CHARACTERIZATION OF A NOVEL IN VITRO MODEL FOR SMOOTH MUSCLE DIFFERENTIATION AND THE ROLE OF DEDICATOR OF CYTOKINESIS 2 IN SMOOTH MUSCLE DIFFERENTIATION, PHENOTYPIC MODULATION, AND VASCULAR REMODELING

by

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(Under the Direction of SHI-YOU CHEN)

ABSTRACT

Smooth muscle cell (SMC) differentiation is an important process during embryonic development. In the project, we developed a novel in vitro model for SMC differentiation from human embryonic stem cell-derived mesenchymal cells (hES-MCs). hES-MCs were differentiated to SMCs by transforming growth factor-β (TGF-β) in dose and time-dependent manners. hES-MC-derived SMCs had an elongated and spindle-shaped morphology and contracted in response to the induction of carbachol and KCl. TGF-β-treated hES-MCs sustained the endothelial tube formation for a longer time due to the sustained SMC phenotype. TGF-β-induced differentiation was both Smad- and SRF/myocardin-dependent. We also identified dedicator of cytokinesis 2 (DOCK2) as one of the factors mediating the SMC differentiation. DOCK2 was important in mediating the TGF-β-induced SMC differentiation. TGF-β induced DOCK2 expression during SMC differentiation. DOCK2 overexpression induced while DOCK2 knockout inhibited SMC early marker gene expression. DOCK2 induced SMC differentiation by

activating phosphorylated p38 MAPK. P38 pathway inhibitor dramatically decreased DOCK2-induced SMC marker gene expression. In vivo, reduced blood vessel formation was observed in the DOCK2-/- embryo yolk sac compared to the WT yolk sac. Hemorrhage was also evident in the DOCK2-/- embryo. Histological analysis revealed defective expression of calponin and α-SMA in dorsal aorta of DOCK2-/- embryo. In addition, DOCK2 played a role in VSMC phenotypic modulation and vascular remodeling. Platelet-derived growth factor-BB (PDGF-BB) induced DOCK2 expression while modulating VSMC phenotype. DOCK2 overexpression inhibited while DOCK2 knockout enhanced SMC marker gene expression in VSMC. DOCK2 regulated VSMC phenotype through suppression myocardin/ SRF-mediated transcription of VSMC marker genes. DOCK2 was essential for PDGF-BB-induced VSMC proliferation and migration. DOCK2 stimulated VSMC migration via induction of focal adhesion contact and stress fiber formation. In a rat carotid artery balloon-injury model, DOCK2 was induced in media layer and neointima VSMCs following vascular injury. Knockdown of DOCK2 inhibited the neointima formation. Knockout of DOCK2 in mice markedly blocked ligation-induced intimal hyperplasia. Therefore, DOCK2 is a novel regulator for VSMC phenotypic modulation and vascular lesion formation following vascular injury. Targeting DOCK2 is a potential therapeutic strategy for the prevention of vascular remodeling in proliferative vascular diseases.

INDEX WORDS: Vascular smooth muscle cells, Human embryonic stem cell-derived mesenchymal cells, Transforming growth factor-β, Differentiation, Dedicator of cytokinesis 2, Platelet-derived growth factor-BB, Migration, Proliferation, Vascular remodeling

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CHAPTER 1

INTRODUCTION

Problem statement

Vascular smooth muscle cells (SMCs) play a pivotal role in angiogenesis and vasculogenesis during embryonic development. Lineage-tracing studies have shown that during embryonic development, SMCs originate from at least eight progenitors including neural crest, secondary heart field, somites, mesoangioblasts, proepicardium, splanchnic mesoderm, mesothelium and various stem cells. SMC differentiation is controlled by different intracellular mechanisms among distinct SMC subtypes. The molecular mechanisms governing SMC differentiation especially the progenitor-specific regulation, however, remain largely unknown. This is due to, at least in part, the lack of in vitro model systems from different progenitors. Therefore, independent model systems using different progenitors are essential for understanding the molecular mechanisms controlling SMC differentiation from different origins. The most common precursor for vascular smooth muscle is poorly defined mesenchymal cells derived from the mesoderm. In order to explore the molecular regulation of SMC differentiation from the mesoderm, we aimed to develop another model system using human embryonic stem cell-derived mesenchymal cells (hES-MCs). hES-MCs are natural SMC progenitors for mesoderm-derived SMCs. hES-MCs were derived from H9 human embryonic stem cells. A large amount of environmental cues such as growth factors/inhibitors, inflammatory mediators, and cell-matrix interactions have been shown to regulate SMC differentiation.

Transforming growth factor-beta (TGF-β) is among the most potent soluble growth factors that activate SMC contractile gene expression in both specified SMC and non-SMC types. It has been well established that the TGF-β/Smad signaling pathway plays critical roles in the SMC differentiation of pluripotent mesenchymal progenitors such as C3H/10T1/2 (10T1/2). However, TGF-β downstream targets that regulate SMC differentiation remain largely unknown. Dedicator of cytokinesis 2 (DOCK2) is an atypical guanine nucleotide exchange factor for the Rho-small guanine triphosphatase. Under physiological conditions, DOCK2 is mainly expressed in hematopoietic cells, and involved in lymphocyte migration and activation via regulating actin cytoskeleton through Rac activation. It is unknown, however, if DOCK2 is involved in regulating SMC differentiation.

Vascular remodeling contributes to the development of a number of vascular disorders including restenosis after angioplasty, transplant vasculopathy, hypertension, and atherosclerosis, etc. Medial VSMC phenotypic modulation from a contractile to a synthetic phenotype triggered by damaging to blood vessel walls followed by VSMC migration and proliferation plays a major role in injury-induced vascular remodeling. Elucidating mechanisms underlying VSMC phenotypic modulation, migration and proliferation, therefore, is critical for understanding the etiology of above-mentioned vascular proliferative disorders and for developing effective therapeutics to block the narrowing of vessel lumen due to vascular remodeling. In this study, we will determine if DOCK2 is a novel regulator of VSMC phenotypic modulation and an essential factor contributing to vascular remodeling.

The hypothesis of the dissertation

Since SMC differentiation is an important process in vascular development, a new robust model system is essential for better understanding of the molecular mechanisms governing SMC differentiation from mesoderm. The model system described in this study is likely an ideal model for SMC differentiation due to the following facts: (1) hES-MCs are likely the natural SMC progenitors because most of the vascular SMCs are derived from mesoderm; (2) hES-MCs are easy to culture. It only needs αMEM and 10% MSC-qualified fetal bovine serum. The cells can be split up to 10 passages; (3) Since hES-MCs are derived from human embryonic stem cells, this model may be used to study SMC differentiation in humans. The **goal** of this part of the project is to establish a model system using human embryonic stem cell-derived mesenchymal cells (hES-MCs).

Another **goal** is to identify TGF-β downstream targets important for SMC differentiation using the newly established SMC differentiation models (hES-MCs and C3H/10T1/2 or 10T1/2). Dedicator of cytokinesis 2 (DOCK2) is an atypical guanine nucleotide exchange factor for the Rho-small guanine triphosphatase. In TGF-β-induced SMC differentiation of hES-MCs and 10T1/2 cells, DOCK2 is upregulated and activated along with SMC markers. We will determine if DOCK2 is involved in regulating SMC differentiation. In this study, we have also found that platelet-derived growth factor-BB (PDGF-BB) induced DOCK2 expression in VSMC while modulating VSMC phenotype. Another **goal** of our project is to demonstrate that DOCK2 is a novel regulator of VSMC phenotypic modulation and an essential factor contributing to vascular remodeling.

The specific aims of the dissertation

The first aim is to establish a novel in vitro model for SMC differentiation from hES-MCs that can be used for studying human SMC differentiation from mesoderm during vascular development. We will: 1) determine if hES-MCs are differentiated to SMCs by TGF-β in dose and time-dependent manners. 2) test if hES-MC-derived SMCs have an elongated and spindle-shaped morphology and contract in response to the induction of carbachol and KCl and if hES-MC-derived SMCs can sustain the endothelial tube formation for a longer time. 3) verify if the TGF-β-induced hES-MCs differentiation is both Smad- and SRF/myocardin-dependent.

The second aim is to identify dedicator of cytokinesis 2 (DOCK2) as one of the factors mediating the SMC differentiation. We will determine: 1) if DOCK2 can be induced by TGF-β during SMC differentiation. 2) if DOCK2 is involved in TGF-β-induced expression of SMC early markers by knockdown of DOCK2 using Ad-shDOCK2 or overexpression of DOCK2. 3) the possible mechanisms that are required for DOCK2 function in inducing SMC differentiation.

The third aim is to determine if DOCK2 play a role in VSMC phenotypic modulation and vascular remodeling. We will test if: 1) PDGF-BB induced DOCK2 expression while modulating VSMC phenotype. 2) DOCK2 is involved with SMC marker gene expression in primary VSMC. 3) myocardin/serum response factor (SRF) is involved in DOCK2 regulated VSMC phenotype. 4) DOCK2 is essential for PDGF-BB-induced VSMC proliferation and migration. 5) DOCK2 can be induced and influence the neointa formation in a rat carotid artery balloon-injury model.

CHAPTER 2

LITERATURE REVIEW

I. Transforming Growth Factor-β and Smooth Muscle Differentiation

Transforming growth factor (TGF)-β family members are multifunctional cytokines regulating diverse cellular functions such as growth, adhesion, migration, apoptosis, and differentiation. TGF-βs elicit their effects via specific type I and type II serine/threonine kinase receptors and intracellular Smad transcription factors. Knockout mouse models for the different components of the TGF-β signaling pathway have revealed their critical roles in smooth muscle cell (SMC) differentiation. Genetic studies in humans have linked mutations in these signaling components to specific cardiovascular disorders such as aorta aneurysm and congenital heart diseases due to SMC defects. In this review, the current understanding of TGF-β function in SMC differentiation is highlighted, and the role of TGF-β signaling in SMC-related diseases is discussed.

TGF-β SIGNALING TRANSDUCTION

Transforming growth factor-β is the founding member of the TGF-β superfamily that comprise TGF-βs, activins, bone morphogenetic proteins (BMPs), and growth and differentiation factors (GDFs) [1]. Three TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3) have been identified in mammals. In most cases, these isoforms exhibit similar functional properties and regulate various cellular activities including cell growth, differentiation,

apoptosis and extracellular matrix synthesis in endothelial cells and vascular smooth muscle cells [2-8].

TGF-β ligands are synthesized as latent precursor molecules (LTGF-β), which are activated via proteolytic cleavage by endoproteases such as furin [9]. Active TGF-β signaling is transmitted through two types of transmembrane serine/threonine protein kinase receptors: TGF-β type I (TβRI) and type II (TβRII) [1, 10-12]. TGF-β first binds to TβRII with the assistance of the membrane-anchored proteoglycan beta glycan TGF-β receptor III (TβRIII) [13], which leads to heterotetrameric complex formation with TβRI, resulting in TβRI phosphorylation [14, 15]. TβRI (also known as activin receptor-like kinase 5; ALK5) transduces TGF-β signaling in most cell types although the signaling can also be mediated by ALK1 or other type I receptors in certain cell types [16, 17]. Activated TβRI propagates signaling by recruiting and phosphorylating receptor-regulated Smad (R-Smad) proteins. ALK5 phosphorylates Smad2 and Smad3, while ALK1 phosphorylates Smad1, Smad5, and Smad8. Activated Smads form a complex with the common Smad (Smad4) and then are translocated into the nucleus, where they regulate target gene expression by binding to regulatory promoter DNA alone or interacting with other transcription factors [18, 19].

Smad3 homomer can form DNA-binding complexes through its MH1 domain independent of Smad4. But Smad2 cannot bind to DNA without Smad4 because of the lack of the additional 30 amino acids present in Smad3 MH1 domain. Smad4 and phosphorylated Smad3 bind multiple 5'-AGAC-3' sequences called Smad binding elements (SBEs) and GC-rich sequences [20]. Smad2 and Smad3 interact with a number of common and distinct transcription factors for SBE selectivity and specific gene

transcription [21]. In most cases, Smad-binding transcription factors can function independent of Smads in controlling a specific gene transcription. However, Smad interacts with these transcription factors to modulate their transcriptional activity by recruiting co-activators or co-repressors [20, 22, 23]. For example, Smads recruit transcription coactivator p300/CBP, which has histone acetyltransferase activity, to facilitate the initiation of transcription [20]. In addition to p300/CBP, various other transcription factors such as Forkhead, homeobox, zinc-finger, AP1, Ets, and basic helix-loop-helix families have also been shown to act in concert with Smad proteins [24, 25]. The diversity of Smad/co-factor combinations enables the regulation of the transcription of a vast amount of target genes. The differential expression of these factors in different cells are thought to contribute, at least in part, to the cell type-specific responses observed upon TGF-β stimulation [19].

TGF-β/Smad signaling pathway is regulated in multiple steps by different factors. SARA (SMAD anchor for receptor activation) presents R-Smads to the activated receptor complexes [26], while TMEPAI (transmembrane prostate androgen-induced protein) sequesters R-Smad proteins from active participation in TGF-β signaling [27]. Inhibitory Smad (I-Smad), Smad6 or Smad7, inhibits R-Smad binding to TGF-β receptor [28-30]. Smad Phosphorylation is reversed by phosphatases such as PPM1A and PDP in order to create a rapid activation-deactivation cycle [31-33]. Moreover, activated Smad proteins may be ubiquitinized by E3 ligases for proteasomal degradation [34, 35]. In addition, transcriptional repressors Ski and SnoN also regulate TGF-β signaling by interacting with Smad proteins [36, 37].

In addition to the canonical Smad signaling pathway that directly regulates the transcription of Smad-dependent target genes, TGF-β function can also be mediated by Smad-independent pathways including MAPK signaling pathways, such as p38 MAPK and c-Jun NH2-terminal kinase, phosphatidylinositol 3-kinase/Akt pathway, and Wnt signaling [38].

TGF-β SIGNALING IN SMOOTH MUSCLE DIFFERENTIATION DURING EMBRYONIC DEVELOPMENT

SMC differentiation is an integral part of embryonic vascular development. Vascular development in the embryo starts with the formation of a primitive vascular network from endothelial precursors through a process known as vasculogenesis. This primary vessel network undergoes angiogenesis to grow into a complex vascular system through branching and remodeling [39]. Recruitment and differentiation of SMC progenitor cells are essential process for both vasculogenesis and angiogenesis. The function of SMCs is to stabilize nascent vessels by inhibiting excessive endothelial cell proliferation and migration. In addition, SMCs express vasoactive peptides, growth factors and cytokines which are important for the overall function of vasculature. After birth, the principal function of SMCs is to regulate pulse pressure and blood flow through contraction [40]. SMCs are capable of reversibly modulating their phenotype during postnatal development and can de-differentiate into proliferative, matrix synthetic cells in response to vascular injury [41, 42]. Abnormal SMC differentiation or function contributes to a number of cardiovascular disorders including congenital heart diseases, aortic aneurysm, atherosclerosis, hypertension, and restenosis [42-51].

TGF-β signaling plays pivotal roles in SMC differentiation during vascular development as well as phenotypic switching in disease states [52]. The importance of TGF-β signaling pathway in SMC differentiation during embryonic development has been demonstrated by numerous studies [53]. Gene-targeting studies in mice have shown that a loss of TGF-β signaling components generally leads to abnormal differentiation and maturation of the primitive vascular network, resulting in defective vessels losing integrity of the vessel wall. One of the defects is the failure of smooth muscle cell recruitment and/or differentiation [54]. 50% of mice with both alleles of TGF-β gene deleted die in utero around 10.5 dpc due to abnormalities in yolk sac vessel development. The vascular defects are caused, at least in part, by the failed differentiation of mesenchymal precursors into vascular SMCs [55]. In young mice with one allele of the TGF-β gene deleted, the levels of both TGF-β and smooth muscle differentiation markers are reduced as compared with that of wild-type mice. This regulation of smooth muscle differentiation by TGF-β also occurs dynamically in the adult animals [56]. Quantitative immunofluorescence data in rat arteries demonstrate that levels of smooth muscle differentiation markers correlate with the levels of TGF-β expression [57].

TβRII is unique and essential for TGF-β signaling[1]. SMC-specific deletion of TβRII gene is the best method to generate mice with ablation of TGF-β signaling in SMCs. Langlois and colleagues have generated mice with conditional deletion of the gene in cells expressing SMC-specific marker SM22α. Their results have shown that all SM22α-Cre/TβRII-floxed embryos die between E14.5 and the end of pregnancy. All mutant embryos display profound vascular abnormalities in the descending thoracic aorta including irregular thickness, occasional aneurysms and elastic fiber disarray.

Importantly, VSMC differentiation is impaired in the descending thoracic aorta in these embryos. TβRII gene deletion in the VSMCs of the descending thoracic aorta diminishes the number of smooth muscle α-actin (α-SMA)-positive VSMCs in the media at E11.5. These results suggest that TGF-β plays an irreplaceable role in the differentiation of VSMCs in the descending thoracic aorta during mouse development [58].

The role of TβRII in SMC differentiation has also been demonstrated by tissue-specific knockout of TβRII gene in neural crest cells. During embryonic development, neural crest cells migrate to various locations within the embryo and differentiate into non-neural tissues. One subpopulation named cardiac neural crest can differentiate to SMCs of ascending aorta and great arteries by a number of growth factors including TGF-β [59, 60]. TGF-β function in this process is demonstrated by neural crest-specific ablation of TβRII using Cre-loxP system. TβRII protein is specifically deleted in neural crest and neural crest-derived cells by mating TβRII-floxed mice with Wnt1-Cre mice. Mouse hearts with TβRII deletion display truncus arteriosus together with ventricular septal defects. In addition, the mutant mice exhibit abnormal patterning of the arteries arising from the aortic arch, the main cause of mortality in human DiGeorge patients. Importantly, although the mutant neural crest cells are able to migrate and form aorto-pulmonary septum at E10.5, they do not contribute to the development of the smooth musculature and fail to adopt a smooth muscle cell fate [61]. The absence of neural crest-derived smooth muscle cells in mutants explains the defective separation of the aorta from the pulmonary trunk, leading inevitably to a truncus arteriosus. Although a later report using the same strategy has failed to identify SMC defect, which is likely due to, as discussed by the authors, an in vivo compensatory mechanism or the use of a different

TβRII-floxed mouse line [62], TGF-β signaling appears to be crucial in SMC differentiation from neural crest cell during embryonic development.

ALK5, a type I receptor, has been shown to be involved in the induction of epicardial to mesenchymal cells, one of the processes by which differentiated smooth muscle cells are produced. Ablation of Alk5 in epicardial lineages using Gata5-Cre mouse lines results in the failure of TGF-β-induced epicardial to mesenchymal cell transition. Late-term mutant embryos lacking epicardial Alk5 display defective formation of the SMC layer around coronary arteries and aberrant formation of capillary vessels in the myocardium [63]. In addition to ALK5, ALK1 is also required for the differentiation and recruitment of vascular SMCs to the vascular endothelium cells because ALK1 knockout embryos contain no VSMCs [64-68]. Mice mutant for ALK1 develops arterio-venous malformations (AVMs), a serious condition characterized by shunting between the arterial and venous circulations.

Endoglin (also known as CD105) is a homodimeric membrane glycoprotein located on cell surface of vascular endothelial cells, hematopoietic cells, neural crest stem cells, etc [69-71]. Endoglin has been identified as a part of the TGF-β receptor complexes and can be co-precipitated with TβRII and TβRI in endothelial and leukemic cells [72-74]. Endoglin has a pivotal function in the development of the cardiovascular system and in vascular remodeling. Mice lacking endoglin gene die during embryonic development due to cardiovascular abnormalities [68, 75]. In contrast to the mice lacking TGF-β or its signaling receptors, the process of vasculogenesis occurs normally in endoglin mutant embryos. However, the second stage of vascular development, angiogenesis, is affected as shown by the absence of organized vessels in yolk sacs. Therefore, endoglin is

important in angiogenesis rather than in vasculogenesis. Importantly, disrupted development of SMC in the yolk sac is observed in endoglin null mice. One important mechanism underlying the limited number of SMC in the vessel walls is the reduced availability of TGF-β protein levels. In endoglin knockout mice, the lacking of TGF-β pathway in endothelial cells of the yolk sac leads to the decreased phosphorylation of Smad2 in the mesothelial layer, which eventually inhibits the recruitment and differentiation of mesenchymal cells into VSMCs [76].

Smad proteins are important components of TGF-β signaling pathway [1, 4, 22, 23]. Smad5 is expressed predominantly in mesenchyme and somites during embryogenesis and in many tissues of the adult. The Smad5 homozygous mutant embryos (Smad5ex6/ex6) exhibit phenotypes similar to those of TGF-β and TβRII knockouts. Smad5ex6/ex6 embryos die at E10.5-11.5 due to defects in angiogenesis which requires extensive interactions of endothelial cells with pericytes or smooth muscle cells. Smad5ex6/ex6 embryos have dilated blood vessels, and the layer of endothelial cells is dissociated from mesenchymal cells, suggesting that the interaction between the endothelial and mesenchymal cells is affected. Many Smad5ex6/ex6 embryos suffer massive apoptosis of mesenchymal cells. The abnormal blood vessels display a decrease in the thickness of SMC layer, indicating that the differentiation of mesenchymal cells into SMC is impaired in Smad5ex6/ex6 mutants [65].

MOLECULAR MECHANISMS OF TGF-β-INDUCED SMC

Differentiation

SMCs are defined by specific molecular markers and contractile functions. Smooth muscle α-actin (α-SMA) and SM22α are early markers of developing SMCs

while calponin, caldesmon, and smooth muscle myosin heavy chain (SMMHC) are late markers. The principal function of SMCs is to regulate pulse pressure and blood flow through contraction [40]. In order to understand the underlying mechanisms of TGF-β-induced SMC differentiation, several *in vitro* models have been developed including primary cultured VSMCs [77], C3H10T1/2 (10T1/2, a multipotent mouse embryonic mesenchymal cell line) [78], and neural crest Monc-1 cells (pluripotent neural crest stem cells) [79], etc. TGF-β has been shown to induce these cells to change into a polarized and elongated SMC morphology accompanied by an up-regulation of SMC contractile proteins [78-84]. These models have significantly contributed to the understanding of transcriptional regulation of genes essential for SMC function. Three TGF-β responsive elements have been identified: the TGF-β control element (TCE), the SBE, and the CArG box. Mutation of any of these elements abolishes TGF-β induction. In addition, crosstalk between TGF-β and Notch signaling is found to be involved in SMC differentiation. Moreover, microRNA is recently emerging as an important regulator for TGF-β-induced SMC differentiation.

TCE/KLF4/KLF5

TCE is a cis-element in SMC promoter region and highly conserved across species in multiple SMC marker genes including α-SMA, SM22α, SMMHC and calponin [85]. Mutation of TCE in α-SMA or SM22α promoter region abolishes TGF-β-induced α-SMA and SM22α promoter activity in cultured SMCs [85-87]. The importance of TCE in the regulation of α-SMA and SM22α promoter activity was further studied in transgenic mice with wild-type and TCE-mutant promoters coupled to a LacZ reporter gene. TCE mutations completely block the promoter activity in directing LacZ transcription in

arterial SMCs [86, 87]. Both α-SMA and SM22α TCE form a TGF-β-dependent complex with nuclear proteins in electrophoretic mobility shift assays (EMSAs) [86, 88]. Mutation of SM22α TCE completely abolishes this complex formation [86]. GKLF/KLF4, a Kruppel-like transcription factor (KLF) containing three C2H2 zinc fingers, specifically binds to SM22α or α-SMA TCE. Interestingly, KLF4 represses rather than activates TCE activity. Overexpression of KLF4 inhibits TGF-β-stimulated increase in SM22α or α-SMA promoter activity in 10T1/2 cells. KLF4-mediated repression of the promoter activity is TCE-dependent because in rat aortic SMCs, KLF4 overexpression inhibits the activity of wild type α-SMA promoter but has no effect on the activity of TCE mutant α-SMA promoter [87]. In addition, inhibition of KLF4 with antisense morpholinos increases α-SMA and SMMHC expression [87]. TGF-β inhibits KLF4 expression in cultured SMCs through induction of microRNA-143 (miR-143) and miR-145, leading to a reduction of KLF4 transcripts and decreased KLF4 protein expression [89].

Studies of KLF4 lead to the finding of another Kruppel like factor, a GKLF-related basic transcriptional element-binding protein (KLF5). KLF5 binds specifically to SM22α TCE [86]. Overexpression of KLF5 enhances TGF-β-dependent SM22-LacZ promoter activity in 10T1/2 cells, while reversing KLF4-mediated repression of α-SMA promoter activity induced by SRF in NIH3T3 cells. These studies suggest that TCE may act as an activator or a repressor of SMC marker genes depending on the stoichiometry of specific binding factors.

SBE/Smad signaling

As mentioned earlier, Smads are major intracellular mediators of TGF-β signaling pathway. When Smad2/Smad3 is phosphorylated, they are translocated into nuclear to

regulate gene transcription. Smads bind to SBE (CAGA or GTCT) to regulate gene transcription. SBE is an important TGF-β responsive element in the promoter region of SMC marker genes and thus regulates SMC differentiation. Mutation of SBE in SM22α promoter inhibits TGF-β-induced SM22α promoter activity in 10T1/2, Balb3T3 and Monc-1 cells [90, 91]. Transgenic embryos with SBE-mutated SM22α promoter shows diminished transcription activation potential of the promoter in the arteries [90]. TGF-β induces a nuclear complex bound to the SBE sequence, and mutation of the SBE blocks this inducible interaction, indicating that SBE is required for the formation of the TGF-β-inducible complex. Smad3 and Smad4 but not Smad2 are present in these inducible complexes. The Smad3 binding to the SBE of SM22α promoter in vivo is demonstrated by chromatin immunoprecipitation assay [90]. It appears that Smad3, but not Smad2 or Smad4, activates SBE activity. Smad3 increases the transactivation of SBE reporter but not the mutant SBE reporter. Therefore, Smad3 is the major mediator of TGF-β-induced SM22α transcription, and SBE in the SM22α promoter is a direct target of Smad3.

In addition to Smad, several other pathways such as RhoA also mediate TGF-β signaling. RhoA is a member of Rho GTPase family that has intrinsic GTPase activity and can shuttle between an inactive GDP-bound state and an active GTP-bound state [92]. RhoA is highly expressed in mature VSMCs. RhoA and p160 Rho kinase (ROCK), a downstream effector of RhoA, regulate the expression of α-SMA and SM22α [93]. Overexpression of RhoA or activation of RhoA in cultured VSMCs causes a contractile phenotype and organized arrangement of actin and myosin. On the other hand, inhibition of RhoA leads to a loss of actin and myosin filaments, indicating that RhoA plays a key role in regulating SMC contractile function [94]. RhoA/ROCK regulates the expression and

nuclear translocation of SRF in SMCs, and ROCK inhibitor decreases SRF enrichment to CArG regions of α-SMA and SMMHC promoters [95, 96]. It appears that RhoA activates SMC marker gene expression via both ROCK-dependent and independent pathways in rat pulmonary artery SMCs [97]. Our studies show that RhoA regulates TGF-β-induced SMC differentiation via modulating Smad signaling. RhoA is activated as early as 5 minutes following TGF-β induction. Inhibition of RhoA blocks TGF-β-induced expression of α-SMA, SM22α and calponin and reverses TGF-β-induced morphology alteration and contractility, indicating that RhoA is essential in TGF-β-stimulated SMC differentiation. Dominant negative RhoA blocks Smad2 and Smad3 phosphorylation, resulting in an impaired nuclear translocation and transcriptional activity, which eventually inhibits SMC marker gene expression. Conversely, constitutively active RhoA significantly enhances Smad-dependent promoter activity [98]. These results suggest that RhoA cross-talks with Smad to regulate TGF-β-induced SMC differentiation.

CArG/SRF and myocardin

Almost all SMC-specific genes have conserved CArG elements with a consensus sequence CC(A/T-rich)$_6$GG in their promoter regions. CArG box, also called serum response element, is involved in TGF-β-induced SMC marker gene expression via binding to SRF [42, 85, 99, 100]. Overexpression of SRF increases SMC marker expression in 10T1/2 cells. Moreover, cell morphology changes from flat to elongated shape in SRF-transfected 10T1/2 cells [100]. TGF-β induces SRF protein expression and enhances its binding activity to the CArG boxes in inducing SMC phenotype [85, 100, 101]. CArG box mutation disrupts SRF binding and completely abolishes TGF-β-induced transcriptional

activation of SMC marker genes [100]. SRF appears to interact with Smad3 upon TGF-β induction and regulates Smad3 transactivation of SM22α promoter.

Myocardin is a transcriptional cofactor of SRF and is highly expressed in aortic medial SMCs [102]. Overexpression of myocardin leads to a high induction of calponin and α-SMA with a cell morphological alteration from flat to spindle shape in several cell lines [103-106]. In addition, overexpression of myocardin stimulates SM22α, α-SMA and SMMHC promoter activities in mouse ES cells [104]. siRNA knockdown of myocardin significantly reduces transcriptional activity of α-SMA, SM22α and SMMHC in aortic SMCs [106]. Myocardin-null embryos die at E10.5 and lack the differentiation of vascular SMCs [107]. Myocardin induces SMC marker transcription in a CArG-dependent manner [106]. Moreover, myocardin alone is sufficient to induce a SMC-like contractile phenotype [108].

Myocardin is also involved in TGF-β-induced SMC differentiation. Myocardin appears to directly interact with Smad3 in a CArG box-independent manner. Myocardin enhances TGF-β-induced alteration of cell morphology and SM22α transcription in 10T1/2 cells [90]. Overexpression of myocardin and Smad3, but not Smad2, leads to a synergistic increase of SBE promoter activity. Moreover, myocardin enhances Smad3-mediated activation of SM22α, SMMHC and α-SMA promoter activities [90]. Taken together, both SRF and its transcription cofactor myocardin play important roles in TGF-β-induced SMC differentiation.

Although myocardin is considered to be a master regulator of SMC differentiation, the expression of some SMC-associated genes such as smoothelin-B is independent of myocardin [109, 110]. In addition, some progenitor cells such as A404 expressing a low level of myocardin are not converted to SMC phenotype without retinoic acid (RA) induction

[106]; Conversely, other SMC progenitors such as 10T1/2 cells can be converted to SMC phenotype by overexpression of myocardin [106], suggesting that a threshold level of myocardin is required for SMC differentiation. In vivo studies show that the expression of early SMC marker genes such as SM22α and α-SMA emerges prior to detectable myocardin mRNA in the embryonic dorsal aorta, indicating that myocardin has a minor role in the initiation of SMC differentiation in some vascular tissues [104, 111-113]. A recent report shows that myocardin null embryonic stem cells can readily form vascular SMCs in the setting of chimeric knockout mice. The results from this study provide novel evidence that myocardin is essential for development of visceral SMCs and ventricular myocytes but is dispensable for development of atrial myocytes and vascular SMCs [113]. Our in vitro studies demonstrate that myocardin may not participate in the initiation of TGF-β-induced SMC differentiation because the early SMC markers are induced preceding the induction of myocardin. It appears that Smad3 activation by TGF-β has blocked the expression of myocardin. Smad3 blocks myocardin transcription by interacting with Nkx2.5, which prevents Nkx2.5 from activating myocardin promoter [114]. Our data suggest that Smad may mediate the initiation of TGF-β-induced SMC differentiation, while myocardin is likely to contribute to the maturation of SMCs during a later stage (Figure 2.1).

TGF-β and Notch Signaling

Like TGF-β, Notch signaling induces SMC differentiation [115-118]. Once ligands (such as Delta-like or Jagged) bind to Notch receptor (Notch1, Notch2, Notch3 and Notch4), the Notch intracellular domain (NICD) is cleaved and translocated into the nucleus to interact with the DNA-binding protein CSL (CBF-1, suppressor of hairless,

and Lag-1, also known as RBP-Jκ), mastermind-like (MAML), and other transcriptional coactivators to modulate the expression of Notch target genes that regulate cell fate decisions [118]. Numerous data show that Notch induces SMC specific marker expression including α-SMA, SM22α, calponin and SMMHC in a number of cell lines [116, 117, 119]. Notch signaling specific inactivation in the neural crest causes cardiac outflow tract defects with decreased expression of SMC markers [116]. Although there are four types of Notch receptor, only Notch1 and Notch3 are expressed in VSMCs. In adult Notch3$^{-/-}$ mice, VSMCs show deficiency in postnatal maturation stage. The expression of late stage SMC marker smoothelin B is significantly inhibited in mutant arteries, suggesting a pivotal role of Notch3 in the maturation of VSMCs [120].

It appears that TGF-β cross-talks with Notch signaling in the regulation of SMC differentiation. TGF-β and Notch have cooperative effect on SMC differentiation [121]. In human SMCs, both Jagged1 and Notch induce SMC marker expression. SMCs embedded within collagen matrix exhibit a greater contractile response with both TGF-β and NICD comparing to individual treatment. CBF1 interacts with Smad2/3, which leads to an increased Smad2/3 transcriptional activity. In addition, Notch increases TGF-β-induced binding of Smad2/3 to SMC marker promoter. As most of SMC marker gene promoters contain CBF1 and Smad consensus binding sites, it is possible that NICD/CBF1 complex binding to adjacent promoter region, which provides a cis regulatory signal to promote Smad binding. In addition to the mature SMCs, TGF-β and Notch also show cooperative activity in SMC differentiation of huMSCs and embryonic stem cells. TGF-β induces Jagged1 expression in huMSCs, suggesting that Notch activation mediates TGF-β signaling during huMSC differentiation into SMC. Knockdown of Jagged1 using shRNA

inhibits TGF-β-induced SMC marker expression in huMSCs [122]. Although TGF-β and Notch cooperate in most of cases, TGF-β appears to inhibit Notch 3 in SMC differentiation of 10T1/2 cells [123], suggesting that TGF-β and Notch signaling pathways interacts in a cell-specific manner.

MicroRNA (miRNA)

MicroRNA (miRNA) are small non-coding RNAs that function as negative regulators of gene expression by associating with the complementary sequences in the 3′ untranslated regions (UTRs) of mRNAs, resulting in mRNA degradation and/or translational inhibition [124, 125]. A number of studies have shown that miRNAs plays a role in VSMC phenotype switch [126-130]. TGF-β/BMP regulate around 20 miRNAs [131], which control expression of protein-coding genes associated with epithelial-mesenchymal transition, skeletal muscle cell differentiation, and cell proliferation, etc [39, 132, 133]. miR-143 and miR-145, which are encoded as a gene cluster, target KLF4 and play a critical role in regulating VSMC phenotype [127, 134, 135]. miR-143 or miR-145 VSMC knock-out mice exhibit abnormal vascular tone and reduced contractile gene expression [134]. The expression of miR-143 and miR-145 is repressed during platelet-derived growth factor (PDGF)-induced VSMC dedifferentiation and during neointimal formation [127]. Recent studies indicate that miR143/145 plays a role in TGF-β-induced SMC differentiation [89, 136]. TGF-β stimulates miR143/145 expression in a dose- and time-dependent manner in VSMC. TGF-β-induced miR143/145 expression is myocardin /SRF-, p38-, and Smad4-dependent [136]. Both CArG box and SBE are essential for TGF-β-dependent activation of miR143/145 enhancer [136]. BMP-4 also induces miR143/145 expression. TGF-β and BMP-4 induction of miR143/145 results in down-regulation of KLF4 [89]. Interestingly,

BMP-4 induces miR143/145 through myocardin-related factor A (MRTFA), but not myocardin, suggesting that TGF-β and BMP4 signaling regulate KLF4 expression through different mechanisms [89].

TGF-β IN SMC-RELATED DISEASES

The principal postnatal function of SMCs is to regulate pulse pressure and blood flow through contraction [40]. SMCs are capable of reversibly modulating their phenotype during postnatal development and can de-differentiate into proliferative, matrix synthetic cells in response to vascular injury [41, 42].TGF-β regulates both SMC differentiation during embryonic development and postnatal phenotypic switching [74, 75, 137]. Overexpression of TGF-β increases the neointimal formation and smooth muscle proliferation and differentiation in balloon injury models [138, 139]. Therefore, it is conceivable that TGF-β plays an important role in the re-differentiation phenomena [140]. TGF-β has been shown to be involved in the development of many cardiovascular diseases including atherosclerosis, congenital heart diseases, aortic aneurysm, hypertension and hereditary hemorrhagic telangiectasia, etc [141-143]. Many of these diseases are due to the failed regulation of SMC function or differentiation.

Atherosclerosis

Atherosclerosis is triggered in response to chronic injury to the vascular endothelium by various risk factors. It is a progressive disease characterized by the formation of a plaque in the inner lining of large arteries. VSMC proliferation, migration, and hypertrophy are involved in the development of atherosclerosis. VSMCs play a maladaptive role in the lesion development and the progression of the disease [144]. TGF-β directs the response of SMC to the injury. In animals, deletion of a single allele of the TGF-β gene increases its

susceptibility to endothelial cell activation and vascular lipid lesion formation in response to pro-atherogenic stimuli such as a lipid-rich diet [145]. TGF-β stimulates SMC proliferation at low concentrations via both PDGF-dependent and -independent manner. Ribozyme oligonucleotides against TGF-β increase vascular inflammation, accelerate lipid lesion formation, and shift the plaque morphology towards an unstable phenotype [146]. Inhibition of TGF-β signaling in ApoE deficient mice, an animal model for atherosclerosis, suggest that the cytokine is critical for the production of extracellular matrix and the maintenance of a stable plaque phenotype through SMC phenotypic regulation [146, 147]. Indeed, SMC in stable lesions express greater amounts of TGF-β than unstable lesions. MacCaffrey *et al* [148] demonstrate that SMCs isolated from atherosclerotic plaque tissue expressed less TβRII than SMCs from healthy vessel wall. These data directly or indirectly show that TGF-β plays a pivotal role in the maintenance of normal blood vessel wall architecture.

Congenital heart diseases

Defective TGF-β signaling causes congenital heart diseases (CHD) during embryonic development [73]. CHD are the most commonly occurring birth defect in humans. Moderate and severe forms of congenital heart disease, including outflow tract defects and aortic arch anomalies, occur in 6 per 1000 live births [149]. Several studies have shown that cardiac neural crest contribute SMCs to the ascending and arch portions of the aorta and the ductus arteriosus. A number of congenital human diseases such as heart and outflow tract malformations are now attributed to failure of cardiac neural crests to differentiate into aortic arch complex [150]. Patients with Alagille syndrome always have CHD with right-sided outflow tract defects and tetralogy of Fallot influenced by cardiac

neural crests.Patients with DiGeorge syndrome (DGS) always have congenital defects with heart and outflow tract malformations influenced by cardiac neural crest [151]. Mice with TβRII mutation in neural crest develop all the morphological features of DGS. The hearts of TβRII-mutant mice display a truncus arteriosus together with a ventricular septum defects (VSD) at E18. Both control and TβRII-mutant neural crest cells are able to populate the pharyngeal apparatus and form aorto-pulmonary septum at E10.5. However, TβRII-mutant neural crest cells in the aorto-pulmonary septum do not develop into smooth muscle cells[61]. The absence of neural crest derived SMCs in mutants explains the defective separation of the aorta from the pulmonary trunk, leading inevitably to a truncus arteriosus. Thus, TGF-β regulation of neural crest differentiation rather than migration plays a crucial role in the etiology of DiGeorge syndrome.

Thoracic aortic aneurysms and dissections

Combination of human molecular genetics and animal modeling has demonstrated the involvement of TGF-β signaling in aortic aneurysm [72]. Mutations in TβRI and TβRII result in a spectrum of genetic conditions, associating with thoracic aortic aneurysms and dissections (TAAD) [152]. Mutations in TβRII are initially identified in individuals with a Marfan-like connective tissue syndrome with TAAD and skeletal features of Marfan syndrome (MFS). TβRI and TβRII mutations are subsequently described in individuals with Loeys-Dietz syndrome (LDS), a syndrome characterized by TAAD in children and young adults, arterial tortuosity, aneurysms and dissections of peripheral arteries. TβRII mutations also lead to descending aortic disease and aneurysms of other arteries. An arginine residue of TβRII at position 460 has been identified as a mutation "hot spot" for TAAD. Structural analysis has revealed that the amino acid substitutions may interfere

with the receptor's ability to transduce signals [153]. In aortic SMCs explanted from patients with TβRII mutations, the expression of SMC contractile proteins is decreased compared with controls. In vivo expression of contractile proteins is also decreased in aortas from patients with TβRII mutations relative to unaffected aortas. The failed expression of SMC contractile proteins in TβRII-mutant SMCs may influence the contractile function of SMCs, which contributes to the pathogenesis of TAAD [154].

Hypertension

Hypertension is defined as a sustained diastolic pressure of >90 mmHg or a systolic blood pressure >140 mmHg. Hypertension is another disease that related to SMC phenotypic switching. Although the etiology is extremely complex and varies among individuals, a common feature in the majority of cases of hypertension is an increase in peripheral resistance as a result of increased SMC contractility and vascular remodeling that are related with the phenotypic switching of SMC [155]. Primary pulmonary hypertension (PPH) is a rare disease with symptoms of fatigue, anorexia, an increase in pulmonary arterial pressure, right ventricular failure and death [156]. PPH is caused by mutations in either of two genes: the BMP type II receptor gene (BMPR-II) and ALK-1 [157-159]. BMPR-II mutations increase the incidence of PPH. BMP-2, -4 and -7 have been shown to inhibit SMC proliferation but increase the SMC marker expression in cultured pulmonary artery SMCs (PASMCs). PASMCs derived from the pulmonary arteries of patients with PPH exhibit abnormal growth responses to TGF-β. TGF- β inhibits serum-induced proliferation of PASMC from healthy individuals while stimulates cell proliferation of PASMCs from patients with PPH [160].

Hereditary hemorrhagic telangiectasia

Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant vascular disorder which always happened in nasal, mucocutaneous, gastrointestinal, pulmonary, cerebral, and hepatic vascular beds. The common syndromes are nose bleeding, skin telangiectases,gastrointestinal bleeding [161]. Pulmonary arteriovenous malformations (PAVM) are always present in 20% of HHT populations. Genetic analyses reveal that Endoglin are responsible for type I HHT [162, 163]. As aforementioned, Endoglin form TGF-β receptor complexes with TβRI and TβRII to modulate the phosphorylation of TβRI and TβRII and plays a pivotal role in angiogenesis as demonstrated in the $Eng^{-/-}$ mice. $Eng^{-/-}$ embryos show a defective development of VSMCs because of the reduced availability of active TGF-β protein. The lack of TGF-β inhibits the recruitment and the differentiation of mesenchymal cells into VSMCs, leading to weak vessel walls, which may contribute to the development of HHT [76]. In addition to Endoglin, ALK-1 and Smad4 are also involved in HHT [164]. ALK-1 gene heterozygous mutation causes type II HHT-2 [164]. Recently, a remarkable ALK1 germinal and somatic mosaicism characterized by the presence of two distinct mutant alleles and a non-mutant ALK1 allele are identified in a woman with HHT and PAH [70]. It is proposed that genetic background and/or environmental factors (second hits), in addition to the mutations in Endoglin and ALK-1 genes, may also play an important role in the development of vascular malformations in HHT patients. Park and colleagues demonstrate using ALK-1-knockout mice that excisional skin wounding, as a second hit, is essential for the development of AVMs in HHT. These results provide new insights for understanding the pathogenesis of HHT [71].

CONCLUSION

SMC differentiation and phenotypic modulation play critical roles in embryonic cardiovascular development as well as pathological conditions in adults. TGF-β and its downstream signaling molecules including receptors, coreceptors and intermediate Smad proteins are all indispensible for the SMC differentiation or phenotypic modulation. As aforementioned, TGF-β itself can activate multiple signaling pathways such as MAPK, PI3K and RhoA. In addition, TGF-β signaling crosstalks with other pathways including Notch and SRF/myocardin. These diversified interactions ensure a precise cell fate determination and maturation of SMCs. Among the various SMC regulators, TGF-β/Smad signaling appears to be critical in regulating the initiation of SMC differentiation. Defective TGF-β signaling leads to development of several prominent cardiovascular diseases including congenital heart diseases, aortic aneurysm, hypertension, neointimal hyperplasia observed in vascular injury and atherosclerosis, etc.

II. Cell Signaling and Function of Guanine Nucleotide Exchange Factor DOCK2

Dedicator of cytokinesis 2 (DOCK2) contains approximately 1830 amino acids (aa) with a molecular mass of 212 kDa. DOCK2 belongs to the CDM family of proteins. CDM is an abbreviation for CED-5 of Caenorhabditis Elegans [165], DOCK180 of mammalian [166] and Myoblast City of Drosophila Melanogaster [167]. The CDM family contains 11 mammalian members (DOCK180, DOCK2-11). The members are organized into four subfamilies, the DOCK A (DOCK1/DOCK180, DOCK2, and DOCK5), DOCK B (DOCK3 and DOCK4), DOCK C (DOCK6, DOCK7, and DOCK8), and DOCK D subfamily (DOCK9, DOCK10, and DOCK11) [168-171]. The CDM family acts as guanine nucleotide exchange factors (GEFs) for the Rho family of small guanine triphosphatase

(GTPase). These small GTPases function as molecular switches by alternating between a GDP-bound "inactive" state and a GTP-bound "active" state [172]. GEF mediates activation of GTPase through exchanging GDP with GTP in response to molecular stimulation [172-177]. Like other small GTPases, Rac alters between a GTP bound and a GDP bound form [178]. DOCK2 is a kind of specific Rac GEF that regulates the loading of GTP to activate Rac. DOCK2 was first characterized by Dr. Nishihara's group from myeloblast cell line KG-1 in 1999 [179]. Currently, DOCK2 has been found in peripheral blood (PB), spleen, and thymus and has been mainly expressed in lymphocytes and macrophages of various organs [179-184].

As a Rac GEF, DOCK2 plays a key role in cell motility, polarity, adhesion, proliferation, and apoptosis. DOCK2 is important for lymphocyte migration and activation [180]. DOCK2 also controls NKT cell and T helper type 2 cell development, neutrophil chemotaxis, and plasmacytoid dendritic cell development through different signaling pathways. In addition, DOCK2 is important for B cell lymphoma and prostate cancer cell proliferation, depending on different signaling regulations. The deletion of DOCK2 enables long-term cardiac allograft survival. DOCK2 is related to Alzheimer's Disease and HIV development as well.

DOCK2 STRUCTURE

DOCK2, as a CDM family protein, contains DOCK homology region (DHR) 1 and DHR2 domains. CDM family proteins, unlike conventional GEFs, lack the typical DH-PH unit. Instead, they share the highly conserved domains DHR1 and DHR2 [181, 185, 186]. The DHR2 domain contains ~500 residues situated in its carboxy-terminal regions. DHR2 domain carries the catalytic activity for Rac and is critical for binding to small

GTPases and catalyzing the nucleotide exchange. Crystal structure analysis of DOCK2DHR2 in complex with Rac revealed that DOCK2DHR2 is composed of three nearly equal sized lobes (lobes A, B, and C) [170]. Lobe A contains six antiparallel α-helices. The anti-parallel interactions of α4 and α5 helices generate the homodimeric interface. The GTPase-binding site and catalytic center are generated from lobes B and C. Lobe B is comprised of two anti-parallel β-sheets and lobe C adopts an almost identical antiparallel α-helical bundle composed of four α-helices. Helix α10 of lobe C is interrupted by a seven-residue insert incorporating the essential and universally conserved Val residue (Val-1540, DOCK2 numbering) that functions as a nucleotide sensor. The majority of contact residues of DOCK2DHR2 with Rac GTPase localized to the α10 of lobe C. This lobe C-GTPase interface is structurally conserved throughout the DOCKDHR2 family. DHR1 domain contains ~200 residues located toward the N terminus and is shown to interact specifically with phosphatidylinositol-3, 4, 5-triphosphate (PtdIns(3,4,5)P3) to mediate signaling and membrane localization [168, 171, 187].

DOCK2, as a DOCK-A subfamily protein, also contains an N-terminal Src-homology 3 (SH3) domain and C-terminal proline-rich motifs. The SH2-SH3 adaptor protein CT10-related kinase II (Crk-II) is associated with the C-terminal proline-rich sequences of DOCK-A proteins [188, 189]. However, DOCK2 does not contain a proline-rich Crk-II-binding motif, PPXLPXK, at the C-terminal region and cannot bind to Crk-II [179, 190]. Instead, another category of proline-like adaptors the engulfment and cell motility proteins (ELMO) bind to the SH3 domain of DOCK2 through their proline-rich sequences [191]. In addition to these typical protein domains, the coiled-coil region has also been detected in some positions of all DOCK180-related proteins including DOCK2.

DOCK2 BINDING PROTEINS AND DOCK2 REGULATION

In the light of the large size of DOCK2 protein with multiple domains and motifs, DOCK2 could interact with numerous partners, which in turn could regulate their intrinsic GEF activity to regulate Rac activation. In addition, there exist intramolecular autoinhibition interactions in DOCK2 involving the N-terminal SH3 domain and C-terminal DHR2 domain. In HEK293T cells overexpressed with DOCK2 N-terminal fragment (Met1-Glu200) and C-terminal fragment (Ser1195-Met1828), N-terminal fragment of DOCK2 can associate with the C-terminal fragment to exhibit an autoinhibition effect in DOCK2.

Phosphoinositides and phosphatidic acid

DOCK2DHR1 domain has been shown to bind to PtdIns(3,4,5)P3, the major product of class IA phosphatidylinositol 3-kinases (PI3Ks) [181, 187]. PIP3 is rapidly produced by PI3Ks in response to activated chemokine receptors (CKRs), and accumulates at the site of the plasma membrane to sense the highest concentration of the extracellular stimulant [192]. By directly binding to the DHR-1 domain, PIP3 recruits DOCK2, which is normally localized mainly in cytosol, to the plasma membrane to activate Rac. When a DOCK2 mutant lacking DHR-1 was expressed in HEK293T cells, PIP3 binding was hardly detected [181]. The PIP3-binding activity allows DOCK2 to translocate to the plasma membrane and to polarize which is important for their Rac GEF activation.

Although the plasma membrane translocation of DOCK2 is initially mediated by PIP3, phosphatidic acid (PA) acts at a late phase to focus DOCK2 localization and to stabilize the leading edge [193]. PA is generated from the hydrolysis of membrane

component phosphatidylcholine by phospholipase D (PLD) [193, 194]. DOCK2 could be accumulated at the plasma membrane when PLD2, a PLD isoform, is coexpressed. However, the catalytically inactive PLD2 mutant failed to alter DOCK2 localization [195]. PA could narrow and enrich the localization of DOCK2 more precisely at the membrane site that will become the growing leading edge. PA controls subcellular localization of DOCK2 by directly binding to the C-terminal polybasic amino acid cluster. The PA binding can be almost totally abolished by deleting the C-terminal 214 amino acid residues. In HEK293T cells, overexpressed DOCK2 mutants failed to localize to the plasma membrane even when PLD2 was coexpressed [195].

Rac

Rac plays a role in the reorganization of cell architecture and gene regulation [196-199]. It is a key regulator of cell motility by controlling F-actin polymerization and lamellipodia formation [178, 200-202]. Up to now, three Rac isoforms have been identified in mammals, Rac1, Rac2, and Rac3. Rac1 is ubiquitously expressed and Rac3 is highly expressed in the brain, whereas Rac2 is largely restricted to the hematopoietic system [203-205]. DOCK2 has been reported to bind to and activate Rac1 and Rac2 to mediate actin polymerization [179, 191, 206-209]. DOCK2 could promote the guanine nucleotide exchange reaction of Rac1 as shown by the increased ratio of GTP/GDP on Rac1 [179, 206]. The Rac1-binding domain has been assigned to DOCK2 DHR2 domain amino acids from 939 to 1323. When truncated mutants of DOCK2 were cotransfected with Rac1 into 293T cells, there was no binding between them and DOCK2 mutant could not activate Rac1. DOCK2 can also regulate Rac2 activity [207]. In 293T cells and Jurkat cells, coexpression of DOCK2 and Rac2 lead to 4-fold higher activation of Rac2. DOCK2 recognizes and

activates Rac2 through divergent residues in Rac2 switch 1 as well as divergent amino acids in Rac2 β2-β3 regions [210]. Mutations in the β1 strand and/or switch 1 and in β2-β3 region of Rac2 lead to decreased level of activation by DOCK2 [210].

Specific adaptor proteins Crk-like (CrkL)

Most of the DOCK A subfamily members can bind with CrkII, an adaptor protein consisting of one SH2 domain and two SH3 domains [211]. However, DOCK2 cannot bind to CrkII because DOCK2 lacks Crk-II binding site [179]. In 293T cells and Jurkat cells, there is no co-immunoprecipitation (Co-IP) of CrkII and DOCK2 even after coexpressing CrkII with DOCK2 [179]. Instead, a signaling adaptor protein Crk-like (CrkL), which shares high sequence homology with Crk-II, acts as the upstream regulatory protein of DOCK2 to regulate Rac1 activity in a number of cell lines [206, 212, 213]. The GST-fusion form of the N-terminal SH3 domain of CrkL can precipitate endogenous DOCK2 in human normal platelets and T-cell lines including Jurkat and MOLT4 [206]. The binding of CrkL and DOCK2 is confirmed by the endogenous CO-IP analysis of DOCK2 to CrkL in Jurkat, MOLT4, and Raji cells [206]. Coexpression of truncated mutants of DOCK2 and CrkL expression plasmid revealed that 2 separate regions of DOCK2 aa 1-515 and aa 939-1476 are contributed to its binding to CrkL-SH3 domain [206]. Overexpression of CrkL and DOCK2 in 293T cells, Jurkat and K562 cell lines all activated Rac1. However, CrkL-induced activation of Rac1 was significantly inhibited by the DOCK2 mutant in 293T cells suggesting the association between CrkL and DOCK2 to activate Rac1 [206]. Immunofluorescent staining of DOCK2 with CrkL and F-actin revealed that DOCK2 was colocalized with CrkL to the cell membrane of Jurkat cells [206].

Engulfment and cell motility protein (ELMO)

ELMO is a kind of scaffold proteins without obvious catalytic activity. They share conserved domain features, including armadillo repeats at N-terminus, an atypical PH domain at 555 to 676 region, and a complex proline-rich region at an extreme C-terminus [214]. ELMO acts as an adaptor molecule that couples Rac to specific downstream effectors [168, 214]. ELMO1 has been shown to interact physically with DOCK2, which is required for DOCK2-mediated Rac signaling [191, 210]. Both DOCK2 and ELMO1 can be detected in T- and B -cell lines [191]. In addition, the endogenous Co-IP results show the association of DOCK2 with ELMO1 in T cells. Further studies showed that DOCK2, through its SH3 domain, interacts with ELMO1 C-terminal region [168]. The association of DOCK2 with ELMO1 was abrogated in mutation of DOCK2 SH3 domain and/or deletion mutants of ELMO1 [191]. The crystal structure analysis of DOCK2 and ELMO1 complex revealed that N-terminal 177-residue fragment of DOCK2 binds to the C-terminal 196-residue fragment of ELMO1 [215]. These structural elements, as well as the PH domain of ELMO1 form a rigid structure for the complex formation. DOCK2 activates Rac and regulates actin cytoskeleton through the interaction with ELMO1 [191]. DOCK2-mediated Rac activation and F-actin assembly were severely impaired in DOCK2 or ELMO1 mutation groups [191]. Upon coexpression of ELMO1 and DOCK2, the intramolecular autoinhibition in DOCK2 was disrupted. The assembly with ELMO1 weakened the intramolecular interaction, relieving DOCK2 from the autoinhibition and allowing Rac accesses to the catalytic DHR-2 domain [215].

T-cell antigen receptor (TCR)

T-cell antigen receptor (TCR) is a molecule found on the surface of T lymphocytes that is responsible for recognizing antigens bound to major histocompatibility complex (MHC) molecules [216, 217]. CD3 stimulation can induce the association of the tyrosine-phosphorylated CD3 ζ-chain and ZAP70 [218]. The adaptor protein CrkL binds to ZAP70 directly or indirectly to transduce signals to downstream effectors [219]. CrkL formed a complex with DOCK2. DOCK2 could coprecipitate with TCR in Jurkat cells [207]. In DOCK2 deficient T cells, antibody induced TCR clustering and lipid raft clustering to the APC interface were impaired and lead to the reduction of antigen-specific T cell proliferation [220]. The signaling pathway of TCR-ZAP70-CrkL-DOCK2-Rac2 has a function in T cells [207]. DOCK2, as downstream of TCR, is essential for TCR-mediated Rac activation and immunological synapse formation [207, 220]. The activated Rac1 and Rac2 were almost totally abolished in splenic T cells and thymocytes of DOCK2-/- mice when stimulated with anti-CD3 antibody (Ab) in the presence or absence of anti-CD28 antibody [220]. DOCK2-induced Rac2 activation promotes the transcription of IL-2. In DOCK2-expressing Jurkat cells, the level of IL-2 gene transcription was increased 2-3-fold. When DOCK2 expressing Jurkat cells were stimulated with anti-CD3 Ab, the levels of IL-2 gene were increased up to 4-5-fold [207].

Apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC)

ASC is an adaptor molecule for inflammasome establishment [221, 222]. ASC specifically regulates DOCK2 expression in myeloid cells and lymphocytes. The absence of ASC does not correlate with a defect in DOCK2 mRNA transcription but instead correlates with a defect in its post-transcriptional regulation. The half-life of DOCK2

transcripts was much lower in Asc-/- BMDCs than in WT cells after the reversible transcription inhibitor actinomycin D treatment [208]. The responses of DCs derived from DOCK2-/- mice mimic those of Asc-/- DCs. ASC expression and inflammasome activation were normal in DOCK2-/- BMDCs. These evidence indicated that ASC may act on upstream of DOCK2 by controlling DOCK2-dependent immune functions [208]. The omission of ASC greatly impaired the natural migratory ability of T and B lymphocytes as shown by lower number of Asc-/- T cells and B cells retrieved from the spleen and lymph nodes of mice. DOCK2 as downstream of ASC is required for antigen uptake by professional phagocytes. The endocytosis of OVA by Asc-/- BMDCs expressing DOCK2 was significantly higher than that by Asc-/- BMDCs alone [208]. Ectopic expression of DOCK2 in Asc-/- lymphocytes and DCs restored ASC-dependent immune functions. Thus, it can be concluded that ASC regulates adaptive immune responses by controlling DOCK2 expression in DCs and lymphocytes.

DOCK2 FUNCTION

DOCK2 plays a critical role in lymphocyte migration and activation and DOCK2 deletion enables long-term cardiac allograft survival. DOCK2 also controls various immunological functions, including NKT cell and T helper type 2 Cell developments, neutrophil chemotaxis, and plasmacytoid dendritic cell migration and type I interferon induction.

The role of DOCK2 in lymphocyte migration

Upon inflammation, tissue cells express multiple chemokines to attract lymphocytes [223-230]. The lymphocyte recruitment into secondary lymphoid organs (SLOs) and peripheral lymph nodes (PLNs) depends on an adhesion cascade including rolling,

firm adhesion, and transmigration [227, 230-232]. Two subfamilies of lymphocyte receptors are needed for lymphocyte trafficking: chemokine receptors (CKR), including CCR7, CXCR4, and CXCR5, and the sphingosine-1-phosphate receptor 1 (S1P1) [233, 234]. SLOs express chemokines, including CCR7 ligands CCL19 and CCL21, CXCR4 ligand CXCL12, and CXCR5 ligand CXCL13 [230, 233, 235]. They are presented on high endothelial venules (HEV) to promote lymphocyte arrest [236]. During migration, lymphocytes acquire membrane polarization, form lamellipodium and uropod, which rely on cytoskeletal protein rearrangement and specific signaling molecules activation [230, 237].

DOCK2 has been shown to play an essential role in lymphocyte migration [180, 232, 238, 239]. DOCK2-/- lymphocytes are impaired in their exit from the thymus as well as in their homing to and migration within both the spleen and lymph nodes [180, 232]. DOCK2-/- mice have defected mature thymocytes emigration to the peripheral blood stream. The CD4+ and CD8+ mature thymocytes emigration from DOCK2-/- mice thymus were significantly reduced compared with the mature thymocytes emigration from the DOCK2+/- thymus [232]. DOCK2-/- mice developed reduced lymphocyte homing to the spleen or PLNs [180, 232, 239]. The amounts of T and B cells in spleen and PLNs of DOCK2-/- mice is significantly decreased compared with those of wild type (WT) mice [180, 232]. When fluorescence-labeled CD4+ T or B cells prepared from DOCK2-/- mice were injected into WT mice, the percentage of the labeled cells that migrated to spleen or PLNs were significantly reduced compared with the levels observed in DOCK2 +/- mice [239]. DOCK2-/- mice developed severe defects in lymphocyte migration in response to chemokines [180, 232]. Chemokines CCL21 and CXCL12-induced actin polymerization and Rac activation were almost totally abolished in DOCK2-/- lymphocytes [180, 232, 238].

Adversely, DOCK2 overexpression in T cells enhanced the formation of GTP-bound Rac and changed cell morphology to a relatively flat and polarized shape [180]. Absence of DOCK2 impaired lymphocytes egress from PLNs. There is decrease of F-actin formation in DOCK2-/- T cells in respond to S1P [239]. In vivo at 12 hr after mAb treatment, the absolute number of control lymphocytes inside the node decreased gradually, whereas DOCK2-/- T and B cell numbers remained essentially constant [239]. Thus, it can be concluded that DOCK2 deficiency increases lymph node dwell time by slowing lymphocyte egress from PLNs.

Integrin-dependent firm adhesion is one important process during lymphocytes migration. Lymphocytes express two major integrin families, α4 and β2. The β2 integrin mediates firm arrest in PLN HEV by binding its ligands and ICAM-2 [227, 240]. α4β1 is needed for lymphocytes migration to bone marrow (BM) and B cells entry into spleen [241]. DOCK2 is required for efficient CXCL12-triggered human peripheral blood T lymphocyte (PBL-T cells) attachment to VCAM-1. In Molt-4 and PBL-T cells, the attachment to VCAM-1 of DOCK2 mutant transfectants was of less magnitude compared with DOCK2 WT transfectants under static conditions [232, 242]. Under flow conditions, Molt-4 T and PBL-T cells transfected with DOCK2 siRNA mainly rolled, with only ~30% of cells displaying stable arrest. Even more, control Molt-4 and PBL-T siRNA transfectants developed a substantially higher resistance to detachment than DOCK2 siRNA transfectants after flow was increased [242]. Once lymphocytes are arrested on their integrin ligands, they embark on a series of cytoskeletal remodeling events to cross the endothelial lining named transendothelial migration (TEM) [227, 240]. DOCK2 is important in chemokine-triggered postarrest lamellipodia formation of T lymphocytes and lateral

locomotion on integrin ligands. DOCK2-/- T lymphocytes arrested on either VCAM-1 or ICAM-1 fail to locomote on these integrin ligands. DOCK2-/- T lymphocytes failed to polarize and spread on these ligands [238]. Mechanism analysis revealed that CCL21 induced microvillar collapse decreased and no lamellipodia extension formed in DOCK2-/- T lymphocytes [238]. Although both WT and DOCK2-/- T lymphocytes cross the endothelial monolayer and take similar time to cross the barrier, less than 20% of successfully transmigrated DOCK2-/- T lymphocytes locomoted away from the site of TEM, suggesting the defect in lateral locomotion of lymphocytes.

The role of DOCK2 in T cell recognition, proliferation, and chemokine mediated T-cell costimulation

Naive T cells traffic to SLOs to search for APCs present of specific peptide Ag on MHC complexes. T cell recognition is mediated by the interaction of TCR with the antigenic peptides bound to MHC [229]. Engagement of TCR by MHC-agonistic peptide induces formation of a highly organized complex between T cells and antigen-presenting cells (APCs), the immunological synapse (IS) [229, 243]. Lipid rafts are also recruited to participate in the IS formation once TCR was stimulated. Chemokines can also act directly as costimulatory factors to stimulate T cells. Chemokines CCL21 and CXCL12 have been shown to act as costimulatory factors during T-cell activation [229, 230].

DOCK2 is associated with the TCR chain and is essential for TCR-mediated Rac activation in T cells [220]. Without DOCK2, IS formation is impaired in 2B4 TCRαβ transgenic (2B4αβ Tg) mice expressing I-Ek (a positively selecting MHC class II molecule). The frequency of the conjugates with polarized TCR in DOCK2-/- 2B4αβ Tg CD4+ T cells only reached the level observed with that of DOCK2+/- 2B4αβ Tg CD4+ T

cells stimulated with an antagonist of TCR. DOCK2 is also important for lipid raft clustering during IS formation. In splenic CD4+ and CD8+ T cells from DOCK2-/- mice, TCR and lipid raft clustering were scarcely observed after incubating with anti-CD3 antibody. Defective IS formation in DOCK2-/- mice results in a significant reduction of antigen-specific T cell proliferation. T cell proliferative response of DOCK2-/- 2B4αβ Tg CD4+ T cells was decreased significantly compared with that of DOCK2+/-2B4αβ Tg CD4+ T cells after stimulation. DOCK2-/-2B4αβ Tg T cells require more antigenic peptides to achieve the proliferative response which is similar to that of DOCK2+/-2B4αβ Tg T cells. T cells lacking DOCK2 showed reduced Rac-GTP formation and ERK phosphorylation after TCR stimulation. CCL21-stimulated CD69 and CD25 expression was also reduced in DOCK2-deficient cells. DOCK2-/- 2B4 TCR-tg T cells did not show any detectable Rac-GTP even when TCR and CCR7 were simultaneously activated [244].

The role of DOCK2 in Vα14 NKT cells and T helper type 2 cells development from their own precursors

Natural killer (NK) T cells are a subset of lymphocytes and play important roles in immune regulation and tumor surveillance. The majority of NKT cells are selected in the thymus through the interaction between CD1d molecules on CD4+CD8+ double-positive (DP) thymocytes and a semi-invariant TCR composed of a Vα14-Jα18 rearrangement [245-247]. α-galactosylceramide (α-GalCer) is a ligand for Vα14 NKT cells. After positive selection, CD44-NK1.1-tetramer+ precursors appear and differentiate into CD44+NK1.1+ mature Vα14 NKT cells through CD44+NK1.1- intermediation [248, 249]. DOCK2 deficiency causes severe reduction of Vα14 NKT cells in the thymus, liver, and spleen. In the thymus and the liver, the numbers of both CD4+ and CD4-CD8- double

negative NKT cells in DOCK2-/- mice were markedly reduced compared with those in DOCK2+/- mice. DOCK2 deficiency modestly reduced the NKT cell population in the spleen [250]. Cell proliferation and cytokine production including IL-4 and IFN-γ were scarcely found with DOCK2-/- spleen cells. DOCK2-/- mice have decreased serum IL-4 and IFN-γ levels in response to α-GalCer compared with DOCK2+/- mice response [250].

During immune responses, antigen-stimulated naive CD4+ T cells differentiate into functionally distinct subsets of T helper (TH) cell, TH1 and TH2 cells [251, 252]. IL-4 promotes TH2 differentiation by binding to its receptor (IL-4R) expressed on naive CD4+ T cells [253-256]. DOCK2 deficiency results in severe TH2 immune responses. Swelling of the footpads of DOCK2 -/- mice was more severe and sustained longer compared with the effects in DOCK2+/- mice after L. major was injected into mice hind footpads. More DOCK2-/- mice developed swelling and erythema of the eyelid than DOCK2+/- mice [257]. There is higher expression of Il4, Il5 and Il13 in DOCK2-/- CD4+ T cells compared with those from DOCK2 +/- mice. DOCK2-/- mice had much higher serum concentrations of IgE and IgG1 than DOCK2+/+. Mechanism analysis revealed that on the surfaces of CD4+ T cells stimulated with anti-CD3 in the presence of anti-IL-4, IL-4Ra was transiently upregulated and this upregulation returned to basal prestimulation amounts within 4 hr but not in DOCK2-/-2B4Tg CD4+ T cells. In normal conditions, once TCR is stimulated, IL-4Ra is internalized and degraded in lysosomal compartments. Lysosomal trafficking of IL-4Ra required microtubule dynamics which regulated by Rac through phosphorylation and inactivation of stathmin [258, 259]. DOCK2-mediated Rac activity regulates lysosomal trafficking of IL-4Ra through microtubule dynamics [257]. Anti-CD3 stimulation resulted in the appearance of stathmin in DOCK2+/+2B4Tg CD4+ T cells but

not in DOCK2-/-2B4Tg CD4+ T cells [257]. Therefore, DOCK2 is essential for the timely degradation of IL-4Ra during the activation of CD4+ T cells.

The role of DOCK2 in neutrophil and neutrophil- like cell motility and chemotaxis

Neutrophils, highly motile leukocytes in immune system, clear pathogens from infected tissue. There is a two-step mechanism for DOCK2 initiation of polarized neutrophil movement: first, PIP3 recruits DOCK2 to the leading edge of the plasma membrane, and then phosphatidic acid (PA) precisely localize DOCK2 to the exact site in the leading edge that will extend the pseudopod [181, 195, 260]. DOCK2-/- neutrophils were less motile than B6 WT neutrophile. In a transwell chemotaxis assay, the percentage of migrating cells of DOCK2-/- BM neutrophils reduced significantly compared with B6 BM neutrophils. Similary results were found in a Zigmond chamber containing N-formyl-methionyl-leucyl-phenylalanine (fMLP) gradient. During neutrophil chemotaxis, DOCK2 regulates leading edge formation by functioning in a PIP3- and Rac-mediated feedback loop. There is severely reduced activation of Rac1 and Rac2 by fMLP in BM neutrophils from DOCK2-/- mice. Most DOCK2-/- neutrophils exhibited aberrant morphology with poorly focused distribution of F-actin [181]. DOCK2-mediated Rac activation regulates the persistent accumulation of PIP3 at the leading edge. An inhibitor of actin polymerization reduces fMLP-mediated PH-Akt, a probe for detecting the spatial distribution of PIP3, translocation in neutrophil-like differentiated HL-60 cells [261]. PA acts selectively in the late phase to control polarized DOCK2 localization during neutrophil chemotaxis [193, 195]. The accumulation of DOCK2 and F-actin at the pseudopods was significantly inhibited in neutrophils from DOCK2-GFP mice treated with FIPI (Phospholipase D 1/2 inhibitor) [195, 262, 263]. The majority of neutrophils treated

with FIPI displayed aberrant morphology with extremely thin lamellae and poorly focused DOCK2 distribution. Treatment with 1-butanol or FIPI impaired localization of activated Rac to the pseudopods [195]. Therefore, PA acted at a late phase to focus DOCK2 localization and to stabilize the leading edge.

PI3K and Src family kinases (SFK) function in parallel in directing cell movement and wortmannin (PI3K inhibitor) and PP2 (SFK inhibitor) combination lead to the complete loss of chemotaxis in response to CXCL8 in HL60 expressing CXCR2 cells (HL60-CXCR2 cells) [264, 265]. Wortmannin and PP2 cotreatment lead to the complete inhibition of ligand-triggered Rac2 activation in dHL60-CXCR2 cells [265]. ELMO1 tyrosine phosphorylation plays an essential role in the function of DOCK2-ELMO1 complex to activite Rac [265]. Its phosphorylation was increased upon CXCL8 stimulation but was ablated by PP2 pretreatment, suggesting that SFK involved ELMO1 tyrosine phosphorylation. Immunostaining showed that the cells pretreated with PP2 alone showed much weaker accumulation of DOCK2 at the leading edge [265]. DOCK2 knockdown HL60 cells showed compromised Rac2 activation, little inhibition of chemotaxis in response to a CXCL8 gradient in the microfluidic gradient chamber assay. A complete inhibition of chemotaxis was observed when DOCK2 knockdown cells were pretreated with wortmannin. If Rac activation is completely inhibited by combined pretreatment of wortmannin and PP2, cells do not undergo chemotaxis [265]. This suggests that the chemotaxis to a CXCL8 gradient in dHL60 cells can utilize either PI3K or DOCK2. There exist parallel SFK-dependent and PI3K-dependent pathways which can produce Rac activation and chemotaxis.

The role of DOCK2 in plasmacytoid dendritic cells migration and function

Dendritic cells (DCs) are specialized antigen-presenting cells in PLNs and SLOs including 2 populations, myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) [266, 267]. DOCK2-/- pDCs, but not DOCK2-/- mDCs, are impaired in their homing to and localization within SLOs. The frequency of labeled DOCK2-/- pDCs migrated to the spleen and PLNs were reduced to half of the WT levels [182]. pDC migration is mediated by CXCR4 and CCR7 [182, 267-269]. In the transwell chemotaxis assay, DOCK2-/- pDCs, however, did not show detectable responses to CXCR4 ligand CXCL12. DOCK2 is a Rac activator indispensable for migration of pDCs. Rac activation was almost completely abolished in DOCK2-/- pDCs in response to CCR7 ligand CCL21. CCL21-induced actin polymerization and polarized F-actin accumulation were both impaired in DOCK2-/- pDCs [182]. Therefore, DOCK2 controls pDC migration by activating Rac.

pDCs have been shown to produce large amounts of type I IFNs (IFN-α and IFN-β) for antiviral immunity [270]. Upon binding of their ligands, both toll-like receptor TLR7 and TLR9 of pDCs recruit a cytoplasmic adaptor MyD88 to form a complex with IFN regulatory factor (IRF) 7 and IκB kinase (IKK) α directly binds to and activates IRF-7 leading to type I IFN induction [271-273]. DOCK2 controls type I IFN induction pathway during TLR7/9-mediated pDC activation. Both TLR7/9 ligands failed to induce IFN-α production in DOCK2-/- mice [209]. The production of IFN-α by DOCK2-/- pDCs was markedly suppressed when DOCK2-/- pDCs were stimulated with influenza A virus and HSV type 2 (HSV-2) which could activate the TLR7 and TLR9 signaling pathways. DOCK2 controls TLR7/9-mediated type I IFN induction in pDCs through Rac activation since Rac activation and F-actin assembly were almost totally abolished in DOCK2-/-

pDCs. This defect in Rac activation was also observed when DOCK2-/- pDCs were stimulated with influenza A virus and HSV-2 [209]. IKK-α carries two serine residues in the activation loop, which are phosphorylated during cellular stimulation. DOCK2-mediated Rac activation controls type I IFN induction partially through phosphorylation and activation of IKK-α. IKK-α phosphorylation could be inhibited in WT pDCs by a cell-permeable dominant-negative Rac mutant [209]. DOCK2 selectively controls type I IFN induction during TLR7/9-mediated pDC activation. Therefore, DOCK2 may be a novel therapeutic target for controlling type I IFN-related autoimmune diseases.

DOCK2 participates in bone marrow lympho-hematopoiesis

Hematopoietic stem/progenitor cells (HSCs/HPCs) are mainly found in BM [274, 275]. CXCL12 and its receptor CXCR4 are the critical retention axis for HSCs [276, 277]. The major effects of CXCR4, such as migration and cytoskeleton reorganization, are highly regulated by DOCK2 in HSCs/HPCs. DOCK2 is broadly expressed in BM hematopoietic compartment. Common lymphoid progenitors also expressed DOCK2 at high level [183].

The number of immature cells, such as B220+IgM- B cell precursors, common lymphoid progenitors (CLPs), and HSCs was completely normal in the absence of DOCK2. BM nucleated cells from WT or DOCK2-/- mice (CD45.2) were mixed with competitor BM cells (CD45.1 congenic) and transplanted into lethally irradiated CD45.1 recipients. WT and DOCK2-/- BM showed similar engraftment one month after transplantation, however, the contribution of DOCK2-/- leukocytes severely decreased two to six months after transplantation [183]. The contribution of CD45.2 HSCs against competitor HSCs one month after transplantation was similar between WT and DOCK2-/- mice. CLPs showed similar trend. DOCK2-/- cells have defective repopulation activity

at pro-T/B cell level. Therefore, the reduction originated from selective reduction in lymphocytes. There was no difference between WT and DOCK2-/- B cells, including B220+IgM- B cell precursors and B220+IgM+ mature B cells one month after transplantation; however, DOCK2-/- cells displayed a significantly lower inferior repopulation activity in this lineage three and six months after transplantation [183].

DOCK2 regulates cell proliferation in B cell lymphoma and CXCL13-stimulated prostate cancer cells through different signaling

There is membraneous and cytoplasmic DOCK2 expression in diffuse large B cell lymphoma and follicular lymphoma in human pathological tissue in 20 cases of human lymphoma specimens [278]. The DOCK2-Rac-ERK signaling pathway is responsible for cell proliferation of B cell lymphoma. Ramos and Raji are two types of B cell lymphoma. The growth rates of Ramos and Raji cells transfected with DOCK2 shRNA were significantly declined compared to the control cells as shown by the growth curve [278]. In vivo tumorigenesis assay using DOCK2 shRNA Ramos cells indicated that the size of subcutaneous xenografts of DOCK2 shRNA Ramos cells was significantly smaller than that of control Ramos cells [278]. There is a decreased phosphorylation level of ERK in DOCK2 shRNA Ramos and Raji cells and Rac1 activiation was significantly decreased in these cells as well [278]. In addition, NSC23766, a specific Rac inhibitor, significantly decreased the phosphorylation level of ERK in WT Ramos cells. It is an alternative target molecule of DOCK2 and Rac for lymphoma therapy.

Chemokines have been shown to play a role in homing and directional migration of CKRs bearing tumor cells to target organs and promote tumor cell survival and serve as growth factors in tumor microenvironment [279-282]. PCa cell lines, PC3, express the

chemokine CXCR5 to support tumor cell invasion [283-285]. CXCR5 binding to their receptor CXCL13 can sustain Akt activation and provide cells with a growth signal that allows them to withstand apoptotic stimuli [286]. DOCK2 was expressed by PC3. [184, 287]. CXCL13-mediated PC3 cells exhibited significant increases in phosphorylation levels of Akt and ERK1⁄2. compared to untreated cells. However, this activation was reduced by DOCK2 knockdown in PC3 cells. CXCL13:CXCR5 stimulated PC3 cell proliferation as shown by the MTT cell proliferation assays [287]. In addition, CXCL13-mediated PCa cell proliferation was inhibited by anti-CXCR5 antibody [184]. A further study indicated that CXCL13-dependent proliferation of PC3 cells was uniquely susceptible to DOCK2 siRNA and apoptosis analysis confirmed that lower cell proliferation observed with DOCK2 siRNA was not the result of cell death, as caspase 3⁄7 activity remained low under these conditions [287]. These data suggest that CXCL13-mediated PC3 cell proliferation is CXCR5 dependent and requires DOCK2.

DOCK2 IMPLICATION IN CLINICAL DISEASE

HIV-1 Nef binds the DOCK2-ELMO1 complex to activate Rac and inhibit lymphocyte chemotaxis

The infectious cycle of primate lentiviruses is linked to interactions between circulating T cells and APC which involve processes that are dependent on cytoskeletal dynamics regulated by the Rho GTPases [199]. HIV-1 pathogenesis partially results from the interplay between the virus and the immune system, especially the regulation of T cell homeostasis and regeneration. Nef, a lentiviral accessory protein, can alter cellular environments to facilitate activation of infected cells and replication in the host [288].

Nef interacts with DOCK2-ELMO1-Rac ternary complex that functions downstream of the TCR. In HEK 293 cells expressed Nef, DOCK2, ELMO1, and Rac2, DOCK2 complexes with ELMO1 (DOCK2-ELMO1) and Rac2 [289]. ELMO1 and Rac2 also copurified with complexes containing both Nef and DOCK2 suggesting that HIV-1 Nef binds DOCK2-ELMO1 complexes that contain Rac2. Nef also activates Rac in T cell lines. Nef activates Rac in CD4+ T lymphocytes in the absence of antigenic stimuli. Primary resting CD4+ T lymphocytes were infected with an HIV-1-derived vector expressing HIV-1 NA7 Nef (H-NA7) or a control nef-deleted vector (H-Δ). Infection with H-NA7 resulted in a readily detectable increase in the steady state level of activated Rac [289]. Robust stimulation of Rac2 activation by Nef requires association with both DOCK2 and ELMO1. Expression of Nef in the absence of ELMO1 or DOCK2 did not increase the fraction of activated Rac in HEK 293 cells. Mutations in Nef that disrupted Rac2 activation affected the association with DOCK2, ELMO1, and Rac2. P72A, P75A and R106A mutations in Nef completely disrupted detectable association with ELMO1 and Rac2, but only weakened that with DOCK2, suggesting that Nef associates with both DOCK2 alone and DOCK2 complexed with ELMO1 and/or Rac2 [289]. Nef deregulates DOCK2 function and affects lymphocyte chemotaxis. Nef disrupts migration to SDF-1 by activating Rac through the DOCK2-ELMO1 module and thus uncoupling Rac activation from CKR signaling [289]. It has been suggested that the chemotaxis of Jurkat T cells coexpressing Nef and GFP was inhibited. P72A, P75A, and R106A mutations diminished the ability of Nef to block migration [289]. Nef binds DOCK2-ELMO1 complexes to inhibit chemotaxis and promote T cell activation and thus provides an important mechanism by which Nef impacts pathogenesis by primate lentiviruses.

DOCK2, a molecular target for controlling transplant rejection-cardiac allograft rejection

Alloreactive T cell infiltration in graft tissue, a process involving a complex cascade of cellular responses, is a hallmark of allograft rejection [290]. Inhibition of the cytoskeletal reorganization in T cells is an approach to attenuate allograft rejection. DOCK2 is essential for chemokine- and antigen-induced Rac activation in T cells by functioning downstream of CKRs and TCRs to reorganize cytoskeletal. In addition, the antigen-induced translocation of TCR and lipid rafts to the APC interface is impaired severely in DOCK2-/- T cells, and results in a substantial reduction of alloreactivity in MLR [220]. DOCK2 deficiency effectively attenuates cardiac allograft rejection, and enables permanent graft survival when combined with certain medications. Cardiac transplantation between BALB/c mice and C57BL/6J (B6) mice with or without DOCK2 expression uncovered that DOCK2 deficiency in the recipients enables long-term survival of cardiac allografts [291]. When BALB/c hearts were transplanted into WT B6 or DOCK2+/- mice, the allografts were rejected with a median survival time (MST). However, BALB/c cardiac allografts survived in DOCK2-/- mice for a longer time [291]. DOCK2 deficiency improves myocardial histopathology of allografts by suppressing priming and activation of naive T cells in SLOs, and by attenuating graft tissue infiltration of activated T cells, the classic signs of acute rejection. The allografts from DOCK2-/- recipient mice showed only mild interstitial mononuclear cell infiltration. Allograft surviving >100 d in a DOCK2-/- recipient showed well-preserved myocardial structure and normal vessels with no interstitial fibrosis [291].

DOCK2 deficiency attenuates intragraft expression of T cell effector molecules. The intragraft expression of cytokines (IL-2, IFN-γ, TNF-α), chemokines (regulated upon activation, normal T cell expressed and secreted), and cytolytic molecules (granzyme B, perforin) were strongly induced in BALB/c grafts from the WT B6 recipient mice on day 6 after transplantation but not in the allografts from DOCK2-/- recipient mice [291]. DOCK2 deficiency attenuates alloreactivity or allocytotoxicity. T cell response to allo-MHC molecules is impaired significantly in DOCK2-/- mice, even after in vivo priming with alloantigens. Splenic T cells from WT B6 recipients showed a proliferative response to the mitomycin C-treated BALB/c spleen cells. The proliferative response of splenic T cells from DOCK2-/- mice was reduced to 40% of the WT level. Splenic T cells from DOCK2-/- T cells harvested on day 15 after transplantation showed poor proliferative response to BALB/c spleen cells [291]. DOCK2 is the downstream of CD28, a costimulatory molecule that is required for full activation of T cells. When DOCK2+/- T cells were stimulated with anti-CD28 mAb, activated Rac was detected readily. However, such Rac activation was totally abolished in DOCK2-/- T cells. DOCK2 deficiency affects graft tissue infiltration of activated alloreactive T cells. T cells from B6 and DOCK2-/- mice were stimulated with the mitomycin C-treated BALB/c spleen cells in vitro in the presence of IL-2, and were adoptively transferred into RAG1-/- recipients with BALB/c heart grafts to examine whether DOCK2 deficiency affects graft tissue infiltration of alloreactive T cells. On day 6 after adoptive transfer of B6 T cells, numerous CD3+ T cells were found in BALB/c cardiac grafts. However, CD3+ T cells infiltrating into the allografts were scarcely detected in the case of DOCK2-/- T cells [291].

There data indicated that DOCK2 deficiency attenuates graft tissue infiltration of T cells, even when the priming defect is bypassed.

DOCK2 is a microglial specific regulator of central nervous system innate immunity found in normal and alzheimer's disease brain

Innate immune activation of the central nervous system is associated with several neurodegenerative diseases including Alzheimer's disease (AD) [292, 293]. Microglia secret a variety of molecules including prostaglandins which could mediate paracrine neurotoxicity. The prostaglandin E2 receptor (EP2) in the mouse brain and primary cultures from mouse brain are potential therapeutic targets for AD [294, 295]. cDNA microarray analysis of EP2-/- and WT microglia after exposure to soluble Aβ1-42 showed that DOCK2 was significantly down-regulated in EP2-/- microglia. DOCK2 protein was only observed in WT microglia [296]. Therefore, the regulation of DOCK2 transcription and protein expression is a consequence of genetic ablation of EP2. DOCK2 is transcriptionally down-regulated in the absence of EP2 [296]. Immunohistochemistry found that DOCK2 is expressed by microglia in vivo. DOCK2 cellular staining is co-localized exclusively with established microglial markers tomato lectin and CD68 [296].

DOCK2 down-regulation had physiological relevance to activated microglial innate immune response. Primary microglia derived from WT and DOCK2-/- mice were exposed to LPS for 24 hr and pro-inflammatory cytokines especially TNF-α and MCP-1 were analyzed since they play important roles in neurotoxicity and microglial chemotaxis, respectively. DOCK2-/- microglia demonstrated a significant reduction in the induction of TNF-α and MCP-1 [296]. As with cytokine induction, DOCK2-/- microglia demonstrated a significant decrease in phagocytosis of 2μm fluorescent spheres. For a direct measure of

microglial DOCK2 effect on paracrine neurotoxicity, WT primary neurons were co-cultured with either WT or DOCK2-/- primary microglia, with or without LPS. Lactate dehydrogenase release by neurons was used as a marker for total neuronal cell death. LPS-stimulated DOCK2-/- microglia showed a significant reduction in bystander neuronal damage when compared with that of the wild-type mice. Six AD patients and six age-matched controls were used for analysis to see if DOCK2 expression was restricted to microglia in the human brain by probing for DOCK2 along with the lectin RCA1 as an established microglial marker. DOCK2 was expressed nearly exclusively in microglia in both the human frontal cortex and hippocampus. The number of DOCK2+ cells was significantly increased in the AD brain compared with normal controls. The total number of microglia determined by DOCK2+/RCA1+ double-labeling was increased in AD compared with normal controls [296]. This increase in DOCK2+ microglia is important because it shows that activated microglia in AD retain DOCK2 expression [296]. Therefore, DOCK2 may be targeted as a way to reduce AD associated neuroinflammation, including bystander neuronal toxicity.

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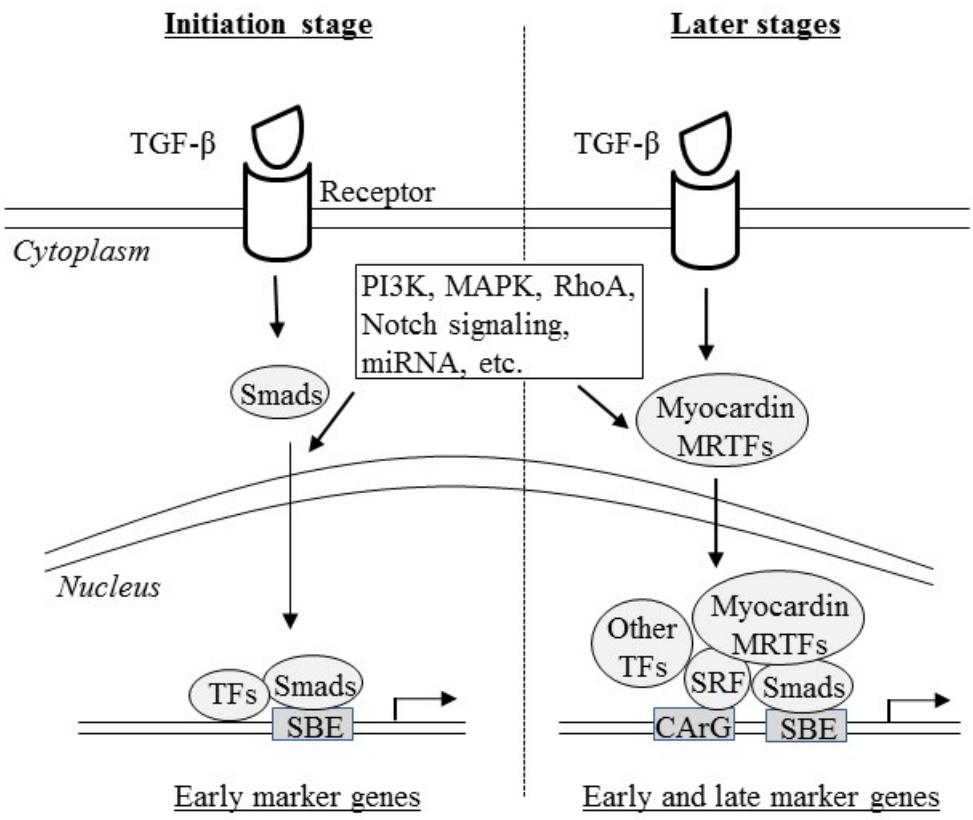

Figure 2.1 TGF-β in SMC differentiation.

Figure legends

Figure 2.1 TGF-β in SMC differentiation. In the initiation stage of SMC differentiation, TGF-β rapidly activates Smad signaling, leading to the activation of SMC early marker genes and cell fate determination by interacting with other transcription factors (TFs). At later stages, myocardin or MRTFs, via interacting with SRF, Smads and other TFs, enforce and accelerate SMC differentiation and maturation. In both the initial or later stages, other signaling pathways including PI3K, MAPK, RhoA, Notch, and miRNA may participate in the regulation of the differentiation process by interacting with TGF-β signaling molecules or downstream targets.

CHAPTER 3

A NOVEL IN VITRO MODEL SYSTEM FOR SMOOTH MUSCLE DIFFERENTIATION FROM HUMAN EMBRYONIC STEM CELL-DERIVED MESENCHYMAL CELLS [1]

[1] Xia Guo, Steven L Stice, Nolan L. Boyd and Shi-You Chen. 2013. *Am J Physiol Cell Physiol*. 304(4): C289–C298. Reprinted here with permission of the publisher.

List of abbreviations

SMC, smooth muscle cell; hES-MCs, human embryonic stem cell-derived mesenchymal cells; SRF, serum response factor; α-SMA, ACTA2, smooth muscle α-actin; SMMHC, smooth muscle myosin heavy chain; shRNA, short hairpin RNA; PFA, paraformaldehyde; TGF-β, transforming growth factor-β; α-MEM, alpha minimal essential medium; CNN1, calponin; TAGLN, SM22α; TβRII, type II receptors; TβRI, type I receptors; EB, embryonic body, ESCs, embryonic stem cells; BM-MSC, bone-marrow-derived mesenchymal stem cells; qPCR, quantitative reverse transcription-polymerase chain;

Abstract

The objective of this study was to develop a novel *in vitro* model for smooth muscle cell (SMC) differentiation from human embryonic stem cell-derived mesenchymal cells (hES-MCs). We found that hES-MCs were differentiated to SMCs by transforming growth factor-β (TGF-β) in dose and time-dependent manners as demonstrated by the expression of SMC specific genes smooth muscle α-actin, calponin and smooth muscle myosin heavy chain. Under normal growth condition, however, the differentiation capacity of hES-MCs was very limited. hES-MC-derived SMCs had an elongated and spindle-shaped morphology and contracted in response to the induction of carbachol and KCl. KCl-induced calcium transient was also evident in these cells. Compared to the parental cells, TGF-β-treated hES-MCs sustained the endothelial tube formation for a longer time due to the sustained SMC phenotype. Mechanistically, TGF-β-induced differentiation was both Smad- and SRF/myocardin-dependent. TGF-β regulated myocardin expression via multiple signaling pathways including Smad2/3, p38 MAPK, and PI3K. Importantly, we found that a low level of myocardin was present in mesoderm prior to SMC lineage determination, and a high level of myocardin was not induced until the differentiation process was initiated. Taken together, our study characterized a novel SMC differentiation model that can be used for studying human SMC differentiation from mesoderm during vascular development.

Key Words: Vascular smooth muscle cells, Human embryonic stem cell-derived mesenchymal cells, Transforming growth factor-β, Differentiation

Introduction

Vascular smooth muscle cells (SMCs) play a pivotal role in angiogenesis and vasculogenesis during embryonic development [1]. SMC functional impairments lead to the development of several prominent cardiovascular diseases including congenital heart diseases, atherosclerosis, hypertension, and restenosis after angioplasty [2-4]. The molecular mechanisms governing SMC differentiation especially the progenitor-specific regulation, however, remain largely unknown. This is due to, at least in part, the lack of *in vitro* model systems from different progenitors.

Lineage-tracing studies have shown that during embryonic development, SMCs originate from at least eight progenitors including neural crest, secondary heart field, somites, mesoangioblasts, proepicardium, splanchnic mesoderm, mesothelium and various stem cells [5]. Interestingly, SMCs from different origins are regulated differently and have distinct functional properties. For example, neural crest-derived SMCs and mesoderm-derived SMCs display dramatically different responses to the stimulation of morphogenetic factors such as transforming growth factor-β (TGF-β) [5]. As a matter of fact, TGF-β stimulates cell growth, extracellular protein expression as well as gene promoter activation in almost opposite ways in these two SMC subtypes [6]. Functionally, mesoderm-derived SMCs cannot rescue the outflow tract defects observed in neural crest-ablated chicken embryos [7]. Additional studies from different laboratories demonstrate that embryonically distinct subpopulations of SMCs are not functionally equivalent and that SMCs within different vascular beds utilize distinct cis-elements and

control regions to regulate SMC marker gene activation [8-12]. These studies suggest that SMC differentiation is controlled by different intracellular mechanisms among distinct SMC subtypes. Therefore, independent model systems using different progenitors especially the natural progenitors are essential for understanding the molecular mechanisms controlling SMC differentiation from different origins.

Previously, we have developed an *in vitro* model using neural crest cell Monc-1 because neural crest cells are the natural progenitors for neural crest-derived SMCs. This model has enabled us to dissect the molecular mechanisms governing SMC differentiation from neural crest progenitor cells [13-16]. In order to explore the molecular regulation of SMC differentiation from mesoderm, the present study was aimed to develop another model system using human embryonic stem cell-derived mesenchymal cells (hES-MCs). hES-MCs are natural SMC progenitors for mesoderm-derived SMCs. hES-MCs were derived from H9 human embryonic stem cells [17]. hES-MCs have the capacity to produce the three lineages associated with mesenchymal stem cells including osteogenic, chondrogenic, and SMC lineages [17]. We found that hES-MCs can robustly differentiate to SMC phenotype upon TGF-β induction. hES-MC-derived SMCs expressed SMC-specific marker genes and exhibited functional SMC morphology. The cells contracted and displayed intracellular calcium transient after stimulation with muscarinic agonist or KCl. Most importantly, these cells were recruited to endothelial tubes and sustained the endothelial tube formation. We also found that myocardin, the master regulator of SMC differentiation, was present at a low level in mesoderm prior to

SMC lineage determination. The high level of myocardin expression was not induced until the SMC differentiation process was initiated. In hES-MCs, TGF-β appeared to induce myocardin expression via multiple signaling pathways.

Methods

Cell culture

hES-MCs were obtained from ArunA Biomedical, Inc (Athens, GA) and characterized by the expression of mesenchymal stem cell surface markers [17]. The cells were maintained in alpha minimal essential medium (αMEM, Cellgro, Fisher Scientific, Pittsburgh, PA) containing 10% mesenchymal stem cell-qualified fetal bovine serum (Hyclone) and 2 mM L-glutamine (Hyclone). For TGF-β-induced SMC differentiation, the cells were grown for 12 h in serum-free medium followed by incubation with TGF-β for various times. Endothelial cells (ECs) C166 and Rat aortic smooth muscle cells (RASMCs) were grown in Dulbecco's modification of Eagle's medium (Invitrogen) supplemented with 10% FBS. Human umbilical vein ECs (HUVECs) were cultured in endothelial basal medium (EGM-2 Bullet Kit, Lonza).

Semiquantitative and quantitative reverse transcription-polymerase chain reaction (RT-PCR and qPCR)

Total RNA from cultured cells was extracted using Trizol Reagent (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized from 1 μg of total RNA by iScript cDNA Synthesis kit (Bio-Rad). Semiquantitative RT-PCR was performed as described [18]. Quantitative PCR analyses were performed with the Stratagene Mx3005P

using SYBR Green (Agilent Technologies). The sequences of the forward and reverse primers were described previously [13-16].

Immunofluorescent staining

hES-MCs were cultured on sterile coverslips in 24-well plates at a density of 10^3 cells/cm^2. Immunostaining was performed as described previously [14]. The primary antibodies for smooth muscle α-actin (α-SMA, ACTA2, Abcam), calponin (CNN1, Santa Cruz), smooth muscle myosin heavy chain (SMMHC, MYH11, Biomedical Technologies Inc.), Smad2 (Cell Signaling), or Smad3 (Santa Cruz) were used at 1:100. The slides were analyzed using a Nikon microscope.

Western blot analysis

hES-MCs were washed twice with PBS followed by protein extraction using RIPA buffer. Western blot analysis was performed as described previously [14]. Antibodies against ACTA2, CNN1, SM22α (TAGLN, Abcam), MYH11 and α-TUBA (Cell signaling) were used for detecting SMC-specific protein expression. Phospho-Smad2 and phospho-Smad3 antibodies were from Cell Signaling.

Contractility assay

hES-MCs were treated with TGF-β for 3 days. The cells were then washed once with PBS followed by stimulation with 1 mM carbachol or 75 mM KCl in the Krebs' solution. Contractility of the cells was observed with an inverted microscope for up to 30 min. Images of the same field before and after carbachol or KCl treatment were snapped and compared. Krebs' solution contained 120 mM NaCl, 5.9 mM KCl, 25 mM $NaHCO_3$,

1.2 mM NaH_2PO_4, 11.5 mM dextrose, 2.5 mM $CaCl_2$, and 1.2 mM $MgCl_2$. The solution was incubated for 30 min at 37°C and 5% (v/v) CO_2 in air to adjust the pH to 7.4. The high KCl depolarizing solution had the same composition as normal Krebs' solution with equal molar substitution of NaCl with KCl.

Measurement of intracellular Ca^{2+} signaling

hES-MCs were cultured on sterile coverslips in 24-well plates. hES-MCs were treated with TGF-β or vehicle for 3 days. The cells were incubated in Fluo-4 Direct™ calcium reagent solution with a probenecid concentration of 5 mM (Invitrogen) for 60 min at 37°C and 5% (v/v) CO_2. The cells were then washed once with PBS and stimulated by 75 mM KCl for 1 min to take image using a Nikon microscope.

ECs and hES-MCs coculture on matrigel

Matrigel (BD Biosciences) was plated in 24-well plates and allowed to polymerize at 37°C and 5% (v/v) CO_2 for at least 30 min before addition of cells. C166 cells were transduced with Ad-GFP (green) for 2 days. hES-MCs were treated with 1 ng/ml TGF-β or vehicle for 3 days. RASMCs, hES-MCs, and TGF-β-treated hES-MCs were labeled with PKH26 (Sigma), a red fluorescent dye used for cell membrane labeling. C166 cells (6×10^4 cells/well) were mixed with PKH26-labeled RASMCs, hES-MCs, or TGF-β-treated hES-MCs (1.2×10^4 cells/well). The mixed cells were added in different wells in a 24-well plate coated with Matrigel and incubated at 37°C for 24 h. Photographs were taken using a Nikon microscope. To detect the SMC differentiation of hES-MCs in coculture with ECs, hES-MCs were treated with 1 ng/ml TGF-β for 3 days. hES-MCs

treated with vehicle or TGF-β were cocultured with HUVEC for 12 h and 24 h. mRNA expression of SMC markers were examined by qPCR.

Transfection and luciferase assay

Acta2 and *Tagln* promoter constructs were transfected into hES-MCs as described previously [16, 18]. Cells were starved in serum-free medium for 12 h followed by 1 ng/ml TGF-β treatment for 24 h. Luciferase assay was performed 36 h post transfection. Experiments were repeated at least three times, and the results from a representative experiment were shown with standard deviations.

Statistical analysis

The data were expressed as mean ±SE. Statistical analysis was performed by ANOVA with pairwise comparisons between groups. A value of $P<0.05$ was considered as statistically significant.

Results

TGF-β induced hES-MCs to express SMC specific genes

SMCs are characterized by a large number of SMC-specific genes including ACTA2, TAGLN, CNN1, MYH11, α-actin, smoothelin B, etc. To test if TGF-β induced hES-MCs to become a SMC phenotype, we detected the expression of *Acta2*, *Cnn1* and *Myh11* because ACTA2 is one of the early differentiation markers; MYH11 is a late marker; and CNN1 is activated in the middle stage of the SMC differentiation [4]. Dose-dependent study showed that SMC marker mRNAs were induced by as little as 0.1 ng/ml of TGF-β. 1 or 5 ng/ml of TGF-β dramatically induced the marker gene expression

(Figure 3.1A). Since 1 ng/ml of TGF-β induced a high level expression of most SMC markers, we chose 1 ng/ml of TGF-β for all the subsequent experiments. As shown in Figure 3.1B, TGF-β induced *Acta2*, *Cnn1* and *Myh11* as early as 2 h, 4 h, and 8 h after TGF-β treatment, respectively, suggesting that hES-MCs can be robustly induced to SMC phenotype by TGF-β. Western blot analysis confirmed that SMC marker proteins were rapidly induced by TGF-β. As shown in Figure 3.1C, ACTA2 and CNN1 were induced as early as 8 h while MYH11 proteins were induced 24 h after TGF-β treatment. Immunostaining of ACTA2, CNN1 and MYH11 showed that at the first day of TGF-β induction, a small portion of hES-MCs were converted to SMC phenotype. However, after 3 days of the induction, more than 90% of the cells expressed SMC marker genes (Figure 3.1D-3.1E). Since SMCs display phenotypic plasticity in culture, we sought to determine if hES-MC-derived SMCs are able to maintain their phenotype after passaging. We found that hES-MC-derived SMCs continued to express ACTA2, CNN1 and TAGLN even after two passages (Figure 3.1F). The marker gene expression in passaged SMCs was independent of TGF-β because these cells were cultured in medium without addition of TGF-β. Therefore, TGF-β appears to induce a long lasting and perhaps heritable differentiation of hES-MCs into a smooth muscle phenotype.

Considering that the complete culture medium containing FBS that may influence the cell phenotype and lead to the spontaneous differentiation of hES-MCs, we detected the spontaneous differentiation of hES-MCs under normal growing condition, and found that early marker *Acta2* was slightly induced after cultured for 4 days (Figure 3.2A).

However, the more SMC-specific marker *Cnn1* was not significantly induced even cultured for up to 6 days (Figure 3.2B). Phenotypically, hES-MCs express a number of mesenchymal cell markers including CD90 and CD105 [19]. High levels of CD90 and CD105 were observed in hES-MCs under normal growing condition (Figure 3.2C-3.2D). However, serum-starvation decreased the expression of CD90 and CD105 but did not induce SMC differentiation (Figure 3.2C-3.2D). SMC differentiation was only induced after TGF-β treatment (Figure 3.1).

hES-MC-derived SMCs exhibited functional SMC morphology and contractility

SMCs display two phenotypes in human or animal bodies, i.e., contractile and synthetic (proliferating) phenotype depending on the physiological or pathological conditions [4]. SMC phenotypic modulation from contractile to synthetic phenotype contributes to a number of vascular diseases including atherosclerosis, hypertension and restenosis after angioplasty. To determine whether TGF-β-treated hES-MCs differentiate to contractile or synthetic SMC phenotype, we observed the morphological alteration after TGF-β induction. As shown in Figure 3.3A, TGF-β induced hES-MCs to become an elongated and spindle-shaped morphology resembling the contractile SMCs observed *in vivo* in the physiological condition. To test whether the hES-MC-derived SMCs are functional, we performed contractility assay using both muscarinic agonist carbachol and KCl to stimulate vehicle- or TGF-β-treated hES-MCs. As shown in Figure 3.3B-3.3E, vehicle-treated cells failed to contract with either carbachol or KCl stimulation. However, both carbachol and KCl induced TGF-β-treated cells to contract as indicated by the

shortening of the cells. Moreover, TGF-β-treated cells, but not the control cells, exhibited strong intracellular calcium transient after KCl stimulation (Figure 3.3F-3.3G). These data suggest that TGF-β converted hES-MCs to a contractile functional SMC phenotype.

TGF-β-treated hES-MCs stablized endothelial tubes

The primary function of SMCs during embryonic development is to participate in vasculogenesis and angiogenesis by attaching to endothelial tubes and regulating growth and function of endothelial cells. To further characterize TGF-β-induced SMC differentiation of hES-MCs, we tested if TGF-β-treated hES-MCs interact with endothelial cells by observing if these cells participate in endothelial tube formation *in vitro*. As shown in Figure 3.4A, 12 h after coculture of actual SMCs, TGF-β-treated, or untreated hES-MCs with C166 cells on Matrigel, endothelial tube networks were developed in all cocultures. SMCs, hES-MCs and TGF-β-treated hES-MCs were recruited to endothelial tubes and closely attached to the tubes (Figure 3.4A). After 24 h, endothelial tubes cocultured with SMCs or vehicle-treated hES-MCs were significantly disrupted (Figure 3.4A and 3.4B, 24 h). Most of the SMCs or vehicle-treated hES-MCs were disassociated with the endothelial tubes and kept with the endothelial aggregates (Figure 3.4A). However, the tubes cocultured with TGF-β-treated hES-MCs were well-maintained (Figure 3.4A and 3.4B, 24 h). TGF-β-treated hES-MCs were still tightly associated with the tubes at this time, similar to 12 h of coculture. These data indicate that hES-MC-derived SMCs play a critical role in stabilizing endothelial tubes.

To determine the mechanism by which TGF-β-treated hES-MCs, but not the vehicle-treated cells, sustained EC tube formation, we examined the phenotype of these cells after cocultured with ECs. hES-MCs treated with vehicle or TGF-β were cocultured with HUVEC for 12 and 24 h, and SMC specific markers *Acta2*, *Cnn1,* and *Myh11* were detected. Interestingly, both vehicle- and TGF-β-treated hES-MCs expressed SMC markers after 12 h of coculture although the expression levels in vehicle-treated cells were lower than TGF-β-treated cells (Figure 3.4B). The marker gene expression in vehicle-treated cells was likely attributed to EC signaling. However, after 24 h of coculture, SMC marker expression in vehicle-treated hES-MCs was significantly reduced to the basal level. The marker expression in TGF-β-treated cells was also reduced but the expression levels were significantly higher than the vehicle-treated cells (Figure 3.4B), and appeared to be sufficient to support the endothelial tube formation. The transient expression of SMC marker genes in vehicle-treated cells is similar to the SMC recruitment and differentiation by ECs observed *in vivo*. However, the maintenance of the SMC phenotype may require additional endocrine support available *in vivo* but is not present in the *in vitro* system.

hES-MC differentiation to SMC was Smad2/3- dependent

TGF-β signals through ligand binding to type II receptors (TβRII), recruitment and phosphorylation of type I receptors (TβRI), and activation downstream signal. To examine if TGF-β signaling components are present in hES-MCs, we examined the expression of the TGF-β receptors. As shown in Figure 3.5A, both type I and type II

receptors were expressed in hES-MCs prior to TGF-β treatment. TGF-β did not significantly alter the receptor expression. TGF-β signaling is predominantly transduced by Smad proteins. Both Smad2 and Smad3 have been shown to play important roles in SMC differentiation from embryonic body, 10T1/2 cells or neural crest cells [20]. To determine if Smad signaling plays a role in TGF-β-induced SMC differentiation of hES-MCs, we first detected if Smad2 and Smad3 are activated in TGF-β-treated cells. As shown in Figure 3.5B, both Smad2 and Smad3 were activated in hES-MCs as early as 30 min after TGF-β induction. TGF-β slightly altered Smad2 and Smad3 protein expression, but the difference was not significant (Figure 3.5B). To test if Smad2 and Smad3 are important for hES-MC differentiation, we knocked down their expression individually and tested if hES-MCs can be induced to SMC phenotype in the absence of Smad2 or Smad3. As shown in Figure 3.5C and 3.5D, knockdown of Smad2 blocked TGF-β-induced ACTA2 expression. This effect is specific because in cells where Smad2 were not knocked down, ACTA2 expression was evident (Figure 3.5D, arrow in left panel). Similar with Smad2, knockdown of Smad3 also blocked TGF-β-induced ACTA2 expression (Figure 3.5E and 3.5F). These results suggest that TGF-β-induced SMC differentiation from hES-MCs was Smad-dependent.

hES-MC differentiation to SMC was SRF/CArG/myocardin-dependent

Expression of most SMC marker genes is regulated by SRF/CArG/myocardin [21]. To determine whether or not TGF-β-induced SMC differentiation from hES-MCs depends on SRF, we analyzed *Acta2* and *Tagln* promoter activity in hES-MCs treated

with or without TGF-β. We found that TGF-β activated both *Acta2* and *Tagln* promoters in hES-MCs (Figure 3.6A and 3.6B). However, CArG box mutations significantly inhibited TGF-β-induced *Acta2* promoter activity (Figure 3.6A) and completely blocked *Tagln* promoter activity (Figure 3.6B). Moreover, SRF knockdown by shRNA significantly blocked TGF-β-induced expression of *Acta2*, *Cnn1* and *Myh11* (Figure 3.6C-3.6F), suggesting that TGF-β-induced hES-MC differentiation is SRF-dependent. Myocardin is a SRF coactivator and is considered as a master regulator of SMC differentiation [22-26]. In hES-MCs, TGF-β induced *Myocd* mRNA expression in a time dependent manner (Figure 3.7A- 3.7B). Myocardin expression reached the highest level at 8 h after TGF-β induction. Previous studies have shown that p38 MAPK is involved in TGF-β-induced myocardin expression in human coronary artery SMCs [27]. In hES-MCs, TGF-β appeared to induce myocardin expression through multiple signaling pathways including p38 MAPK, PI3K and Smad2/3 pathways because pathway-specific inhibitors for p38 MAPK or PI3K blocked myocardin expression (Figure 3.7C). This inhibitory effect was extended to the expression of SMC marker calponin (Figure 3.7D). In addition to p38 MAPK and PI3K, both Smad2 and Smad3 were also important to TGF-β-induced myocardin expression (Figure 3.7E-3.7F).

Since myocardin is very important to SMC differentiation, we sought to determine in what SMC developmental stage myocardin is induced. As aforementioned, hES-MCs were derived from mesoderm that was differentiated from H9 human embryonic stem cells [17]. We detected myocardin expression in these three cells with

different developmental stages and found that myocardin was barely detectable in H9 stem cells (Figure 3.7G). However, a low level of myocardin was present in mesoderm, and a slightly higher expression was found in hES-MCs (Figure 3.7G-3.7H). Of importance, TGF-β treatment robustly induced myocardin expression (Figure 3.7G-3.7H). These data indicate that myocardin expression is gradually activated along the differentiation stages. A surge of myocardin expression may be needed for the effective SMC differentiation.

Discussion

Since SMC differentiation is an important process in vascular development, much effort has been made to illustrate the molecular mechanisms underlying the differentiation process using various *in vitro* model system including C3H10T1/2 cells, neural crest cells, A404, embryonic body (EB), and embryonic stem cells (ESCs) [14, 28-33]. Although these models have significantly contributed to our understanding of SMC differentiation, each of these models has its limitations. For example, C3H10T1/2 cell is a very convenient model, but the definitive SMC marker MYH11 cannot be readily induced in these cells [20]. Neural crest cells are the demonstrated SMC progenitors *in vivo*, but culture of neural crest cell line Monc-1 and Joma 1.3 requires a complex defined medium making culture difficult [34, 35]. A404 is a great model, but it is not a natural SMC progenitor found *in vivo* because it was developed by introducing *Acta2* promoter into the cells [31]. EB recapitulates the embryonic development, but the purity of SMC lineage in the induced EB cannot be clearly defined. SMC differentiation from embryonic stem cells

reflect the normal process of the differentiation, but maintaining the pure undifferentiated ESC clones takes a lot of effort and may be a challenge to many laboratories [36, 37]. In addition, previous studies have shown that human ES cells can be differentiated to both EC and SMC populations in the same differentiation conditions. These cells are excellent for *in vivo* neoangiogenesis and regeneration of blood vessel. However, they may not be ideal for studying the mechanism of SMC differentiation especially the progenitor-specific regulation because the SMCs differentiated from ES cells are heterogenic and thus a mixed populations. Therefore, a new robust model system is essential for better understanding of the molecular mechanisms governing SMC differentiation from mesoderm. Several studies have used bone-marrow-derived mesenchymal stem cells (BM-MSC) to study SMC differentiation, but they are not well-characterized, and no model system has been established using BM-MSCs [38, 39]. It is also unclear whether or not BM-MSCs are natural SMC progenitors *in vivo*. The model system described in this study is likely an ideal model for SMC differentiation due to the following facts: (1) hES-MCs are likely the natural SMC progenitors because most of the vascular SMCs are derived from mesoderm [5]. Characterization of the mesenchymal cells used in this study indicates they are derived from mesoderm [17] and the mesenchymal cell markers are diminished during the SMC differention. Moreover, the spontaneous differentiation of hES-MCs is minimal due to the low expression of *Acta2* and *Cnn1* under normal growing condition; (2) hES-MCs are easy to culture. It only needs αMEM and 10% MSC-qualified fetal bovine serum. The cells adhere to the culture dish and can

be split up to 10 passages; (3) one single cytokine, TGF-β, is sufficient for the induction of SMC differentiation. Moreover, TGF-β can induce a rapid SMC differentiation. 3 days of TGF-β treatment converts most of hES-MCs into SMC phenotype; (4) SMCs differentiated from this model are functional. They can contract in response to muscarinic agonist carbachol as well as KCl. Most importantly, hES-MC-derived SMCs can be recruited to endothelial tube network and help stabilize the tubes; (5) since hES-MCs are derived from human embryonic stem cells, this model may be used to study SMC differentiation in human.

During embryonic development, the SMC marker ACTA2 appears very early during the vascular development. CNN1, a later marker, expresses after the initiation of SMC differentiation. MYH11 appears at the late stage of vascular development [40-42]. The TGF-β-induced SMC differentiation from hES-MCs recapitulates the normal SMC differentiation process. *Acta2* is activated at 2 h, *Cnn1* at 4 h while *Myh11* at 8 h after TGF-β induction of hES-MCs, which mimics SMC marker gene expression pattern observed during normal SMC development [42]. TGF-β-induced expression of SMC markers in hES-MCs appears to depend on both Smad signaling and SRF/CArG/myocardin because knockdown of Smad2, Smad3, or SRF blocks SMC marker expression. Consistently, mutation of SRF-binding CArG box blocks SMC marker promoter activity. These results indicate that TGF-β induction of hES-MC may serve as a physiologically-relevant model for SMC differentiation.

Interestingly, in addition to TGF-β-treated cells, untreated hES-MCs are also recruited to endothelial tubes in 12 h after coculture. A previous study suggests that notch signaling is important for pericyte/SMC recruitment to endothelial tubes [43, 44]. It is likely that notch signaling is activated in these untreated cells, leading to their recruitment to endothelial tubes. Alternatively, hES-MCs may be stimulated by cytokines from ECs or presented in the Matrigel including TGF-β. Indeed, control hES-MCs cocultured with ECs transiently express SMC marker genes in the initial period of recruitment (12 h) but the differentiation is blocked in a prolonged coculture (24 h), resulting in disruption of endothelial tube network after 24 h of coculture. The unsustained expression of SMC marker genes in control hES-MCs is probably due to the lack of endocrine support required for a complete SMC differentiation that is only available *in vivo*. The endothelial tubes cocultured with actual SMCs also disrupted after 24 h of incubation, probably because these SMCs have underwent phenotypic modulation in culture, i.e., become non-contractile SMCs that have lost their function in maintaining endothelial integrity [4]. hES-MC-derived SMCs are able to preserve the endothelial tubes probably because they have acquired the contractile property of functional SMCs.

TGF-β induces myocardin expression, which is important for the SRF/CArG-mediated SMC marker gene activation. It appears that TGF-β induces hES-MCs to express myocardin via mechanism different from other cells because multiple signaling pathways including Smads, p38 MAPK and PI3K are all required for TGF-β-induced myocardin expression in hES-MCs [27, 45]. Although myocardin is

considered as a master regulator of SMC differentiation, several reports indicate that its role in SMC development is not as critical as previously anticipated because the expression of early SMC marker genes such as *Tagln* and *Acta2* emerges prior to detectable myocardin mRNA in the embryonic dorsal aorta; this may suggest that myocardin has a minor role in the initiation of SMC differentiation in some vascular tissues [40, 45-48]. In order to better understand myocardin function in SMC development, especially in human SMC differentiation, we have detected the dynamic expression of myocardin in different phases of the differentiation from mesoderm. We found that myocardin expression is not obvious in human embryonic stem cells. However, myocardin mRNA is detectable at a low level in mesoderm, and its expression was increased when mesoderm is differentiated to hES-MCs. These low levels of myocardin appear not to be able to activate SMC differentiation. However, when hES-MCs are treated with TGF-β, myocardin mRNA is dramatically induced. These results suggest that a threshold expression is required for myocardin to be functionally involved in SMC differentiation. This notion is also supported by a previous report showing that a low level of myocardin is expressed in A404 cells, but it is not converted to SMC phenotype until retinoic acid induction [26].

In addition to serving as a model for SMC differentiation, hES-MC-derived SMCs have potential to be used for tissue engineering because of their human origin. Since hES-MCs are derived from mesoderm, these cells are potentially important resources for regenerating human SMCs that are mesoderm-origin including most vascular SMCs.

Acknowledgments

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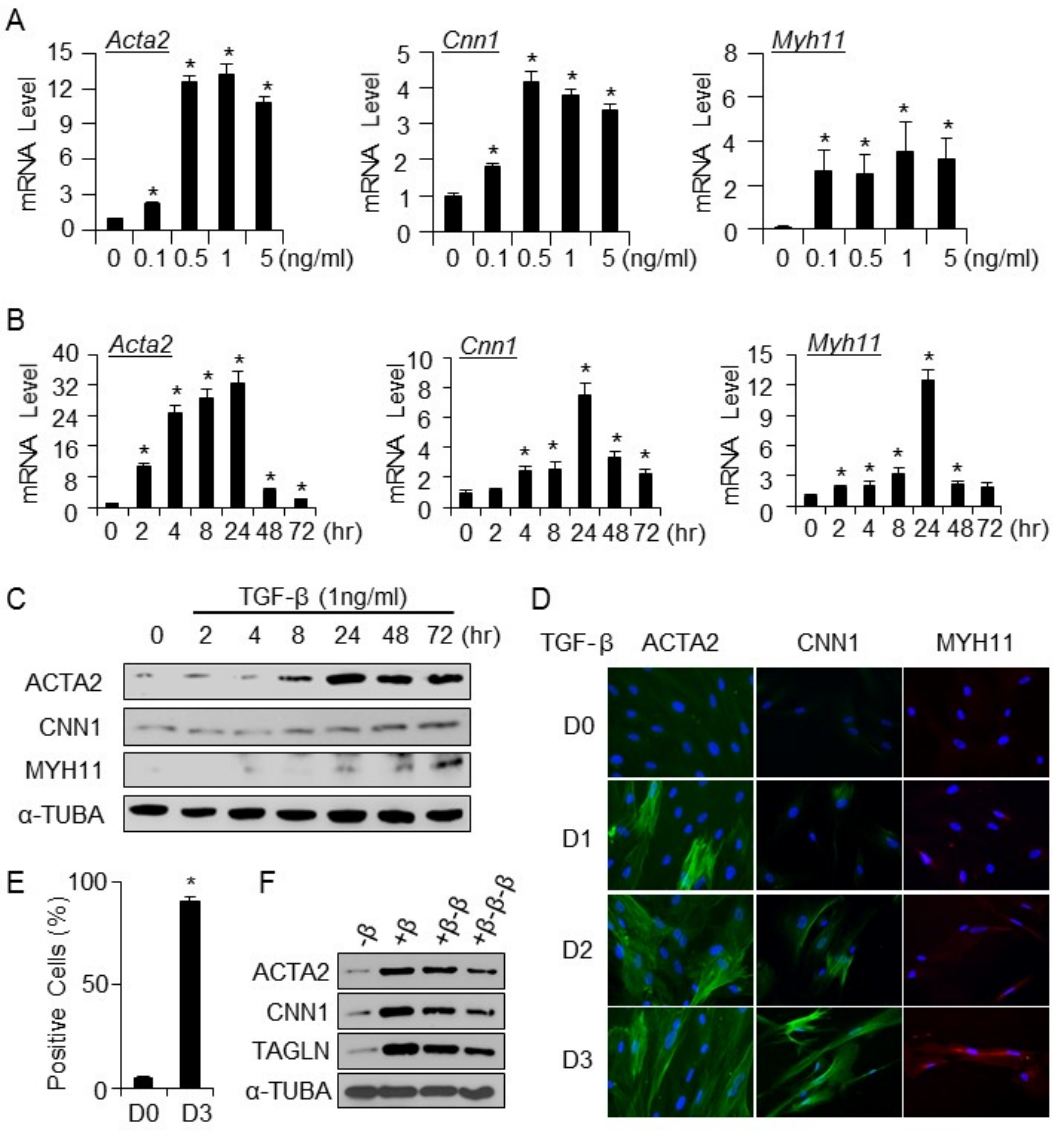

Figure 3.1. TGF-β induced SMC specific gene expression in hES-MCs.

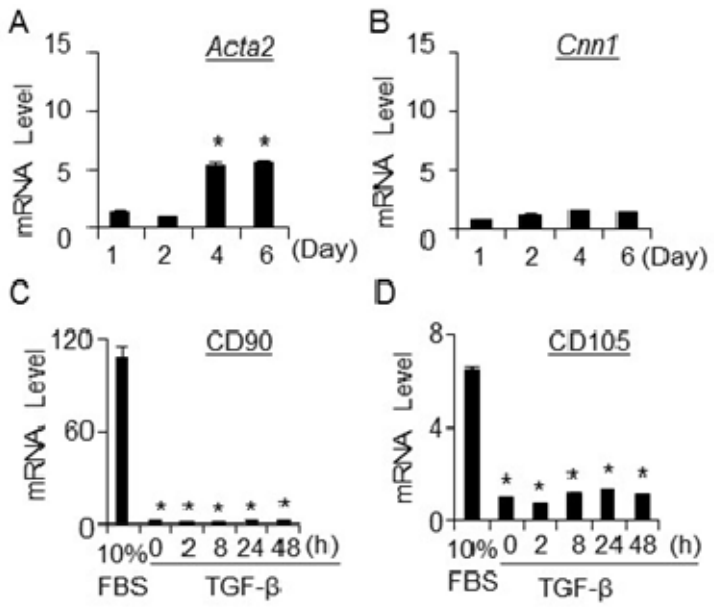

Figure 3.2. Characterize hES-MC differentiation under normal growing condition.

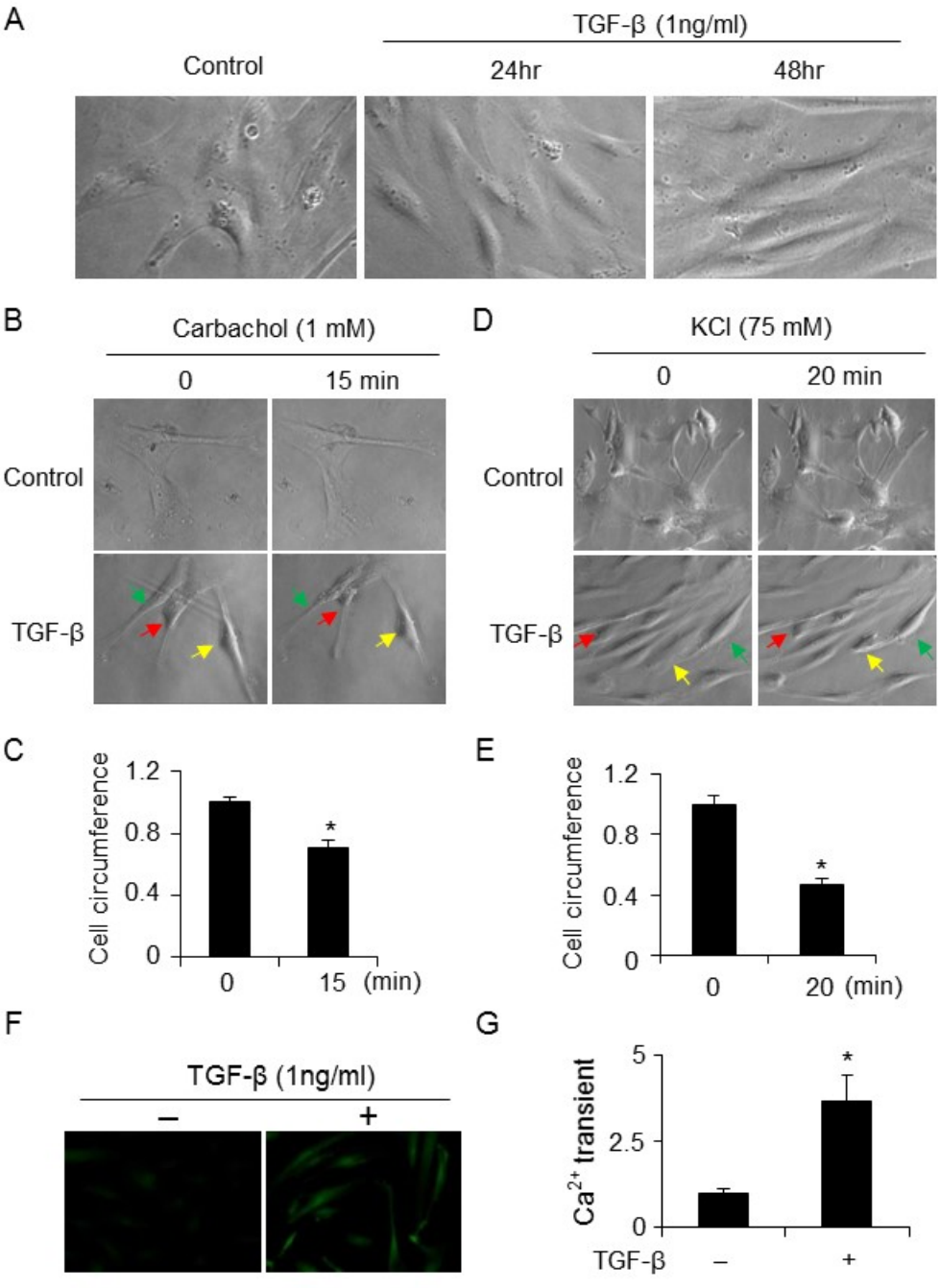

Figure 3.3. TGF-β converted hES-MCs to a contractile SMC phenotype.

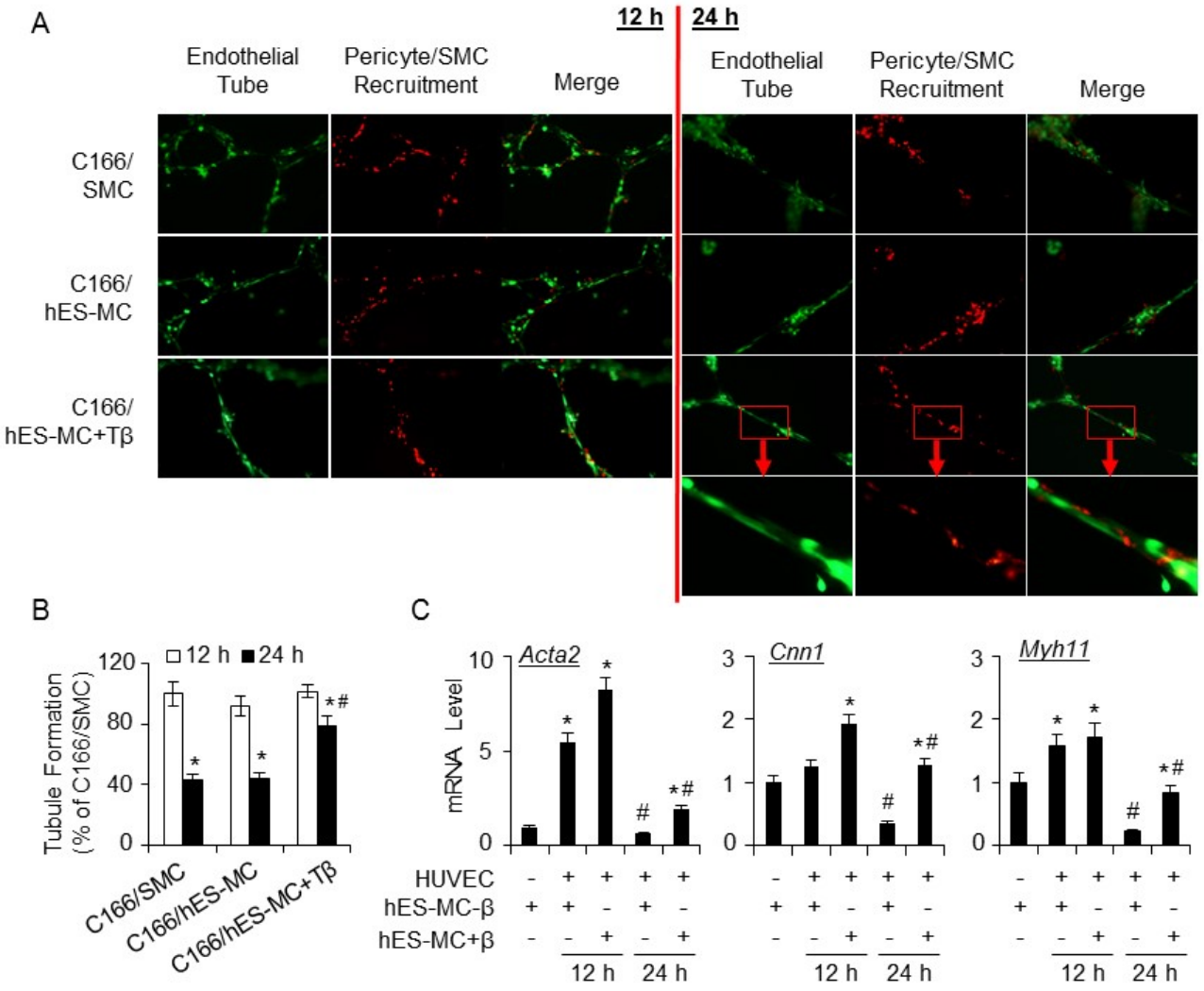

Figure 3.4. Recruitment of hES-MCs to endothelial tubes.

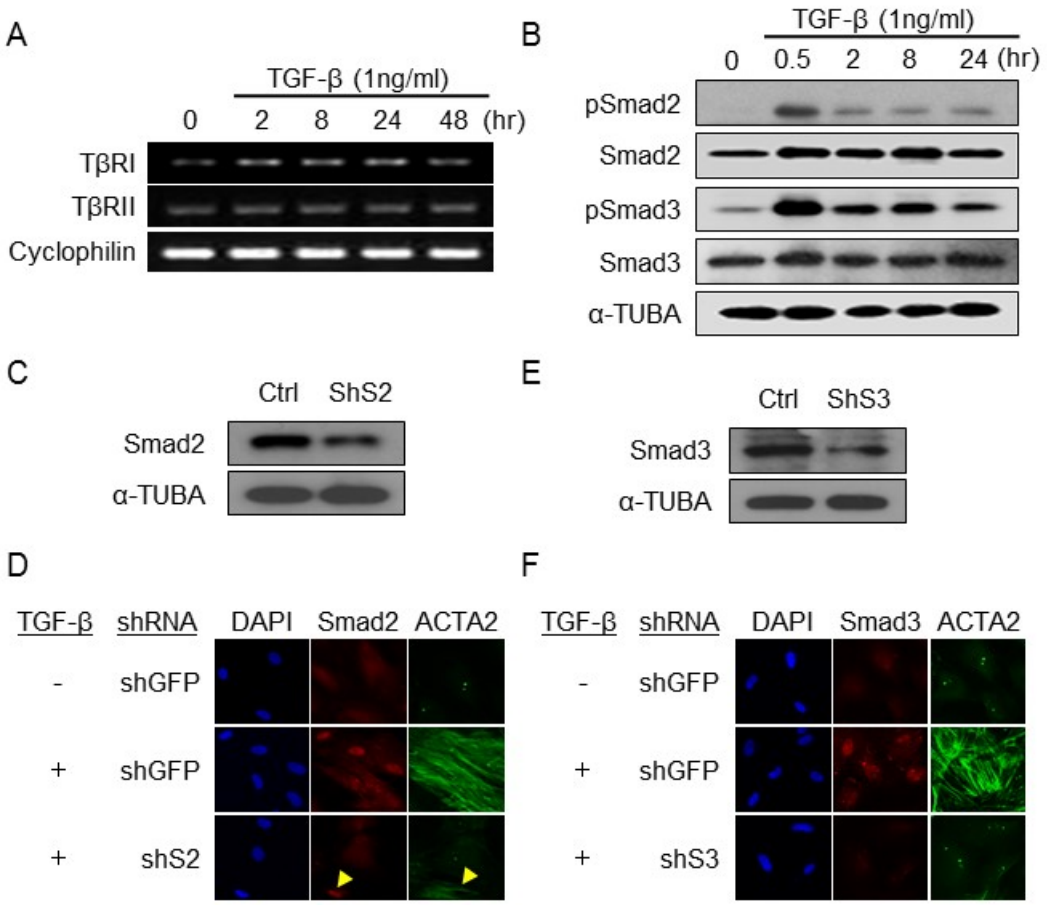

Figure 3.5. TGF-β-induced SMC differentiation of hES-MCs was Smad-dependent.

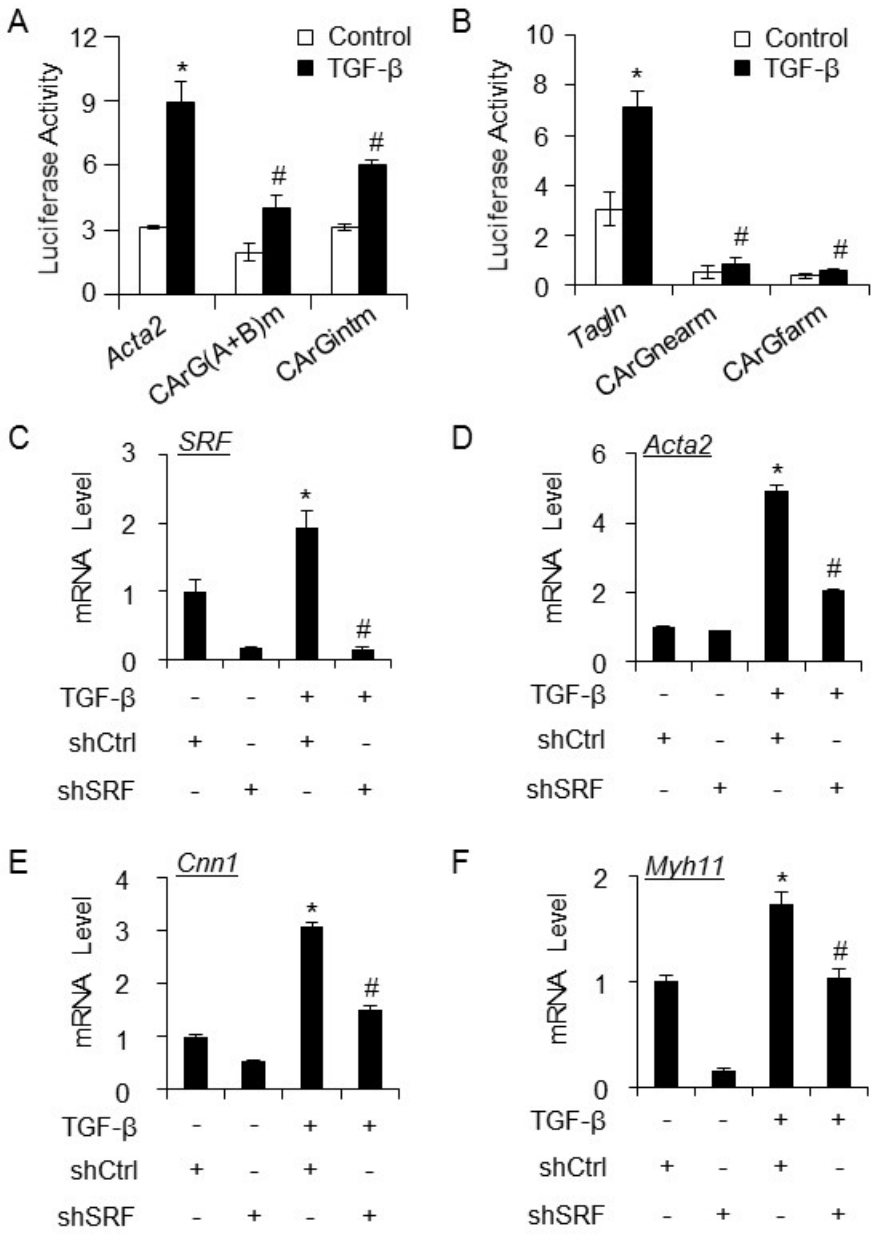

Figure 3.6. TGF-β-induced SMC differentiation of hES-MCs was SRF-dependent.

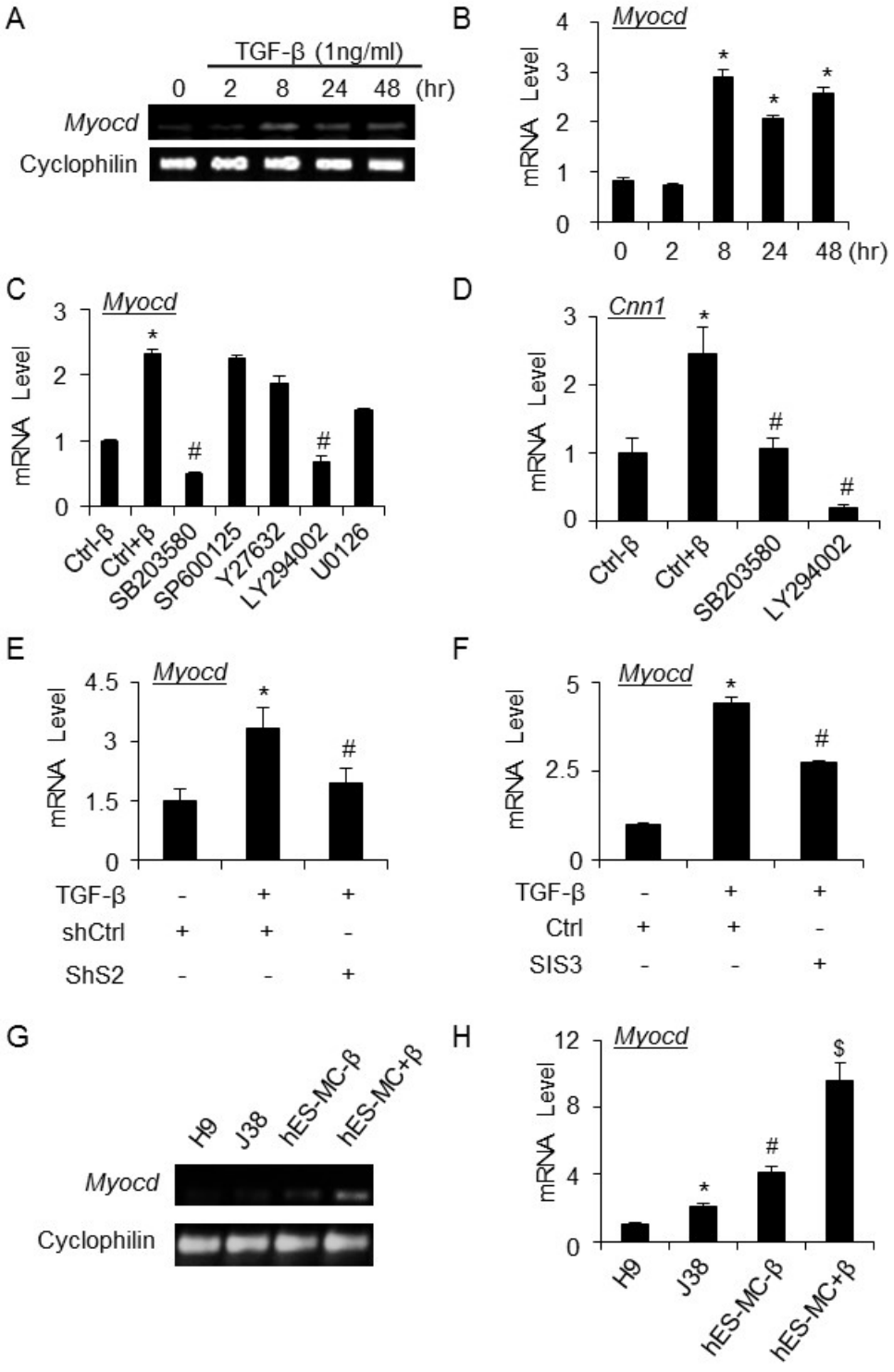

Figure 3.7. Myocardin expression in SMC differentiation of hES-MCs.

Figure legends

Figure 3.1. TGF-β induced SMC specific gene expression in hES-MCs. A, TGF-β stimulates SMC marker mRNA expression in a dose-dependent manner. hES-MCs were treated with various dosages of TGF-β as indicated for 24 h. mRNA expression of SMC markers were examined by qPCR. *P<0.05 compared to the untreated group for each individual marker. **B,** TGF-β stimulates SMC marker mRNA expression in a time-dependent manner. hES-MCs were treated with 1 ng/ml TGF-β for various time points as indicated. qPCR was performed as in A. *P<0.05 compared to the untreated group for each individual marker. **C,** TGF-β induces SMC marker protein expression. hES-MCs were treated with 1 ng/ml TGF-β for various time points as indicated and western blot was performed. α-TUBA was an internal control. **D**, Global expression of SMC-specific genes in TGF-β-treated hES-MCs. hES-MCs were treated with 1 ng/ml TGF-β for 0 (D0) 1 (D1), 2 (D2), and 3 days (D3) to detect SMC marker expression by immunostaining. **E,** Percentage of α-SMA positive cells before and after 3 days of TGF-β treatment. *P<0.05 compared to untreated cells (D0). **F**, Stability of SMC phenotype after trypsinization and reculture. hES-MCs were treated with vehicle (-β) or 1 ng/ml TGF-β (+β) for 3 days. Cells were then trypsinized and recultured without TGF-β (+β-β). After 2 days of incubation, cells were trypsinized again and recultured without TGF-β for an additional 2 days (+β-β-β). Marker expression was examined by western blot.

Figure 3.2. Characterize hES-MC differentiation under normal growing condition. A-B, *Acta2* (A) and *Cnn1* (B) mRNA expression in hES-MCs under normal growing

condition (10% FBS) for the times indicated. *P<0.05 compared to the cells cultured for one day. **C,** CD90 mRNA expression in hES-MCs under normal growing condition or treated with TGF-β for various times as indicated. *P<0.01 compared to the cells with 10% FBS. **D**, CD105 mRNA expression in hES-MCs under normal growing condition or treated with TGF-β for various times as indicated. *P<0.01 compared to the cells with 10% FBS.

Figure 3.3. TGF-β converted hES-MCs to a contractile SMC phenotype. A, TGF-β converted hES-MCs to an elongated and spindle-shaped functional SMC morphology. **B,** TGF-β-treated hES-MCs contracted in response to carbachol induction. hES-MCs were cultured on silicon-coated coverslips and treated with vehicle (Control) or 1 ng/ml TGF-β for 3 days. Photographs were taken before and after 1 mM carbachol induction for 15 min. Arrowheads indicate representative contracted cells in the TGF-β-treated group. **C,** Quantification of the circumference of TGF-β-treated hES-MCs before and after carbachol induction. *P<0.05 compared to the carbachol-untreated cells (0 min). **D,** TGF-β-treated hES-MCs contracted in response to KCl stimulation. hES-MCs were treated with vehicle (Control) or 1 ng/ml TGF-β for 3 days. Photographs were taken before and after 75 mM KCl stimulation for 20 min. Arrowheads indicate representative contracted cells in the TGF-β-treated group. **E,** Quantification of the circumference of TGF-β-treated hES-MCs before (0 min) and after KCl stimulation (20 min). *P<0.05 compared to the KCl-untreated cells (0 min). **F,** TGF-β-treated hES-MCs exhibited intracellular Ca^{2+} transient in response to KCl induction. hES-MCs were treated with

vehicle (-) or 1 ng/ml TGF-β (+) for 3 days. Cells were preloaded in Fluo-4 Direct™ calcium reagent with a probenecid concentration of 5 mM (Invitrogen) for 60 min and then stimulated with 75 mM KCl for 1 min before taking images. **G,** Quantification of intracellular Ca^{2+} transient after KCl stimulation. **P*<0.05 compared to vehicle-treated cells (-).

Figure 3.4. Recruitment of hES-MCs to endothelial tubes. A, hES-MCs participated in endothelial tube formation. C166 cells were transduced with Ad-GFP (green) for 2 days. hES-MCs were treated with vehicle or 1 ng/ml TGF-β (hES-MC+Tβ) for 3 days. hES-MCs and RASMCs (SMC) were labeled with a red fluorescent dye PKH26. C166 cells were mixed with SMCs, hES-MCs, or hES-MC+Tβ, and cocultured on 2D Matrigels in individual wells in a 24-well plate. Images were obtained by fluorescent microscopy. Images of the PKH26-labeled cells were overlaid (Merge) with GFP-labeled endothelial tubes after 12 h (top) and 24 h (bottom) of coculture. Bottom images in the 24 h group were higher magnifications of the endothelial tubes in the rectangle boxes in C166/hES-MC+Tβ coculture. **B**, Endothelial tube formation was quantified by assessing tube length. **P*<0.05 compared to the corresponding coculture for 12 h; $^{\#}P$<0.05 compared to C166/SMC coculture for 24 h. **C,** Transient differentiation of hES-MCs to SMC phenotype in coculture with ECs. hES-MCs treated with vehicle or TGF-β (1 ng/ml) for 3 days were cocultured with HUVEC for 12 and 24 h. mRNA expression of SMC markers were examined by qPCR. **P*<0.05 compared to hES-MCs without coculture; $^{\#}P$<0.05 compared to the corresponding group with coculture for 12 h.

Figure 3.5. TGF-β-induced SMC differentiation of hES-MCs was Smad-dependent. **A,** TGF-β receptor expression in hES-MCs. hES-MCs were treated with vehicle (0) or 1 ng/ml TGF-β for various time points as indicated. TGF-β type I (TβRI) and type II receptor (TβRII) was detected by RT-PCR. Cyclophilin was an internal control. **B,** Smad2 and Smad3 activation in TGF-β-treated hES-MCs. hES-MCs were treated vehicle (0) or 1 ng/ml TGF-β for the times indicated. Western blot was performed to detect Smad2 and Smad3 expression and phosphorylation. **C,** Smad2 knockdown by Smad2 shRNA (shS2) was detected by western blot. **D,** Smad2 knockdown by shS2 blocked TGF-β-induced ACTA2 expression, as examined by immunostaining. GFP shRNA (shGFP) served as a control. Arrowhead indicates cells in which Smad2 was not knocked down expressed ACTA2. Photographs were taken at the same area for DAPI (blue), Smad2 (red), and ACTA2 (green). **E,** Smad3 knockdown by Smad3 shRNA (shS3) was detected by western blot. **F,** Smad3 knockdown by shS3 blocked TGF-β-induced ACTA2 expression, as examined by immunostaining. GFP shRNA (shGFP) served as a control. Photographs were taken at the same area for DAPI (blue), Smad3 (red), and ACTA2 (green).

Figure 3.6. TGF-β-induced SMC differentiation of hES-MCs was SRF-dependent. A, TGF-β-induced *Acta2* promoter activity was CArG-dependent. *Acta2* promoter (from -2.6 to +2.8 kb) constructs with wild type or mutant CArG box either in the promoter region (CArG(A+B)m) or in the first intron (CArGintm) were transfected into hES-MCs followed by vehicle (Control) or 1 ng/ml TGF-β treatment for 24 h. Luciferase assay was performed. $*P<0.01$ compared to wild type promoter with vehicle treatment; $^{\#}P<0.01$

compared to TGF-β-induced wild type promoter. **B,** TGF-β-induced *Tagln* promoter activity was CArG-dependent. *Tagln* promoter constructs with wild type or mutant CArG box in the two SRF binding sites (CArGnearm or CArGfarm) were transfected into hES-MCs followed by vehicle (Control) or 1 ng/ml TGF-β treatment for 24 h. Luciferase assay was performed. **P*<0.01 compared to wild type promoter with vehicle treatment; $^{\#}$*P*<0.05 compared to TGF-β-induced wild type promoter. **C,** SRF knockdown by SRF shRNA (shSRF) was detected by qPCR. **P*<0.01 compared to vehicle-treated group. $^{\#}$*P*<0.01 compared to TGF-β-treated group with control shRNA (shCtrl) transfection. **D-F,** SRF knockdown blocked *Acta2* (D), *Cnn1* (E), and *Myh11* (F) expression. **P*<0.01 compared to vehicle-treated group; $^{\#}$*P*<0.05 compared to TGF-β-treated group with shCtrl transfection in individual gene.

Figure 3.7. Myocardin expression in SMC differentiation of hES-MCs. A, TGF-β induced myocardin (Myocd) mRNA expression as detected by RT-PCR. **B,** TGF-β induced *Myocd* expression as detected by qPCR. **P*<0.05 compared to vehicle-treated cells (0 h). **C,** TGF-β induced *Myocd* expression via p38 MAPK and PI3K signaling pathways. hES-MCs were treated with pathway-specific inhibitors for p38 (SB203580), JNK (SP600125), RhoA (Y27632), PI3K (LY294002), or ERK1/2 (U0126) for 30 min prior to vehicle (Ctrl-β) or TGF-β (1 ng/ml) treatment for 8 h. *Myocd* expression was detected by qPCR. **P*<0.05 compared to vehicle-treated group; $^{\#}$*P*<0.05 compared to TGF-β-treated group without inhibitor (Ctrl+β). **D,** TGF-β-induced *Cnn1* expression was blocked by p38 MAPK and PI3K inhibitors. **P*<0.01 compared to vehicle-treated group

(Ctrl-β); $^{\#}P<0.01$ compared to TGF-β-treated group without inhibitor (Ctrl+β). **E-F,** Both Smad2 and Smad3 were required for TGF-β-induced *Myocd* expression. Knockdown of Smad2 expression by shRNA (E, shS2) or blockade of Smad3 activity by Smad3 inhibitor (F, SIS3-Smad3 inhibitor) inhibited *Myocd* expression. $*P<0.01$ compared to vehicle-treated group; $^{\#}P<0.05$ compared to TGF-β-treated group with control shRNA (E, shCtrl) or SIS3 vehicle (F, Ctrl). **G,** *Myocd* expression in different developmental stages. Total RNA was extracted from hES cells (H9), mesoderm (J38), hES-MCs, and TGF-β-treated hES-MCs. *Myocd* expression was detected by RT-PCR. **H,** Quantitative analysis of *Myocd* expression shown in G by normalizing to cyclophilin. $*P<0.05$ compared to H9 cells, $^{\#}P<0.05$ compared to J38 mesoderm; $^{\$}P<0.001$ compared to all other cells.

CHAPTER 4

DEDICATOR OF CYTOKINESIS 2 IS ESSENTIAL FOR THE INITIATION OF TRANSFORMING GROWTH FACTOR-BETA-INDUCED SMOOTH MUSCLE DIFFERENTIATION [1]

[1] Xia Guo, Ning Shi, Yung-Chun Wang, Kun Dong, Shi-You Chen. To be submitted to *Cardiovascular Research*.

List of abbreviations

SMC, smooth muscle cell; hES-MCs, human embryonic stem cell-derived mesenchymal cells; α-SMA, smooth muscle α-actin; Ad-shRNA, adenovirus short hairpin RNA; PFA, paraformaldehyde; TGF-β, transforming growth factor-β; α-MEM, alpha minimal essential medium; TβRII, TGF-β type II receptors; TβRI, TGF-β type I receptors; EB, embryonic body, ESCs, embryonic stem cells; BM-MSC, bone-marrow-derived mesenchymal stem cells; DOCK2, dedicator of cytokinesis 2; KO, Knock out; WT, wild type; FBS, fetal bovine serum; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; DPC, days post-coitum

Abstract

Smooth muscle cell (SMC) differentiation is an important process during embryonic development. The underlying mechanisms regulating the SMC differentiation process, however, remain largely unknown. The present study identified dedicator of cytokinesis 2 (DOCK2) as one of the factors mediating the SMC differentiation. DOCK2 was important in mediating the transforming growth factor-β (TGF-β)-induced SMC differentiation from human embryonic stem cell derived mesenchymal cells (hES-MCs) and 10T 1/2 pluripotent embryonic mesenchymal cells. TGF-β induced DOCK2 expression during SMC differentiation as shown by the SMC marker expression including α-SMA, SM22α and calponin. DOCK2 overexpression induced while DOCK2 knockout inhibited SMC early marker gene expression. Mechanistically, DOCK2 induced SMC differentiation by activating phosphorylated p38 MAPK. DOCK2 overexpression induced while DOCK2 knockdown inhibited the level of phosphorylated p38. P38 pathway inhibitor dramatically decreased DOCK2-induced SMC marker gene expression. In vivo, reduced blood vessel formation was observed in the DOCK2-/- embryo yolk sac compared to the WT yolk sac. Hemorrhage was also evident in the DOCK2-/- embryo. Histological analysis revealed defective expression of calponin and α-SMA in dorsal aorta of DOCK2-/- embryo. Taken together, our studies demonstrated that DOCK2 is an important novel factor regulating SMC differentiation.

Keywords: Dedicator of Cytokinesis 2, TGF-β, Smooth Muscle Cell, Differentiation.

Introduction

Vascular smooth muscle cells (SMCs) originate from multiple sources during vascular development. The most common precursor for vascular smooth muscle is poorly defined mesenchymal cells derived from the mesoderm [1]. A large number of environmental cues such as growth factors/inhibitors, inflammatory mediators, cell-cell and cell-matrix interactions have been shown to regulate smooth muscle cell differentiation. Transforming growth factor-beta (TGF-β) is among the most potent soluble growth factors that activate SMC contractile gene expression in both specified SMC and non-SMC types [2-7]. It is well established that the TGF-β/Smad signaling pathway plays critical roles in the SMC differentiation of pluripotent mesenchymal progenitors such as C3H/10T1/2 (10T1/2) [8, 9]. However, TGF-β downstream targets that mediate the SMC differentiation, remain largely unknown.

Dedicator of cytokinesis 2 (DOCK2) is an atypical guanine nucleotide exchange factor for the Rho-small guanine triphosphatase [10]. In adult animal or human, DOCK2 is mainly expressed in hematopoietic cells, and involved in lymphocyte migration and activation via regulating actin cytoskeleton through Rac activation [10-12]. DOCK2 controls various immunological functions including helper T cell differentiation, neutrophil chemotaxis and type I interferon induction [13-16]. It is unknown, however, if DOCK2 is involved in regulating SMC differentiation.

In this study, we found that DOCK2 is an important regulator in TGF-β-induced SMC differentiation of hES-MCs and 10T1/2 cells. DOCK2 was upregulated and activated along with SMC markers. Both gain and loss of function studies showed that DOCK2 had a dramatic effect on the transcription and protein expression of SMC

markers. Most significantly, we found that during the initiation of TGF-β-induced SMC differentiation, DOCK2 activated p38 MAPK signaling. In vivo studies demonstrated that DOCK2 is essential for SMC differentiation during embryo development. DOCK2 deficiency caused less vascular formation in the yolk sac and hemorrhage on the body surface of mouse embryo.

Methods

Cell culture and transfection

hES-MCs were obtained from ArunA Biomedical, Inc (Athens, GA). The cells were maintained in alpha minimal essential medium (α-MEM, Cellgro, Fisher Scientific, Pittsburgh, PA) containing 10% mesenchymal stem cell-qualified fetal bovine serum (FBS) (Hyclone) and 2 mM L-glutamine (Hyclone) [17]. C3H10T1/2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Hyclone). For transfections, cells were cultured in 12 or 6-well plates for 24 h (about 80% confluence), and then transfected using Lipofectamine LTX (Invitrogen) according to the Manufacture's instructions. For transduction with adenoviral short hairpin RNA (Ad-shRNA) constructs, cells at 50% confluence were transduced with adenoviral constructs for 16 h followed by plasmid transfection.

Preparation of shRNA adenoviral vector

Adenoviral vectors expressing scramble (Ad-shctrl) or DOCK2 short hairpin RNA (Ad-shDOCK2) were constructed, and the viruses were purified as described previously [18]. Human, mouse, and scramble shDOCK2 forward and reverse sequences were shown in table 1. Double-stranded DNA was cloned into pRNAT-H1.1/Adeno shuttle vector (Genscript) digested with Mlu I and Hind III. Recombinant adenoviral

vector was produced by homologous recombination in AD-1 competent cells. The resultant recombinant vector digested with Pac I was transfected into AD-293 cells to package viral particles. The adenovirus was purified by $CsCl_2$ gradient ultracentrifugation and dialyzed in dialysis buffer.

Immunofluorescent staining

E16.5 mouse embryo was collected for analysis. The embryo section containing the aorta were rehydrated, blocked with 10% goat serum and permeabilized with 0.01% Triton X-100 in PBS, and incubated with primary antibody α-SMA (Sigma) at 1:100 in blocking buffer consisting of 1% bovine serum albumin (BSA) in PBS at 4℃ overnight followed by incubation with TRITC-conjugated secondary antibody (1:100). Stained cells were imaged with a Nikon microscope.

Western blotting

Cells were cultured in DMEM and treated with TGF-β or p38 pathway inhibitor SB203580 for various times. Cells were then washed with PBS for two times, followed by protein extraction using RIPA buffer (50 mmol/L Tris-HCI, pH 7.4, 1% Triton X-100, 0.25% wt/vol sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EGTA, 0.1% SDS, protease inhibitors, phosphatase inhibitors). Protein concentration was measured using BCA Protein Assay Reagent (Thermo Scientific). 5 or 10 μg of the lysates was resolved by SDS-PAGE and transferred to PVDF (Bio-Rad) or nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% nonfat dry milk for regular antibodies or 5% BSA for anti-phospho antibodies, and then incubated for 1 to 2 h with primary antibodies in blocking buffer followed by incubation with HRP-conjugated secondary antibody for 1 h (Sigma). Primary antibodies used were: anti-DOCK2 (Abcam), anti-α-SMA, anti-

SM22α (Abcam), anti-calponin (Cell signaling), anti-α-tubulin (Cell signaling). Detection was performed with enhanced chemiluminescence (Millipore).

Mating and tissue collection

DOCK2+/– mice, obtained from Yoshinori Fukui's lab, were genotyped using primers listed in Table 2. The DOCK2+/– male mice were mated with the DOCK2+/– female mice. Gestational age was determined based on the presence of a vaginal plug, with the morning of detection designated as day 0.5. Plugged females were removed from the males and housed in separate cages. The pregnant females were euthanized at 13.5 or 16.5 days post-coitum (DPC) by CO2 inhalation (~2.0 L/min with mouse cage) followed by cervical dislocation to ensure the mice were dead and would not regain consciousness. The uteri were dissected. The vasculatures on yolk sacs and embryo were captured using a Cannon camera. Embryonic tissue was used for genotyping.

Statistical analysis

All values are expressed as mean ± S.E. Data were analyzed using ANOVA with pair wise comparisons between groups. A P value <0.05 was considered statistically significant.

Results

TGF-β induced DOCK2 expression during SMC differentiation.

TGF-β is an important determinant for SMC lineage. We found that DOCK2 expression was significantly increased along with the activation of SMC early markers α-SMA, SM22α and calponin. TGF-β induced the DOCK2 expression in a time-dependent manner in 10T1/2 cell while the SMC markers α-SMA and SM22α were induced. There was a four-fold increase of DOCK2 expression after 24 h of TGF-β treatment (Fig. 4.1A-

4.1B). In hES-MCs, TGF-β induced DOCK2 protein expression in a dose-dependent manner during the SMC differentiation as shown by the SMC marker expression α-SMA and calponin (Fig. 4.1C-4.1D). These data suggest that DOCK2 may be involved in the TGF-β-induced SMC differentiation.

Knockdown of DOCK2 blocked TGF-β-induced SMC differentiation.

To test if DOCK2 is required for TGF-β-induced SMC differentiation, we used DOCK2 specific shRNA to knockdown DOCK2 expression in 10T1/2 and hES-MCs. Cells were infected with Ad-Ctrl and Ad-shDOCK2 for 48 h and then treated with TGF-β for 24 h individually. As shown in Figure 4.2A-4.2D, Ad-shDOCK2 dramatically inhibited the expression of DOCK2 as well as SMC markers in both 10T1/2 cells (Figure 4.2A-4.2B) and hES-MCs (Figure 4.2C-4.2D), demonstrating an essential role of DOCK2 in SMC differentiation.

DOCK2 was sufficient for the early phase of SMC differentiation.

To test if DOCK2 alone can induce SMC differentiation, we tested whether or not DOCK2 overexpression induces SMC marker gene expression. As shown in Figure 4.3A-4.3B, forced expression of DOCK2 in 10T1/2 cell promoted α-SMA and SM22α protein expression. In hES-MCs, DOCK2 overexpression induced SMC marker calponin and SM22α expression (Fig. 4.3C-4.3D).

DOCK2 induced SMC differentiation via p38 MAPK signaling.

Previous studies have shown that several signaling pathways including ERK, JNK, p38 MAPK, and PI3K were phosphorylated during TGF-β-induced SMC differentiation. Since DOCK2 was important for TGF-β-induced SMC differentiation (Fig. 4.3), we sought to determine which signaling mediated the DOCK2 activity. Thus,

we overexpressed DOCK2 in 10T1/2 cells and detected the phosphorylation of p38, Akt, Creb, Smad2. As shown in Figure 4.4A-4.4B, DOCK2 overexpression only increased the phosphorylation of p38. Conversely, knockdown of DOCK2 using Ad-shDOCK2 blocked TGF-β-induced p38 phosphorylation (Fig. 4.4C-4.4D), indicating that DOCK2 is required for TGF-β-induced p38 phosphorylation.

In order to determine if DOCK2-induced differentiation is mediated by p38 MAPK signaling, we used p38-selective inhibitor SB203580 to treat 10T1/2 cells while overexpressing DOCK2. As shown in Figure 4.5A-4.5B, DOCK2 significantly activated α-SMA and SM22α protein expression. SB203580, however, blocked the DOCK2-induced effects. These results suggest that p38 MAPK signaling is essential for DOCK2 induction of SMC differentiation (Figure 4.5C).

DOCK2 is essential for SMC differentiation during embryonic development.

To determine if DOCK2 is important for SMC differentiation during the development of the systemic vasculature, blood vessel formation was analyzed at E13.5 yolk sac. DOCK2-/- yolk sacs exhibited fewer and smaller vessels and increased vascular space compared with the wild-type yolk sacs (Figure 4.6A-4.6B). When the embryo vasculatures were examined, approximately 20% of DOCK2-/- embryos displayed hemorrhages, especially in the midbrain region, as compared to the wild type littermates (Figure 4.6C-4.6D). Embryo dorsal aorta immunostaining revealed a reduced expression of α-SMA and calponin in the DOCK2-/- embryo compared with wild-type embryos (Figure 4.6E-4.6F), indicative of a defective SMC differentiation during the embryonic development.

Discussion

SMC differentiation is an important process during embryonic development. Abnormal SMC differentiation and phenotypic switching contribute to the pathogenesis and progression of several prominent cardiovascular diseases including congenital heart diseases, aortic aneurysm, atherosclerosis, hypertension, and restenosis [19-22]. Understanding the molecular mechanisms that control SMC differentiation will provide fundamental insights into the pathological processes of these cardiovascular diseases. VSMCs originate from multiple sources during vascular development [1]. The most common precursor for vascular smooth muscle is poorly defined mesenchymal cells derived from mesoderm. A large number of environmental cues such as growth factors/inhibitors, inflammatory mediators, cell-cell and cell-matrix interactions have been shown to regulate smooth muscle cell differentiation [19]. Transforming growth factor-beta (TGF-β) is among the most potent soluble growth factors that activate SMC contractile gene expression in both specified SMC and non-SMC types [6, 7, 23, 24]. Genetic analysis in the mouse has demonstrated that knockout of TGF-β, its receptors, coreceptor endoglin, or downstream signaling molecules all lead to abnormal vascular development. The vascular defects are caused, at least in part, by the failed differentiation of mesenchymal precursors into vascular SMCs [5, 25-27]. However, the molecular mechanisms underlying TGF-β function, especially TGF-β downstream targets responsible for the activation of SMC differentiation and/or SMC maturation remain largely unknown. In the present study, by using the pluripotent mesenchymal progenitors C3H/10T1/2 (10T1/2) and hES-MCs that we established in chapter 3, we found that DOCK2 is able to regulate the differentiation of SMC progenitor hES-MCs and 10T1/2 cells. DOCK2 is induced

along with the differentiation of hES-MCs and 10T1/2 cells. Knockdown of DOCK2 attenuates SMC differentiation. However, DOCK2 overexpression induced SMC marker gene expression. Most importantly, DOCK2-/- causes a reduction in blood vessel formation and an increase in avascular space as compared to wild-type yolk sacs. Moreover, DOCK2-/- embryos displayed hemorrhages at 13.5 DPC. It appears that the hemorrhage is due to a defects in SMC differentiation because reduced expression of SMC markers α-SMA and calponin was evident in the dorsal aorta of DOCK2-/- embryos. These results demonstrate that DOCK2 is essential for SMC differentiation in vivo during the embryonic development.

Emerging evidence has demonstrated an essential role of p38 MAPK in regulating TGF-β1-induced SMC differentiation [24, 28]. We found that DOCK2 increases p38 phosphorylation, and knockdown of DOCK2 significantly inhibits TGF-β-induced p38 phosphorylation. Importantly, p38 inhibitor dramatically decreases DOCK2-induced expression of SMC markers α-SMA and SM22α in hES-MCs, thus providing new insight into the mechanism of TGF-β1-p38 MAPK in modulating SMC differentiation. Future studies should identify downstream substrates of DOCK2 in activating p38 MAPK activity.

In summary, we have identified a novel role of DOCK2 in regulating SMC differentiation. Our results shed a new light on the molecular mechanisms governing SMC differentiation and maturation, which may contribute to the development of novel therapeutics for treating the SMC-related cardiovascular disorders.

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Table 4.1: Primer sequences for adenovirus construction

Primer	Forward	Reverse
Huamn Ad-shDOCK2	5'-CGCGTCGACGGAAATATCTTGGACCCTGATAATACTTCAAGAGAGTATTATCAGGGTCCAAGATATTTCCGTCTTTTTTCCAAA-3'	5'-AGCTTTTGGAAAAAAGACGGAAATATCTTGGACCCTGATAATACTCTCTTGAAGTATTATCAGGGTCCAAGATATTTCCGTCGA-3'
Mouse Ad-shDOCK2	5'-CGCGTCGTTCAGTCCATGAATGAGATCGTGCAGAGTTCAAGAGACTCTGCACGATCTCATTCATGGACTGAACTTTTTTCCAAA-3'	5'-AGCTTTTGGAAAAAAGTTCAGTCCATGAATGAGATCGTGCAGAGTCTCTTGAACTCTGCACGATCTCATTCATGGACTGAACGA -3'
Scramble Ad-shCtrl	5'-CGCGTCGATCGATGATTCGCCCGGCGTCTTCATAATTCAAGAGATTATGAAGACGCCGGGCGAATCATCGATCTTTTTTCCAAA-3'	5'-AGCTTTTGGAAAAAAGATCGATGATTCGCCCGGCGTCTTCATAATCTCTTGAATTATGAAGACGCCGGGCGAATCATCGATCGA-3'

Table 4.2: Primer sequences for DOCK2 mice genotyping

Primer	Sequence
DOCK2SC1	5' -ATCTGTCTGCATGATGGATGCTT-3'
DOCK2SC2	5'-AATGCCTGCTCTTTACTGAAGG-3'
DOCK2SCB2	5'-AAGTGACCTTACCTGTGACAG-3'

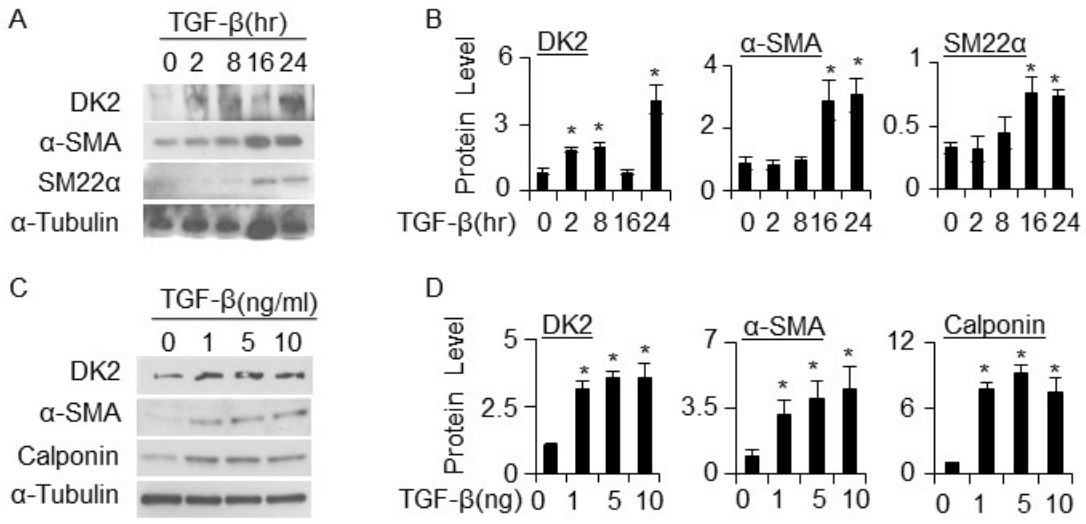

Figure 4.1. TGF-β induced DOCK2 expression while promoting SMC differentiation.

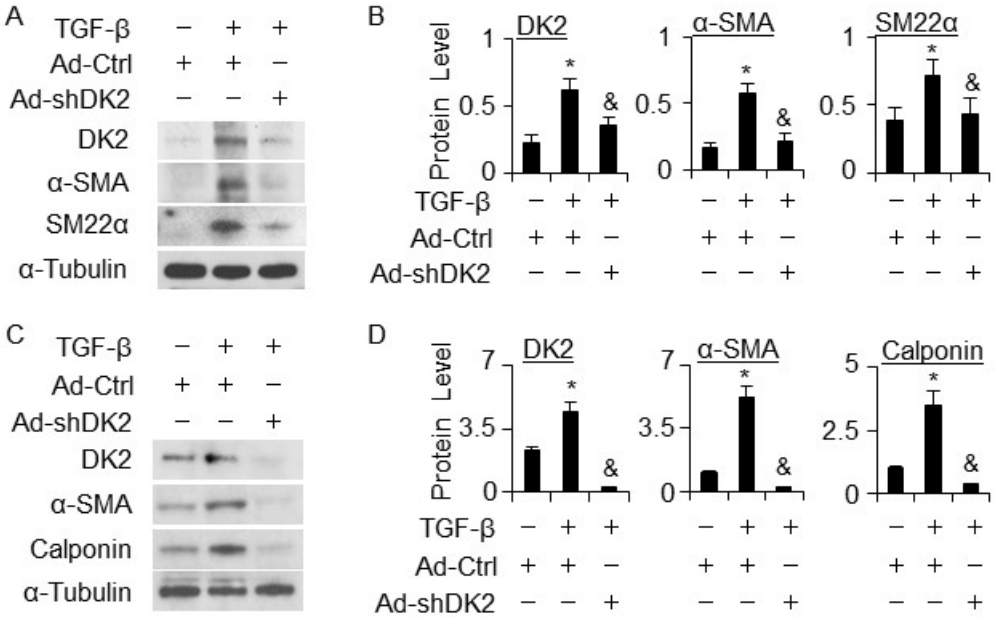

Figure 4.2. DOCK2 is essential for TGF-β-induced SMC differentiation-shRNA.

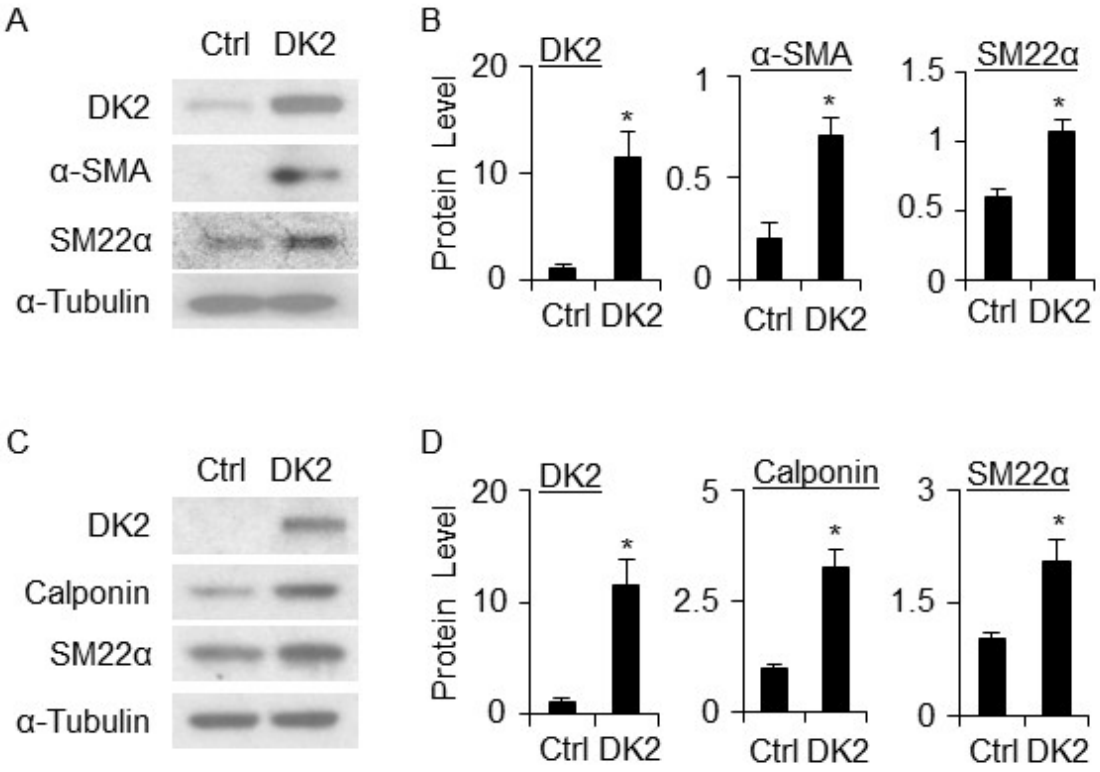

Figure 4.3. DOCK2 overexpression induced SMC differentiation.

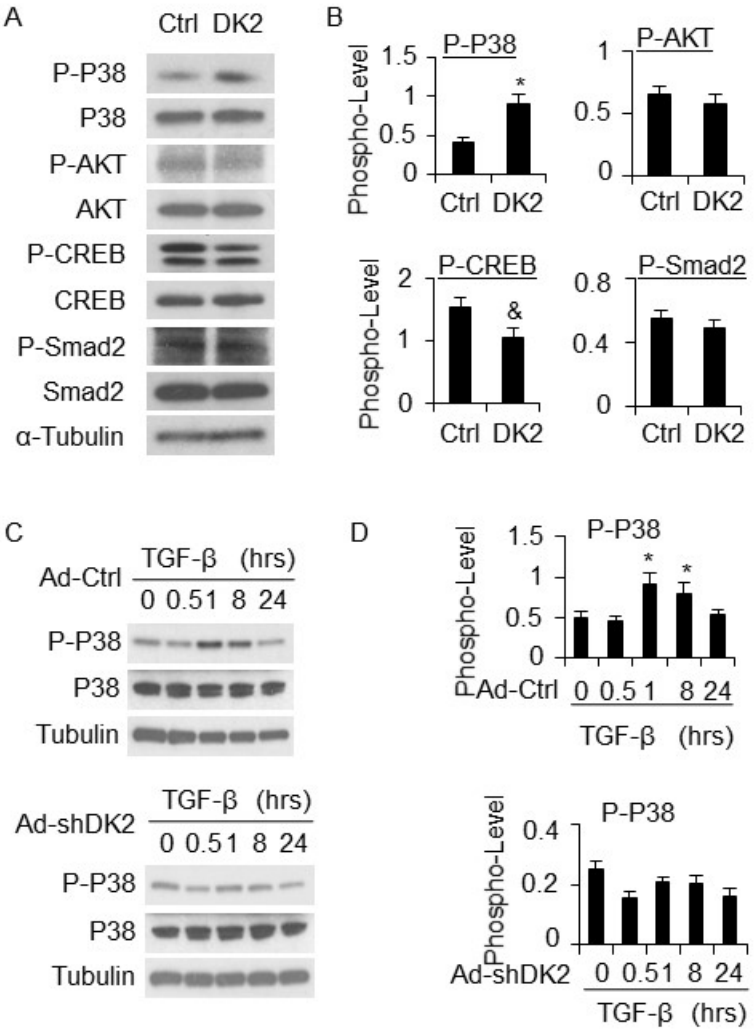

Figure 4.4. DOCK2 regulate P38 phosphorylation.

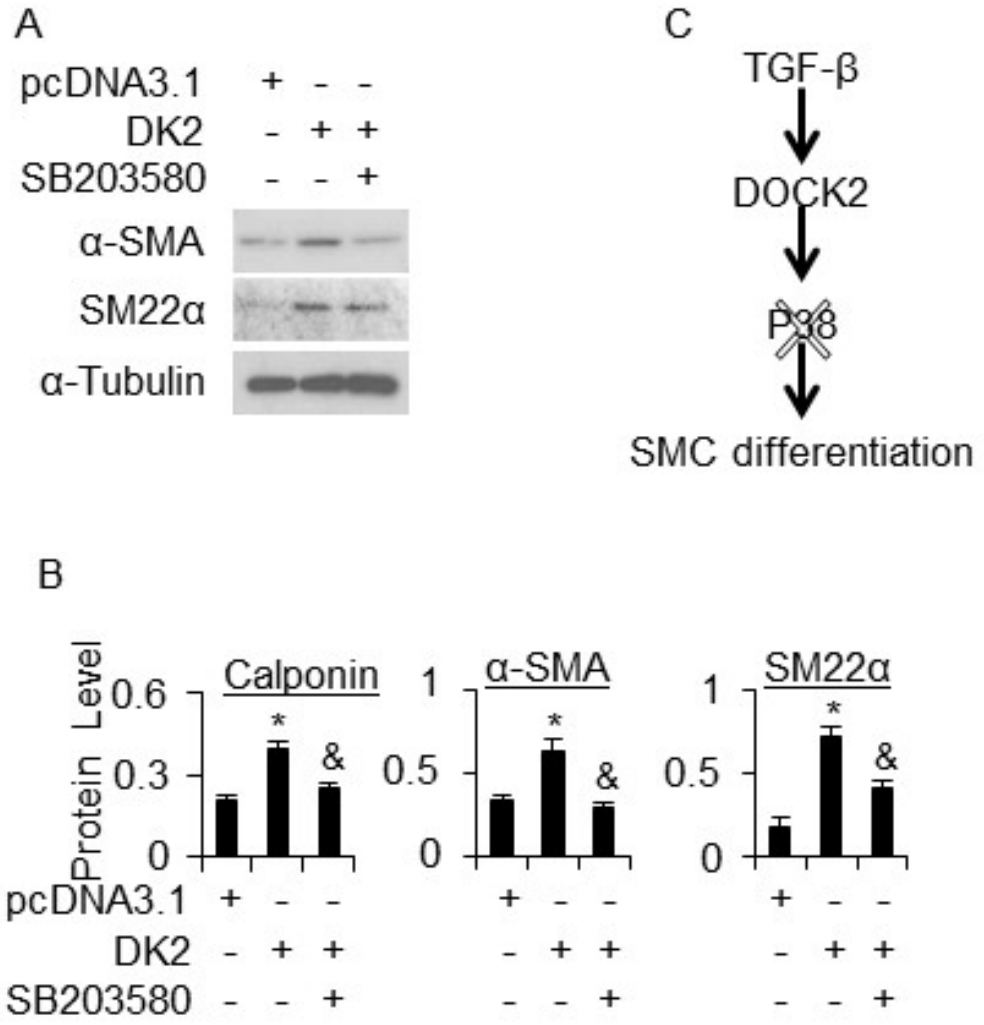

Figure 4.5. p38 phosphorylation is required in DOCK2 induced SMC differentiation.

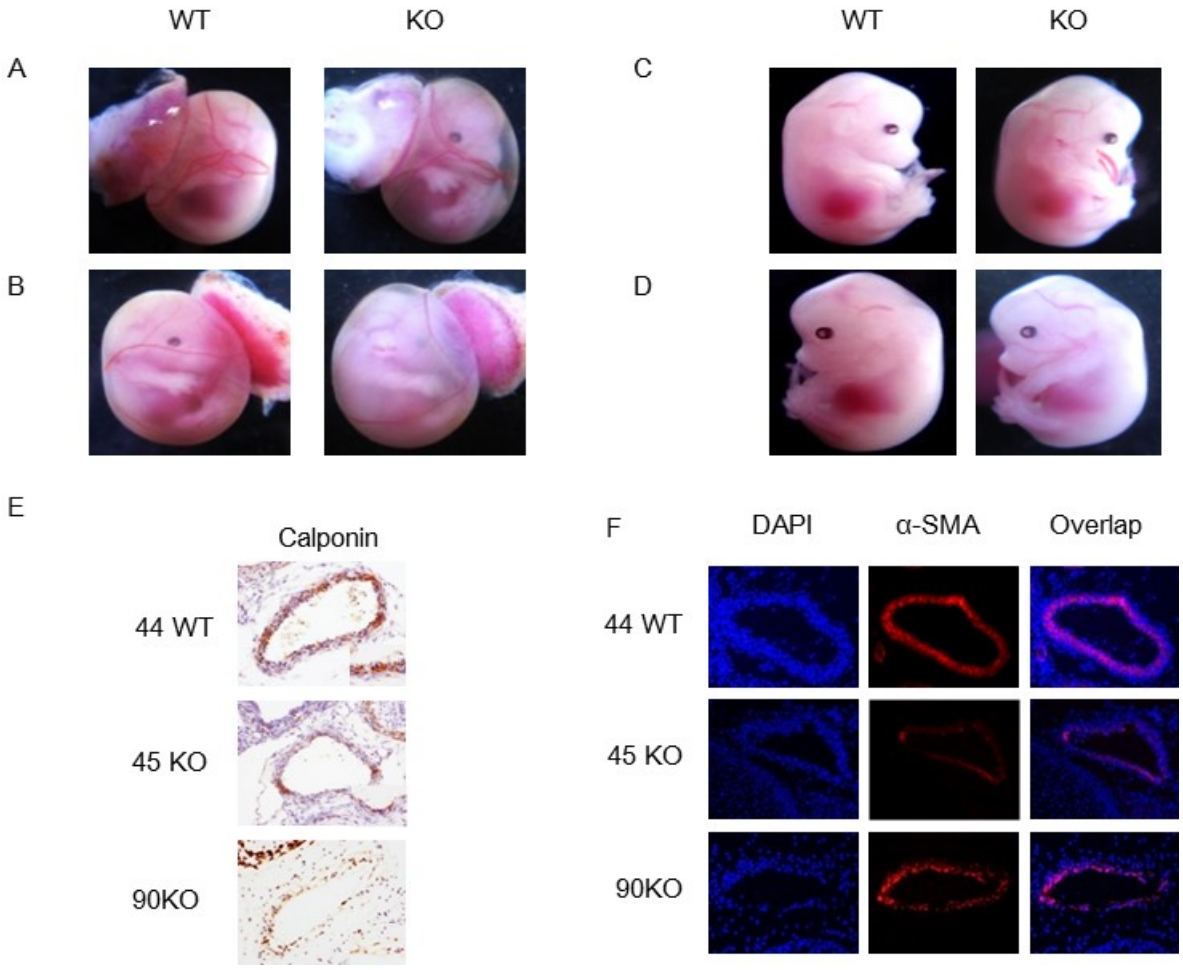

Figure 4.6. DOCK2 is essential for SMC differentiation during embryonic development formation.

Figure Legends

Figure 4.1. TGF-β induced DOCK2 expression while promoting SMC differentiation. A, 80% confluent 10T1/2 cells were starved with 0.5% serum-containing medium for 12 h followed by TGF-β treatment (5 ng/ml) for the indicated times. Cell lysates were collected and western blot was performed to examine the expression of DOCK2 and SMC marker proteins as indicated. **B,** Quantitative analysis of DOCK2 and SMC marker expression. The protein expression was normalized to α-Tubulin expression. *P<0.01 compared to vehicle-treated group (0 h). **C,** hES-MCs cells were starved with 0.5% serum-containing medium for 12 h followed by TGF-β treatment (5 ng/ml) for the indicated times. Cell lysates were collected and western blot was performed to examine the expression of DOCK2 and SMC marker proteins as indicated. **D,** Quantitative analysis of DOCK2 and SMC marker expression. The protein expression was normalized to α-Tubulin expression. *P<0.01 compared to vehicle-treated group (0 h).

Figure 4.2. DOCK2 is essential for TGF-β-induced SMC differentiation. A, DOCK2 knockdown diminished expression of SMC early markers in 10T1/2 cell. 10T1/2 cells were infected with Ad-control or Ad-DOCK2 shRNA for 24 h followed by vehicle or TGF- β treatment (5 ng/ml) for 24 h. Western blot was performed to detect DOCK2 and SMC marker protein expression. **B,** Quantitative analysis of protein expression shown in A by normalizing to α-Tubulin. *, *P*<0.05 compared with vehicle-treated Ad-control group; &, P<0.05 compared with TGF-β-treated Ad-control group. **C**, DOCK2 knockdown diminished TGF-β induced expression of SMC markers in hES-MCs cell. hES-MCs were infected with Ad-control or Ad-DOCK2 shRNA for 24 h followed by vehicle or TGF- β treatment (5 ng/ml) for 24 h. Western blot was performed to detect

DOCK2 and SMC marker protein expression. **D**, Quantitative analysis of protein expression shown in C by normalizing to α-Tubulin. *, P<0.05 compared with vehicle-treated Ad-control group; &, P<0.05 compared with TGF-β-treated Ad-control group.

Figure 4.3. DOCK2 alone induced SMC differentiation. **A,** DOCK2 enhanced SMC early marker expression in 10T1/2 cells. 10T1/2 cells were transfected with control or DOCK2 plasmid followed by serum-starvation for 24 h. Cell lysates were collected for western blot to detect SMC marker expression. **B,** Quantitative analysis of the protein expression in panel A by normalizing to α-Tubulin. *P<0.05 compared to the control plasmid-transfected group. **C,** DOCK2 enhanced SMC early marker expression in hES-MCs. hES-MCs cells were transfected with control or DOCK2 plasmid followed by serum-starvation for 24 h. Cell lysates were then collected for western blot to detect SMC marker expression. **D,** Quantitative analysis of the protein expression in panel C by normalizing to α-Tubulin. *P<0.05 compared to the control plasmid-transfected group.

Figure 4.4. DOCK2 regulated p38 MAPK phosphorylation. (A) DOCK2 overexpression effectively induced p38 MAPK phosphorylation. 10T1/2 cells were transfected with pcDNA and DOCK2 for 24 h. Cell lysates were collected for western blot to detect phosphorylation of different signaling pathway kinases as indicated. **B**, Quantitative analysis of the protein expression in panel A by normalizing to α-Tubulin. *P<0.01 compared to control group. **C**, 10T1/2 cells were infected with Ad-shCtrl or Ad-shDOCK2 for 24 h and then treated with TGF-beta for the indicated times. Cell lysates were collected for western blot to detect p38 phosphorylation. **D**, Quantitative analysis of the protein expression shown in panel C by normalizing to α-Tubulin. *P<0.01 compared to vehicle-treated Ad-Ctrl group.

Figure 4.5. p38 phosphorylation was required for DOCK2-induced SMC differentiation. A, p38 MAPK inhibitor decreased DOCK2 induced SMC marker expression. pcDNA or DOCK2-transfected hES-MCs cells were treated with vehicle or p38 MAPK inhibitor SB203580 (10μM) for 10 min. Cell lysates were collected for western blot to detect SMC marker expression. **B**, Quantitative analysis of protein expression shown in A by normalizing to α-Tubulin. *, $P<0.05$ compared with vehicle-treated control group; &, $P<0.05$ compared to SB203580-treated DOCK2 group. **C,** A schematic mechanism by which DOCK2 regulates SMC differentiation. TGF-β stimulation increased the expression of DOCK2, leading to p38 MAPK phosphorylation, which in turn induces SMC differentiation.

Figure 4.6. DOCK2 was essential for SMC differentiation during embryonic development. A-B, Gross vasculatures of two comparable locations of the wild-type and DOCK2–/– yolk sacs at 13.5 DPC. **C-D**, DOCK2-/- embryos at E13.5 displayed hemorrhage. **E**, Defective calponin expression in DOCK2-/- embryo dorsal aorta as compared to wild type embryo. DOCK2-/- or wild type embryo dorsal aorta was detected by immunohistochemistry staining using calponin antibody and DAB visualization. **F**, Defective α-SMA expression in DOCK2-/- embryo dorsal aorta as compared to wild type embryo dorsal aorta. DOCK2-/- or wild type embryo dorsal aorta was detected by immunostaining using α-SMA antibody (red color). DAPI stains nucleus (blue color).

CHAPTER 5

DEDICATOR OF CYTOKINESIS 2, A NOVEL REGULATOR FOR SMOOTH MUSCLE PHENOTYPIC MODULATION AND VASCULAR REMODELING [1]

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List of abbreviations

VSMC, vascular smooth muscle cell; PDGF-BB, Platelet-derived growth factor; DOCK2, dedicator of cytokinesis 2; SRF, serum response factor; α-SMA, smooth muscle α-actin; shRNA, short hairpin RNA; MTT, 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide; HE, hematoxylin and eosin; PFA, paraformaldehyde; IHC, immunohistochemistry; PCNA, proliferating cell nuclear antigen

Abstract

VSMC phenotypic modulation and vascular remodeling contributes to the development of a number of vascular disorders such as restenosis after angioplasty, transplant vasculopathy, and atherosclerosis. The mechanisms underlying these processes, however, are still largely unknown. Objective: The objective of this study is to determine the role of dedicator of cytokinesis 2 (DOCK2) in vascular smooth muscle cell (VSMC) phenotypic modulation and vascular remodeling. Methods and Results: Platelet-derived growth factor-BB (PDGF-BB) induced DOCK2 expression while modulating VSMC phenotype as indicated by reduced VSMC marker gene expression. DOCK2 overexpression inhibited while DOCK2 knockout enhanced SMC marker gene expression in primary VSMC. Mechanistically, DOCK2 regulated VSMC phenotype through suppression myocardin/serum response factor (SRF)-mediated transcription of VSMC marker genes. DOCK2 blocked myocardin, but not SRF, expression, inhibited myocardin binding to SRF, and dramatically attenuated myocardin-induced smooth muscle α-actin promoter activity. In addition, DOCK2 was essential for PDGF-BB-induced VSMC proliferation and migration. DOCK2 stimulated VSMC migration via induction of focal adhesion contact and stress fiber formation. In a rat carotid artery balloon-injury model, DOCK2 was induced in media layer VSMC initially and neointima VSMC subsequently following vascular injury. Knockdown of DOCK2 dramatically inhibited the neointima formation by 60%. Most importantly, knockout of DOCK2 in mice markedly blocked ligation-induced intimal hyperplasia. Conclusions: Our studies identified DOCK2 as a novel regulator for VSMC phenotypic modulation and vascular lesion formation following vascular injury. Therefore, targeting DOCK2 is a

potential therapeutic strategy for the prevention of vascular remodeling in proliferative vascular diseases.

Key Words: Dedicator of cytokinesis 2, Vascular smooth muscle cells, Migration, Proliferation, Vascular remodeling

Introduction

Vascular remodeling contributes to the development of a number of vascular disorders including Restenosis after angioplasty, vein graft stenosis, transplant vasculopathy, and atherosclerosis, etc [1, 2]. Although neointimal formation may be controlled by different mechanisms, medial VSMC phenotypic modulation from a contractile to a synthetic phenotype triggered by damaging to blood vessel walls followed by VSMC migration and proliferation plays a major role in injury-induced vascular remodeling [3-12]. Elucidating mechanisms underlying VSMC phenotypic modulation, migration and proliferation, therefore, is critical for understanding the etiology of above-mentioned vascular proliferative disorders and for developing effective therapeutics to block the narrowing of vessel lumen due to vascular remodeling.

Dedicator of cytokinesis 2 (DOCK2) is an atypical guanine nucleotide exchange factor for the Rho-small guanine triphosphatase [13]. Under physiological conditions, DOCK2 is mainly expressed in hematopoietic cells, and involved in lymphocyte migration and activation via regulating actin cytoskeleton through Rac activation [13-15]. Deletion of DOCK2 enables long-term cardiac allograft survival via suppressing graft tissue infiltration of alloreactive T cells [16]. DOCK2 also controls various immunological functions including helper T cell differentiation, neutrophil chemotaxis and type I

interferon induction [17-20]. It is unknown, however, if DOCK2 is involved in regulating vascular function.

In this study, we found that platelet-derived growth factor-BB (PDGF-BB) induced DOCK2 expression in VSMC while modulating VSMC phenotype. Ectopic expression of DOCK2 inhibited VSMC marker smooth muscle α-actin (α-SMA) and calponin expression while knockout of DOCK2 (DOCK2-/-) enhanced the marker gene expression in primary VSMCs isolated from DOCK2-/- mice. DOCK2 appeared to modulate VSMC phenotype by suppressing myocardin/serum response factor (SRF)-mediated transcription of VSMC marker genes. In vivo study using rat carotid artery balloon-injury model showed that DOCK2 is essential for injury-induced neointima formation because knockdown of DOCK2 dramatically inhibited the neointima formation by 60%. Most importantly, knockout of DOCK2 in mice markedly blocked ligation-induced intimal hyperplasia. Our study demonstrates that DOCK2 is a novel regulator of VSMC phenotypic modulation and an essential factor contributing to vascular remodeling.

Methods

Animals

Male Sprague-Dawleyrats weighing 450-500g were purchased from Harlan. DOCK2-/- mice were previously described [14]. All animals were housed under conventional conditions in the animal care facilities and received humane care incompliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals.

Animal surgical procedures were approved by the Institutional Animal Care and Use Committee of The University of Georgia.

Cell culture

VSMCs were cultured by enzyme digestion method from rat or mice thoracic aorta as described previously [21-23]. The primary cultured VSMCs were confirmed by the expression of smooth muscle α-actin (α-SMA) and SM22α.

Construction of adenovirus

Adenoviral vectors expressing scramble (control) or DOCK2 short hairpin RNA (shRNA) (shDOCK2) were constructed, and the viruses were purified as described previously [24]. ShDOCK2 sequences were as follows: 5'-CGCGTCGGACCTAATT GCATGGCAGATCCCCTTCCTTCAAGAGAGGAAGGGGATCTGCCATGCAATTAG GTCCTTTTTTCCAAA-3' (top strand) and 5'-AGCTTTTGGAAAAAAGG ACCTAATTGCATGGCAGATCCCCTTCCTCTCTTGAAGGAAGGGGATCTGCCATG CAATTAGGTCCGA-3' (bottom strand). Control shRNA (shCtrl) sequences were as follows: 5'-CGCGTCGATCGATGATTCGCCCGGCGTCTTCATAATTCAAGAGATTA TGAAGACGCCGGGCGAATCATCGATCTTTTTTCCAAA-3' (top strand) and 5'-A GCTTTTGGAAAAAAGATCGATGATTCGCCCGGCGTCTTCATAATCTCTTGAATT ATGAAGACGCCG GGCGAATCATCGATCGA-3' (bottom strand). Both strands were annealed and ligated into pRNAT-H1.1/Adeno (Genscript corporation) digested with Mlu I and Hind III. Recombinant adenoviral vector was produced by homologous recombination in AD-1 competent. The resultant recombinant vector pAd-shDOCK2 digested with Pac I was transfected into AD-293 cells to package viral particles

expressing shDOCK2 (Ad-shDOCK2). The adenovirus was purified using gradient density ultracentrifugation of cesium chloride and dialyzed in dialysis buffer.

Western blot analysis

Western blot was performed as described previously [25]. Cultured VSMCs were lysed in RIPA buffer (50 mmol/L Tris-HCI, pH 7.4, 1% Triton X-100, 0.25% wt/vol sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EGTA, 0.1% SDS) containing protease inhibitors. VSMCs from Rat or mice thoracic aorta were homogenized in RIPA buffer containing protease inhibitors. Cell lysates or proteins collected from arteries were resolved on a 10% SDS-PAGE and were transferred to PVDF membrane (Bio-Rad). Antibodies against DOCK2 (Millipore), α-SMA (Sigma), calponin (Abcam), SM22α (Abcam), SRF (Santa Cruz Biotechnologies), Flag (Sigma), GAPDH (Sigma), andα-Tubulin (Sigma) were used for immunoblotting.

Real-time quantitative PCR (qPCR)

Quantitative PCR was performed as described previously [26]. Total RNA from cells was extracted using Trizol reagent (Invitrogen) and reverse transcribed to cDNA using iScript cDNA Synthesis kit (Bio-Rad). qPCR was performed on a Stratagene Mx3005 qPCR thermocycler using SYBR Green master mix (Agilent Technologies, La Jolla, CA). The sequences of the forward and reverse primers used in this study were listed in Table S1.

Transfection and luciferase assay

α-SMA promoter constructs were co-transfected with myocardin and DOCK2 plasmid individually or in combination into VSMCs as described previously [27]. Cells were then starved in serum-free medium for 12 hr followed by 10 ng/ml PDGF-BB

treatment for 24 hr. Luciferase assay was performed as described previously [26]. Experiments were repeated for three times, and results from a representative experiment were shown with standard deviations.

Cell proliferation assay

VSMC cell proliferation was evaluated with 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazoliumbromide (MTT) assay by TACS MTT Cell Proliferation Assay Kit (Trevigen). The optical density at 570nm was measured.

Wound healing assay

Wound healing assay was performed using CytoSelect Wound Healing Assay Kit (Cell Biolabs) as previously described [21, 28, 29]. Briefly, cells transduced with adenovirus expressing control or DOCK2 shRNA were suspended and added to both side of wound healing inserts followed by incubation overnight. The inserts were then removed, and PDGF-BB (10 ng/ml) was added to induce cell migration. Images of wound healing were taken using a dissection microscope, and cell migration distances were measured.

Transwell assay

Transwell assay was performed using a modified Boyden chamber method with microchemotaxis chambers and polycarbonate filters (Nunc, Rochester, NY, USA) with a pore size of 8.0 μm [30]. Control or DOCK2 shRNA adenovirus-transduced VSMCs were harvested and seeded onto the upper chamber at a concentration of 5×10^4 cells/100 μl. PDGF-BB as a chemoattractant was added to the lower chamber. Cells were allowed to migrate for 24 hr. The non-migrating cells on the upper surface were removed. Migrated cells on the lower surface of the membrane were stained with DAPI and counted with a

Nikon fluorescence microscope (Nikon America Inc). The number of cells was recorded from at least 8 fields per well. Experiments were performed in triplicate.

Rat carotid artery injury model and adenoviral gene transfer

Rat carotid artery balloon injury was performed as described previously [31, 32]. A 2F Fogarty arterial embolectomy balloon catheter (Baxter Edwards Healthcare) was used to denudate the endothelium. The method that introduces adenovirus into rat balloon-injured carotid artery has been previously described [24, 33]. The injured artery was washed with saline, and incubated with 100 μl saline or adenovirus ($5x10^9$pfu) for 20 minutes. 3, 7, and 14 days later, the balloon-injured and adenovirus-dwelled segments were removed and fixed with 4% paraformaldehyde (PFA) and embedded in paraffin. Subsequent morphometric analyses were performed in a double-blinded manner.

Mouse carotid artery ligation-injury model

The mouse carotid artery ligation model was described previously [34, 35]. Mice were anesthetized with ketamine hydrochloride (80 mg/kg IP) and xylazine (5 mg/kg IP). The left common carotid artery was exposed and ligated by a 6-0 propylene suture about 2 mm proximal from the carotid bifurcation. Four weeks later, the mice were sacrificed and the ligation-injured segments were removed and fixed with 4% PBS-buffered PFA and embedded in paraffin. Subsequent morphometric analyses were performed in a double-blinded manner.

Histomorphometric analysis and immunohistochemistry (IHC) staining

Vessel segments were cut for analysis. The dissected arteries were stained with modified hematoxylin and eosin (HE) or Elastica van Gieson staining and captured using a Nikon microscope. The circumference of lumen, internal elastic lamina, and external

elastic lamina were measured by Image-pro Plus Software. For immunohistochemistry, sections were rehydrated, blocked with 10% goat serum and permeabilized with 0.01% Triton X-100 in PBS, and incubated with primary antibody DOCK2 or proliferating cell nuclear antigen (PCNA, Santa Cruz Biotechnology) at 4℃overnight followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody. The sections were counterstained with hematoxylin.

Immunofluorescent staining

VSMCs were grown on glass coverslips in 24-wellcell culture plates at a density of 10^3 cells/cm^2 and incubated overnight in normal cell growth conditions. 24 hr later, the cells were infected with control or DOCK2 shRNA for 24 hr and then were treated with vehicle or PDGF-BB (10 ng/ml) for 24 hr. Cells were fixed with 4% PFA, or 1% PFA for 1 min and permeabilized with 0.01% Triton X-100 in PBS for vinculin (Santa Cruz Biotechnology) or phalloidin (Invitrogen) staining. Anti-vinculin was used at 1:100 in blocking buffer containing 3% BSA in PBS. Cells were then incubated with TRITC-conjugated secondary antibodies (1:100). Phalloidin staining was performed following the standard protocol. Stained cells were imaged using a Nikon microscope.

Statistical analysis

All data were evaluated with a 2-tailed, unpaired Student *t* test or compared by one-way ANOVA followed by Fisher *t* test and are expressed as mean±SD. A value of $P<0.05$ was considered statistically significant.

Results

DOCK2 was essential for VSMC phenotypic modulation

PDGF-BB is a potent stimulator of VSMC phenotypic modulation [36, 37]. Although DOCK2 is expressed in a very low level in normal VSMC, its expression was significantly induced in VSMCs when the cells were treated with PDGF-BB. Dose-dependent study showed that 5 ng/ml of PDGF-BB significantly elevated DOCK2 expression, while 10 ng/ml of PDGF-BB induced a high level of DOCK2 expression (Fig. 5.1A-5.1B). Therefore, we used 10 ng/ml of PDGF-BB for all subsequent experiments. Since VSMC phenotypic modulation is characterized by the reduction of VSMC contractile proteins, we detected if DOCK2 induction by PDGF-BB correlates with the attenuation of VSMC marker proteins. As shown in Fig. 5.1C-5.1D, PDGF-BB induced DOCK2 expression in a time-dependent manner while inhibiting the expression of VSMC contractile protein SM22α and calponin. These data suggest that DOCK2 is involved in PDGF-BB-mediated VSMC phenotype modulation.

To determine if DOCK2 is important for VSMC phenotypic modulation, we tested if DOCK2 regulates VSMC marker gene expression. We found that ectopic expression of DOCK2 in VSMC significantly blocked α-SMA and calponin mRNA expression (Fig. 5.2A), suggesting that DOCK2 is able to alter VSMC phenotype. To confirm this new function of DOCK2, we cultured aortic VSMCs from wild type and DOCK2-/- mice and treated the cells with PDGF-BB for 36 hr followed by detection of VSMC contractile protein levels. As shown in Figure 5.2B-5.2C, PDGF-BB induced DOCK2 protein expression while decreased VSMC contractile marker α-SMA and calponin. However, DOCK2 knockout dramatically increasedα-SMA and calponin

protein expression even in basal condition, suggesting that a low level of DOCK2 is essential for maintaining contractile protein homeostasis. PDGF-BB treatment did not block the contractile protein expression in DOCK2-/- VSMC (Fig. 5.2B-5.2C), further demonstrating that DOCK2 plays an essential role in PDGF-BB-induced VSMC phenotypic modulation.

DOCK2 regulated SMC phenotypic modulation through suppression of myocardin/SRF-mediated transcriptional activation of SMC marker genes

SRF and its co-activator myocardin are well-known factors regulating VSMC contractile protein expression [38-40]. PDGF-BB has been shown to induce VSMC phenotypic modulation by inhibiting the expression of SRF and myocardin or blocking SRF-myocardin interaction. In order to determine the mechanisms underlying DOCK2 function in PDGF-BB-induced VSMC phenotypic modulation, we first tested if DOCK2 regulates SRF or myocardin expression. We found that PDGF-BB inhibited myocardin and SRF mRNA expression. Knockout of DOCK2, however, dramatically increased myocardin but not SRF mRNA expression. PDGF-BB treatment was unable to reduce myocardin expression in DOCK2-/- VSMCs, suggesting that DOCK2 can block myocardin expression (Fig. 5.3A). SRF expression was inhibited by PDGF-BB in DOCK2-/- VSMCs, similar to that observed in wild type VSMCs (Fig. 5.3A), suggesting that DOCK2 is not involved in SRF expression. Conversely, overexpression of DOCK2 blocked myocardin, but not SRF, expression (Fig. 5.3B), further demonstrating that DOCK2 plays an important role in PDGF-BB-induced down regulation of myocardin, but not SRF, in VSMC phenotypic modulation.

Since PDGF-BB treatment also alters myocardin binding to SRF, we sought to determine if DOCK2 mediates PDGF-BB-induced VSMC phenotypic modulation by influencing myocardin-SRF interaction. Since high quality of myocardin antibody is not readily available, we transfected Flag-tagged myocardin plasmid into VSMC and detected the interaction of Flag-tagged myocardin with endogenous SRF. As shown in Figure 5.3C and 5.3D, myocardin indeed interacted with SRF in VSMCs, and PDGF-BB inhibited their interaction. However, when DOCK2 was knocked down by shRNA, the reduced myocardin-SRF interaction by PDGF-BB was restored. These data suggest that DOCK2 mediated PDGF-BB-induced reduction of mycardin binding to SRF. To confirm DOCK2 function in blocking myocarin-SRF binding, we overexpressed DOCK2 in VSMCs without PDGF-BB treatment and found that DOCK2 indeed significantly blocked the interaction between myocardin and SRF (Fig. 5.3E and 5.3F).

Because myocardin functions to activate VSMC marker gene transcription [40, 41], we sought to determine if DOCK2 is able to alter myocardin-activated VSMC marker gene promoter activity. As shown in Figure 5.3G, myocardin strongly induced the transcription of α-SMA promoter. PDGF-BB inhibited the promoter activity probably due to PDGF-BB-induced DOCK2 expression. Myocardin-induced promoter activity was not completely blocked by PDGF-BB because of the limited DOCK2 expression induced by PDGF-BB. However, overexpression of DOCK2 almost completely blocked myocardin-activated α-SMA promoter activity. These data demonstrate that DOCK2 regulates VSMC phenotype modulation through inhibiting both myocardin expression and myocardin binding with SRF, resulting in the reduction of VSMC contractile gene expression.

Previous studies in lymphocytes suggest that DOCK2 is mainly involved in lymphocyte migration, a function related to its cytoplasm location [14, 42]. Because DOCK2 plays a critical role in modulating VSMC marker gene transcription, we hypothesized that DOCK2 may be a nuclear factor in VSMCs. Indeed, immunostaining of DOCK2 showed that PDGF-BB-induced DOCK2 were located both on the cytoplasm membrane and in the nuclei of VSMCs (Fig. 5.3H), consistent with its function in inhibiting both the myocardin-SRF interaction and myocardin-activated α-SMA promoter activity.

DOCK2 was essential for VSMC migration and proliferation

Phenotypic modulated VSMCs gain enhanced migratory ability. The VSMC migration from the media into the intimal surface of blood vessels is a critical aspect of pathological remodeling in response to vascular injury. Because PDGF-BB induced DOCK2 expression on the spreading cytoplasm membrane (lamellipodium) of VSMCs, we postulated that DOCK2 plays a role in VSMC migration, similar to its role in lymphocytes. Thus, we used DOCK2 shRNA to knock down its expression in VSMCs (Fig. 5.4A-5.4B), and tested if blockade of DOCK2 alters PDGF-BB-induced VSMC migration using both wound healing (Fig. 5.4C-5.4D) and Transwell migration assays (Fig. 5.4E-5.4F). PDGF-BB strongly induced VSMC migration. DOCK2 knockdown, however, blocked the migration, demonstrating an essential role of DOCK2 in VSMC migration.

A key component of cell migration is cytoskeleton reorganization, in which focal adhesion contact and stress fiber formation play critical role [5]. In order to determine the mechanism by which DOCK2 promotes VSMC migration, we tested if DOCK2affects cytoskeletal organization and focal adhesion formation. Because vinculin is a critical

factor in assembling the focal adhesion contact, we detected if DOCK2 plays a role in vinculin expression and its cellular distribution. As shown in Figure 5.4G, PDGF-BB strongly induced vinculin expression and distribution to the lamellipodia of VSMCs, consistent with previous reports [43, 44]. Knockdown of DOCK2, however, significantly blocked the PDGF-BB-induced vinculin expression. Although PDGF-BB down-regulated overall expression of actin cytoskeleton in VSMCs, it enhanced the accumulation of F-actin on the membrane of VSMCs (Fig. 5.4H), which is required for VSMC migration. Knockdown of DOCK2, however, blocked PDGF-BB-induced actin cytoskeleton reorganization (Fig. 5.4H). These data demonstrate that DOCK2 regulates VSMC migration by altering focal adhesion contact and stress fiber reorganization.

In addition to enhanced motility, phenotypic modulated VSMCs also exhibit proliferative phenotype, which is another major contributing factor in neointima formation following arterial injury [6, 7]. Thus, we tested if DOCK2 plays a role in VSMC proliferation. As shown in Figure 5.4I, PDGF-BB induced VSMC proliferation. However, when DOCK2 expression was blocked by its specific shRNA, PDGF-BB-induced VSMC proliferation is completely blocked. These data demonstrate that DOCK2 plays a vital role in VSMC proliferation.

DOCK2 expression was activated during injury-induced VSMC phenotypic modulation

Mechanical injury to artery causes phenotypic modulation of medial layer VSMC followed by neointimal formation. DOCK2 is clearly important for PDGF-BB-induced VSMC phenotypic modulation *in vitro*. To determine if DOCK2 is involved in VSMC phenotypic modulation and vascular remodeling *in vivo*, we used balloon catheter to

mechanically injure rat carotid artery. As shown in Figure 5.5A, balloon injury induced a progressive neointima formation, consistent with previous reports [31, 32]. DOCK2 expression is barely detectable in VSMCs of the normal artery, but was highly induced in the medial layer VSMCs 3 days after the injury (Fig. 5.5B), a time when VSMC phenotypic modulation occurs but without evident neointima formation. 7 or 14 days after the injury, DOCK2 was expressed in the neointimal VSMCs (Fig. 5.5B). Time course quantitative analysis of DOCK2 expression revealed that DOCK2 protein was highly induced as early as 3 days after the injury, and the high level of expression persisted until 14 days after the injury (Fig.5.5C-5.5D). These data indicate that DOCK2 is involved in vascular lesion formation.

DOCK2 was essential for injury-induced vascular remodeling

To test if DOCK2 plays a role in balloon injury-induced vascular remodeling, we used adenovirus to deliver DOCK2 shRNA to the injured arteries. As shown in Figure 5.6A, DOCK2 shRNA effectively blocked DOCK2 expression in neointima VSMCs. Saline or control shRNA did not affect the injury-induced neointima formation. However, knockdown of DOCK2 dramatically blocked the neointima formation (Fig. 5.6B). Morphometric quantification of elastic-stained sections showed that DOCK2 shRNA inhibited the neointima formation by 60% (0.050±0.006 versus 0.123±0.011 mm^2; $P<0.01$, n=6, Fig. 5.6C). The Intima/Media area ratios (Fig.5.6D) showed similar results as in Figure 5.6C. These results demonstrate that DOCK2 is a novel regulator essential for injury-induced vascular remodeling.

Since we have found that DOCK2 is important for VSMC proliferation *in vitro*, we sought to determine if DOCK2 is involved in VSMC proliferation in neointima

formation *in vivo*. Thus, we examined the expression of proliferating cell nuclear antigen (PCNA) in artery sections treated with control or DOCK2 shRNA. As shown in Figure 5.6E, vascular injury induced PCNA expression in neointima VSMCs. DOCK2 knockdown, however, markedly decreased the number of PCNA-positive VSMCs (Fig. 5.6E). These data indicate that DOCK2 plays an important role in VSMC proliferation *in vivo*, which contributes to the neointima formation following vascular injury.

Knockout of DOCK2 in mice blocked ligation injury-induced intimal hyperplasia

Although adenovirus-mediated shRNA delivery is a powerful and convenient approach to knock down gene expression in injured arteries, it may not be able to fully reveal DOCK2 function in vascular remodeling because adenoviral vector can only modify DOCK2 expression in cells with a direct contact with the virus. Therefore, we used DOCK2-/- mice and carotid artery ligation injury model to further confirm the role of DOCK2 in vascular remodeling. As shown in Figure 5.7, knockout of DOCK2 (Fig. 5.7A) dramatically inhibited ligation injury-induced neointima formation (Fig. 5.7B). Morphometric quantification of the elastic-stained sections showed that the neointima was reduced by 80% in carotid arteries of DOCK2-/- mice as compared with the wild type mice (0.009±0.001 versus 0.048±0.001 mm^2; $P<0.01$, n=6, Fig. 5.7C). The Intima/Media area ratios (Fig. 5.7D) showed similar results as in Figure 5.7C. These data further demonstrate a critical role of DOCK2 in injury-induced vascular remodeling.

Discussion

VSMC phenotypic modulation and vascular remodeling play critical roles in the development of a number of proliferative vascular disorders. The molecular mechanisms governing these two processes, however, remain largely unknown. In the present study,

we demonstrate DOCK2 as a new player in VSMC phenotypic modulation and vascular remodeling. DOCK2 is scarcely detectable in normal VSMCs. However, it is induced by PDGF-BB. DOCK2 appears to be critically important for PDGF-BB-induced phenotypic modulation because knockout of DOCK2 dramatically blocks PDGF-BB activity in down-regulating VSMC contractile protein expression. On the other hand, ectopic expression of DOCK2 inhibits VSMC marker expression. Interestingly, DOCK2 knockout in PDGF-BB-untreated VSMCs also causes a dramatic increase of contractile protein levels, suggesting that a low level of DOCK2 present in quiescent VSMCs is required for maintaining the proper level of contractile proteins. Excessive DOCK2 expression results in VSMC phenotype alteration, similar to the treatment with PDGF-BB. In vivo, DOCK2 is also barely detectable in the normal arterial VSMCs, but is induced in medial VSMCs initially, and neointima VSMCs subsequently following vascular injury. The initial expression of DOCK2 in media VSMCs most likely contributes to the phenotypic modulation in injured arteries because at this time (1-3 days after injury), VSMCs are responding to injury-triggered serum factors such as PDGF-BB to modulate its phenotype. The absence of intimal hyperplasia during the initial expression of DOCK2 also suggests that DOCK2 may transform VSMCs from a contractile to a synthetic phenotype at this stage, consistent with its function *in vitro*. Blocking the initial expression of DOCK2 immediately after injury using adenovirus-delivered shRNA effectively blocks injury-induced neointima formation, suggesting that DOCK2-induced VSMC phenotype modulation plays a major role in injury-induced vascular remodeling. The definitive role of DOCK2 in vascular remodeling is supported by the blockade of ligation injury-induced intimal hyperplasia in DOCK2-/- mice.

DOCK2 appears to induce VSMC phenotypic modulation by inhibiting myocardin/SRF-mediated VSMC marker gene transcription. DOCK2 regulates myocardin/SRF function via two pathways. Firstly, DOCK2 inhibits the expression of myocardin, but not SRF. Knockout of DOCK2 dramatically increases myocardin expression, similarly as the contractile proteins, even in quiescent VSMCs, suggesting that a low level of DOCK2 inhibits excessive myocardin expression in the normal VSMCs. Although PDGF-BB down regulates myocardin expression in normal VSMCs, it does not alter myocardin expression in DOCK2-/- VSMCs, suggesting that PDGF-BB inhibition of myocardin is mediated by DOCK2. Secondly, DOCK2 blocks myocardin binding to SRF, a prerequisite for myocardin to activate VSMC marker gene transcription. PDGF-BB inhibits myocardin-SRF interaction. However, this inhibition is blocked when DOCK2 is knocked down by shRNA, suggesting that DOCK2 mediates PDGF-BB inhibition of myocardin-SRF binding. Indeed, overexpression of DOCK2 alone is able to block myocardin-SRF interaction, consistent with the functional analysis showing that DOCK2 expression significantly blocks myocardin-activated VSMC marker gene transcription.

In addition to VSMC phenotypic modulation, DOCK2 is important for VSMC migration and proliferation, two processes contributing to the neointima formation following artery injury. DOCK2 regulates VSMC migration through altering focal adhesion contact formation and actin cytoskeleton reorganization, consistent with its role as an atypical guanine exchange factor. DOCK2 regulates focal adhesion contact by enhancing vinculin expression and its distribution to the leading edge of migrating cells while regulating cytoskeleton by promoting F-actin formation in the lamellipodia of

VSMCs. Whether or not DOCK2 regulates VSMC cytoskeleton reorganization through Rac or RhoA is a subject for future study. DOCK2 is known to regulate Rac activation in lymphocytes. In addition to migration, DOCK2 is also required for VSMC proliferation both *in vitro* and *in vivo* although the underlying mechanisms remain to be determined. DOCK2 has been shown to regulate cell proliferation through Rac and ERK activation in B cell lymphoma [45]. Whether or not DOCK2 uses the same mechanism to regulate VSMC proliferation is an interesting subject for future study.

Interestingly and uniquely, VSMCs use DOCK2 to achieve its two distinct roles, i.e., gene transcription and VSMC migration, by locating them at two different subcellular compartments. Although it is established that DOCK2 membrane localization mediates lymphocyte migration and thus regulates inflammation responses [14, 18, 19, 46]. This study discovers a novel function of DOCK2 in modulating VSMC gene transcription, supported by its nuclear localization, interaction with nuclear factors, and modulation of α-SMA promoter activity. Therefore, DOCK2 is likely to be a novel transcription factor or cofactor that may also have functions in many other cellular processes beyond VSMC phenotypic modulation.

Taken together, our study has identified DOCK2 as a novel regulator for VSMC phenotype modulation and vascular remodeling. DOCK2 regulates vascular remodeling by modulating VSMC phenotype and promoting VSMC migration/proliferation. Blocking DOCK2 expression, therefore, may be an effective approach to treat proliferative vascular disorders. Moreover, since DOCK2 is functionally involved in inflammation, an essential process contributing to neointima formation, targeting

DOCK2 may hamper both VSMC proliferation and inflammation simultaneously to achieve a better vascular repair.

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Table 5.1: Primer sequences for qPCR

Primer	Forward	Reverse
DOCK2	5'TTTGATGCCAAGTTGCTCAG3'	5'GATGCTGTTGAGCAGTCCCA3'
α-SMA	5'TTCAATGTCCCTGCCATGTA3'	5'GAAGGAATAGCCACGCTCAG3'
Calponin	5'ATGGGCACCAATAAGTTTGC3'	5'GACCTGGCTCAAAGATCTGC3'
Myocardin	5'GGCAGAAAGATCCATTCCAA3'	5' TTGGAGAGGCTCACCTCAGT3'
SRF	5'CCTAGTCCCCATGCAGTGAT3'	5'TGCTGTGAGGAACACCTGAG3'

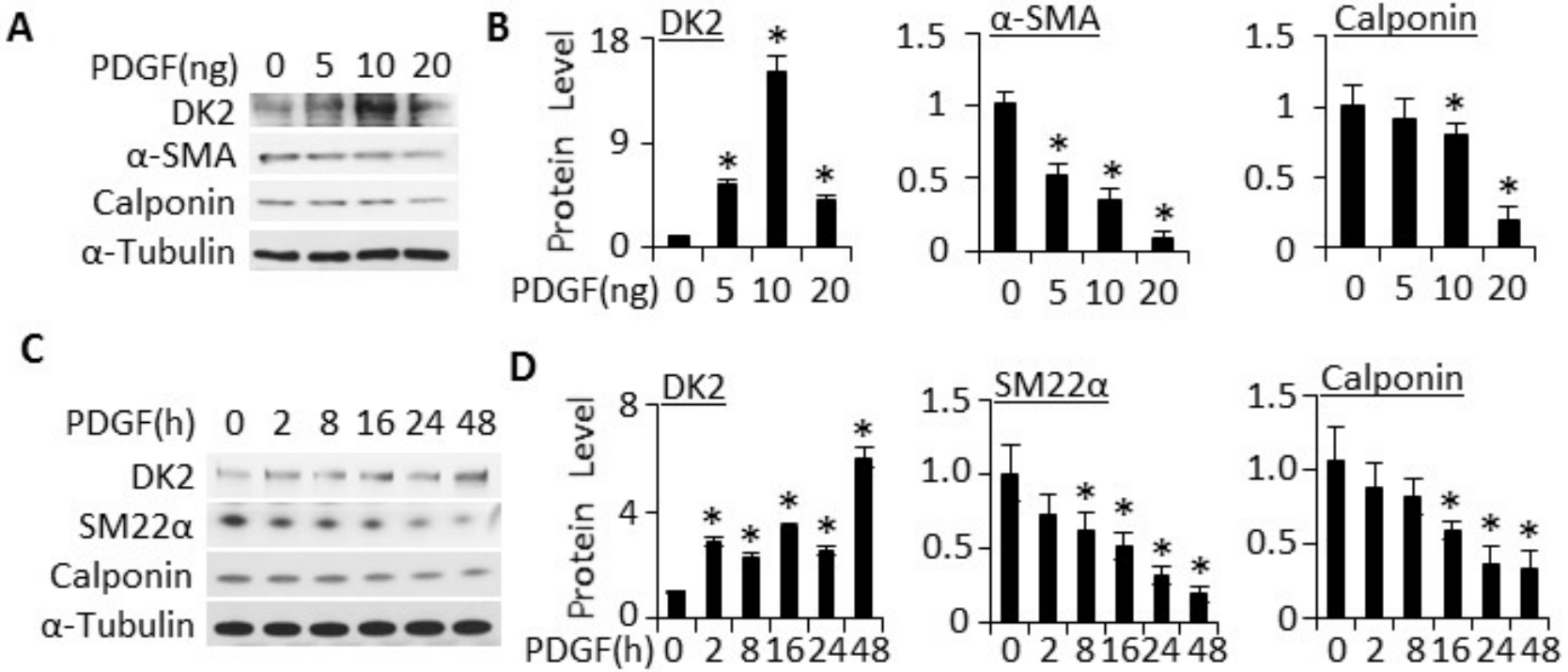

Figure 5.1. PDGF-BB induced DOCK2 expression along with VSMC phenotypic modulation.

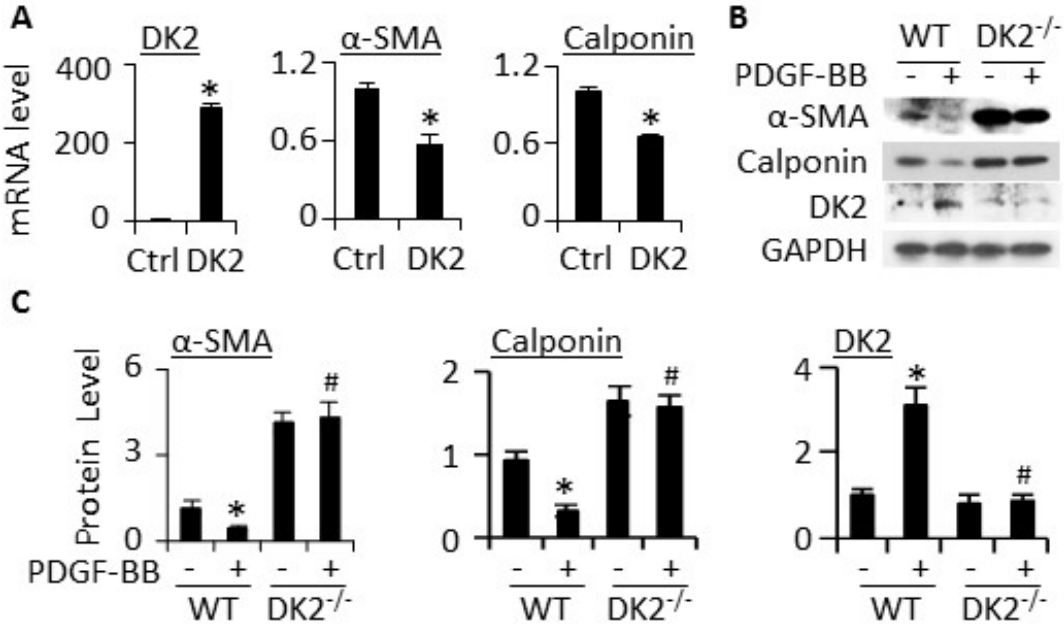

Figure 5.2. DOCK2 mediated VSMC phenotypic modulation.

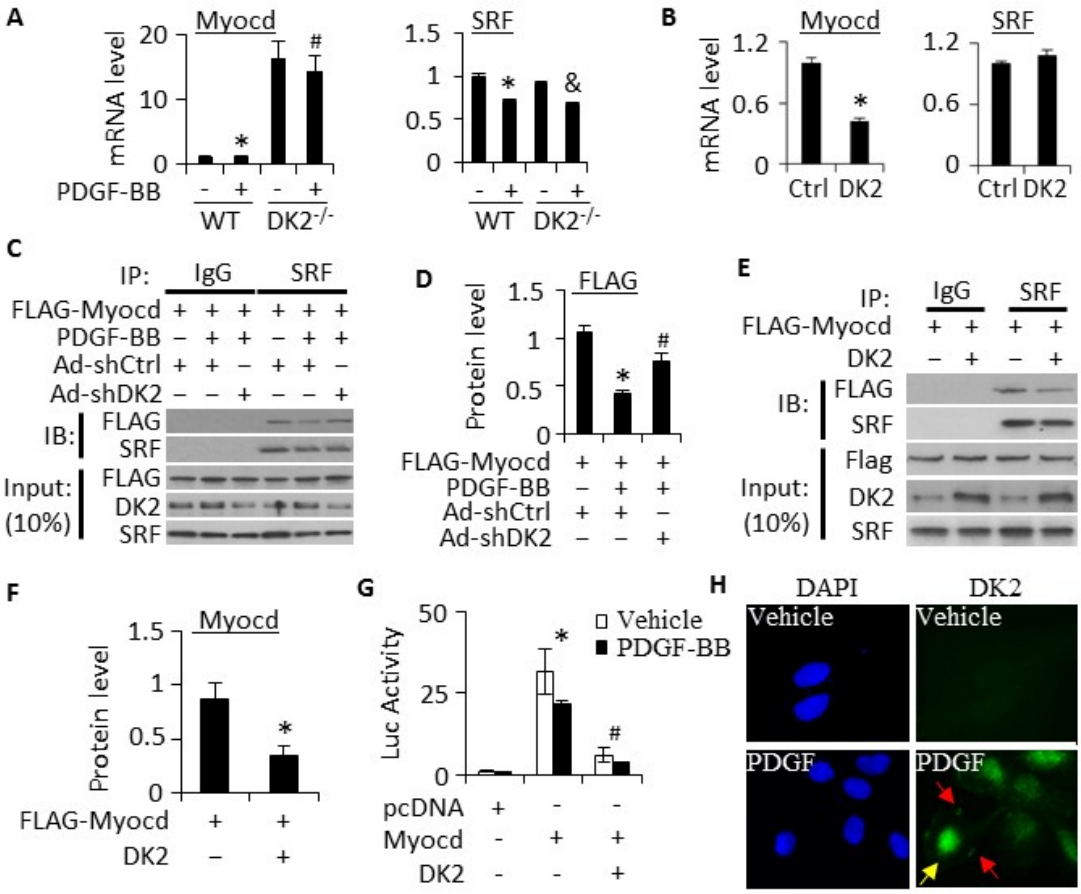

Figure 5.3. DOCK2 regulated VSMC phenotype by suppressing myocardin/SRF-mediated VSMC marker gene transcription.

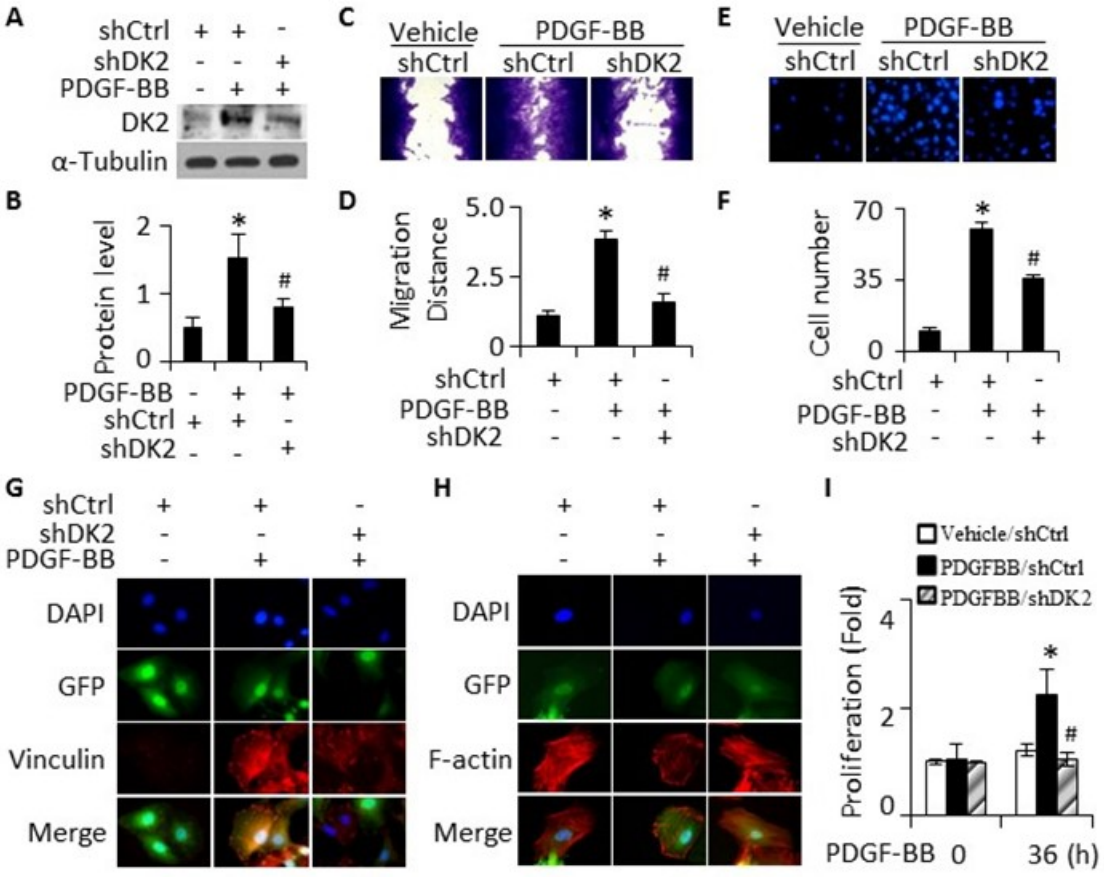

Figure 5.4. DOCK2 was essential for PDGF-BB-induced VSMC migration and proliferation.

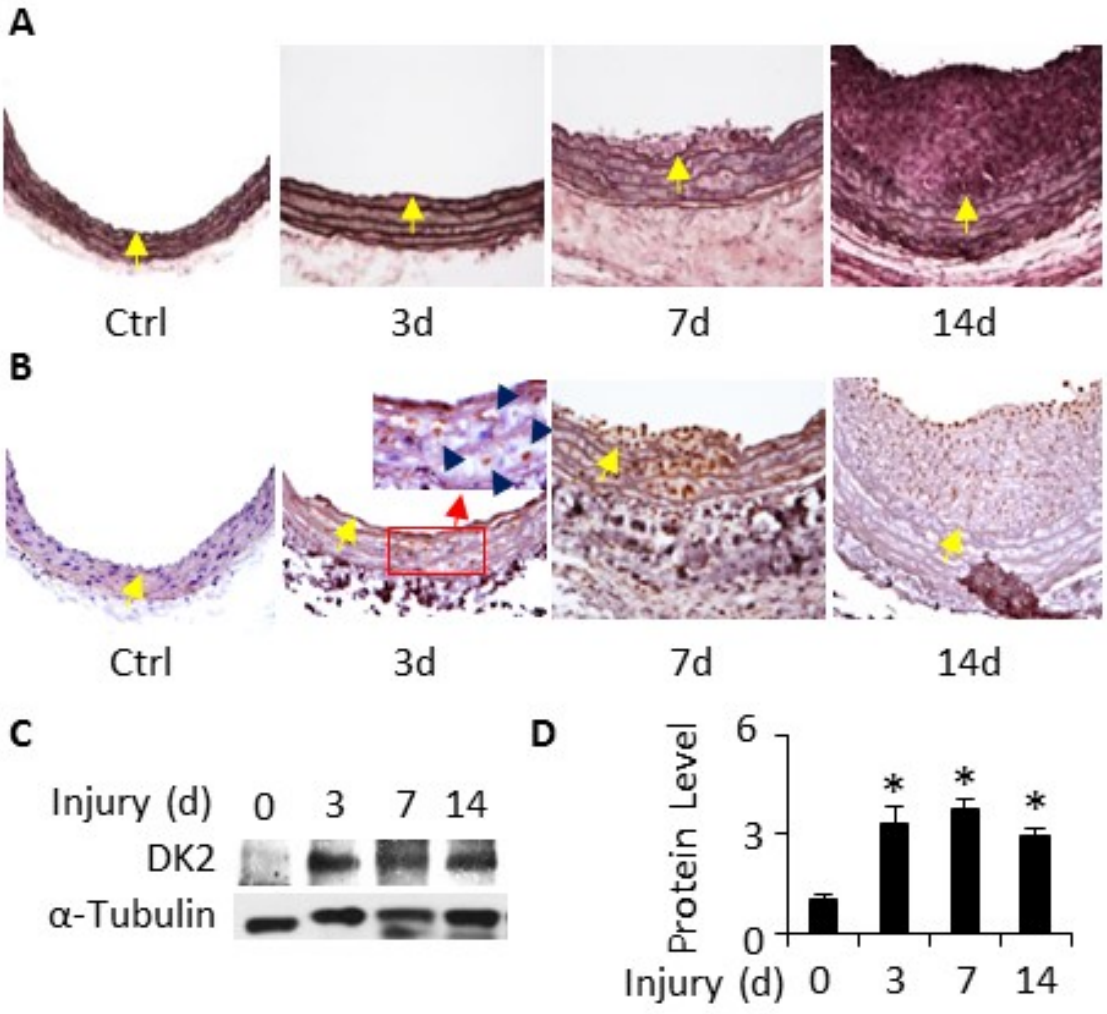

Figure 5.5. Balloon injury induced DOCK2 expression in tunica media and neointima VSMCs.

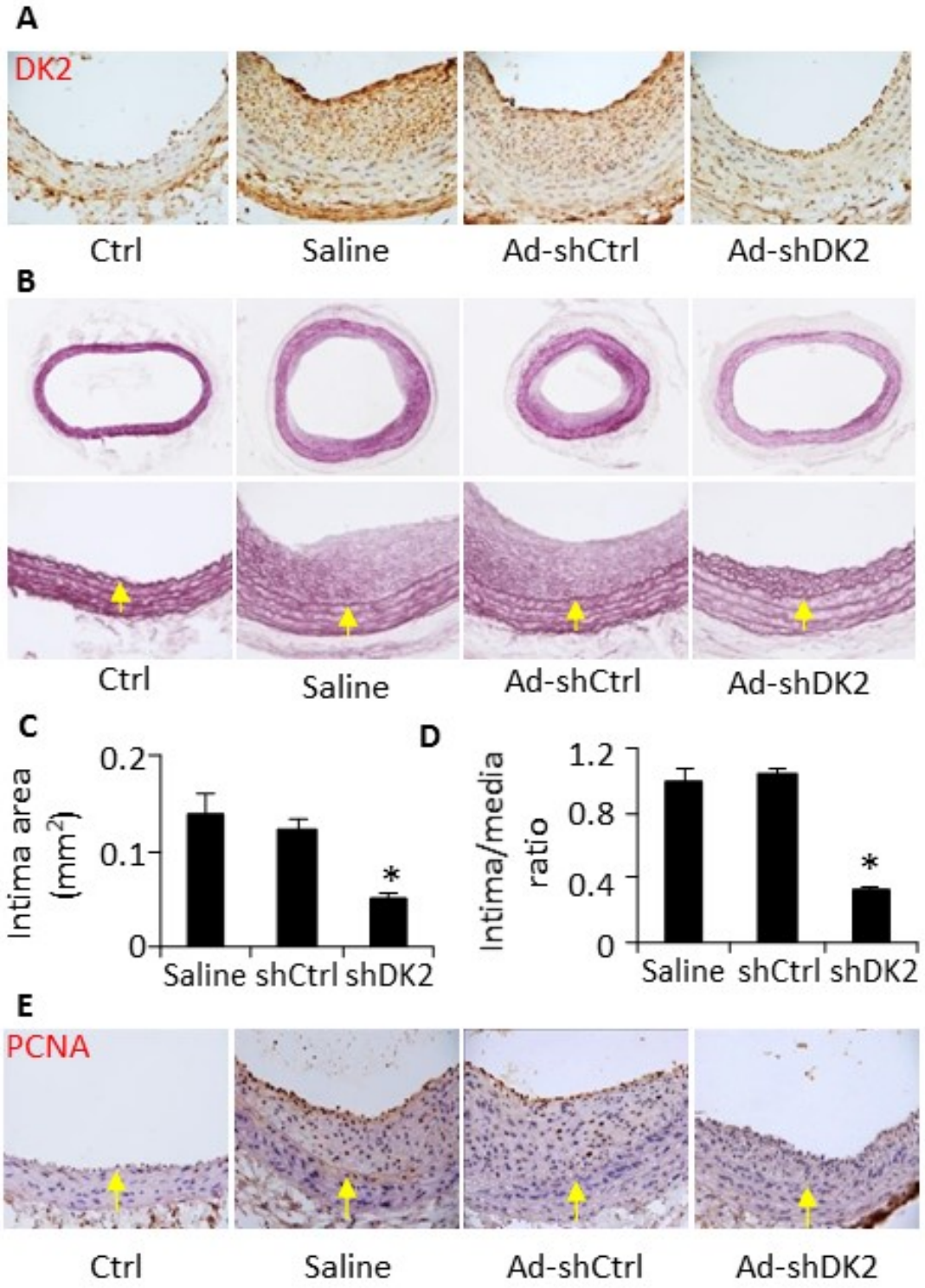

Figure 5.6. Knockdown of DOCK2 blocked neointima formation and VSMC proliferation in vivo.

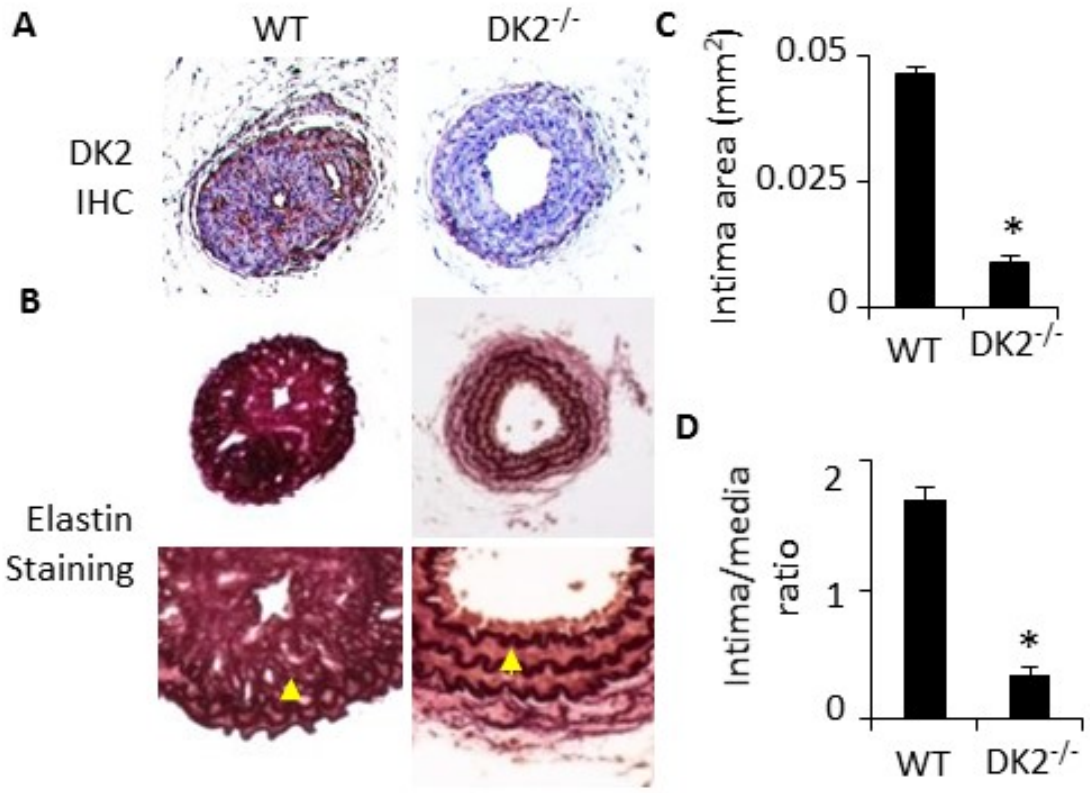

Figure 5.7. Knockout of DOCK2 blocked ligation injury-induced neointima formation in mice.

Figure Legends

Figure 5.1. PDGF-BB induced DOCK2 expression along with VSMC phenotypic modulation. A, PDGF-BB induced DOCK2 protein expression in a dose-dependent manner. VSMCs were treated with different dosages of PDGF-BB for 48 hr. The protein expression of DOCK2 (DK2) and VSMC marker α-actin (α-SMA) and calponin was detected by western blot. B, Quantitative analysis of protein expression shown in A by normalizing to α-Tubulin. *, $P<0.05$ vs vehicle-treated group (0 ng). C, Time-dependent induction of DOCK2 (DK2) and VSMC markers by PDGF-BB (10 ng/ml). D, Quantitative analysis of protein expression shown in C by normalizing to α-Tubulin. *, $P<0.05$ vs vehicle-treated group (0 hr).

Figure 5.2. DOCK2 mediated VSMC phenotypic modulation. A, DOCK2 inhibited VSMC marker expression. VSMCs transfected with pcDNA3.1 (Ctrl) or DOCK2 (DK2) cDNA were starved for 24 hr. DK2 and VSMC marker mRNA expression was detected by qPCR. *, $P<0.05$ compared with Ctrl groups. B, VSMCs isolated from wild type (WT) and DOCK2 knockout ($DK2^{-/-}$) mice were treated with vehicle (-) or PDGF-BB (10 ng/ml) for 36 hr. The protein expression of DK2 and VSMC marker α-SMA and calponin was detected by western blot. C, Protein level of each protein shown in B was quantified by normalizing to GAPDH. *, $P<0.05$ compared with vehicle-treated (-) WT group; #, $P>0.05$ compared with vehicle-treated $DK2^{-/-}$ group.

Figure 5.3. DOCK2 regulated VSMC phenotype by suppressing myocardin/SRF-mediated VSMC marker gene transcription. A, PDGF-BB inhibited

myocardin (Myocd) expression in wild type (WT) but not DOCK2 knockout ($DK2^{-/-}$) VSMCs. VSMCs isolated from WT or $DK2^{-/-}$ mice were treated with 10 ng/ml of PDGF-BB for 24 hr. mRNA expression of indicated genes was detected by qPCR. *, $P<0.05$ compared with vehicle-treated (-) WT group, #, $P>0.05$ compared with vehicle-treated $DK2^{-/-}$ group. &, $P<0.05$ compared with vehicle-treated $DK2^{-/-}$ group. B, DOCK2 (DK2) inhibited Myocardin (Myocd) but not SRF expression. Myocardin and SRF expression was detected by qPCR. *, $P<0.05$ compared with control (Ctrl) group. C, Knockdown of DOCK2 restored PDGF-BB-blocked Myocardin-SRF interaction. VSMCs were infected with adenovirus expressing scramble (Ad-shCtrl) orDOCK2 shRNA (Ad-shDK2) and transfected with Flag tagged Myocd cDNA. Cells were then treated with PDGF-BB. Cell lysates were immunoprecipitated (IP) with normal IgG or SRF followed by immunobloting (IB) with Flag or SRF antibody. D, Quantification of SRF-bound Myocd by normalizing to the input Myocd level in each treatment. *, $P<0.05$ vs PDGF-BB-untreated group (-); #, $P<0.05$ vs PDGF-BB and Ad-shCtrl-treated groups. E. DOCK2 overexpression suppressed Myocardin-SRF interaction. DK2 and/or Flag-tagged Myocd were transfected into VSMCs as indicated followed by Co-IP with the same antibodies used in C. F. SRF-bound Myocd was quantified similarly as in D. *, $P<0.05$ vs control plasmid-transfected group (-). G, DOCK2 blocked Myocd-activated transcription of α-SMA promoter. pcDNA3.1, Myocd, or DK2 plasmid were co-transfected with α-SMA promoter as indicated into VSMCs followed by PDGF-BB treatment for 24 hr. Luciferase assay was performed. *, $P<0.05$ vs pcDNA-transfected

group; #, $P<0.05$ vs Myocd alone-transfected group. H, VSMCs were treated with vehicle or PDGF-BB for 24 hr. DOCK2 expression was detected by immunostaining. DK2 was expressed on both cytoplasm membrane (red arrows) and nuclei (yellow arrow) of VSMCs upon PDGF-BB treatment.

Figure 5.4. DOCK2 was essential for PDGF-BB-induced VSMC migration and proliferation. A, DOCK2 shRNA knockdown efficiency was detected by western blot. B, DOCK2 expression shown in A was normalized to α-Tubulin. *, $P<0.05$ vs shCtrl/vehicle-treated group (-), #, $P<0.05$ vs shCtrl/PDGF-BB-treated group. C, Representative microphotographs of wound healing assay in VSMCs transduced with adenovirus expressing scramble (shCtrl) or DOCK2 shRNA (shDK2) and treated with vehicle or PDGF-BB. D, Quantification of the migration distance. *, $P<0.05$ vs shCtrl with vehicle treatment (-), #, $P<0.05$ vs shCtrl with PDGF-BB treatment. DOCK2 knockdown inhibited PDGF-BB-induced VSMC migration. E, Representative photomicrographs of Transwell assay. The migrated cells were stained with DAPI. F, The number of the migrated cells was counted and averaged from 8 different fields in each of three independent experiments. *, $P<0.05$ vs shCtrl with vehicle treatment (-), #, $P<0.05$ vs shCtrl with PDGF-BB treatment. G, Knockdown of DOCK2 by shRNA (shDK2) blocked PDGF-BB-induced focal adhesion contact formation. The focal adhesion contacts were labeled with vinculin antibody followed by staining with tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antibody. Shown are the representative results of 3 independent experiments. H, DOCK2 mediated

PDGF-BB induced accumulation of F-actin stress fibers on cell membrane. The F-actin stress fibers were labeled with phalloidin. Shown are the representative results of 3 independent experiments. I, DOCK2 was essential for PDGF-BB-induced VSMC proliferation. Cell proliferation was measured by MTT assay. Shown are the proliferation fold changes. *, $P<0.05$ vs vehicle/shCtrl at 36 hr; #, $P<0.05$ vs PDGF-BB/shCtrl at 36 hr.

Figure 5.5. Balloon injury induced DOCK2 expression in tunica media and neointima VSMCs. A, Balloon injury induced progressive neointima formation. Rat carotid arteries were sham-operated (Ctrl) or injured for 3, 7, and 14 days as indicated. Artery sections were stained with Elastica van Gieson solution. Yellow arrows indicate the internal elastin lamina. B, DOCK2 was induced in tunica media initially and neointima VSMCs subsequently following injury. Artery sections were incubated with DOCK2 antibody followed by horseradish peroxidase-conjugated secondary antibody and DAB staining. Dark blue arrows in the enlarged image of the red box in the 3d sections show representative VSMCs expressing DOCK2. Yellow arrows indicate the internal elastin lamina. C, DOCK2 expression in injured arteries was detected by western blot. Data shown are a representative result of 3 independent experiments. D, Quantification of DOCK2 expression by normalizing to α-Tubulin. *, $P<0.01$ compared to uninjured arteries (0 d).

Figure 5.6. Knockdown of DOCK2 blocked neointima formation and VSMC proliferation *in vivo*. A, DOCK2 expression was efficiently blocked by adenovirus

delivery of DOCK2 shRNA. Immediately after balloon injury, the injured rat carotid arteries were incubated with sterile saline solution, adenovirus expressing scramble (Ad-shCtrl), or DOCK2 shRNA (Ad-shDK2) as indicated. 14 days later, artery sections were stained with DOCK2 antibody. DOCK2 (DK2) expression was visualized by DAB staining. B, DOCK2 knockdown blocked injury-induced neointima formation. Artery sections were stained with Elastica van Gieson solution. Yellow arrows indicate internal elastic lamina. C and D, Quantification of intima area and intima/media ratio. *, $P<0.05$ compared with saline- or Ad-shCtrl-treated arteries (n=6). E, DOCK2 knockdown inhibited VSMC proliferation *in vivo* as shown by the expression of proliferating cell nuclear antigen (PCNA).

Figure 5.7. Knockout of DOCK2 blocked ligation injury-induced neointima formation in mice. A, Ligation injury induced DOCK2 expression in wild type (WT), but not DOCK2 knockout ($DK2^{-/-}$) mouse left carotid arteries. Mouse left carotid arteries were injured by ligation for 30 days. DOCK2 (DK2) expression was detected by immunohistochemistry (IHC) staining using DK2 antibody and DAB visulization. B, DK2 knockout blocked ligation-induced neointima formation. Artery sections were stained with Elastica van Gieson solution. Arrows indicate internal elastic lamina. C-D, Quantification of intima area and intima/media ratio of the injured arteries.. *, $P<0.05$ compared to WT carotid arteries with ligation (n=6).

CHAPTER 6

CONCLUSION

This dissertation work was dedicated to develop a new robust model system using human embryonic stem cell-derived mesenchymal cells (hES-MCs) for the following reasons: (1) hES-MCs are likely the natural SMC progenitors because most of the vascular SMCs are derived from mesoderm. (2) hES-MCs are easy to culture and can be split up to 10 passages; (3) this model may be used to study SMC differentiation in human. Identify the TGF-β downstream targets that are important in regulating SMC differentiation using the newly established SMC differentiation model is another objective of the dissertation. Dedicator of cytokinesis 2 (DOCK2) is an atypical guanine nucleotide exchange factor for the Rho-small guanine triphosphatase. In this dissertation, we have determined if DOCK2 is involved in regulating SMC differentiation and phenotypic modulation. Several conclusions can be drawn from this dissertation:

1. The TGF-β-induced SMC differentiation from hES-MCs recapitulates the normal SMC differentiation process. Acta2 is activated at 2 h, Cnn1 at 4 h while Myh11 at 8 h after TGF-β induction of hES-MCs, which mimics SMC marker gene expression pattern observed during normal SMC development.

2. TGF-β-induced expression of SMC markers in hES-MCs appears to depend on both Smad signaling and SRF/CArG/myocardin because knockdown of Smad2, Smad3, or SRF blocks SMC marker expression. Consistently, mutation of SRF-binding CArG

box blocks SMC marker promoter activity. These results indicate that TGF-β induction of hES-MC may serve as a physiologically-relevant model for SMC differentiation.

3. hES-MC-derived SMCs are able to preserve the endothelial tubes probably because they have acquired the contractile property of functional SMCs.

4. TGF-β induces myocardin expression, which is important for the SRF/CArG-mediated SMC marker gene activation. It appears that TGF-β induces hES-MCs to express myocardin via a mechanism different from other cells because multiple signaling pathways including Smads, p38 MAPK and PI3K are all required for TGF-β-induced myocardin expression in hES-MCs.

5. DOCK2 is scarcely detectable in normal VSMCs. However, PDGF-BB induced DOCK2 expression while modulating VSMC phenotype as indicated by a reduced VSMC marker gene expression. DOCK2 appears to be critically important for PDGF-BB-induced phenotypic modulation because ectopic expression of DOCK2 inhibited VSMC marker α-SMA and calponin expression while knockout of DOCK2 (DOCK2-/-) enhanced the marker gene expression in primary VSMCs isolated from DOCK2-/- mice.

6. DOCK2 regulated VSMC phenotype through suppression myocardin/serum response factor (SRF)-mediated transcription of VSMC marker genes. DOCK2 blocked myocardin, but not SRF, expression. Knockout of DOCK2 dramatically increased myocardin expression. Although PDGF-BB down regulates myocardin expression in normal VSMCs, it does not alter myocardin expression in DOCK2-/- VSMCs. DOCK2 blocks myocardin binding to SRF. PDGF-BB inhibits myocardin-SRF interaction. However, this inhibition is blocked when DOCK2 is knocked down by shRNA, suggesting that DOCK2 mediates PDGF-BB inhibition of myocardin-SRF binding.

7. DOCK2 stimulated VSMC migration via induction of focal adhesion contact and stress fiber formation. DOCK2 regulates focal adhesion contact by enhancing vinculin expression and its distribution to the leading edge of migrating cells while regulating cytoskeleton by promoting F-actin formation in the lamellipodia of VSMCs. In addition to migration, DOCK2 is also required for VSMC proliferation both in vitro and in vivo although the underlying mechanisms remain to be determined.

8. In vivo study using rat carotid artery balloon-injury model indicates that DOCK2 is induced in media layer VSMC initially and neointima VSMC subsequently following vascular injury. Blocking the initial expression of DOCK2 immediately after injury using adenovirus-delivered shRNA effectively blocks injury-induced neointima formation by 60%. Most importantly, knockout of DOCK2 in mice markedly blocks ligation-induced intimal hyperplasia.

9. Collectively, our studies identified DOCK2 as a novel regulator for VSMC phenotypic modulation and vascular lesion formation following vascular injury. Therefore, targeting DOCK2 is a potential therapeutic strategy for the prevention of vascular remodeling in proliferative vascular diseases.