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# REDOX-SENSITIVE REGULATION OF MRTF-A PHOSPHORYLATION VIA PALLADIN IN VASCULAR SMOOTH MUSCLE CELL DIFFERENTIATION

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# ABSTRACT

## REDOX-SENSITIVE REGULATION OF MRTF-A PHOSPHORYLATION VIA PALLADIN IN VASCULAR SMOOTH MUSCLE CELL DIFFERENTIATION

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Vascular smooth muscle cells (VSMCs) undergo phenotypic changes in cardiovascular diseases such as atherosclerosis and restenosis. Differentiated VSMCs have robust stress fibers and express contractile proteins, such as smooth muscle $\alpha$-actin (SMA), necessary for vascular function. Loss of the differentiated phenotype has been observed in disease processes. Understanding the mechanisms involved in these changes is important in understanding and treating these diseases. Our previous studies indicate that transforming growth factor $\beta$-1 (TGF-$\beta$) induces reactive oxygen species (ROS) through NADPH oxidase 4 (Nox4), which modulate activity of myocardin-related transcription factor A (MRTF-A) and serum response factor (SRF), transcription factors that regulate expression of prodifferentiation genes such as SMA. Regulation of these factors is incompletely understood, but evidence exists for an interaction of MRTF-A with the actin-binding protein, palladin, although how this interaction affects MRTF-A function is unclear. In this thesis, we tested the hypothesis that Rho kinase (ROCK)-mediated phosphorylation of MRTF-A is a key event in the regulation of SRF responsive genes in VSMCs and that this phosphorylation is dependent upon Nox4-mediated palladin expression

and interaction with phosphatase PP2A. Knockdown of Nox4 using siRNA decreases TGF-β-induced palladin expression and MRTF-A phosphorylation suggesting redox-sensitive regulation of the proteins. The phosphorylation of MRTF-A is mediated by ROCK and inhibition of this kinase resulted in a decrease in SMA expression. Palladin also regulates this phosphorylation, because knockdown of palladin decreases MRTF-A phosphorylation. Co-immunoprecipitation assay showed palladin binds to protein phosphatase 2A (PP2A), suggesting the idea that palladin may sequester PP2A from MRTF-A, which results in prevention of MRTF-A dephosphorylation. These data suggest that ROCK-mediated phosphorylation of MRTF-A is important in regulation of SRF responsive gene expression. Furthermore, the data support the idea that the MRTF-A phosphorylation is controlled by Nox4-mediated palladin expression and interaction with PP2A. Therefore, the novel role of Nox4 and palladin in phosphorylation of MRTF-A and subsequent differentiation gene expression in VSMCs may offer a new therapeutic target for atherosclerosis and restenosis.

# REDOX-SENSITIVE REGULATION OF MRTF-A PHOSPHORYLATION VIA PALLADIN IN VASCULAR SMOOTH MUSCLE CELL DIFFERENTIATION

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**M.S., Kyungpook National University, 2008**

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A thesis submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Master of Science

in

Molecular and Systems Pharmacology
Graduate Division of Biological and Biomedical Sciences
2013

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| | |
|---|---|
| MAPK | Mitogen Activated Protein Kinase |
| MRTF | Myocardin-Related Transcription Factor |
| NAC | N-acetyl Cysteine |
| Nox | NAPDH oxidase |
| PP1 | Protein Phosphatase 1 |
| PP2A | Protein Phosphatase 2A |
| ROCK | Rho kinase |
| ROS | Reactive Oxygen Species |
| SOD | Superoxide Dismutase |
| SMA | Smooth Muscle $\alpha$-Actin |
| SM-MHC | Smooth Muscle Myosin Heavy Chain |
| SM22$\alpha$ | Smooth Muscle 22 alpha |
| SRF | Serum Response Factor |
| TGF-$\beta$ | Transforming Growth Factor-$\beta$1 |
| VSMC | Vascular Smooth Muscle Cell |

# CHAPTER 1

## Introduction

## 1.1 Diseases: Atherosclerosis, In-stent restenosis

In the vasculature, differentiated vascular smooth muscle cells (VSMCs) are critical for physiological homeostasis. VSMCs provide structural support for the vasculature and modulate contractility to regulate blood flow and pressure. However, VSMCs retain remarkable plasticity even in adult animals, which means that they modulate their phenotype from differentiated to dedifferentiated in response to extracellular stimuli, whereas the skeletal muscle and cardiac muscle cells are terminally differentiated. Differentiated VSMCs express SMC-specific contractile proteins including smooth muscle α-actin (SMA) (Rovner et al., 1986), smooth muscle myosin heavy chain (SM-MHC) (Gabbiani et al., 1981; Miano et al., 1994; Rovner et al., 1986), smooth muscle 22 alpha (SM22$\alpha$) (Lees-Miller et al., 1987), and calponin (Takahashi et al., 1986). However, VSMCs undergo the process of dedifferentiation, which is characterized by decreased differentiation marker gene expression and increased proliferation, migration, and matrix synthesis, in various cardiovascular diseases such as atherosclerosis and in-stent restenosis.

The best-studied example of a disease related to VSMC phenotypic switching is atherosclerosis, which causes nearly 50% of all deaths in developed countries. The majority of differentiated VSMCs stay in the medial region in the vessels; however, upon exposure to proatherogenic cytokines, they undergo phenotypic modulation into dedifferentiated cells. The dedifferentiated VSMCs in the medial region migrate into the intima, where they proliferate and produce

extracellular matrix contributing to formation of intimal atherosclerotic lesions (Ross, 1993, 1999).

Despite decades of research, the molecular mechanisms required for the maintenance of differentiated VSMC phenotype (and therefore prevention of de-differentiation) remain poorly understood, but are crucial to rational design of pharmacological strategies to prevent the disease.

## 1.2 Reactive Oxygen Species and NADPH Oxidase Signaling

Reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, are linked to the development of many cardiovascular disease pathologies, including atherosclerosis and restenosis. ROS were originally thought to have a detrimental role in oxidation of proteins, lipids, and DNA; however, recent research has shown that ROS act as second messengers in numerous signal transduction pathways. ROS such as superoxide and hydrogen peroxide modify the intracellular redox state and regulate physiologic and pathophysiologic processes in vascular system (Griendling et al., 2000a). Superoxide can be produced by mitochondria and several enzymatic systems such as NADPH oxidases (Griendling et al., 2000b) and also rapidly converted to hydrogen peroxide by superoxide dismutase (SOD). Hydrogen peroxide is one of the most stable and robust oxidizing ROS implicated in the regulation of signaling pathways including VSMC growth, differentiation, migration, and inflammation (Lassegue and Clempus, 2003). Although hydrogen peroxide is not a free radical, it reacts with thiol-containing proteins resulting in redox-sensitive

signals (Winterbourn and Hampton, 2008). Hydrogen peroxide initially reacts with cysteine residues in proteins to form a disulfide bond (-SSR) or a protein sulfenic acid (-SOH). Then, sulfinic acid (-$SO_2H$) and sulfonic acid (-$SO_3H$) can be formed which are irreversible modifications (Barford, 2004; Forman et al., 2002).

NADPH oxidases are major sources of ROS in VSMCs (Rajagopalan et al., 1996; Zafari et al., 1998). The NADPH oxidase family consists of Nox1, Nox2, Nox3, Nox4, Nox5, Duox1 and Duox2 (Lassegue and Clempus, 2003). Most cell types express multiple NADPH oxidases, which are differentially regulated by their expression level and subcellular localization. VSMCs from large arteries predominantly express Nox1 and Nox4, while resistance and coronary arteries express Nox2 (Gupte et al., 2009; Touyz et al., 2002), and only human VSMCs express Nox5 (Banfi et al., 2001). Nox 4 is highly expressed in focal adhesions (Hilenski et al., 2004), the nucleus (Kuroda et al., 2005), the endoplasmic reticulum (Ambasta et al., 2004), and mitochondria within the cell (Block et al., 2009; Kim et al., 2012), whereas Nox1 is found in caveolae on the plasma membrane (Hilenski et al., 2004) and Nox2 exists in phagosomes (Lambeth, 2004; Vignais, 2002) and on the leading edge of lamellipodia (Ushio-Fukai, 2006), indicative of their diverse roles within the cell.

Nox1, Nox2, and Nox3 all form an integral membrane complex with p22phox, a transmembrane protein that stabilizes the catalytic subunit (Nox) and serves as a docking site for cytosolic subunits (Ambasta et al., 2004; Lassegue and Griendling, 2010). These isoforms complex with cytosolic regulatory

subunits such as p47phox, NoxA1, NoxO1, p67phox, and Rac to produce ROS, primarily superoxide, whereas Nox4 does not require cytosolic subunits to produce ROS, but rather binds with polymerase delta interacting protein-2 (Podip2) (Lyle et al., 2009). Like Nox1, Nox2, and Nox3, Nox4 also interacts with p22phox (Ambasta et al., 2006; Hanna et al., 2004; Ushio-Fukai et al., 1996). The N-terminus of p22phox is essential for Nox4/p22phox localization (von Lohneysen et al., 2008); however, a p22phox mutant harboring alterations in the proline-rich region does not affect Nox4 activity but decreases Nox1 activity (Kawahara et al., 2005) suggesting that Nox4 is constitutively active (Ellmark et al., 2005), at least from the perspective of not requiring traditional cytosolic activators. It has been suggested that the major mechanism of Nox4 regulation is transcriptional (Bedard and Krause, 2007).

Nox4 was originally found in the kidney (Geiszt et al., 2000), but is expressed in many cell types including mesangial cells (Gorin et al., 2003), hepatocytes (Carmona-Cuenca et al., 2006), smooth muscle cells (Ellmark et al., 2005; Hilenski et al., 2004; Pedruzzi et al., 2004), endothelial cells (Ago et al., 2005; Van Buul et al., 2005), fibroblasts (Cucoranu et al., 2005; Dhaunsi et al., 2004), keratinocytes (Chamulitrat et al., 2004), and neurons (Vallet et al., 2005). Nox4 is required for VSMC differentiation marker gene expression. Our previous research has shown that knockdown of Nox4 reduced TGF-β-induced SMA and calponin mRNA and protein expression in VSMCs (Clempus et al., 2007; Martin-Garrido et al., 2011). Because Nox4 has been found in the nucleus (Hilenski et al., 2004), these studies suggested that the transcription factors associated with

differentiation marker gene expression could be regulated by Nox4-mediated ROS production. Although a role of ROS in VSMC differentiation has been proposed, the specific mechanisms require further investigation.

### 1.3 TGF-β-mediated Smooth Muscle Cell Differentiation Signaling

Transforming growth factor-β1 (TGF-β) increases contractile gene expression (such as SM-MHC, SM22α, calponin, and SMA) at both the transcriptional and translational levels. Among the smooth muscle genes, SMA is the most abundant in SMC and the best characterized (Owens and Thompson, 1986), and has been used by others as a prototypical SMC-specific gene.

Signaling pathways regulating TGF-β-induced gene expression can largely be divided into Smad-dependent and Smad-independent pathways. Many studies have shown that TGF-β increases Smad3 phosphorylation, which results in upregulation of contractile gene expression including SMA (Cucoranu et al., 2005; Hu et al., 2003; Yu et al., 2002). Recently, non-Smad pathways have been implicated as well, including various mitogen-activated protein kinase (MAPK) pathways, Rho-like GTPase signaling pathways, and phosphatidylinositol-3-kinase (PI3K)/AKT pathways. The best-studied non-Smad pathway is the p38MAPK pathway. In rat pulmonary arterial smooth muscle cells, p38MAPK mediates TGF-β-induced SMA expression (Deaton et al., 2005) and in the TGF-β-induced epithelial-mesenchymal transition, inhibition of p38MAPK decreased SMA expression (Rhyu et al., 2005; Yu et al., 2002).

TGF-β has been shown to increase Nox4 activity in stem cells, fibroblasts, and pulmonary artery smooth muscle cells. Our previous research showed that TGF-β induces production of ROS through Nox4, and that Nox4 is necessary for TGF-β-induced SMA expression and stress fiber formation in VSMCs (Clempus et al., 2007; Martin-Garrido et al., 2011). Nox4 exerts its effects in part through p38MAPK, which is known to be redox-sensitive (Ushio-Fukai et al., 1998) and to be downstream of Nox4 (Li et al., 2006; Martin-Garrido et al., 2011). Nox4/p38MAPK increases activity of the SMC-specific transcription factor SRF (serum response factor) by increasing its phosphorylation (Martin-Garrido et al., 2011). In addition, Poldip2, which binds to Nox4, activates RhoA in VSMCs (Lyle et al., 2009), an additional signaling pathway that has been linked to SMA expression (Lyle et al., 2009). However, the precise pathways by which Nox4 regulates SMA gene expression in response to TGF-β remain incompletely understood.

### 1.4 MRTF-A and SRF

As noted, VSMC contractile gene transcription is largely regulated by SRF, which binds to highly conserved CArG cis-elements (CC(A/T)6GG) that are present in the promoter of SMC-specific genes. SRF is a dual function transcription factor, which depends on its co-activators for specificity. For example, PDGF increases proliferative and migratory gene expression via inducing ternary complex factor binding to SRF, whereas TGF-β increases differentiation marker gene expression by inducing myocardin or myocardin-

related transcription factors (MRTFs) - A and - B binding to SRF. Myocardin is exclusively expressed in cardiomyocytes and smooth muscle cells, whereas MRTFs are more broadly expressed than myocardin (Wang et al., 2002). Myocardin is constitutively expressed in the nucleus and regulates transcriptional activity via SRF. Deletion of myocardin is embryonic lethal at day 10.5 due to a defect in heart development causing hemorrhage.

MRTF-A, also termed megakaryoblastic leukemia 1 (MKL1), megakaryocytic acute leukemia (MAL), and basic, SAP and coiled-coil (BSAC), was originally identified as the chromosome 22-encoded protein altered by the t(1;22) translocation of acute megakaryoblastic leukemias in infants and children (Ma et al., 2001; Mercher et al., 2001). The functional role of each domain of MKL1 has been explored. MKL1 has two RPEL domains at N-terminus that are important for monomeric G-actin binding. Basic regions of MKL1 are essential for nuclear localization and interaction with SRF. Basic regions are sufficient for SRF interaction, but glutamine rich region (Q-domain) accelerate the interaction with SRF (Zaromytidou et al., 2006). The SAP domain in myocardin is required for activation of a subset of SRF (Wang et al., 2001), but mutation of the SAP domain in MRTF-A does not affect SRF activity (Miralles et al., 2003). The leucine zipper domain (coiled-coil region) mediates dimerization of MRTF-A. MRTF-A preferentially binds SRF as a dimer (Miralles et al., 2003). The C-terminal region of MKL1 contains a transcriptional activation domain that is critical for gene expression (Cen et al., 2003). MRTF-B possesses similar domains and interacts with SRF in a similar manner to MRTF-A (Wang et al.,

2001); however, MRTF-B is less potent in inducing differentiation marker gene transcription than MRTF-A (Wang et al., 2002). Therefore, we focused on examining the functional role of MRTF-A in VSMC differentiation rather than the role of MRTF-B.

In fibroblasts, MRTF-A resides in the cytosol but is translocated into the nucleus to initiate SMC-specific differentiation gene transcription upon serum stimulation (Miralles et al., 2003). Binding of monomeric G-actin is an effective regulator for MRTF-A translocation from cytosol into the nucleus in fibroblasts. Activation of RhoA induces actin polymerization, which results in release of monomeric G-actin from MRTF-A. The dissociated MRTF-A translocates into the nucleus via importins located on the nucleus membranes (Pawlowski et al., 2010). However, in some SMCs, the majority of MRTF-A is found in the nucleus (Du et al., 2004; Hinson et al., 2007), suggesting that other signaling mechanisms are needed for the activation of MRTF-A.

Upon serum stimulation, MRTF-A is phosphorylated in NIH3T3 cells. C3 transferase, a Rho-kinase inhibitor, and U0126, an ERK inhibitor, decreased the serum-induced molecular weight shift of MRTF-A (indicative of phosphorylation), while a PKC inhibitor did not affect it (Miralles et al., 2003). The authors also showed that phosphorylation is not a leading factor for nuclear accumulation by serum, but suggest instead that phosphorylation may be important for its activation. In Hela cells, MRTF-A is phosphorylated at Serine454 by ERK in response to serum and mutation of the phosphorylation site constitutively localized MRTF-A in the nucleus, indicating that MRTF-A phosphorylation may

be important in nuclear export (Muehlich et al., 2008). However, how phosphorylation contributes to MRTF-A activity in SRF-responsive gene expression remains unclear, especially in VSMCs.

### 1.5 Palladin

Palladin is an actin-associated protein widely expressed in actin-based subcellular structures (Otey et al., 2005) including stress fibers, focal adhesions, cell-cell junctions, and embryonic Z-lines (Mykkanen et al., 2001; Parast and Otey, 2000). Seven isoforms of palladin have been found in different types of cells. The 90-kDa isoform of palladin is highly expressed in SMCs (Wang and Moser, 2008) and its overexpression induces stress fibers (Rachlin and Otey, 2006) whereas its knockdown triggers disordered stress fibers in SMCs (Liu et al., 2007). The 140-kDa isoform is widely expressed with the exception of liver, muscle, and skin (Parast and Otey, 2000; Rachlin and Otey, 2006; Wang and Moser, 2008), and it is neo-expressed by TGF-$\beta$ in myofibroblasts (Ronty et al., 2006).

In vivo, loss of palladin causes embryonic lethality at day 15.5 due to an apparent cell migration defect resulting in defective neural tube and ventral closure (Luo et al., 2005). A few studies have shown that palladin has an important role in SMC differentiation. Palladin mRNA is induced at day 20 in an in vitro embryoid body differentiation system (Jin et al., 2009b) and knock out of palladin appears to impair differentiation into SMC by regulating smooth muscle marker gene expression (Jin et al., 2010). It is worthwhile to note that palladin is

important in the organization of the SMC cytoskeleton and in regulating contraction (Jin et al., 2010; Jin et al., 2007).

Retinoic acid markedly induces palladin expression as well as SMA and SM22$\alpha$ expression in A404 cells and overexpression of palladin without retinoic acid significantly induces SMA and SM22$\alpha$ gene expression in undifferentiated A404 cells (Jin et al., 2010). To determine how palladin regulates the expression of SMC contractile proteins, Jin *et al.* (Jin et al., 2009b) compared palladin null cells to WT cells measuring GTP•RhoA activity, Rho kinase expression, and SRF expression, but no significant difference was observed. However, co-immunoprecipitation assays (co-IP) in HEK 293 cells and in vitro GST pull down assays provided evidence that palladin binds to MRTFs but not myocardin. Immunocytochemistry assays clearly show that the C-terminal fragment, but not the N-terminal fragment, of palladin co-localizes with MRTF-A in the nucleus (Jin et al., 2010). This suggests that the C-terminus of palladin may have a distinctive role, whereas the N-terminus supports stress fibers and binding to many actin related proteins including $\alpha$-actinin (Ronty et al., 2004), profilin (Boukhelifa et al., 2006), Spin90 (Ronty et al., 2007), ArgBP2 (Ronty et al., 2005), Eps8 and Src (Goicoechea et al., 2006; Ronty et al., 2007). The fact that palladin interacts with many of these actin related proteins suggests that palladin serves as a cytoskeleton scaffold and signaling mediator. Evidence that the C-terminus directs palladin to the nucleus suggests that palladin may possess another role in regulation of transcriptional activity by modulating transcription

factors or other molecules such as co-factors or translational modification enzymes.

### 1.6 Overall Hypothesis

Nox4 is a necessary enzyme for maintenance of the differentiated VSMC phenotype, yet its function in the biological system is not fully understood. Based on the above-mentioned studies implicating Nox4 and SRF in VSMC differentiation marker gene expression, demonstrating a requirement for an interaction between MRTF-A and SRF for induction of VSMC differentiation marker gene transcription, and supporting a role of palladin in VSMC differentiation and its potential interaction with MRTF-A, this thesis explores the link between Nox4, MRTF-A and palladin in the regulation of differentiation marker gene expression in VSMCs. In particular, how MRTF-A activation is managed in VSMCs has not been explored yet; therefore, we investigated the specific role of palladin in the regulation of MRTF-A activity in response to TGF-β-induced Nox4 activation.

In the work described here, **we tested the hypothesis that Rho kinase (ROCK)-mediated phosphorylation of MRTF-A is a key event in regulation of SRF responsive genes in VSMCs and that this phosphorylation is dependent upon Nox4-mediated palladin expression and interaction with phosphatase PP2A.** To address this hypothesis, we developed two specific aims.

The first aim of this research is to determine if TGF-β-mediated induction of MRTF-A phosphorylation and palladin expression is ROS sensitive. The second aim is to determine how palladin regulates phosphorylation of MRTF-A in the control of differentiation marker gene expression. Throughout, we have used SMA as a prototypical marker gene.

Figure 1.1 represents a schematic diagram of the detailed working model used to test our hypothesis. We propose that TGF-β-induced Nox4 activation produces $H_2O_2$ and that this regulates phosphorylation of MRTF-A and expression of palladin. The phosphorylation of MRTF-A is mediated by Rho kinase (ROCK) and its dephosphorylation is mediated by protein phosphatase 2A. Phosphorylation of MRTF-A is maintained by sequestration of protein phosphatase 2A by formation of a complex with palladin.

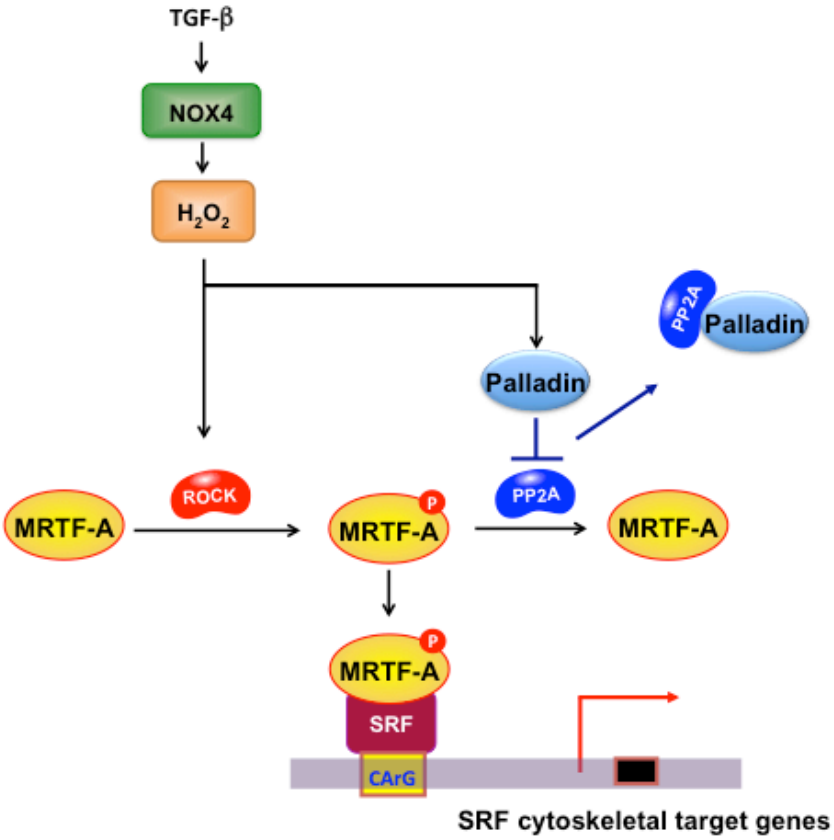


**Figure 1.1 TGF-β-induced Redox-Sensitive Regulation of MRTF-A Phosphorylation via Sequestration of PP2A by Palladin in VSMC Differentiation**

TGF-β-mediated $H_2O_2$ production from Nox4 increases phosphorylation of MRTF-A and expression of palladin. MRTF-A phosphorylation is mediated by ROCK and dephosphorylation of MRTF-A is prevented by sequestration of PP2A via palladin.

# CHAPTER 2

## Role of Palladin, Nox4 and MRTF-A in Vascular Smooth Muscle Cell Differentiation Gene Expression

## 2.1 Introduction

VSMCs are not terminally differentiated and have plasticity; that is, they are able to undergo the process of dedifferentiation. Dedifferentiated VSMCs contribute to neointima formation by migrating into intima from the media in the vessel and proliferating, causing vessel occlusion. To prevent neointima formation, maintenance of the differentiated status of VSMCs is important. TGF-β is one of the most potent stimuli that induces contractile gene expression and supports maintenance of the differentiated phenotype (Hautmann et al., 1997; Liu et al., 2003). Our previous publication shows that TGF-β-induced ROS production via Nox4 activation regulates VSMC differentiation (Clempus et al., 2007; Martin-Garrido et al., 2011).

SRF is a transcription factor that is under the control of TGF-β-induced Nox4 signaling (Martin-Garrido et al., 2011). SRF regulates two types of functionally different genes depending on which co-factors bind to it. MRTF-A, one of the most powerful transcription factors, interacts with SRF and is required for differentiation marker gene expression. In fibroblasts, nuclear translocation of cytosolic MRTF-A is prohibited by monomeric G-actin binding (Miralles et al., 2003). RhoA activation by extracellular stimulation such as serum leads to the depletion of cytosolic monomeric G-actin as it is recruited to actin polymers. Low concentrations of G-actin in the cytosol result in fewer interactions with MRTF-A, and the free MRTF-A translocates to the nucleus where it binds to SRF and increases expression of differentiation genes such as smooth muscle α-actin (SMA). In VSMCs, however, MRTF-A exists in the nucleus without any

extracellular stimulation (Du et al., 2004; Hinson et al., 2007); therefore, the concepts discovered in fibroblasts cannot be applied to VSMCs. A potential mode of regulation of MRTF-A activity is phosphorylation.

One protein that has been proposed to regulate MRTF-A activity in VSMCs is palladin, an actin associated protein known to bind to MRTF-A (Jin et al., 2010). However, the signaling pathways by which palladin itself is regulated and the precise mechanisms by which palladin might alter MRTF-A activity are unknown. Based on our previous work implicating Nox4 and ROS in TGF-β-induced activation of SRF-mediated SMA expression (Martin-Garrido et al., 2011), we tested the hypothesis that Nox4 is responsible for the expression and/or the activity of MRTF-A and palladin in regulation of expression of VSMC differentiation marker genes, using SMA as a prototypical gene. We further explored the function of palladin in regulating MRTF-A phosphorylation.

## 2.2 Methods

### *2.2.1 Cell Culture*

Human aortic smooth muscle cells (here referred to as VSMCs) were obtained from Invitrogen (Mountain View, CA). Cells were cultured as recommended by the manufacturer and used between passages 5 and 9.

### *2.2.2 Materials*

TGF-β was obtained from R&D Systems (Minneapolis, MN, USA). MRTF-A (C-19) and p300 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA); palladin, SMA, and β-tubulin antibodies (Sigma, St. Louis, MO, USA); ERK and

phospho-ERK antibodies (Cell Signaling, Danvers, MA); and PP2A antibodies (Millipore, Billerica, MA) were used for Western blot. siNox4, siMRTF-A and All-Star negative control siRNA (siNeg) (Qiagen, Valencia, CA), Lipofectamine RNAiMAX (Invitrogen, Mountain View, CA), and OPTI-MEM (Gibco) were used for siRNA transfection. ROCK inhibitor (Y-27632), and ERK inhibitor (U0126) (Sigma, St. Louis, MO, USA) were used for kinase inhibition.

### *2.2.3 RNA Isolation and Quantitative RT-PCR*

Total RNA was extracted from cells using RNeasy plus kit (Qiagen), per the manufacturer's recommendations. Superscript II (Invitrogen) and random primers were used for reverse transcription. Message expression of human Nox4 (primer sequences: CTGGAGGAGCTGGCTCGCCAACGAAG and GTGATCATGAGGAATAGCACCACCACCATGCAG) and human hypoxanthine phosphoribosyl transferase 1 (hHPRT1) (primer sequences: TGACACTGGCAAAACAATGCA and GGTCCTTTTCACCAGCAAGCT) were measured by amplification of human VSMC cDNA using the LightCycler (Roche) real-time thermocycler and SYBR green dye. Specific Nox4 primers were used to measure mRNA and normalized to hHPRT1.

### *2.2.4 siRNA transfection*

For transfection with siRNA, human VSMCs were transfected with 25 nM small interfering RNA against Nox4 (siNox4#1; 5'-AAACTGAGGTACAGCTGGATG-3', siNox4#2; 5'-CAGCATCTGTTCTTAACCTCA-3'), MRTF-A (siMRTF-A; 5'-ATGGAGCTGGTGGAGAAGAAC-3') or with the All-Star negative control siRNA

(siNeg) using Lipofectamine RNAiMAX in OPTI-MEM. Cells were then cultured in serum-free medium for 24 hr before treatment. For siRNA against palladin (siPall; 5'-ATCAGTTGTACTGGACGGCTA-3'), 1 x $10^6$ cells were nucleotransfected with 200 pmol of siPall or siNeg using the U-025 program of Amaxa. After attaching, the cells were cultured in serum-free medium for 24 hr before treatment.

### *2.2.5 Western blot and Immunoprecipitation*

VSMCs were lysed in 1% Triton X-100 buffer (25 mM Hepes, 25 mM NaCl, 2.5 mM EDTA, 10 mM Na-pyrophosphate, 10 mM NaF, 0.1 mM $Na_3VO_4$, 1% Triton X-100, and protease inhibitors) for all experiments. Whole cell lysates were utilized for Western blot and immunoprecipitation experiments, as described previously (Clempus et al., 2007; Hanna et al., 2004). Protein concentration was measured using the Bradford Assay. Proteins were separated using SDS-PAGE and transferred to nitrocellulose membranes, blocked, and incubated with appropriate primary antibodies. Proteins were detected by ECL (Amersham). Band intensity was quantified by densitometry using Image J 1.46 software.

### *2.2.6 Nuclear and Cytosolic Fractionation*

VSMCs were lysed in Buffer A (10 mM Hepes (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM NaF, 0.2 mM $Na_3VO_4$, 0.4 mM PMSF, and 10 μg/ml leupeptin) with 0.625% Nonidet-P40. Cells were vortexed vigorously for 15 sec, followed by centrifugation at 21,000 x g at 4°C for 1 min to isolate the cytosolic fraction. The pellet was washed with buffer A three times. Then, to

isolate nuclear fraction, the pellets were incubated in buffer B (10 mM Hepes (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 0.2 mM NaF, 0.2 mM $Na_3VO_4$, 0.4 mM PMSF, 10 μg/ml leupeptin, and 10% sterile glycerol) for 30 min on ice and centrifuged at 20,000 x g 4°C for 20 min.

### *2.2.7 Alkaline Phosphatase treatment*

Cells were lysed in Hepes-Triton lysis buffer (Boston Bioproducts, Ashland, MA) (25 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 1% Triton-X 100) containing halt protease inhibitor EDTA-free 100x and halt phosphatase inhibitor 100x (Pierce, Rockford, IL). Lysates were either not treated or treated with 20 units of alkaline phosphatase (Fermentas) at 37ºC for 30 min and subjected to western blot analysis.

### *2.2.8 Statistical analysis*

Results are expressed as mean ± SEM from at least three independent experiments unless otherwise indicated. Significance of statistical comparisons was assessed using analysis of variance (ANOVA), followed by Bonferroni's multiple comparison post-hoc test. A value of $p<0.05$ was considered significant.

## 2.3 Experimental Results

### *2.3.1 TGF-β induces Palladin and SMA Expression*

To determine whether TGF-β alters palladin expression in VSMCs, we treated TGF-β for 24 hr and performed western blot analysis. As shown in Figure 2.1, TGF-β increased expression of the 90-kDa isoform and caused neo-expression of the 140-kDa isoform of palladin. As previously reported (Martin-

Garrido et al., 2011), TGF-β also increased SMA expression (Figure 2.1). This result supports the idea that increased palladin may be related to SMA expression.

### *2.3.2 TGF-β-induced SMA Expression Requires Palladin.*

To elucidate if TGF-β-induced SMA expression requires palladin, siRNA against palladin was transfected into human VSMCs. Knockdown of both forms of palladin was confirmed using palladin antibody, and led to decreased SMA expression (Figure 2.2). Thus, palladin is required for TGF-β-induced SMA expression.

### *2.3.3 Induction of Palladin by TGF-β is Redox-Sensitive.*

Our previous observation that TGF-β induces ROS production via Nox4, leading to regulation of SRF activity (Martin-Garrido et al., 2011) led us to test if palladin induction is regulated by ROS. We used the antioxidant N-acetylcysteine (NAC) to block ROS and measured palladin expression. We have previously shown that 20 mM NAC completely scavenges NAPDH oxidase-derived ROS in these cells (Lyle et al., 2009; Martin-Garrido et al., 2011). NAC treatment significantly reduced TGF-β-induced palladin and SMA expression (Figure 2.3). These data suggest that the induction of palladin by TGF-β requires ROS.

### *2.3.4 siNOX4 Blocks Palladin and SMA Expression.*

To determine if the ROS production that controls palladin expression is derived from Nox4, we used two different siRNA sequences against Nox4. Transfection of siNox4 diminished its mRNA level (Figure 2.4) and decreased

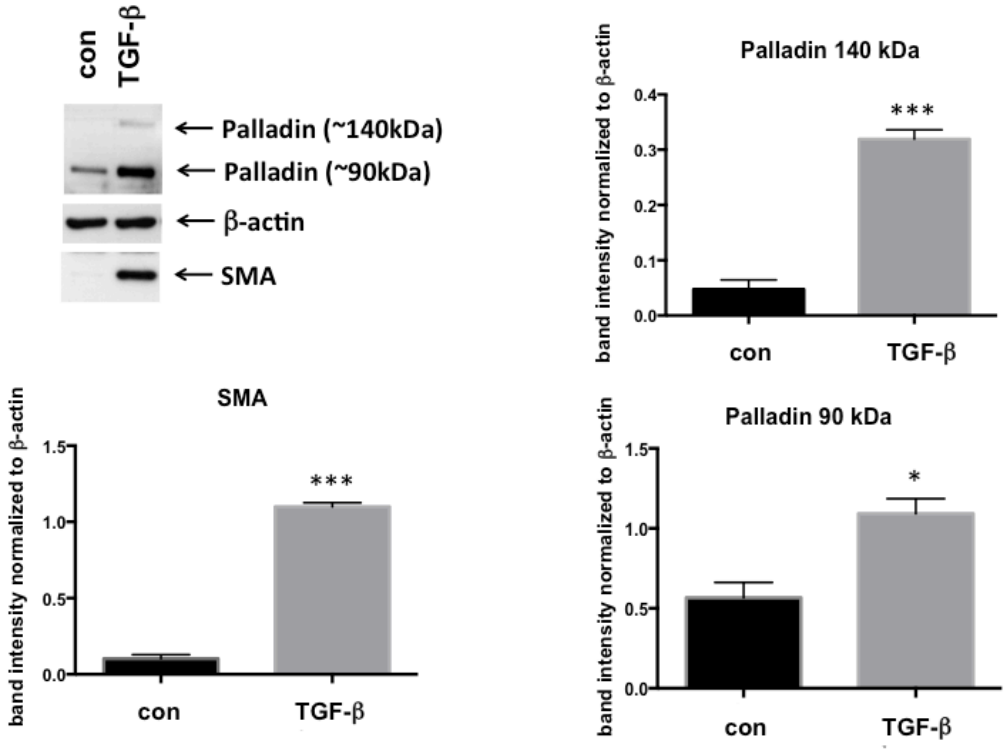


**Figure 2.1 TGF-β Induces Palladin and SMA Expression.**

Human VSMCs were incubated with TGF-β (2 ng/ml) for 24 hr. Total protein was extracted and levels of palladin and SMA were analyzed using specific antibodies. β-actin was used as a loading control. Bars are means ± SEM of 3 independent experiments. *p<0.05 vs con and ***p<0.001 vs con. Con=control cells untreated with TGF-β.

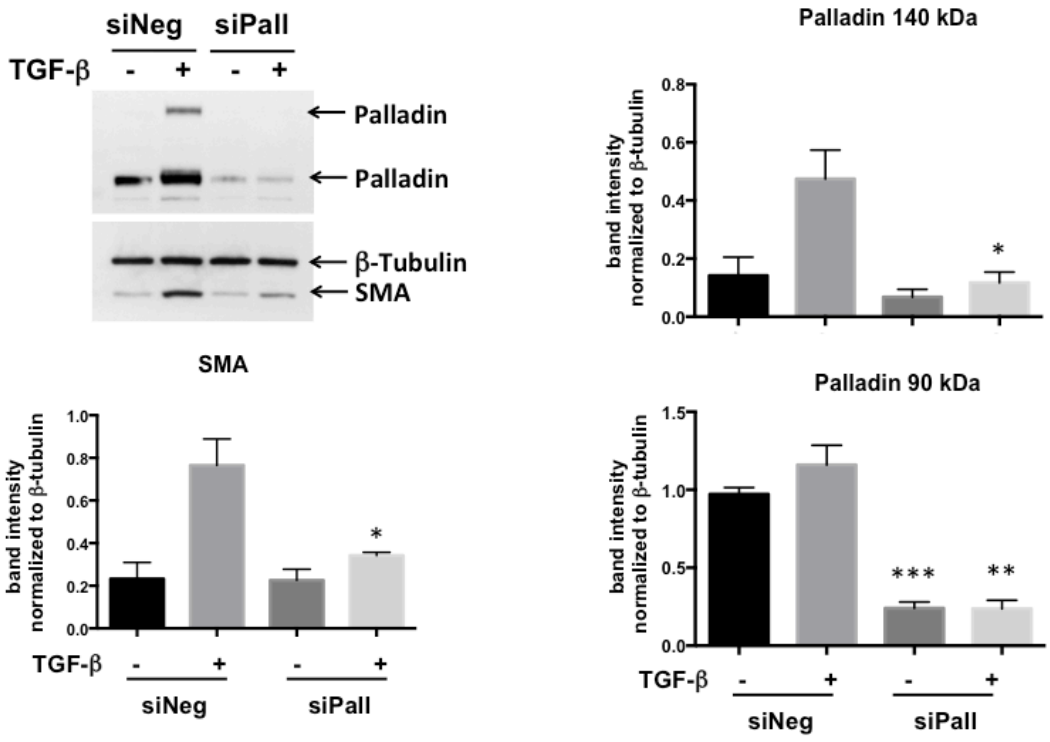


**Figure 2.2 TGF-β-Induced SMA Expression Requires Palladin.**

Human VSMCs were transfected with control siRNA (siNeg) or siRNA against palladin (siPall). After 48 hr, the cells were treated with TGF-β (2 ng/ml) for 24 hr. Total protein was extracted and levels of palladin and SMA were analyzed using specific antibodies. β-tubulin was used as a loading control. Bars are means ± SEM of 3 independent experiments. *p<0.05 vs con and ***p<0.001 vs con.

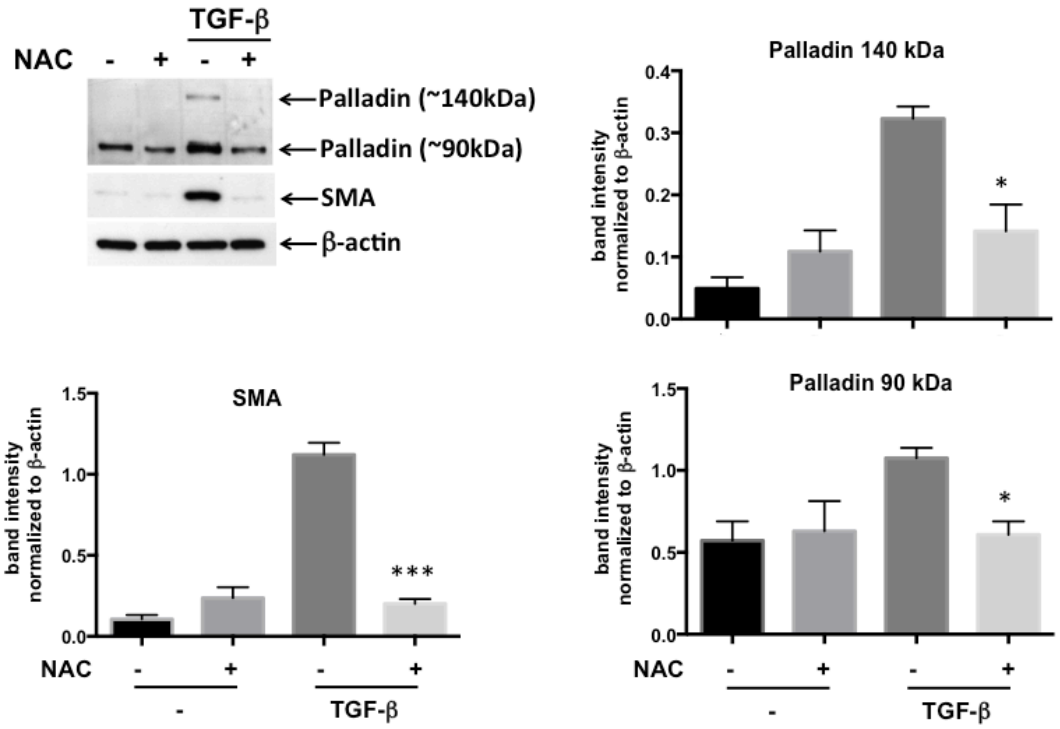


**Figure 2.3 Induction of Palladin by TGF-β is Redox-Sensitive.**

Human VSMCs were preincubated with 20 mM NAC for 1 hr and then stimulated with TGF-β (2 ng/ml) for 24 hr. Total protein was extracted and levels of palladin and SMA were analyzed using specific antibodies. β-actin was used as a loading control. Bars are means ± SEM of 3 independent experiments. *p<0.05 vs con and ***p<0.001 vs con.

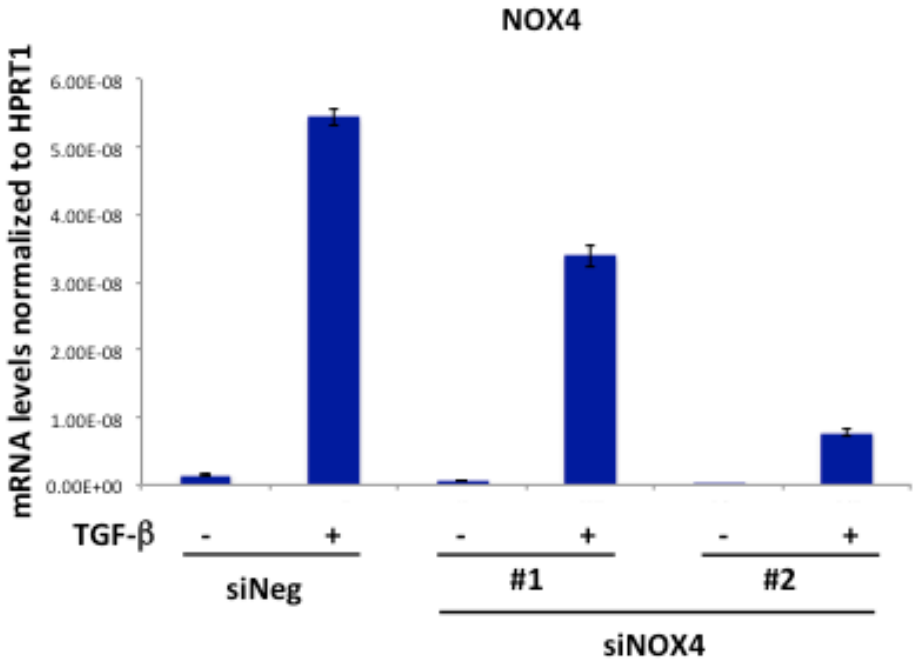


**Figure 2.4 siNOX4 Decreases mRNA Levels of NOX4.**

Human VSMCs were transfected with control siRNA (siNeg) or siRNA against NOX4 (siNOX4). Two different sequences for siNOX4 were used. After 48 hr, the cells were treated with TGF-β (2 ng/ml) for 24 hr. NOX4 mRNA was analyzed by real-time RT-PCR. Bars are means ± SEM of duplicate measurements of one experiment.

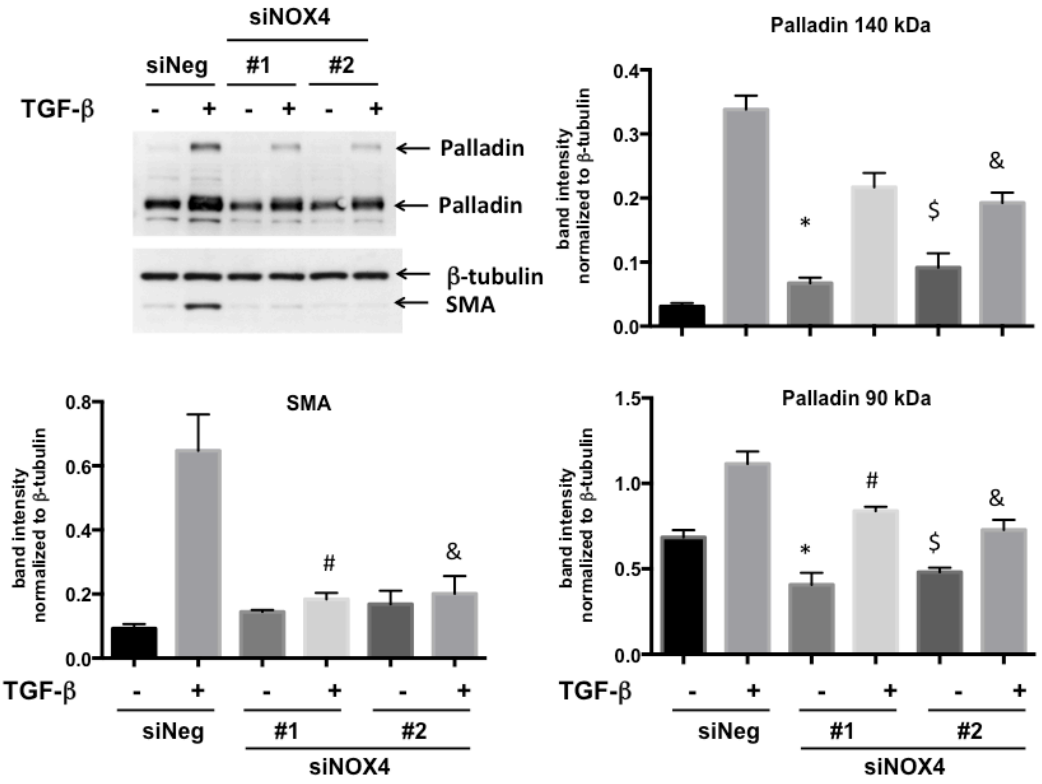


**Figure 2.5 siNOX4 Inhibits Palladin and SMA Expression.**

Human VSMCs were transfected with control siRNA (siNeg) or siRNA against NOX4 (siNOX4). Two different sequences for siNOX4 were used. After 48 hr, the cells were treated with TGF-β (2 ng/ml) for 24 hr. Total protein was extracted and levels of palladin, and SMA were analyzed using specific antibodies. β-tubulin was used as a loading control. *$p<0.05$ vs siNeg, #$p<0.05$ vs siNeg + TGF-β, \$$p<0.05$ vs siNeg, &$p<0.05$ vs siNeg + TGF-β.

TGF-β-induced palladin and SMA expression (Figure 2.5). These data show that Nox4 controls palladin and SMA expression.

### *2.3.5 TGF-β-induced MRTF-A Phosphorylation is Abolished by siPalladin.*

Jin *et al.* (Jin et al., 2010) have shown that palladin binds to MRTF-A, but did not demonstrate the function of that binding. Based on this finding, we tested if knockdown of palladin affects the expression level of MRTF-A. Surprisingly, the data show no effect of palladin depletion on MRTF-A expression, but knockdown of palladin prevented the TGF-β-stimulated molecular weight shift in MRTF-A (Figure 2.6).

MRTF-A is known to be phosphorylated on multiple sites (Miralles et al., 2003), suggesting that this shift could be due to phosphorylation. To test this possibility, we incubated alkaline phosphatase with the TGF-β treated/untreated lysate. The result shows that alkaline phosphatase treatment successfully abolished molecular weight shift of MRTF-A suggesting that the upward molecular weight shift is due to phosphorylation of MRTF-A (Figure 2.7).

To confirm that palladin acts upstream of MRTF-A and not downstream, siMRTF-A was transfected into VSMCs. siMRTF-A transfection did not change palladin expression (Figure 2.8), even though it successfully knocked down MRTF-A. TGF-β-induced SMA expression was also decreased by siMRTF-A, which confirms that MRTF-A has a key role in regulating SMA expression. These results imply that palladin acts upstream of TGF-β-induced MRTF-A phosphorylation.

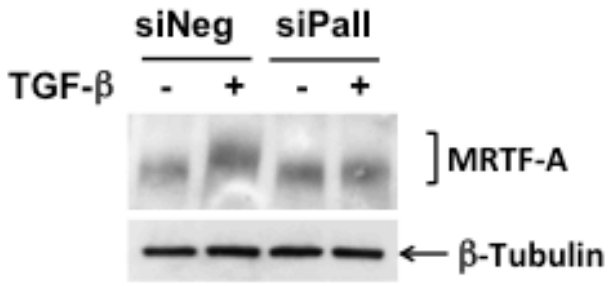


**Figure 2.6 TGF-β-induced MRTF-A Molecular Weight Shift is Abolished by siPalladin.**

Human VSMCs were transfected with control siRNA (siNeg) or siRNA against palladin (siPall). After 48 hr, the cells were treated with TGF-β (2 ng/ml) for 24 hr. Total protein was extracted and levels of MRTF-A were analyzed using specific antibody. β-tubulin was used as a loading control. This figure is representative of 3 independent experiments.

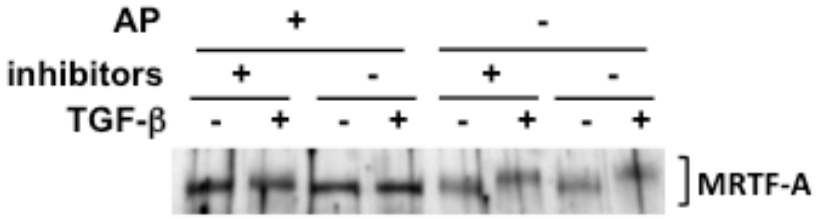


**Figure 2.7 Alkaline Phosphatase Diminishes Molecular Weight Shift in MRTF-A.**

Human VSMCs were treated with TGF-β (2 ng/ml) for 24 hr. Lysates were incubated with or without alkaline phosphatase at 37ºC for 30 min. Western blot was performed using MRTF-A antibody. This figure is representative of 3 independent experiments.

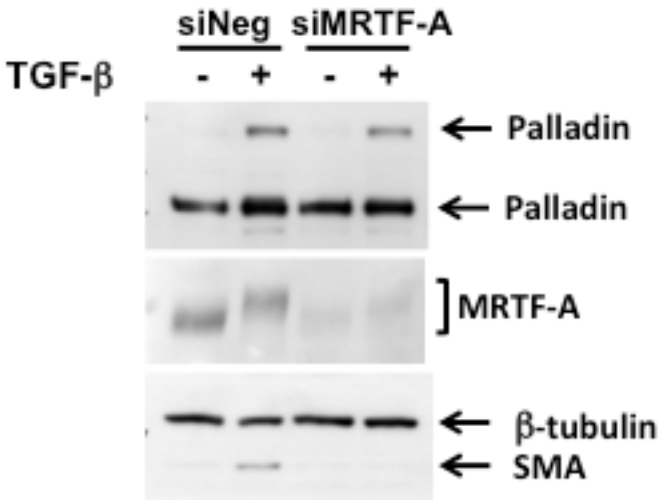


**Figure 2.8 Knockdown of MRTF-A Does Not Alter TGF-β-induced Palladin Expression.**

Human VSMCs were transfected with control siRNA (siNeg) or siRNA against MRTF-A (siMRTF-A). After 48 hr, the cells were treated with TGF-β (2 ng/ml) for 24 hr. Total protein was extracted and levels of palladin, MRTF-A and SMA were analyzed using specific antibodies. β-tubulin was used as a loading control. Blot is representative of 2 independent experiments.

### *2.3.6 TGF-β-induced MRTF-A Phosphorylation is Redox-Sensitive.*

Because TGF-β-induced palladin expression is redox-sensitive, we predicted that MRTF-A phosphorylation also requires ROS. VSMCs were treated with NAC prior to TGF-β addition and then MRTF-A phosphorylation was measured using the molecular weight shift on western blot. MRTF-A phosphorylation by TGF-β was abolished by NAC treatment (Figure 2.9). These data show that MRTF-A phosphorylation is regulated by ROS.

### *2.3.7 siNOX4 Blocks MRTF-A Phosphorylation and its Expression.*

To see if the ROS that regulate MRTF-A phosphorylation are released from Nox4, we transfected our two different sequences of siNox4 and then observed MRTF-A phosphorylation. The result indicates that MRTF-A phosphorylation is diminished by knockdown of Nox4 and is expression level also decreased (Figure 2.10). These data suggest that MRTF-A phosphorylation is under the control of ROS produced from Nox4.

### *2.3.8 TGF-β-induced MRTF-A Phosphorylation is Mediated by ROCK.*

Next, we explored which kinase mediates the phosphorylation of MRTF-A. Since palladin itself is not a kinase, we investigated other options. In NIH3T3 cells, C3 transferase (Rho GTPase family inhibitor) and U0126 (MEK1/2 inhibitor) treatment abolished the upward shift in MRTF-A molecular weight in response to fetal calf serum (Miralles et al., 2003), implicating Rho Kinase (ROCK) and the ERK pathway in MRTF-A phosphorylation. We used Y-27632, more specific ROCK inhibitor than C3 transferase, and U0126 to determine if either of these pathways mediate MRTF-A phosphorylation in response to TGF-β in VSMCs. Y-

27632 treatment reduced MRTF-A phosphorylation, while U0126 alone had no effect (Figure 2.11). This result shows that in VSMCs, MRTF-A phosphorylation is mediated by ROCK but not by ERK, and raises the possibility that palladin may be involved in regulating ROCK activity. The effect of palladin on ROCK activity remains to be measured.

### *2.3.9 Palladin Does Not Affect Nuclear Localization of MRTF-A*

There are several other potential modes of regulation of MRTF-A phosphorylation by palladin including regulation of nuclear localization or phosphatase activity. To test the possibility that palladin affects nuclear localization of MRTF-A, we fractionated the cells into cytosolic and nuclear fractions after TGF-β treatment. The result shows that the majority of palladin and MRTF-A resides in the nucleus and siPall does not affect the amount of MRTF-A in the nucleus, suggesting it does not have a role in translocation of MRTF-A. As expected, siPall did reduce MRTF-A phosphorylation in the nucleus (Figure 2.12).

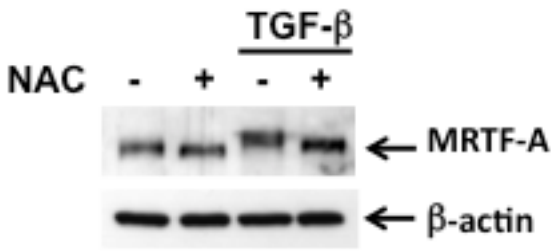


**Figure 2.9 TGF-β-induced MRTF-A Phosphorylation is Redox-Sensitive.**

Human VSMCs were preincubated with 20 mM NAC for 1 hr and then stimulated with TGF-β (2 ng/ml) for 24 hr. Total protein was extracted and levels of MRTF-A were analyzed using specific antibodies. β-tubulin was used as a loading control. This figure is representative of 3 independent experiments.

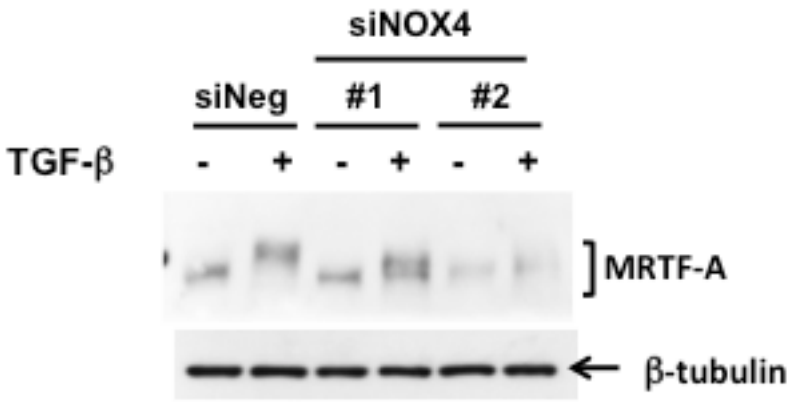


**Figure 2.10 siNOX4 Blocks MRTF-A Phosphorylation and its Expression.** Human VSMCs were transfected with control siRNA (siNeg) or siRNA against NOX4 (siNOX4). Two different sequences for siNOX4 were used. After 48 hr, the cells were treated with TGF-β (2 ng/ml) for 24 hr. Total protein was extracted and levels of MRTF-A were analyzed using specific antibodies. β-tubulin was used as a loading control. This figure is representative of 3 independent experiments.

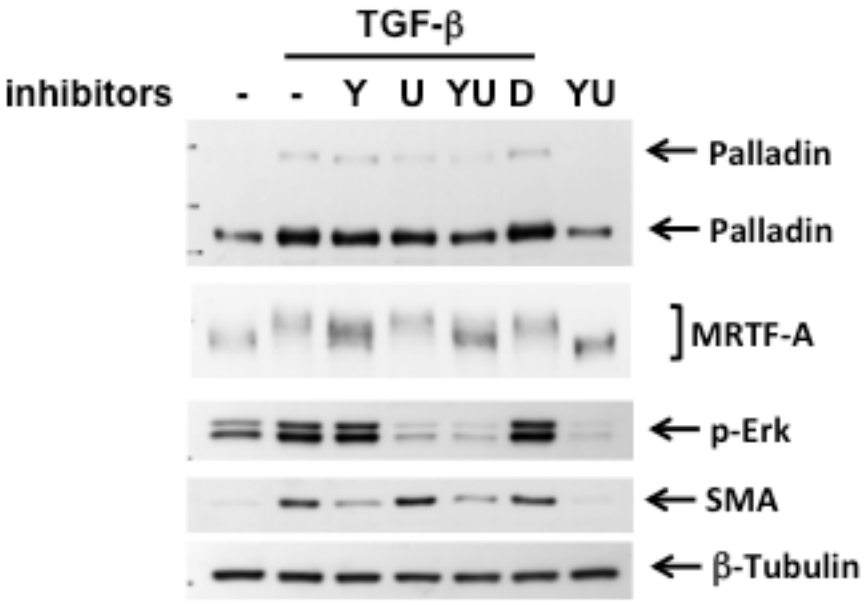


**Figure 2.11 TGF-β-induced MRTF-A Phosphorylation is Mediated by ROCK.** Human VSMCs were preincubated with 10 μM ROCK inhibitor (Y-27632) for 1 hr or 30 μM ERK inhibitor (U0126) for 30 min. Then the cells were treated with TGF-β (2 ng/ml) for 24 hr. Total protein was extracted and levels of palladin, MRTF-A, phospho-ERK, and SMA were analyzed using specific antibodies. β-tubulin was used as a loading control. Y; Y-27632, U; U0126, D; DMSO vehicle control. This figure is representative of 3 independent experiments.

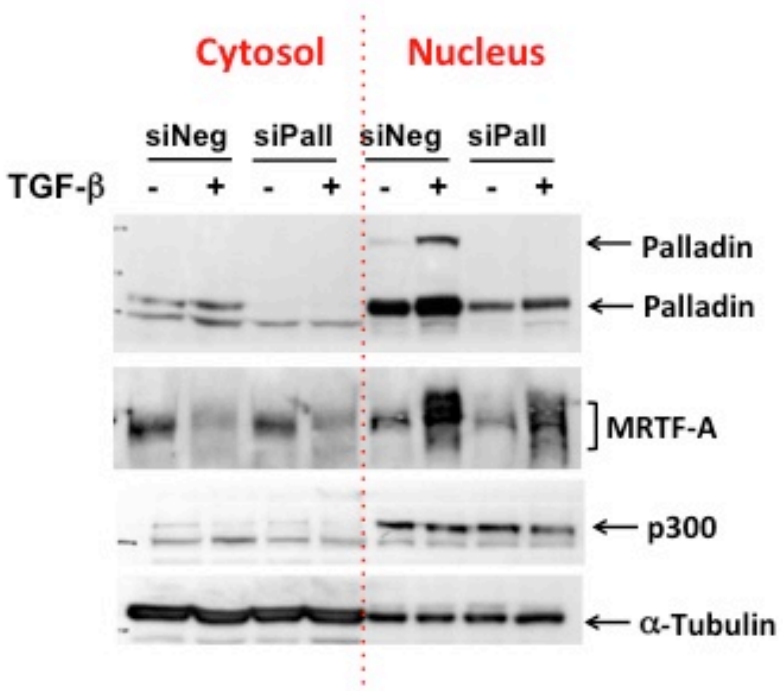


**Figure 2.12 Palladin Does Not Affect Nuclear Localization of MRTF-A.** Human VSMCs were transfected with control siRNA (siNeg) or siRNA against palladin (siPall). After 48 hr, the cells were treated with TGF-β (2 ng/ml) for 24 hr. The cells were fractionated into cytosol and nucleus. Total protein was extracted and levels of palladin, and MRTF-A using specific antibodies. p300 and α-tubulin were used as loading controls. This figure is representative of 3 independent experiments.

### *2.3.10 Palladin may sequester PP2A from MRTF-A by Binding to PP2A.*

Another possibility to explain MRTF-A phosphorylation by palladin is via inhibition of phosphatase(s). We focused on protein phosphatase 2A (PP2A), a serine/threonine phosphatase with broad substrate specificity that exists in both cytosol and nucleus (McCright et al., 1996). We first tested the hypothesis that palladin might form a complex with the phosphatase, preventing its proximity to MRTF-A. We immunoprecipitated palladin and blotted for PP2A. The result shows that palladin binds to PP2A, which suggests that palladin may sequester PP2A from MRTF-A resulting in prevention of blocking MRTF-A phosphorylation (Figure 2.13). The slightly increased binding of PP2A in TGF-β treated cells supports the idea that TGF-β-induced palladin expression increases sequestration of PP2A from MRTF-A. Further experiments are necessary to test this hypothesis.

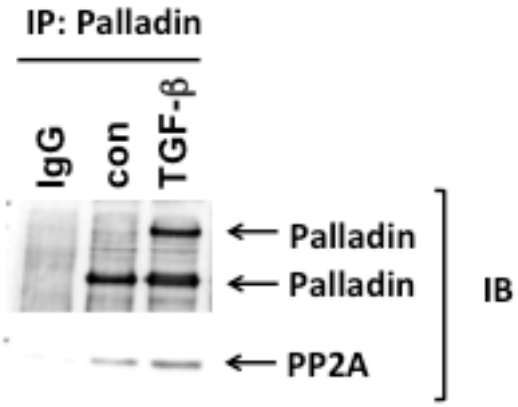


**Figure 2.13 Palladin Binds to PP2A**

Human VSMCs were treated with TGF-β (2 ng/ml) for 24 hr. The cells were immunoprecipitated (IP) with palladin antibody and immunoblotted (IB) with palladin (*upper*), and PP2A (*lower*) antibodies.

## 2.4 Discussion

In this study, we demonstrated that 1) TGF-β-induced ROS production from Nox4 regulates palladin expression and MRTF-A phosphorylation; 2) knockdown of palladin abolishes MRTF-A phosphorylation and SMA expression; 3) the kinase regulating MRTF-A phosphorylation is ROCK; 4) palladin has no effect on nuclear localization of MRTF-A, but may sequester PP2A from MRTF-A to control phosphorylation of MRTF-A.

Palladin has multiple isoforms created by alternative transcription initiation and splicing that are differentially expressed in cells and tissues (Otey et al., 2009). The 90-kDa form of palladin was previously reported to be upregulated and the 140-kDa isoform of palladin to be neo-expressed by TGF-β during fibroblast-to-myofibroblast conversion (Ronty et al., 2006). Palladin has several functions in actin-based subcellular structures (Otey et al., 2005), acting as a molecular scaffold and actin cross linker (Boukhelifa et al., 2001; Dixon et al., 2008; Goicoechea et al., 2006). In addition, recent findings suggested its involvement in transcriptional regulation, showing that palladin is essential for SMC marker gene expression (Jin et al., 2010; Jin et al., 2009b). How palladin participates in transcriptional regulation is not clear; however, palladin was found in the nucleus binding with MRTF-A (Jin et al., 2010), raising the possibility that palladin may have a role in regulation of MRTF-A activity for SMC marker gene expression.

Previously, hydrogen peroxide was used to see if ROS alter palladin expression in SMCs (Jin et al., 2009a). The authors found that extracellular

application of 100 μM $H_2O_2$ decreased palladin expression after 24 hr of treatment. In contrast, we found that the intracellular production of likely smaller amounts of $H_2O_2$ is actually required for TGF-β-induced palladin expression. The discrepancy between these studies most likely relates to the lack of specificity of extracellularly applied $H_2O_2$, which easily diffuses through membranes, is not locally produced and is applied in amounts in great excess of those produced locally by NADPH oxidases.

Our results show that both palladin and MRTF-A are under the regulation of Nox4-dervied ROS, suggesting possible cross-talk in the signaling. Knockdown of palladin using siRNA abolishes MRTF-A phosphorylation but not expression, which indicates that palladin is an upstream protein regulating the phosphorylation of MRTF-A. The fact that knockdown of MRTF-A did not affect the expression levels of palladin confirms the idea that palladin is an upstream protein. Since palladin does not have kinase activity, we experimented to find downstream kinases that mediate MRTF-A phosphorylation. Our results show that ROCK regulated phosphorylation of MRTF-A but ERK did not have an effect. This was surprising because ERK has been implied as an effective kinase in phosphorylation of MRTF-A in NIH3T3 and Hela cells (Miralles et al., 2003; Muehlich et al., 2008). Possible explanations for this discrepancy include differences in cell type or in subcellular localization, since in VSMCs MRTF-A is in the nucleus in unstimulated cells, while in fibroblasts, it resides in the cytosol. The huge molecular weight change of MRTF-A in response to TGF-β suggests

that MRTF-A is phosphorylated at multiple sites, which leaves the possibility that additional kinases phosphorylate MRTF-A.

Based on previous data, the role of palladin in MRTF-A phosphorylation could be narrowed down to three likely possibilities. First, palladin may act to regulate the kinase activity. Therefore, the effect of palladin on ROCK activity should be tested in the future. Second, palladin may help to localize MRTF-A to the nucleus. However, knockdown of palladin only changed MRTF-A's phosphorylation status but not its translocation. These data and the treatment with kinase inhibitors (Figure 2.11) show that the inhibition of phosphorylation is not a key factor for localization of MRTF-A. Third, palladin may regulate phosphatase activity. We observed binding of palladin with PP2A and a slight increase of binding in the presence of TGF-β. One highly possible role of palladin in regulation of MRTF-A phosphorylation thus may be the sequestration of PP2A from MRTF-A. Okadaic acid, an inhibitor of PP1- and PP2A-like phosphatases, prevented the dephosphorylation of MRTF-A in neurons (Kalita et al., 2006), supporting the concept that PP2A may be a crucial enzyme for maintenance of MRTF-A phosphorylation. Future work will be necessary to dissect the precise relationship among palladin, PP2A and MRTF-A.

PP2A is an attractive candidate as the link between palladin and MRTF-A also because it has been shown to be regulated by ROS. PP2A activity was inhibited by $H_2O_2$ in Caco-2 cells (Rao and Clayton, 2002) and in VSMCs (Rocic et al., 2003), suggesting that Nox4-derived ROS might be an important regulator not only for palladin expression, but also for PP2A activity. Thus, it is likely that

TGF-β-induced ROS production from Nox4 has multiple roles: it may increase palladin expression, decrease PP2A activity, and increase sequestration of PP2A from MRTF-A. This is supported by the data in Figure 2.5, which shows that siNox4 was more effective against SMA than palladin, suggesting Nox4 has multiple roles and palladin is only one of them.

Phosphorylation is a rapid and often transient event commonly observed in a few minutes after extra- or intra- cellular stimulation. However, MRTF-A phosphorylation occurs within an hour after TGF-β treatment and lasts at least for 24 hr (data not shown). Therefore, the sequestration of PP2A by palladin may be a key step in maintaining the phosphorylation status. Nevertheless, exploring other possible phosphatases will be required for better understanding of Nox4/palladin/MRTF-A signal transduction in VSMC differentiation.

In conclusion, these data demonstrate that ROCK-mediated MRTF-A phosphorylation is crucial in regulation of SRF responsive genes in VSMCs, and that this event is regulated by Nox4-mediated palladin expression and its interaction with PP2A. These findings may contribute to the discovery of new targets for prevention and recovery from cardiovascular diseases such as atherosclerosis and in-stent restenosis.

# CHAPTER 3

## Discussion

The data presented in Chapter 2 clearly demonstrate a novel important role for Nox4-mediated regulation of MRTF-A and palladin in VSMC differentiation marker gene expression. Specifically, we illustrate that TGF-β-induced ROS production from Nox4 is required for increased expression of palladin and phosphorylation of MRTF-A. We also describe that palladin is necessary for the increase of TGF-β-induced phosphorylation of MRTF-A. The kinase involved in MRTF-A phosphorylation by TGF-β is ROCK, as assessed by inhibition of ROCK activity during TGF-β treatment. Furthermore, interaction between palladin and PP2A raises the possibility that PP2A may mediate dephosphorylation of MRTF-A. Thus, the potential role of palladin in mediating differentiation gene expression is sequestration of PP2A from MRTF-A, which results in sustained MRTF-A phosphorylation.

Although the data presented here support the hypothesis shown in Figure 1.1, further experiments are needed. The potential interaction between palladin and PP2A in TGF-β treated and untreated cells should be confirmed. siRNA against PP2A should be used to confirm that PP2A dephosphorylates MRTF-A in VSMCs. Additionally, identifying regions of palladin that may be important for binding with PP2A could provide insight as to how palladin regulates PP2A and consequently MRTF-A phosphorylation. Moreover, PP2A might be directly regulated by Nox4 based on the previous findings (Rao and Clayton, 2002; Rocic et al., 2003). PP2A activity should be measured in siNox4 transfected cells to see if PP2A activity is controlled by Nox4. To demonstrate if Nox4 directly oxidizes PP2A, point mutations at predicted oxidation sites in PP2A should be

made, and the mutants should be transfected into the cells to determine if Nox4-mediated oxidation on PP2A is a key event in the regulation of its activity. Finally, the relevance of these pathways to expression of other SMC-specific differentiation genes should be investigated.

The role of palladin may not be restricted to regulation of PP2A. There are other possible roles of palladin in regulation of MRTF-A phosphorylation. First, palladin may also interact with other phosphatases to prevent dephosphorylation of MRTF-A. Previous experiments using okadaic acid (Kalita et al., 2006) indicate that both PP1 and PP2A may have a role in prevention of MRTF-A phosphorylation. Therefore, the possible involvement of PP1 should be tested. siPP1 could be used to see if PP1 dephosphorylates MRTF-A and then an immunoprecipitation assay should be conducted to determine if palladin and PP1 interact. Identifying their binding sites by expressing fragments of the protein and using mutagenesis to confirm which regions of the proteins are important for interaction would be informative. Mutants could then be tested for their effect on dephosphorylation of MRTF-A. Second, palladin may modulate kinase activity. Since the ROCK inhibitor interferes with phosphorylation of MRTF-A, future studies should test TGF-β-induced ROCK activity using siPalladin to determine if siPalladin disrupts activation of ROCK. Third, palladin may bind to MRTF-A and prevent phosphatase access. The N-terminus of palladin is known to bind to the N-terminus of MRTF-A, and palladin does not compete with G-actin, which means it does not bind to the RPEL domain in MRTF-A (Jin et al., 2010). Mutation at the expected binding site on palladin

would determine if MRTF-A phosphorylation is prevented when palladin does not bind to MRTF-A.

Phosphorylation of MRTF-A is closely related to its activity. The ROCK inhibitor decreased TGF-β-induced phosphorylation of MRTF-A (Figure 2.11). However, due to the large molecular weight shift of MRTF-A, it is conceivable that multiple kinases are involved in phosphorylation of MRTF-A or that multiple sites on MRTF-A are phosphorylated. There are 8 highly possible ROCK phosphorylation sites predicted by GPS 2.1 program. Two occur 5' of the RPEL1 domain: one serine (16Ser) and one threonine (22Thr). In between the RPEL 2 domain and the basic domain four possible phosphorylation sites were found; 215Ser, 220Ser, 223Ser, and 318Ser. Within the transcriptional activation domain, there are two prospective phosphorylation sites, 743Ser and 744Ser. Point mutation on the 8 potential phosphorylation sites would be an ideal experiment to explore the functional role of MRTF-A phosphorylation. However, since C-terminal deletion mutants have been shown to lose MRTF-A activity possibly due to the loss of a transcriptional activation domain (Cen et al., 2003), the last two sites (743Ser and 744Ser) are possibly related to direct regulation of MRTF-A activity. Once these mutants are made, they should be transfected into VSMCs to see if they alter TGF-β-induced SMA expression. Based on the results, a custom antibody for phospho-MRTF-A at the most likely sites could be generated. Because palladin-regulated phosphorylation sites should also be sensitive to Nox4 inhibition and loss of palladin expression, the phospho-antibody should be used in TGF-β-stimulated cells transfected with siNox4 or siPalladin,

with the expectation that loss of either Nox4 or palladin would block MRTF-A phosphorylation on that particular site.

Future studies designed to identify other phosphorylation sites on MRTF-A induced TGF-β would add to our understanding of how phosphorylation of this important transcription factor globally regulates differentiation gene expression. To do this, mass spectrometry may be an efficient tool. Discovery of which kinases may be involved based on sequence of the phosphorylation sites found by mass spectrometry would generate testable hypotheses. Inhibitor treatment for the predicted kinases could then be performed to confirm that the kinases phosphorylate MRTF-A. Also, the identified phosphorylation sites could be mutated and checked for their ability to inhibit SMA or other differentiation gene expression to determine the function of each site.

The studies described in this thesis rely solely on cell culture models to investigate signaling pathways leading to differentiation gene expression. Future studies to determine the physiological and pathological relevance of the identified pathways should be performed using Nox4 knockout (KO) mice and palladin KO mice. One model that could be used is redifferentiation of SMCs after vascular injury. Fourteen to 21 days after wire or balloon injury of rat or mouse arteries, respectively, the neointima is fully developed and the resident SMCs begin to revert to the differentiated phenotype and re-express differentiation genes (Clempus et al., 2007; Martin-Garrido et al., 2011). Typically, at 7 days after wire or balloon injury, VSMC differentiation has not occurred; with the progression of the injury, at day 21, differentiated VSMCs should be observed. Therefore, at 7,

14 and 21 days after wire or balloon injury, the whole artery should be collected and lysed for western blot and sectioned for immunohistochemistry and the difference in expression levels of MRTF-A, palladin, and SMA in the KO and wild-type (WT) mice should be analyzed. Also, phosphorylation of MRTF-A should be evaluated to elucidate the physiological implications of the phosphorylation. Based on our cell culture data, we would expect that neointimal area would be greater in both knockout animals, since Nox4 and palladin would enhance VSMC redifferentiation and thus limit further neointimal formation. In WT mice, MRTF-A, palladin, and SMA expression should be increased at day 21 compared to day 7, but Nox4 KO mice may have a smaller induction than the WT. Palladin KO mice may have a similar phenotype except palladin expression should be not be detected. The whole arteries from each KO mice and WT mice could also be processed for a PP2A activity assay. PP2A activity would be expected to be decreased in a time dependent manner in WT mice. In Nox4 KO mice, PP2A activity may be increased at day 21 compared to WT mice. In palladin KO mice, the activity may not be changed if the regulation of its activity is solely from Nox4. However, if palladin regulates PP2A activity directly, palladin KO mice may have increased PP2A activity at day 21 compared to WT mice.

In summary, the data provided in this thesis link Nox4-mediated ROS production to MRTF-A phosphorylation and palladin expression in the control of differentiation marker gene expression in VSMCs. Maintenance of VSMC differentiation is clinically important in prevention of cardiovascular diseases such as restenosis after angioplasty and atherosclerosis. The present study provides

information on signaling pathways important in the maintenance of differentiation, or prevention of dedifferentiation, of VSMCs, namely phosphorylation of MRTF-A, its regulating kinases or phosphatases and their interacting partners.

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