D., Seidman, J. G., and Seidman, C. E. (2013) Genetics of congenital heart disease: the glass half empty. *Circulation research* **112**, 707-720

APPENDIX: EXPANDED MATERIALS AND METHODS

CELL CULTURE:

The following cell lines were utilized in the above studies, A10, COS7, C3H10T1/2, and VMSC mouse aortic primary cultures.

Reagents:

- 0.125% Trypsin-EDTA (Gibco) diluted in PBS
- DMEM supplemented with Penicillin-Streptomycin (Gibco) and L-glutamine (Gibco) added as required
    - Growth Medium: 10% fetal bovine serum (FBS)
    - Low Serum: 5% horse serum (HS)
- Freezing Medium: Growth media supplemented with 10% DMSO (5% DMSO for A10s)

Cell Passaging:

- Remove media from established stock cultures
- Rinse the cell monolayer briefly with 10 ml of 1xPBS
- Add 1.0ml of 0.125% Trypsin-EDTA (0.125% Trypsin for A10 cell line) solution to 100mm dish or 0.5ml to 60mm dish, incubate at 37°C for 1-4 min.
- Inactivate the Trypsin by adding 5.0 ml of DMEM
- Pipette the cells up and down several times to ensure complete removal of the cells from the dish.
- Centrifuge at 1500 g for 5 minutes. Remove media and re-suspend pellet in 5mL of media

- Count the cells in a haemocytometer (optional) and seed a dilution of cells that allows for sufficient cell (1:10 dilution for 10T1/2 cells, 1:8 dilution of COS7 cells, 1:3 for A10 cells and 1:5 for primary cell cultures).

Inducing Muscle Cell Quiscence or 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 (CH3 10T1/2), or Serum free medium (A10, Primary VSMC)
- Incubate cells for 6-12 hours prior to pharmacological treatment, 24hours for smooth muscle quiescence, or 96 hours for conversion assay (10T1/2 cells)

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 in -80 °C freezer and freeze overnight.
- Remove vials from polystyrene box and place in -80 °C freezer for short-term storage, or 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 10ml of growth medium.
- Plate cells in 100 mm culture dish.

TRANSFECTION OF MAMMALIAN CELLS WITH DNA

Calcium-mediated transfections were performed in 35/100 mm cell culture dishes. The below protocol is for transfection of 100 mm plates; reagents were scaled proportional to surface area for transfection of 35 mm plates. Lipofectamine transfections were performed in 35/100mm cell culture dishes. The below protocol is for transfection of 100mm plates, and reagents were scaled proportional to surface area for transfectin of 35mm plates.

Reagents:

- 2x HEBS (2.8 M NaCl, 15mM Na2HPO4, 50mM HEPES). pH to 7.15, filter sterilized, store at -20 °C.
- 2.5 M $CaCl_2$, filter sterilized, store at -20 °C.

Calcium transfection:

- Plate cells 24 hours prior to transfection so that they are 50-70% confluent at time of transfection.
- Re-feed cell cultures with growth media 2-3 hours prior to addition of DNA.
- Add 0.5 ml of 2x HEBS to each sterile tube
- Prepare DNA-$CaCl_2$ solution: add 25µg DNA to 450µl of ddH_20, mix, add 50µl 2.5 M $CaCl_2$, mix.
- While vortexing HEBS at low speed add DNA-$CaCl_2$ solution in a drop-wise manner.
- Incubate at room temperature for 15mins

- Add DNA mix drop-wise to cell cultures. Incubate for 16 hours at 37°, 5%CO_2
    - 10T1/2 cells, Primary VSMC : 1mL transfection mixture to 100mm dish
    - COS7 cells: 300µL transfection mixture to 100mm dish
- Wash cells twice with PBS and re-feed with growth media for 24 hours prior to harvesting or pharmacologically treating cells.

Lipofectamine in A10 cells:

- Seed cells at 80% confluence in 10 cm plates 24 hours prior to transfection
- 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 DNA-DMEM and Lipofectamine-DMEM mixtures, mix and incubate for 15-30 minutes.
- Add 1.6 mL of serum- and antibiotic-free media to the mix.
- Wash plated cells twice with 1xPBS and re-feed cells in 3.2 mL of serum- and antibiotic-free media.
- Add the DNA/Lipo mix to cell culture dishes and gently rock.
- Incubate of 2 hours at 37°C and 5% CO_2
- Wash 2x in PBS and re-feed in growth media for 24 hours prior to harvesting or pharmacological treatment

Lipofectamine in 10T1/2 and primary smooth muscle cells:

- Seed cells at 80% confluence in 10 cm plates 24 hours prior to transfection
- Dilute 10 µg of DNA in 500 µl serum- and antibiotic-free media.
- Mix 40 µl of Lipofectamine reagent in 500 µl serum- and antibiotic-free media.

- Combine DNA-DMEM and Lipofectamine-DMEM mixtures, mix and incubate for 15-30 minutes.
- Add 3 mL of serum- and antibiotic-free media to the mix.
- Wash plated cells twice with 1xPBS and re-feed cells in 4.0 mL of serum- and antibiotic-free media.
- Add the DNA/Lipo to cell culture dishes and gently rock.
- Incubate of 5 hours at 37°C and 5% CO_2
- Supplement media with 20% FBS overnight, wash 2x in PBS and re-feed in growth media and allow for recovery for 24 hours prior to harvesting, or pharmacological treatments.

LUCIFERASE EXTRACTS AND 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:

- Luciferase Lysis buffer: (20 mM Tris, pH 7.4, 0.1% Triton-X 100 in 1xPBS)
- Firefly and Renilla Luciferase substrate (Promega)

Harvesting:

- Wash adherent cells with cold 1x PBS.
- Add 200-300 µL (A10 cells) or 400-600 µL (10T1/2, COS7, primary VSMC cells) of lysis buffer per dish.
- Incubate for 5 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 and transfer 50µl to Luciferase assay tube.
- Obtain luciferase readings using Lambat (LB 958) luminometer

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).

Reagents:

- 1xPBS (keep cold)
- NP-40 Lysis buffer
- 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 (7.5%)

Whole Cell Extracts:

- Remove media from cells, wash with cold 1xPBS, repeat.
- Add 1.0 mL cold 1xPBS and gently scrape cells with rubber policeman, transfer to new tube.
- Centrifuge cells at 1500xg for 5 min at 4°C

- Remove PBS, approximate the cell pellet volume and dilute with five times that volume in NP-40 lysis buffer.
- Vortex cells briefly every 10 min for 40 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 6min, centriguge briefly, chill on ice for five minutes, store at - 80 °C.

Nuclear and Cytoplasmic Protein Extracts (NE-PER kit, Pierce):

- 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 ANALYSIS:

Reagents:

- 1XPBS
- 10% Resolving gel (ddH_2O, 1.5M Tris pH8.8, 30% acrylamide, 10% SDS,n 10% APS, TEMED)
- Stacking gel (ddH_2O, 1.0M Tris pH6.8, 30% acrylamide, 10% SDS,n 10% APS, TEMED)
- 10X Laemmli (1L)

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:

- 1XTransfer buffer (100ml) (methanol, glycine, Tris Base)
- Methanol
- 1xPBS and/or 1xTBS
- Primary and Secondary antibody (as required)

Methods:

- Following SDS PAGE, transfer protein to Immobilon-P (Millipore) membrane by wet transfer at 20 V for 16-20 h, or at 100V for 1h.
- Block membrane with 5 % (w/v) skim milk powder in PBS/TBS (depending on primary antibody manufacturers's requirements)
- Incubate membrane with primary antibody diluted 1:100-1:10 000 in blocking solution overnight at 4 °C, as per manufacturer's instructions.
- Wash membrane with PBS/TBST (3 X 10min).
- Incubate membrane with secondary antibody 1:1000-1:100 000 in blocking solution for 2 hours at room temperature
- Wash membrane with PBS/TBST (3 X 10min).
- Develop blot with chemiluminescence reagent, expose blot to film, and develop.

CO-IMMUNOPRECIPITATION ASSAY (EXACTACRUZ PROTOCOL):

- Prepare cell lysates as described in protein extracts section.
- To 500µL cold 1xPBS add 40µL of ExactaCruz beads (species specific to primary antibody) and 1-5µg of primary antibody. Nutate overnight at 4°C
- After overnight nutation, wash IP matrix beads 3x in 1xPBS (cold) by centrifugation.
- To ExactaCruz IP matrix beads, add 1 ml of NP-40 lysis buffer and 250-1000µg total cell lysate protein. Nutate overnight at 4°C
- After overnight nutation, wash IP matrix beads 3x in 1xPBS (cold) by centrifugation
- Resuspend pellet in 40 µl of 2 X SDS sample buffer and boil for 5 min

- Load supernatant into SDS-PAGE gel for Western analysis

RNA ISOLATION:

- Add 1 ml of Trizol to 100 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.
- Use *One Taq RT-PCR Kit* (NEB E5310S) for cDNA and PCR reactions

IMMUNOFLUORESCENCE:

- Wash cells several times with cold PBS.
- Fix cells with 4 % paraformaldehyde in PBS for 10 min at room temperature.

- Wash cells several times with PBS.
- Permeabilize cells with 90% methanol (cold) for 6 mins at -20°C
- Block cells with 10% goat serum in PBS
- Incubate cells with primary antibody (1:50 –1:500 dilution), overnight at 4°C
- Wash cells several times with PBS.
- Incubate cells with secondary antibody (1:200) (species-specific to primary antibody, conjugated with either FITC or TRITC)
- Wash cells several times with PBS, add a drop of appropriate mounting media (with DAPI), and cover slip and analyse using fluorescence microscopy.

CHROMATIN IMMUNOPRECIPITATION:

- Fix approximately 1x 10^7 cells with 1% formaldehyde for 10 minutes at room temperature.
- Fixing is quenched by Glycine at a final concentration of 0.125M for 5 minutes at room temperature.
- Collect cells in PBS containing PMSF and protease inhibitor cocktail and centrifuge at 5000 rpm for 5 minutes at 4^oC.
- Lyse cells with Wash Buffer I (10mM HEPES pH 6.5, 0.5M EGTA, 10mM EDTA, 0.25% Triton X-100, protease inhibitor cocktail, PMSF) for 5 minutes on ice.
- Collect nuclei and resuspend in Wash Buffer II (10mM HEPES pH 6.5, 0.5 mM EGTA, 1 mM EDTA, 200 mM NaCl, protease inhibitor cocktail, PMSF) for 10 min on ice. Colect nuclei and treat with nuclear lysis buffer (50mM Tris-HCl pH 8.1, 10 mM EDTA, 1% SDS).

- Shear chromatin using a sonicatorto produce 500 bp fragments. Collect the crosslinked sheared chromatin following a 15 minute spin at maximum speed. Set aside twenty percent of total chromatin for the input. Dilute sheared crosslinked chromatin 1:10 with IP dilution buffer (0.01% SDS, 1.1% Triton-X 100, 1.2 mM EDTA, 16.7 mM Tris-HCL pH 8.1, 167 mM NaCl) and incubate with antibody overnight at 4^{o}C with rocking.
- Block Protein G Dynabeads with 20 µg salmon sperm DNA in IP dilution buffer (15 µl beads + 135 µl IP dilution buffer + 20 µg salmon sperm DNA per IP) overnight at 4^{o}C with rocking. Incubate 152 µl of pre-blocked beads with the IP reaction at 4^{o}C for 1 hr.
- Wash dynabead-bound antibody:chromatin complexes using IP Wash Buffer I (20 mM Tris pH 8.1, 2mM EDTA, 150 mM NaCl, 1% Triton-X 100, 0.1% SDS), Wash Buffer II (20 mM Tris pH 8.1, 2 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.1% SDS) and Wash Buffer III (20 mM Tris pH 8.1, 250 mM LiCl, 1% NP-40, 1% deoxycholate and 1 mM EDTA), each incubated for 10 minutes at 4^{o}C, and followed with two washes in TE buffer at 4^{o}C.
- Free Protein:DNA complexes from Dynabeads through the addition of elution buffer (0.1 M NaHCO3, 1% SDS) for 30 minutes at room temperature. Treat samples with 12 µl of 5 M NaCl (BioShop) at 65^{o}C overnight.
- Add Proteinase K, EDTA, Tris pH 6.5 for 1 hr at 45^{o}C. Purify DNA samples using a PCR clean up kit.