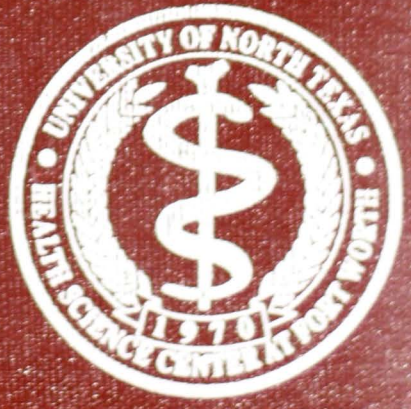
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Differentiated vascular smooth muscle cells (SMCs) exhibit a work phenotype characterized by expression of several well-documented contractile apparatus-associated proteins. However, when exposed to mitogens such as serum or growth factors, SMCs retain the ability to de-differentiate into an "immature" proliferative phenotype, in which they lack contractile myofilaments. Proliferation of SMCs is involved in the formation of atherosclerotic plaques as well as arterial restenosis following balloon angioplasty. Thus, understanding the mechanisms involved in maintaining SMC differentiation process is critical to the development of therapies and treatments for the abnormal growth seen in these disease states. In this study, the molecular mechanisms through which transforming growth factor-beta 1 (TGF-β1) induces differentiation of SMCs were examined. The data presented demonstrate that TGF-β1 stimulates actin re-organization, up-regulation of SM-specific marker gene expression and inhibition of cell proliferation of PAC-1 SMCs These characteristics are indicative of the differentiated phenotype. The effects of TGF-β1 can be blocked by pretreatment of the cells with either HA1077 or Y-27632, which inhibit the functions of the kinases downstream of RhoA. Moreover, TGF-β1 induced differentiation is correlated with an increase in the activity of RhoA and its downstream target, PKN. Over-expression of active PKN alone is sufficient to increase the transcriptional activity of the SM α-actin, SM-MHC and SM22 promoters in PAC-1

cells. In addition, the activity of SRF, GATA and MEF2, three transcription factors that are known to regulate expression of SM-specific marker genes, are also increased by PKN. Finally, examination of MAPK signaling cascades demonstrates that TGF-β1 increases the activity of MKK3/6 and p38 MAPK and decreases the activity of ERK1/2 and JNK1/2. Co-expression of dominant negative p38 MAPK is sufficient to abolish PKN-mediated activation of SRF, GATA and MEF2 as well as PKN-mediated activation of SMC marker gene promoters. Taken together, these results identify components of an important intracellular signaling pathway through which TGF-β1 activates RhoA and PKN to promote differentiation of SMCs.

CHARACTERIZATION OF THE ROLE OF PKN IN

TGF-BETA 1-MEDIATED DIFFERENTIATION OF

VASCULAR SMOOTH MUSCLE CELLS

Rebecca Ann Deaton, B.S.

APPROVED:

Major Professor

Committee Member

Committee Member

Committee Member

University Member

Chair, Department of Biomedical Sciences

Dean, Graduate School of Biomedical Sciences

CHARACTERIZATION OF THE ROLE OF PKN IN TGF-BETA 1-MEDIATED DIFFERENTIATION OF VASCULAR SMOOTH MUSCLE CELLS

DISSERTATION

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Rebecca Ann Deaton, B.S.

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CHAPTER I

INTRODUCTION

Alterations in vascular smooth muscle cell (SMC) phenotype contribute to the pathophysiology of a number of human diseases including atherosclerosis, restenosis, hypertension, cancer progression and asthma. Specifically, increases in SMC proliferation (hyperplasia), cell size (hypertrophy), migration and deposition of extracellular matrix proteins are known to occur during these vascular disease states. Considerable effort has focused on understanding the role of SMC phenotypic switching during the development of atherosclerosis, which is the major cause of cardiovascular disease in western societies. Treatment of atherosclerosis often involves balloon angioplasty to open the occluded artery. However, the success of this treatment is greatly limited by the recurrent hyperplasia of SMCs within the recently opened vessel.

Over the last ten years, considerable strides have been made towards understanding the molecular mechanisms that control changes in SMC phenotype. There are now several candidate proteins such as transforming growth factor-beta 1 (TGF-β1) and platelet derived growth factor (PDGF) that are thought to initiate changes (either positive or negative) in SMC phenotype. In addition, much is known regarding the transcription factors that control changes in the expression of genes altered during phenotypic switching of SMCs. Despite the progress made toward understanding the molecular mechanisms leading to changes in SMC phenotype, the signal transduction

pathways through which these stimuli work remain unknown. The focus of this dissertation was to identify the signaling pathway through which TGF-β1 regulates changes in SMC phenotype, with an emphasis on the control of transcriptional regulation of several SMC differentiation-specific marker genes.

The following section will provide a detailed description of SMC phenotype with respect to both normal SMC function as well as phenotypes that contribute to the pathophysiology of vascular disease. A review of the relevant known molecular mechanisms involved in eliciting changes in SMC phenotype will also be provided.

Differentiated Smooth Muscle Cell Phenotype

Vascular smooth muscle cells are a unique subset of muscle cells whose primary function is contraction leading to alterations in arteriolar tone, which ultimately regulates blood flow and pressure. The healthy adult artery is comprised of three main layers: tunica intima, tunica media and tunica adventitia (Figure 1). The tunica intima, which is the innermost layer of the vessel, consists of a single layer of endothelial cells. The tunica media is comprised primarily of SMCs. The outermost layer is the tunica adventitia, which contains connective tissue, blood vessels and nerves. Each layer is separated by elastic lamina.

a) Vascular Development

During development, vascular SMCs are derived from two distinct embryonic origins: mesenchyme and neural crest, with the majority of SMCs arising from

mesenchyme (62, 106). Initially, the primitive blood vessel consists of a single layer of endothelial cells (144). Precursor SMCs are recruited from the surrounding mesenchyme where they are stimulated to differentiate into SMC through yet undefined mechanisms thought to be regulated in part through contact with endothelial cells themselves (56, 57, 61). The process of SMC differentiation from precursor cells involves exit of the cells from the cell cycle (resulting in a reduction in the rate of proliferation) as well as temporal up-regulation of cell-type specific genes required for the proper SMC function. The adult SMCs in the tunica media exist primarily in the differentiated phenotype, characterized by their extremely low rate of proliferation and their expression of a distinct set of contractile apparatus-associated proteins, which are required for their contractile function (129, 163). Collectively, the genes that encode these proteins are referred to as SM-specific marker genes, as they are expressed upon the onset of SMC differentiation. Although some are transiently expressed in other cell types during development, including cardiac and skeletal muscle, their expression is highly specific for SMCs in adult animals.

b) Smooth Muscle-specific Marker Genes

SM-specific markers include smooth muscle α-actin (SM α-actin) (37, 62, 95), smooth muscle γ-actin (SM γ-actin) (21), smooth muscle myosin heavy chain (SM-MHC) (97, 108), SM22α (28, 86), h_1-calponin (28, 36), h-caldesmon (36) and smoothelin (176, 177). As previously mentioned, these proteins are expressed in a time dependent fashion during development (Figure 2) (129). Expression of these SM-specific marker genes

results in the increased myofibrillar organization required for contraction. The most important and well characterized of these markers includes smooth muscle α-actin, smooth muscle myosin heavy chain and SM22α.

Smooth muscle α-actin

SM α-actin is the first SM-specific marker to be expressed in SMCs during development (36). It is one of six actin isoforms expressed in mammalian cells (178). Importantly, it is the most abundant actin isoform expressed in differentiated vascular SMCs, accounting for more than 70% of the total actin present (31). It is also ranks as the most abundant protein expressed in differentiated vascular SMC, making up approximately 40% of the total protein found in each cell (31). In the adult, its expression is highly specific for differentiated SMCs, although it is known to be expressed transiently in other muscle types during development (37, 186).

Smooth muscle myosin heavy chain

SM-MHC is arguably the most definitive SM-specific marker of differentiated SMCs, as it appears to be the most tissue-specific of all the markers identified to date (97, 108). In contrast to SM α-actin, SM-MHC is one of the last SM-specific markers to be expressed (80). There are two main SM-specific MHC isoforms, designated SM1 and SM2, which are produced by alternative splicing (121, 153). In addition, SMCs can also express a non-muscle form of MHC, designated NM-B MHC or SMemb, which is

expressed in fetal SMC and re-appears in neointimal SMCs of an atherosclerotic lesion (see below) (79).

SM22α

SM22α is a 22-kDa cytoskeletal protein that contains some sequence homology to calponin (124). While calponin is known to bind to proteins of the contractile apparatus, such as actin, it is controversial whether SM22α associates with actin (112). To date, no known physiological function has been ascribed to SM22α. Moreover, SM22α knockout mice are phenotypically normal, with no apparent vascular abnormalities (196). It has been recently reported that knockout of SM22α in ApoE-/- deficient mice (which readily develop atherosclerosis when fed a high fat/high cholesterol diet) results in enhanced atherogenesis (32). This argues that SM22α plays an important role in the regulation of SMC phenotype.

c) Morphology of Differentiated SMCs

In addition to the expression of SM-specific markers, differentiated SMCs undergo other morphological changes that are required for their contractile function. First and foremost, up-regulation of the SM-specific marker genes correlates with increased myofibrillar organization, characterized by actin stress fiber formation in differentiated SMCs (44, 129). Secondly, SMCs switch from a "fibroblast"-like shape to an elongated, "spindle-like" shape upon differentiation (61). Lastly, there is a drastic

reduction in the abundance of synthetic organelles such as golgi and ER, which correlates with the reduced need for protein synthesis in differentiated SMCs (Figure 3) (173).

Phenotypic Modulation and Vascular Disease

Unlike other cells of myogenic lineages (such as skeletal and cardiac muscle), differentiated SMC retain the ability to alter their phenotype in response to extracellular stimuli, which has been termed phenotypic switching or phenotypic modulation. Although the characteristics of distinct SMC phenotypes are not mutually exclusive, they can be generally classified into three main groups, differentiated (contractile), proliferative (synthetic) or hypertrophied. As previously discussed, most SMCs in adult animals are of the differentiated phenotype. Phenotypic modulation of a differentiated SMC to the proliferative phenotype is characterized by a reversion to a more "embryonic" phenotype. Specifically, this involves: 1.) down-regulation of the SMC-specific marker genes (contractile proteins) (3, 76, 77); 2.) up-regulation of proliferation markers such as proliferating cell nuclear antigen (PCNA) (143); 3.) hyperplasia (proliferation) (151); 4.) a change in cell morphology from a "spindle-like" shape to a "fibroblast-like" shape (76, 77); and 5.) an increase in synthetic organelles such as golgi and ER (173). In contrast, phenotypic modulation of differentiated SMCs to a hypertrophied phenotype does not involve cell proliferation, but involves an increase in overall cell size without concomitant cell division (128). Moreover, hypertrophied SMCs display an increased rate of protein synthesis, including increased expression of the SM-

specific marker genes. In addition, many hypertrophied SMCs are polyploid, displaying an increase in the average content of DNA per cell (128).

The normal, healthy adult artery contains predominantly differentiated SMCs. However, there is an increase in proliferative and hypertrophied SMCs during vascular disease states including atherosclerosis, restenosis and hypertension (Figure 4). These aberrant phenotypes have been shown to have both beneficial and detrimental effects on the pathology of these diseases as will be described below.

a) Atherosclerosis and Restenosis

Cardiovascular disease is the #1 killer in the United States, regardless of race or gender. It claims more lives that the next five leading causes of death combined (cancer, chronic lower respiratory disease, accidents, diabetes mellitus and influenza/pneumonia) (6). Moreover, atherosclerosis accounts for nearly 75% of all deaths from cardiovascular disease (6). Atherosclerosis is characterized as a vascular inflammatory disease resulting from complex interactions between many factors including oxidized lipids (low-density lipoproteins or LDL), immune cells (macrophage), endothelial cells, vascular SMCs and platelets (40). These interactions subsequently result in the development of vascular lesions referred to as plaques. Plaque development causes gradual occlusion of the affected artery, thereby resulting in a narrowing of the vascular lumen. Decreased lumen diameter greatly reduces the normal flow of blood through the occluded artery. If not corrected, plaque rupture or thrombosis will result in myocardial infarction or stroke and potentially, death.

Formation of an atherosclerotic lesion is complex and involves many risk factors including hypercholesterolemia, cigarette smoking, hypertension, physical inactivity, obesity, diabetes mellitus, metabolic syndrome and family history (6, 40). Much evidence suggests that the formation of an atherosclerotic lesion is initiated by endothelial injury, which is manifested by uptake of oxidized LDL (oxLDL) (151). Enhanced trapping of oxLDL by endothelial cells stimulates the expression of cell adhesion molecules, which recruit circulating monocytes (151). The monocytes migrate into the endothelial layer, where they differentiate into macrophages. While this is initially thought to be an immunoprotective effect, the macrophages also accumulate high levels of oxLDL to form foam cells, which create the initial "fatty streak" of the atherosclerotic lesion (151). In addition to macrophage, other cells including T lymphocytes and platelets accumulate in this damaged region. These cells can secrete a variety of growth factors and cytokines (such as platelet derived growth factor, or PDGF) that are mitogenic to the underlying SMCs, causing them to "de-differentiate" and adopt a more proliferative phenotype. The pathological proliferation of SMCs within an atherosclerotic lesion is classified as neointimal hyperplasia, which contributes to the occlusion of the vascular lumen in the damaged area (Figure 5). Interestingly, these proliferating SMCs also synthesize extracellular matrix proteins that contribute to the formation of a fibrous cap, which has been hypothesized to temporarily stabilize the plaque (40, 127, 151). As the lesion progresses, the fibrous cap becomes thinner and ultimately unstable (referred to as a vulnerable plaque), leading to rupture and thrombosis (Figure 6). Thus, SMC proliferation contributes to the formation of the vascular

occlusion through neointimal hyperplasia. However, in late lesion formation these SMCs may play a potentially beneficial role in plaque stabilization.

Treatment of atherosclerosis usually involves balloon angioplasty, also known as pericutaneous transluminal coronary angioplasty (PTCA), to clear out the blocked artery. According to the American Heart Association, more than 500,000 PTCA procedures are performed in the United States annually (6). However, the success of this procedure is limited by the recurrent hyperplasia of the underlying SMCs, known as restenosis (8, 127). This causes re-occlusion of the vessel recently cleared by angioplasty. Restenosis usually occurs within 3 to 6 months post-angioplasty in 25-40 percent of patients undergoing this form of treatment (26, 161). As a result, complications from atherosclerosis are the most common cause of death in Western societies (6, 40). Understanding the molecular signaling pathways that maintain or promote SMCs differentiation will be important for the treatment of aberrant smooth muscle proliferation associated with atherosclerosis and restenosis.

b) Hypertension

Hypertension, also referred to as high blood pressure (HBP), is defined as systolic pressure of 140 mm of Hg or higher and a diastolic pressure of 90 mm Hg or higher (6). Nearly 1 out of every 4 adults is hypertensive (6). Moreover, hypertension is associated with a 2-3 times higher risk of developing congestive heart failure (CHF) and precedes CHF in 91% of all cases (6).

The etiology of hypertension is undoubtedly quite complex, however, it has been well established to involve increased peripheral vascular resistance due to vascular remodeling and potentially increased vascular contractility (17, 174). Vascular remodeling can involve phenotypic switching of the SMCs. Indeed, studies have shown that both the proliferative and hypertrophied phenotypes can contribute to an increased media:lumen ratio depending upon the model of hypertension as well as the size of blood vessel examined. For instance, remodeling of large conduit arteries is associated primarily with a hypertrophy of the existing SMCs whereas smaller resistance arteries display an increase in SMC hyperplasia, but not hypertrophy (120, 131, 132).

c) Other vascular diseases

In addition to atherosclerosis, restenosis and hypertension, phenotypic modulation of SMCs is associated with other vascular diseases. First, it has been well documented that angiogenesis is critical for the development and growth of most solid tumors and plays an important role in metastasis of tumors from one location (or organ) to another (20). In addition to cancer progression, phenotypic modulation of SMCs also plays an important role in the pathology of asthma. Airway SMCs in patients with asthma are hyper-responsive to contractile agonists, leading to exaggerated contraction of the airway. This response may be due to alterations in the response of SMCs to contractile agonists or to increased SMC mass due to hyperplasia or hypertrophy (7, 81, 160).

In summary, it is quite obvious from the preceding discussion that phenotypic modulation of SMCs plays an important role in the pathology of several important

diseases in man. As such, delineating the molecular mechanisms that control the differentiation of SMCs will ultimately provide novel therapies designed to maintain the contractile phenotype, thereby blocking the contribution of aberrant SMC phenotypes to these vascular diseases.

Transcriptional regulation of SM-specific marker gene expression

The hallmark of SMC differentiation is the increased expression of SM-specific marker genes. As previously mentioned, the proteins encoded by these genes include contractile apparatus-associated proteins, which, when expressed, ultimately provide the cell with the ability to contract and work. Therefore, it is critical to understand the molecular mechanisms involved in driving the expression of these genes. Recent studies by numerous labs have identified key regulatory elements within the promoter regions of these genes that are essential for their proper expression both *in vitro* and *in vivo* (137). Moreover, studies of the transcription factors and cofactors that bind to these elements have provided further insight into the possible signaling cascades that may regulate the expression of SM-specific marker genes (Table 1). The key transcription factors and the DNA elements to which they bind are summarized below.

a) Serum Response Factor (SRF)

SRF is the most highly studied and characterized transcription factor with respect to SM-specific marker gene expression. It is a MADS (MCM1, Agamous, Deficiens, SRF) box transcription factor that binds to the consensus sequence $CC(A/T_6)GG$,

otherwise known as a CArG box (107). Nearly all SM-specific marker genes studied to date are thought to depend on one or more CArG boxes (Table 2). Indeed, mutation of the CArG boxes in the SM α-actin, SM-MHC and SM22α promoters almost completely abrogates their transcriptional activity (73, 95, 100). Moreover, the importance of these CArG boxes is underscored by the fact that these elements have been evolutionarily conserved among many different species (107). The expression of SRF in SMCs coincides with the expression of SM-specific marker genes (18, 82). Importantly, ablation of SRF activity, through the use of a dominant negative form of SRF, can block differentiation of SMCs from precursor cells *in vitro* (82). Its activity is regulated in part by binding to other transcription factors (including GATA, Mhox and NK family members) and co-factors (including myocardin and CRP family members) (22, 51, 125, 142, 181, 191, 192). SRF is also subjected to post-translational modification, such as phosphorylation, although the relative effect on SRF activity is controversial (149, 150). Lastly, SRF may also be regulated by subcellular compartmentalization as it has been recently reported that SRF translocation from cytosol to nucleus is associated with an increase in its activity (19, 90).

b) GATA

The GATA family of transcription factors consists of zinc-finger proteins that bind to the consensus DNA sequence (A/T)GATA(A/G) (also referred to as WGATAR) (111). There are 6 members of the GATA family: GATA-1/2/3 isoforms are expressed in hematopoietic cells whereas GATA-4/5/6 isoforms are expressed predominantly in

cardiovascular tissues. Of the latter, GATA-6 is the predominant isoform expressed in adult vascular SMCs and plays a role in SMC-specific marker gene expression and cell cycle arrest (125, 141, 180). Interestingly, expression of GATA-6 is down-regulated in proliferating SMCs following balloon angioplasty and restoration of GATA-6 expression in these areas lessens neointimal formation (101). Taken together, these data imply that GATA plays an important role in maintaining the differentiated phenotype of SMCs. GATA is phosphorylated by mitogen activated protein kinases (MAP kinases), which increases its transcriptional activity and its ability to bind DNA (23, 88). GATA can also partner with a number of other transcription factors such as SRF, NK family members and MEF2 to drive expression of both cardiac and SM-specific marker genes (113, 125).

c) Myocyte enhancer factor 2 (MEF2)

MEF2 proteins are a family of MADS-box transcription factors that are expressed in developing skeletal, cardiac and smooth muscle cells (14). There are four members of the MEF2 family (MEF2A/B/C/D) that bind to A/T-rich DNA sequences in order to regulate the expression of muscle-specific genes in these tissues. It has been well established that MEF2 plays a critical role in differentiation of skeletal muscle as well as mediating the hypertrophy response of cardiac myocytes (123, 138). Its role in SMC differentiation is less clear, although MEF2C deficient mice die from vascular abnormalities primarily due to the failure of SMC to differentiate (89). Moreover, MEF2B has been reported to increase SM-MHC gene expression (71). Taken together, these results argue that MEF2 may play an important role in regulating SM phenotype,

particularly during development. MEF2 proteins are regulated by phosphorylation (via MAP kinases such as p38 MAP kinase and BMK, also known as ERK5) as well as through partnering with other transcription factors such as GATA and basic helix-loop-helix (bHLH) proteins such as MyoD (14).

d) Others

Several other transcription factors have been implicated in the control of SM-specific gene expression (Table 1). Importantly, two of these families of proteins lie downstream of TGF-β1 signaling. These include the classic downstream signaling target of TGF-β1, the Smads family of transcription factors, as well as proteins that bind what has been termed the TGF-β control element (TCE) (24, 50, 70). Proteins that bind these elements include Krüppel-like transcription factors such as BTEB2 (1).

Extracellular Stimuli that regulate SM phenotype

As can be seen from the previous section, much is known regarding the key transcriptional elements that regulate SM-specific marker gene expression, including the transcription factors and co-activators that bind to these regions within the promoters of these genes. This information is important for understanding the control of gene expression as it pertains to changes in SMC phenotype. However, unraveling the extracellular stimuli that are responsible for *initiating* these changes as well as the other morphological changes that are required for phenotype switching is perhaps even more critical. Numerous factors have been characterized to play a role in altering the

phenotype of SMCs. These include contractile agonists such as Angiotensin II (AngII) and Endothelin 1 (ET-1), growth factors such as transforming growth factor beta-1 (TGF-β1) and platelet derived growth factor (PDGF), cell-cell interactions between SMCs and/or endothelial cells, cell-matrix interactions and mechanical or hemodynamic forces (129). Of these, much work has focused on the effects of PDGF and TGF-β1 on SMC phenotype.

a) Platelet Derived Growth Factor (PDGF)

PDGF is the best recognized SMC mitogen. It is known to stimulate both proliferation and migration of SMC (54). In addition, PDGF induces down-regulation of many SMC-specific marker genes in cell culture including SM α-actin, SM-MHC, SM22α, calponin and h-caldesmon (52, 58, 69). The molecular mechanisms through which PDGF work to down-regulate SM-specific marker genes are not completely clear. However, it has been shown that PDGF stimulation of SMC is associated with a decrease in SRF binding to consensus CArG boxes as well as an increase in cytoplasmic localization of SRF, where it would be transcriptionally inactive (68). As SRF binding and activity are known to be critical for proper expression of nearly all SM-specific marker genes, it stands to reason that a loss of SRF activity would produce global changes in gene expression in SMC. In addition to the inactivation of transcription factors that increase SM-specific marker gene expression (such as SRF), PDGF may also stimulate repressors that actively decrease the expression of these genes, although this hypothesis remains to be tested.

In addition to these *in vitro* studies, PDGF has been shown to stimulate SMC proliferation and migration *in vivo* following experimental models of balloon angioplasty in animals (33, 67). Moreover, inhibition of the PDGF receptor in ApoE-/- knockout mice fed a high fat diet (an animal model for atherosclerotic lesion development) decreased the lesion size by 67% and decreased neointimal proliferation of SMCs by 80% (155). This demonstrates a functional role for PDGF in phenotypic modulation of SMC associated with vascular disease.

b) Transforming Growth Factor Beta-1 (TGF-β1)

A role for TGF-β1 in the differentiation of SMCs has been demonstrated both *in vitro* and *in vivo* although the signal transduction pathways required for these effects are not completely clear. First, TGF-β1 can up-regulate SM-specific marker genes. *In vivo,* TGF-β1 expression and activity is highly correlated with the extent of SMC-specific marker gene expression (42). Moreover, *in vitro*, TGF-β1 increases expression of SM-MHC, SM α-actin, SM γ-actin, calponin and SM22α in smooth muscle cells using promoter-reporter assays and/or northern blot analysis and cell staining (1, 50, 55). Much like PDGF, the molecular mechanisms through which TGF-β1 modulate SMC-specific gene expression are not completely clear. However, TGF-β1 increases the expression and DNA binding activity of SRF, which as previously discussed, is a critically important transcription factor for expression of SM-specific marker genes (55). The signaling mechanisms through which TGF-β1 regulates SRF remain unknown.

In addition to up-regulating the expression of SM-specific marker genes, TGF-β1 suppresses SMC proliferation. *In vitro,* TGF-β1 inhibits the increase in DNA synthesis associated with PDGF-induced proliferation of cultured SMCs and has been implicated in blocking cell cycle events in SMCs as well as other cell types (13, 49, 128). When taken together, the ability of TGF-β1 to decrease proliferation of SMC and to up-regulate the expression of SM-specific marker genes makes it a strong candidate for a pro-differentiation factor. This point is further emphasized by the ability of TGF-β1 to drive several multipotential stem cell lines to a SMC fate (57, 162), arguing its importance during SMC development. Moreover, TGF-β1 and TGF-β1 type I receptor knockout mice die during development due to abnormalities in vascular development caused primarily by a failure of SMCs to differentiate (83, 103).

The role of TGF-β1 in response to vascular disease is less clear and has been confounded by contradictory reports. In contrast to the anti-proliferative effects previously described, TGF-β1 expression is up-regulated in neointimal smooth muscle cells following vascular injury (98). Moreover, inhibition of TGF-β1 by use of neutralizing antibodies lessens neointimal hyperplasia of SMCs and extracellular matrix deposition in a rat carotid injury model (148, 185). However, in agreement with studies demonstrating a pro-differentiated effect of TGF-β1, ApoE-/- deficient mice treated with TGF-β1 neutralizing antibody display increased atherosclerotic lesion size associated with a higher percentage of infiltrating inflammatory cells (99). Lastly, inhibition of TGF-β1 signaling by use of a soluble TGF-β receptor II protein in ApoE-/- deficient mice also displayed increased inflammatory cell infiltration as well as drastically decreased

extracellular matrix deposition leading to frequent plaque rupture due to the loss of fibrosis (94). Due of these confounding reports, it remains unclear whether TGF-β1 function in vascular disease is helpful or harmful.

Signal Transduction Pathways Involved in TGF-β1-mediated SMC Differentiation

The molecular signaling pathway(s) through which TGF-β1 induces differentiation of SMCs is unclear. The classic signaling cascade downstream of TGF-β1 involves the activation of the Smads family of transcription factors (110). However, there have been relatively few studies concerning the role of Smads in TGF-β1-mediated differentiation of SMCs. Moreover, TGF-β1 signaling has recently been linked to the activation of the small GTPase RhoA in other cell types (12, 29, 104). RhoA signaling is known to induce many of the same phenotype changes in SMCs as TGF-β1 and thus may be a novel target involved in producing the downstream effects of TGF-β1 in SMCs.

a) Smads

As previously mentioned, Smad proteins constitute the major downstream target of the TGF-β superfamily (110). TGF-β1 binds to two distinct receptors: type I and type II. While both types of receptor contain serine/threonine kinase activity, only the type II receptor is constitutively active. Binding of TGF-β1 ligand to the receptor induces a hetero-tetrameric complex consisting of two type I and two type II receptors. Type II receptors phosphorylate the type I receptors, thereby stimulating their intrinsic kinase activity. The active type I receptor is then responsible for activation of Smads signaling.

The Smads family can be divided into three classes, R-Smads, Co-Smads and I-Smads (110). The R-Smads are those that are activated directly by the type I receptor and include Smad2 and Smad3 for TGF-β1. The Co-Smad (or common Smad) is Smad4, which serves to partner with the activated R-Smads, stimulating translocation of the active complex to the nucleus. In the nucleus, the Smads can activate gene transcription through direct binding to CAGA elements of a promoter or through interaction with other transcription factors. Finally, I-Smads serve to inhibit TGF-β1 signaling by binding and repressing the function of either the active receptor or R-Smads. These include Smad6 and Smad7, respectively.

A role for Smads in TGF-β1-mediated effects on SMC phenotypic modulation has been recently demonstrated through the use of the I-Smad, Smad 7. TGF-β1 stimulation of rat aortic SMCs caused a decrease in SMC proliferation as well as an increase in SM α-actin and SM-MHC expression. Both effects could be blocked by exogenous expression of Smad7 (70). Moreover, it has also recently been shown that Smads contribute to TGF-β1-mediated activation of SM22α expression in three multipotent stem cell lines, which take on a SMC phenotype when treated with TGF-β1 (24). Since the consensus Smad binding element (CAGA) has not been characterized to be required for SM-specific marker gene expression, it is unknown how Smads might contribute to the up-regulation of these genes. Interestingly, Smads have been shown to partner with other transcription factors such as GATA-3 in hematopoietic cells and MEF2 in skeletal muscle cells, and it is easy to speculate that interaction of Smads with these factors in SMCs might contribute to SMC-specific gene expression (16, 146). Moreover,

during the completion of this work, Qiu *et al.* discovered that Smad3 was important for the up-regulation of SM22α in 10T1/2 cells in cooperation with SRF (145). However, the authors also show that TGF-β1 can activate SM22α in the absence of Smad3, which argues that factors other than Smad3 can mediate the effects of TGF-β1 on SM22α. Thus, although Smads signaling may contribute to the effects of TGF-β1 in SMC phenotypic modulation it appears that they are not the only downstream targets through which TGF-β1 elicits its effects.

b) RhoA

The small GTPase RhoA is normally found in an inactive state complexed to GDP dissociation inhibitors (GDIs) in the cytosol. Guanine nucleotide exchange factors (GEFs) activate RhoA by catalyzing GDP to GTP exchange. Classically this was thought to occur in response to G-protein coupled receptors (GPCRs), however, it has become increasingly clear that multiple receptors and/or signaling molecules may also stimulate RhoA through activation of Rho-specific GEFs (Figure 7) (156, 157, 183). Of interest, it was recently shown that TGF-β1 could up-regulate expression of NET-1, a known RhoA GEF, providing a possible mechanistic link between TGF-β1 and activation of RhoA signaling, although this mechanism has yet to be tested in SMCs (164).

As previously mentioned, activation of RhoA produces many of the same effects on SMC phenotype as TGF-β1, which argues that RhoA plays an important role in SMC differentiation. It is well documented that RhoA promotes myofibrillar organization in multiple cell types including cardiac, skeletal and smooth muscle myocytes (44, 59, 96,

137, 171). In adult primary smooth muscle cell cultures, RhoA expression and activity are initially low following isolation when the cells are actively proliferating. Interestingly, RhoA expression and activity increase and peak two days post-confluency when the cells re-adopt the differentiated phenotype (187). This implies that RhoA activity may be associated with SMC differentiation. In agreement with these findings, it has also recently been reported that RhoA is required for coronary SMC differentiation from proepicardial cells (91). Other studies have demonstrated that RhoA increases expression of SMC-specific marker genes through the activation of the transcription factor SRF (96). RhoA can also activate GATA-dependent transcription in cardiac myocytes (23). While these studies provide strong evidence for the potential involvement of RhoA in SMC differentiation, other researchers have suggested a role for RhoA in mediating vascular growth responses such as increased DNA synthesis, migration and cell hypertrophy associated with vascular injury and/or hypertension (158, 190).

The signal transduction pathways through which RhoA elicits its downstream effects have yet to be completely elucidated. Two key downstream targets of RhoA are Rho kinase (also known as ROCK) and PKN. While Rho kinase has been established to participate in the up-regulation of SMC-specific marker gene expression, use of a dominant negative form of Rho kinase cannot completely block the effects of active RhoA (64). Moreover, constitutively active Rho kinase does not completely recapitulate the phenotype seen with active RhoA (154). This implies that there are additional

signaling molecules (PKN or others) through which RhoA mediates its downstream effects.

PKN structure and function

PKN is member of a novel serine/threonine protein kinase family whose catalytic domains are quite similar to protein kinase C (PKC) (118). As such, these proteins have also been called protein kinase C related kinases (PRKs) (134, 136). Mukai *et al.*, who originally cloned PKN, recently reviewed the PKN family of protein kinases, designating the three isoforms PKNα, PKNβ and PRK2/PKNγ, with PKNα being the originally cloned PKN (Table 3) (115). While some structural homology to PKC exists, it is limited to the C'-terminal catalytic domain. Moreover, PKN cannot be activated by phorbol esters, diacylglycerol, calcium or phosphatidylserine, which emphasizes that its N'-terminal regulatory domain is unique compared to PKC (116, 134). However, among the different PKN family members there are several conserved regions within the regulatory domain, which are important for activation of PKN through interaction with other proteins (Figure 8). Most importantly, there are three conserved antiparallel coiled-coil (ACC) regions, the first of which directly binds GTP-bound (active) RhoA to increase the activity of PKN (5, 115, 182). In addition, the conserved C2-like domain contains an auto-inhibitory domain that is sensitive to arachidonic acid, which also regulates the activity of PKN (116, 134, 193). PKN has several autophosphorylation sites within its regulatory domain (not shown) although their relative importance for the regulation of PKN kinase activity is not completely clear (193). However, there are two critical

residues within the catalytic domain that are required for the kinase activity of PKN. Threonine 774 lies within the activation loop of the catalytic domain and its phosphorylation is required for activation of PKN (193). Moreover, lysine 644 is required for ATP binding within the catalytic domain, which is necessary for the kinase activity of PKN (193).

Effects attributed to PKN activation include: i.) hypertrophy of primary rat neonatal cardiomyocytes; ii.) cell cycle arrest of cycling *Xenopus* embryos; iii.) Ca^{2+}-sensitization in SMCs via inactivation of myosin phosphatase; iv.) direct binding to cytoskeletal proteins such as α-actinin and vimentin; and v.) activation of SRF-dependent transcription in bovine arterial SMCs (43, 45, 105, 109, 114, 119). The signal transduction pathways through which PKN elicits its downstream functions have not been specifically determined; however, PKN has been recently shown to bind and activate members of the p38 mitogen activated protein kinase (MAP kinase) family (169). Moreover, in NIH-3T3 cells, PKN alters gene expression through the activation of p38 MAP kinase demonstrating the function of p38 MAP kinase as a downstream target for PKN-mediated effects (102).

Project Hypothesis and Specific Aims

It has been well documented that TGF-β1 can induce differentiation of SMCs both *in vitro* and *in vivo*. However, the signal transduction pathways required for TGF-β1 effects are not completely clear.

The goal of this project was to identify the signaling pathway through which TGF-β1 regulates SMC differentiation. We hypothesized that PKN, a downstream target of RhoA, plays a central role in mediating the effects of TGF-β1 through a p38 MAPK dependent mechanism. This pathway and its effects on SMC phenotype are illustrated in Figure 9. The significance of this study lies in understanding the molecular mechanisms that control SMC phenotype. Ultimately, this understanding provides potential therapeutic targets to prevent the aberrant proliferation of SMCs associated with vascular disease. To test the proposed role of PKN in mediating TGF-β1 signaling, the following specific aims were addressed:

a) <u>Specific Aim #1</u>: Identification of TGF-β1 as a pro-differentiation factor of the PAC-1 SMC line and determination of the requirement of RhoA signal transduction cascades for mediating the downstream effects of TGF-β1.

PAC-1 smooth muscle cells were cultured in the presence of TGF-β1 or a vehicle control. Following treatment, cells were examined for changes in phenotype indicative of differentiation. Specifically, we monitored; i.) actin organization, ii.) SM-specific marker gene expression, iii.) promoter activity of SM-specific marker genes and iv.) the rate of cellular proliferation. To determine whether changes in SMC phenotype require TGF-β1-mediated activation of RhoA and its downstream targets, we assessed the ability of specific inhibitors of these enzymes to block TGF-β1 effects.

b) <u>Specific Aim #2</u>: Elucidation of the potential roles of RhoA and PKN in TGF-β1-mediated activation of SM-specific marker gene expression.

PAC-1 smooth muscle cells were cultured in the presence of TGF-β1 or a vehicle control and the activity of endogenous RhoA and PKN was measured by immunoblotting. Furthermore, we determined whether increasing the activity of PKN *alone* (independent of other targets downstream of TGF-β1 or RhoA) was sufficient to induce the promoter activity of three SM-specific marker genes as well as increase the activity of three important transcription factors.

c) <u>Specific Aim #3</u>: Elucidation of the role of p38 MAP kinase signaling for TGF-β1- and PKN-mediated effects on SM-specific marker gene expression.

PAC-1 smooth muscle cells were cultured in the presence of TGF-β1 or a vehicle control and the phosphorylation status of endogenous MAP kinases (ERK, JNK and p38 MAPK and MKK3/6) was measured by immunoblotting. The kinase activity of p38 MAP kinase was determined by *in vitro* kinase assay. Lastly, we determined whether the use of a dominant negative form of p38 MAP kinase could block PKN-mediated effects on SM-specific marker gene expression and transcription factor activation.

Figure 1. Schematic drawing of a healthy adult artery and its layers

In the adult animal, a normal artery consists of three layers: intima, media and adventitia. Each layer is separated by elastic lamina. Taken from Junqueira, L.C., Carneiro, J. and Kelly, R.O. (1998) *Basic Histology*, 9th ed, Appleton & Lange, A Simon & Schuster Company, Stamford, Connecticut, p. 203.

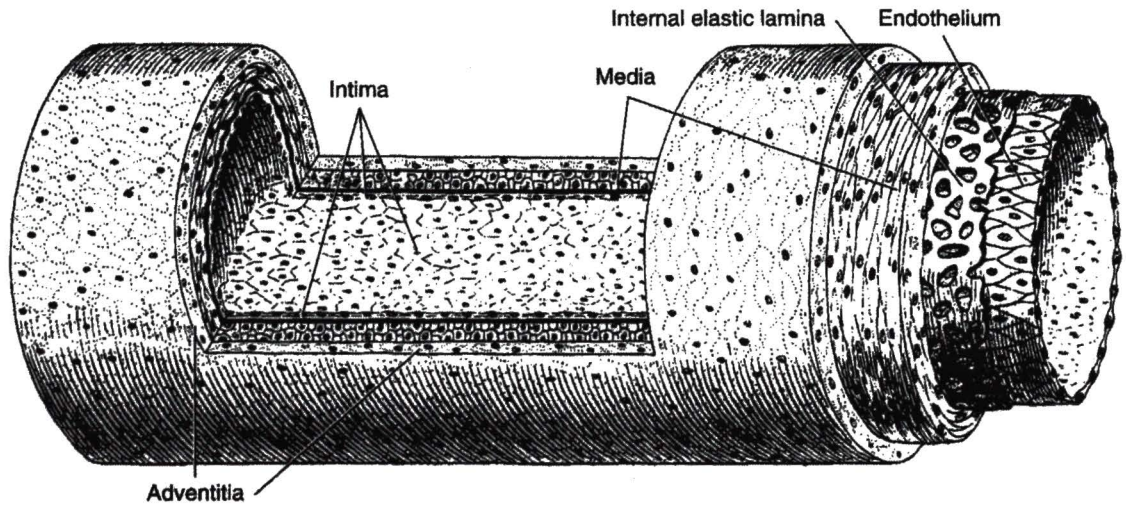

Internal elastic lamina
Endothelium
Intima
Media
Adventitia

Figure 2. Expression of smooth muscle-specific marker genes during development

During vascular development, precursor SMCs are stimulated to differentiate into SMCs, characterized by the time-dependent expression of several contractile apparatus-associated proteins. The expression of SM-specific marker genes is a hallmark of SMC differentiation. Taken from Owens, GK. Regulation of Differentiation of Vascular Smooth Muscle Cells. *Physiol Rev.* 1995;75:487-517.

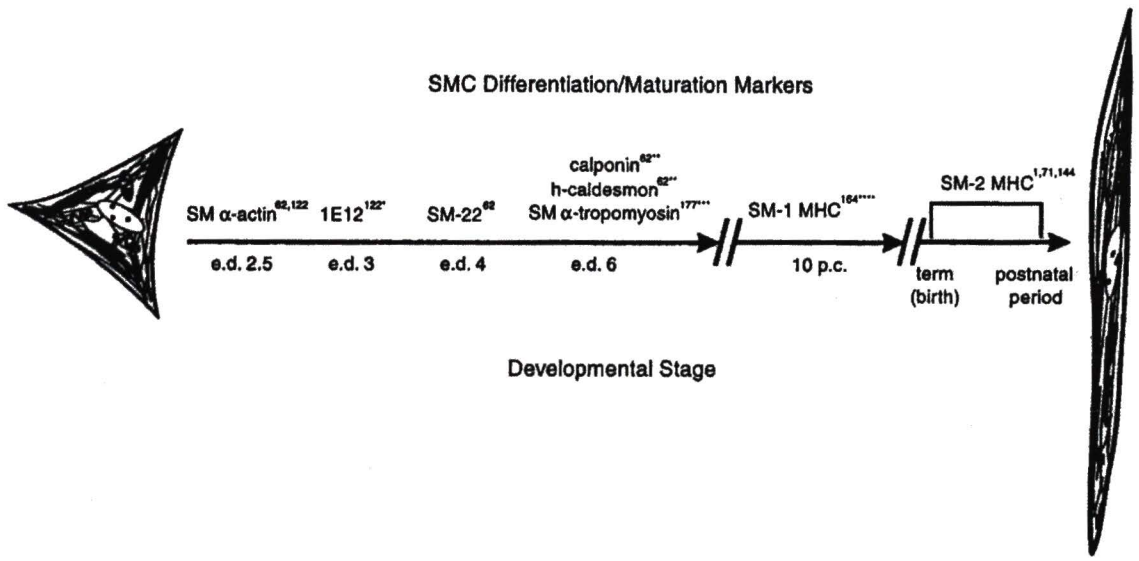

SMC Differentiation/Maturation Markers
SM α-actin[62,122]
1E12[122*]
SM-22[62]
calponin[62**]
h-caldesmon[62**]
SM α-tropomyosin[177***]
SM-1 MHC[164****]
SM-2 MHC[1,71,144]
e.d. 2.5
e.d. 3
e.d. 4
e.d. 6
10 p.c.
term (birth)
postnatal period
Developmental Stage

Figure 3. Morphological comparison of the proliferative and differentiated smooth muscle cell phenotypes

During vascular development, SMCs switch from a proliferative, synthetic phenotype to a differentiated, contractile phenotype. This involves a decrease in synthetic organelles, such as ER and Golgi, as well as an increase in myofibrillar organization due to the up-regulation of contractile-apparatus associated proteins. Taken from Thyberg J, Hedin, U, Sjölund M, Palmberg K and Bottger BA. Regulation of Differentiated Proteins and Proliferation of Arterial Smooth Muscle Cells. *Arteriosclerosis*. 1990;10:966-990.

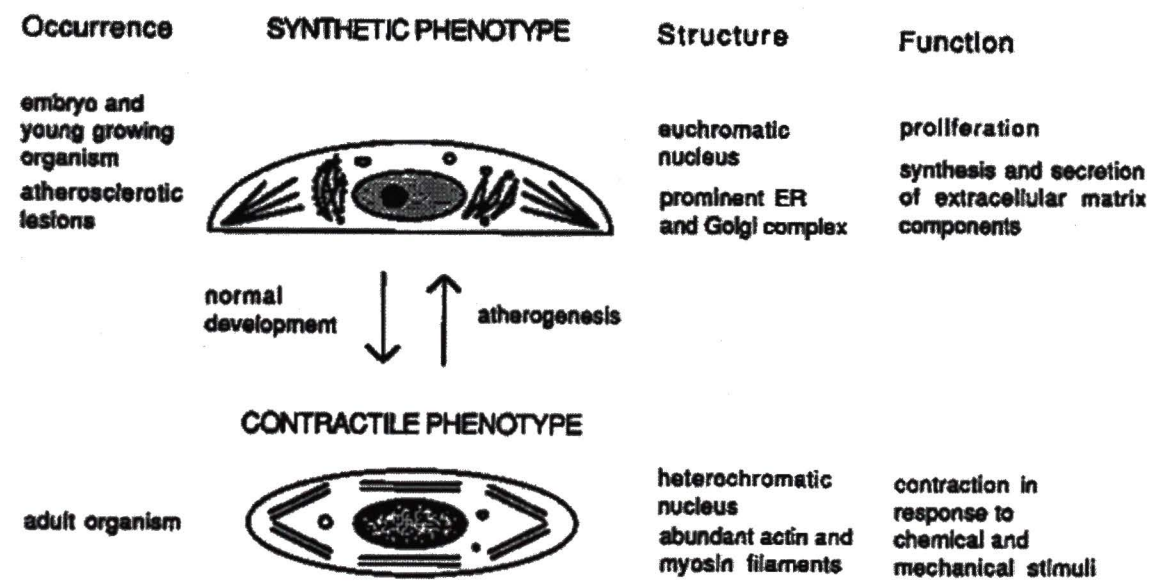

Occurrence
SYNTHETIC PHENOTYPE
Structure
Function
embryo and young growing organism
atherosclerotic lesions
euchromatic nucleus
prominent ER and Golgi complex
proliferation
synthesis and secretion of extracellular matrix components
normal development
atherogenesis
CONTRACTILE PHENOTYPE
adult organism
heterochromatic nucleus
abundant actin and myosin filaments
contraction in response to chemical and mechanical stimuli

Figure 4. Phenotypic modulation of smooth muscle cells during vascular disease

Differentiated SMCs retain the ability to modulate their phenotype in response to extracellular stimuli. The prevalence of these phenotypes is increased with several vascular disease states, including atherosclerosis/restenosis (proliferative phenotype) and hypertension (hypertrophied phenotype). Alterations in phenotype involve changes in SM-specific gene expression.

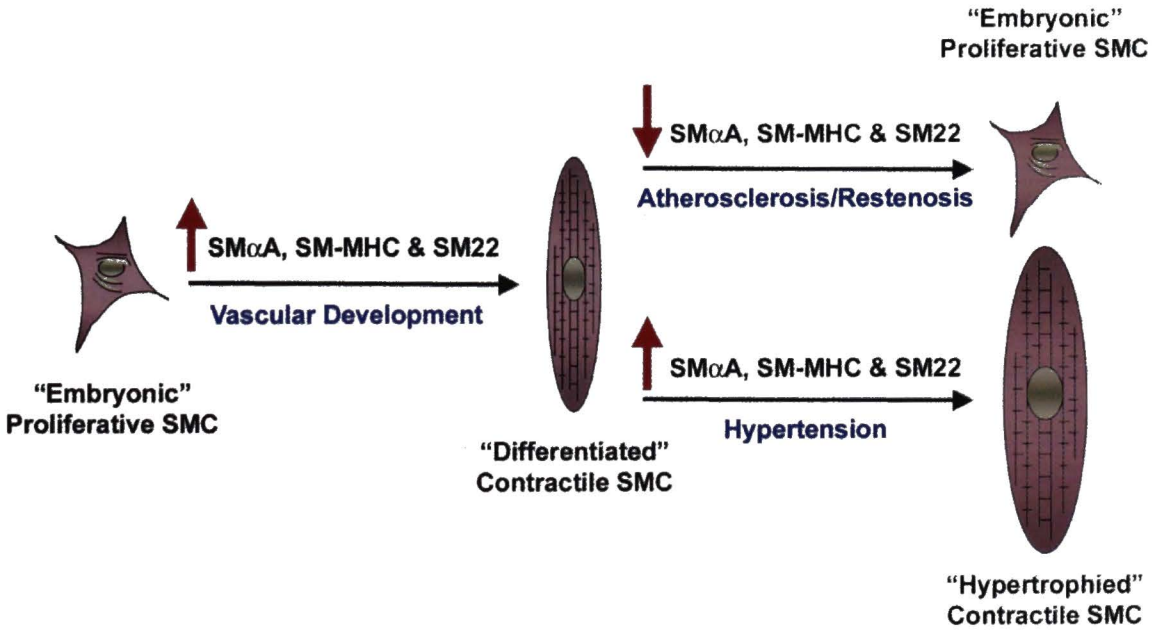

"Embryonic"
Proliferative SMC
SMαA, SM-MHC & SM22
Atherosclerosis/Restenosis
SMαA, SM-MHC & SM22
Vascular Development
"Embryonic"
Proliferative SMC
"Differentiated"
Contractile SMC
SMαA, SM-MHC & SM22
Hypertension
"Hypertrophied"
Contractile SMC

Figure 5. Contribution of smooth muscle cell proliferation to atherosclerotic lesion formation

Cytokines and growth factors secreted from the infiltrated inflammatory cells contribute to the activation of SMC phenotypic modulation. SMCs migrate from the media to the intima where they readily proliferate. This process is termed neointimal formation and contributes to the narrowing of the vascular lumen during disease progression. Taken from Glass GK, Witztum, JL. Atherosclerosis: The Road Ahead. *Cell.* 2001;104:503-516.

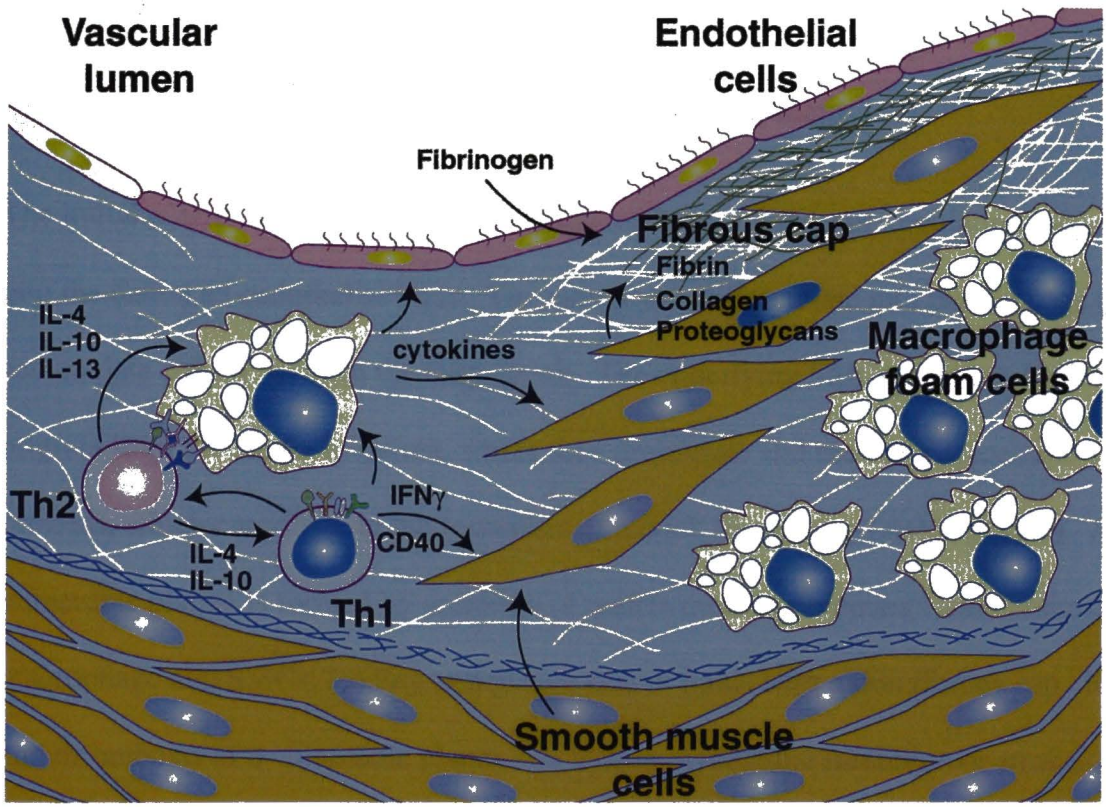

Vascular lumen
Endothelial cells
Fibrinogen
Fibrous cap
Fibrin
Collagen
Proteoglycans
IL-4
IL-10
IL-13
cytokines
Macrophage foam cells
Th2
IFNγ
CD40
IL-4
IL-10
Th1
Smooth muscle cells

Figure 6. Progression of atherosclerotic lesion development

The initial atherosclerotic lesion is characterized by infiltration of circulating monocytes into the damaged endothelial layer. The monocytes differentiate into macrophage and accumulate oxLDL to form the fatty streak. The underlying SMCs are stimulated to migrate from the media to intima, where they proliferate and secrete extracellular matrix proteins, which contribute to the formation of a thick fibrous cap (stable plaque). A vulnerable plaque is formed by secretion of matrix metalloproteinases and apoptosis of macrophage and SMCs, leading to a plaque rich in lipids with a thin fibrous cap. Rupture and thrombosis of a vulnerable plaque can lead to myocardial infarction or stroke. Taken from Libby P. Inflammation in Atherosclerosis. *Nature*. 2002;420:868-874.

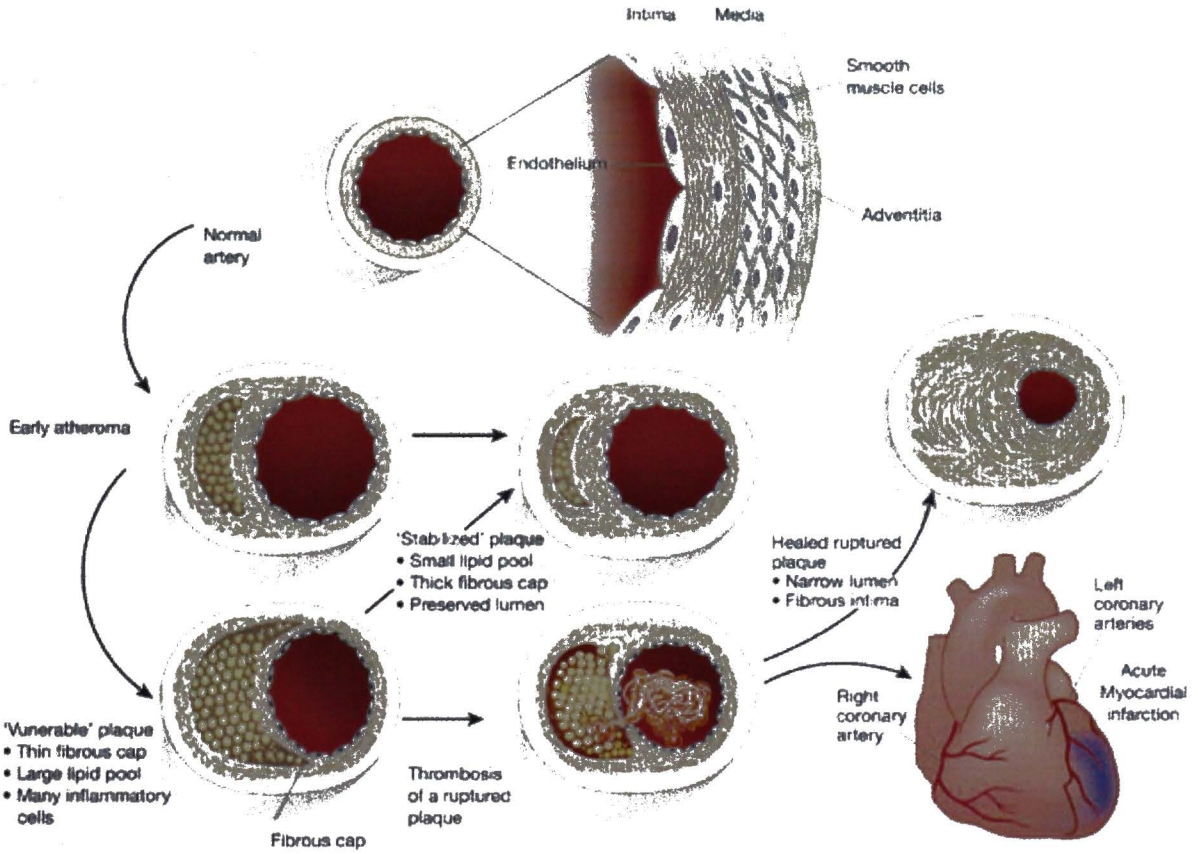

Intima
Media
Smooth muscle cells
Endothelium
Adventitia
Normal artery
Early atheroma
'Stabilized' plaque
• Small lipid pool
• Thick fibrous cap
• Preserved lumen
Healed ruptured plaque
• Narrow lumen
• Fibrous intima
'Vunerable' plaque
• Thin fibrous cap
• Large lipid pool
• Many inflammatory cells
Fibrous cap
Thrombosis of a ruptured plaque
Right coronary artery
Left coronary arteries
Acute Myocardial infarction

Table 1. Transcription Factors that regulate SMC phenotype

In the differentiated phenotype, SMC express a distinct set of contractile proteins that are required for contraction, including smooth muscle α-actin (SM α-actin), smooth muscle myosin heavy chain (SM-MHC) and SM22α. Expression of these genes is coordinately regulated by a number of specific transcription factors. Taken from Kumar, MS and Owens GK. Combinatorial Control of Smooth Muscle-Specific Gene Expression. *Arterioscler Thromb Vasc Biol.* 2003;23:737-747.

Transcription Factors Known or Likely to be Involved in Regulation of SMC Differentiation/Proliferation

Transcription Factor	Target Gene(s)
MADS Box Proteins	
SRF	↑ numerous SMC differentiation markers[4,6]
MEF2B	↑ SM MHC[101]
MEF2C	SMC specific targets unknown[102]
Homeodomain Proteins	
Phox1/Mhox	↑ SM α-actin[44]
Barx1b	↑ β-Tropomyosin[48]
Barx2b	SMC specific targets unknown[45]
Nkx3.1	↑ SM γ-actin[46]
Nkx3.2	↑ α_1 integrin, SM22α, and caldesmon[49]
Hox B7	↑ SM22α and calponin[103]
Hex	↑ SMemb/NMHC-B[104]
Gax (Mox2)	↑ p21; ↓ proliferation[98]
GATA family (GATA 4/5/6)	↑ numerous SMC differentiation markers[49,92,105]
	↑ p21; ↓ proliferation[95,106]
Myocardin	↑ numerous SMC differentiation markers[50,51]
Kruppel-like Zinc Finger Proteins	
Sp1/3	↑ ACLP[12]; ?↓ SM MHC and SM22α[95,107]
GKLF/KLF4	↓ SM22α and SM α-actin[30]
	↑ p21; ↓ proliferation[108,109]
BTEB2/KLF5	↑ SMemb/NMHC-B[110]; ?↑ SM22α[30]
Helix-Loop-Helix (HLH) Proteins	
USF	↑ osteopontin[111], SM α-actin[76], APEG-1[74]
CHF/HRT/gridlock	SMC specific targets unknown[112–114]
dHAND/HAND2	SMC specific targets unknown[115]
eHAND/HAND1	SMC specific targets unknown[116]
capsulin/epicardin	?↑ SM α-actin[117]
Id2/3	↓ p21; ↑ proliferation[99,118,119]
Twist	↓ SM22α[91]
Single-Stranded DNA Binding Proteins	
Purα, Purβ, and MSY1	?↓ SM α-actin[83,84]
Others	
Egr-1	↑ proliferation (numerous targets)[100]
TEF-1	?↑ SM α-actin[83,84]
SSRP1	↑ SM22α[47]
Mrf2α/β	↑ SM α-actin and SM22α; ↓ proliferation[28]
AP-1	↑ osteopontin[111]
YY1	↑ SM22α[37]
p53	↑ SM α-actin[120]
c-myb	↑ SM α-actin[121]
CRP1/2	SMC specific targets unknown[9,122]
NFATc1	↑ SM MHC[123]

Table 2. Evolutionarily conserved CArG box elements

Smooth muscle α-actin (SM α-actin), smooth muscle myosin heavy chain (SM-MHC) and SM22α each have multiple CArG box elements that are conserved among species. These elements have been demonstrated to be required for the proper tissue specific expression of these genes. Adapted from Miano, JM. Serum response factor: toggeling between disparate programs of gene expression. *J Mol Cell Cardiol.* 2003;35:577-593.

CArG Box Dependent SM-specific marker gene Regulatory Elements

SM-specific marker gene	*CArG box element*	*CArG designation*	*Reference*
SM α-actin	CCCTATATGG CCTTGTTTGG CCTAATTAGG	CArG B CArG A CArG Int	(95)
SM-MHC	CCTTTTTGGG CCTTTTATGG CCTTGTATGG	CArG B CArG A CArG Int	(100)
SM22α	CCATAAAAGG CCAAATATGG	CArG B CArG A	(73, 87)

Figure 7. Activation of the small GTPase RhoA

Multiple receptors activate RhoA, including G-protein coupled receptors (GPCRs) and growth factor receptors (such as the TGF-β1 receptors). This occurs through the up-regulation or activation of a RhoA-specific guanine exchange factor (GEF). GEFs catalyze GTP-exchange, which results in the activation of RhoA. GTP-bound RhoA can then bind to its downstream targets, including Rho kinase and PKN. GTPase activating proteins (GAPs) inactivate RhoA by increasing its intrinsic GTPase activity. Taken from Wettschureck N and Offermanns S. Rho/Rho-kinase mediated signaling in physiology and pathphysiology. *J Mol Med.* 2002;80:629-638.

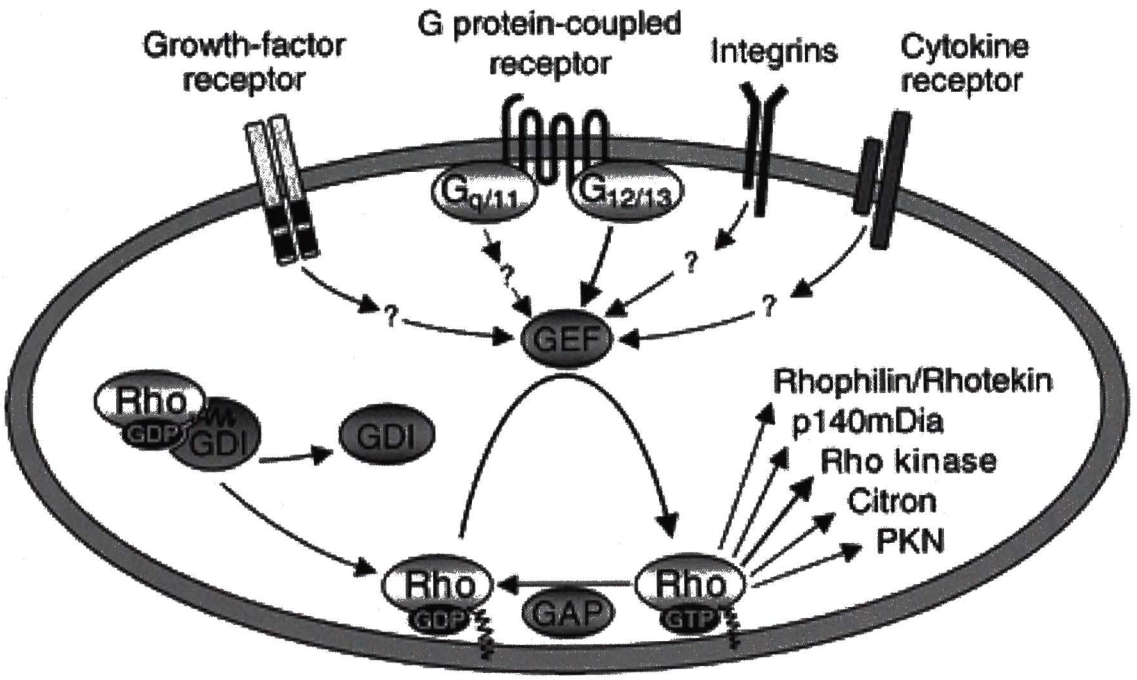

Growth-factor receptor
G protein-coupled receptor
Integrins
Cytokine receptor
$G_{q/11}$
$G_{12/13}$
?
?
?
?
GEF
Rho
GDP
GDI
GDI
Rhophilin/Rhotekin
p140mDia
Rho kinase
Citron
PKN
Rho
GDP
GAP
Rho
GTP

Table 3. Nomenclature of the PKN family of proteins

PKN was originally cloned in 1994 by Mukai *et al.* (118). In addition, several other labs also cloned PKN and related isoforms in multiple tissues .(93, 117, 126, 135, 140, 167, 194) Because of this, PKN family members have multiple different names. This table summarizes the different nomenclature and defines the names of these proteins, as will be used herein. Adapted from Mukai, H. The structure and function of PKN, a protein kinase having a catalytic domain homologous to that of PKC. *J Biochem (Tokyo).* 2003;133:17-27.

PKN Family Nomenclature

Species	cDNAs		
Human	PKN	PKNβ	
Human	PRK1		PRK2
Rat	PKN		
Rat	PAK-1		PAK-2
Frog	PKN		
Starfish			PRK2
Fly		PKN[a]	
Mukai *et al.* (2003)	**PKNα**	**PKNβ**	**PRK2/PRKγ**
Nomenclature used here	**PKN**	**PKNβ**	**PRK2**

[a]It is difficult to classify this clone into the corresponding isoform.

Figure 8. Comparison of the structure of PKN family members

Among the different PKN family members, there are several conserved regions within the regulatory and catalytic domains. These regions are important for the activation of PKN through interaction with other proteins. In the regulatory domain, the first antiparallel coiled-coil (ACC) region is important for binding to RhoA (residues 33-111 of PKN). In addition, the C2-like domain contains the autoinhibitory region. The catalytic domain contains both the activation loop phosphorylation site (threonine 774 for PKN) as well as the ATP binding domain (lysine 644 for PKN).

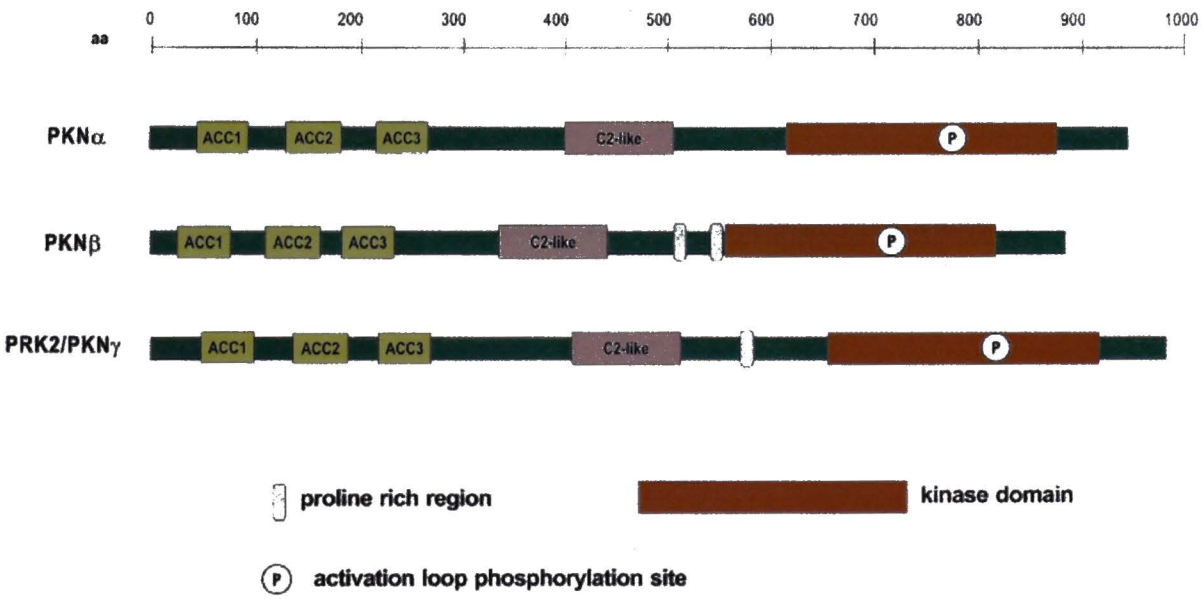
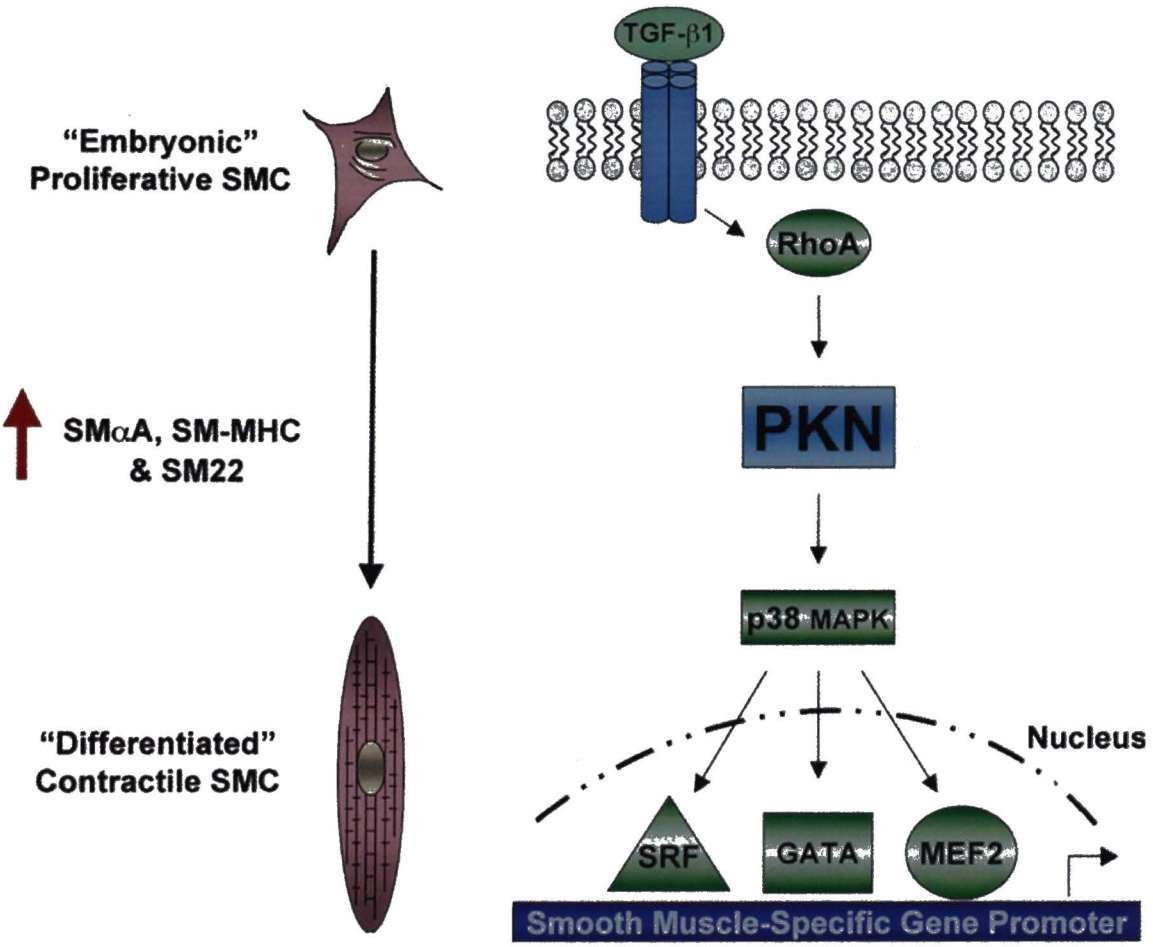

0
100
200
300
400
500
600
700
800
900
1000
aa
PKNα
ACC1
ACC2
ACC3
C2-like
P
PKNβ
ACC1
ACC2
ACC3
C2-like
P
PRK2/PKNγ
ACC1
ACC2
ACC3
C2-like
P
proline rich region
kinase domain
P
activation loop phosphorylation site

Figure 9. Schematic drawing of the proposed signaling pathway through which TGF-β1 stimulates SMC differentiation

TGF-β1 may regulate SMC differentiation through the activation of RhoA and PKN, a downstream target of RhoA. PKN may play a central role in mediating the effects of TGF-β1 on SM-specific marker gene expression through a p38 MAPK dependent mechanism.


"Embryonic"
Proliferative SMC
SMαA, SM-MHC
& SM22
"Differentiated"
Contractile SMC
TGF-β1
RhoA
PKN
p38 MAPK
Nucleus
SRF
GATA
MEF2
Smooth Muscle-Specific Gene Promoter

CHAPTER II

MATERIALS AND METHODS

Cell culture and reagents

Rat pulmonary arterial smooth muscle cells (PAC-1 cells) have been previously described (152). PAC-1 cells were cultured in media 199 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA) and Cellgro® gentamicin (Fisher, Hampton, NH) (unless otherwise specified). All experiments were performed on cells between pass 3 and pass 15. TGF-β1 was purchased from Sigma-Aldrich (St. Louis, MO). HA1077, Y-27632 and SB203580 were purchased from Calbiochem® (EMD Biosciences, San Diego, CA).

Plasmids

The smooth muscle α-actin promoter-reporter (SMP-2-luc, containing 767bp of DNA from –724 to +43) was provided by J. Cook (Ochsner Clinic Foundation, New Orleans, LA) (9). The smooth muscle myosin heavy chain promoter-reporter (SM-MHC1.2-luc, containing 1249bp of DNA from –1161 to +88) was provided by S. White (University of Vermont, Burlington, VT) (184). The SM22α promoter-reporter (p441 SM22α-luc, containing 482bp of DNA from -441 to +41) was a gift from M. Parmacek (University of Pennsylvania, Philadelphia, PA) (165). The 6 x GATA-luciferase enhancer-reporter ($p(G1)_6$-tk-luc) was provided by T. Yamagata (Joslin Diabetes Center,

Harvard Medical School, Cambridge, MA) (189). The 3 x MEF2-luciferase enhancer-reporter and the 4 x SRF-luciferase enhancer-reporter (4xSM22CArG-luc) were obtained from E. Olson (UT Southwestern Medical Center, Dallas, TX) (92, 181). Expression vectors encoding active PKN (PKN-AF3), which encodes amino acids 561-942 (catalytic domain), and kinase dead PKN (PKN-AF3(K644E)), which contains a mutation in the ATP-binding domain of PKN, were gifts from Y. Ono (Kobe University, Japan) (170, 193). Expression vectors encoding constitutively active MKK6 (MKK6(E)) and dominant negative p38α ($p38\alpha_{AF}$) were obtained from J. Han (Scripps Research Institute, La Jolla, CA) (46, 147).

Western blot analysis

Proteins were boiled at 95°C for 5 minutes prior to loading on a Tris-glycine SDS-PAGE gel (10% acrylamide, except for detection of PKN, in which 6% gels were used). Proteins were separated by electrophoresis and transferred to either nitrocellulose or PVDF membrane using an XCell *SureLock*™ Mini-Cell (Invitrogen, Carlsbad, CA) per manufacturer's settings. Following transfer, membranes were washed for five minutes in TBS containing 1% Tween-20 (TBST) then blocked in TBST plus 5% nonfat milk (1 hour at room temperature, except for detection of PKN, in which membranes were blocked over night at 4°C). After blocking, membranes were washed three times with TBST (5-10 minutes each wash) followed by the addition of primary antibody over night at 4°C (except for rabbit anti-PKN which was incubated for 3 hours at room temperature following over night blocking). Primary antibodies were diluted in TBST

plus 1% BSA at a concentration based on manufacturer's recommendations. Primary antibodies used for western blotting are listed within the methods section for each specific experiment. After incubation in primary antibody, membranes were washed three times with TBST (5-10 minutes each wash). Proteins were visualized using HRP-linked secondary antibodies and LumiGLO reagent (Cell Signaling Technology, Beverly, MA) per manufacturer's instructions. (For specific details on the western blotting of RhoA following immunoprecipitation see the section entitled *RhoA activity assay* below).

Immunocytochemistry

Actin re-organization was assessed using indirect immunocytochemistry. PAC-1 cells were cultured in 12-well tissue culture plates at a starting density of 1 x 10^4 cells/well on 1.5% gelatin-coated coverslips in media 199 supplemented with 10% FBS plus gentamicin. 24 hours post-plating, cells were pre-treated with HA1077 (20μM), Y-27632 (10μM) or vehicle control (water) for three hours prior to the addition of TGF-β1 (2.5ng/mL diluted in 4mM HCl + 0.1% BSA) or vehicle control (4mM HCl + 0.1% BSA) for 48 hours. As a positive control, cells were stimulated to differentiation by serum withdrawal (media 199 supplemented with 0.5% FBS) for 48 hours. Following treatment, cells were fixed in 1:1 acetone:methanol solution for 20 minutes at -20°C and were subsequently washed three times for 10 minutes each with ice cold Hank's Balanced Salt Solution (HBSS, HyClone®)(Fisher, Hampton, NH). The coverslips were blocked with 5% BSA in HBSS for one hour at room temperature, then incubated in primary antibody (monoclonal anti-α smooth muscle actin, Sigma-Aldrich, St. Louis,

MO) diluted in HBSS + 1% BSA overnight at 4°C with gentle shaking. Coverslips were washed three times for 10 minutes each with ice cold HBSS followed by incubation in secondary antibody (AlexaFluor® 594 goat anti-mouse IgG, Molecular Probes, Eugene, OR) for 1 hour at 37°C in HBSS + 1% BSA under dark conditions. Coverslips were then washed 4 times for 5 minutes each with HBSS + 0.1% Tween-20, followed by one 5 minute wash with HBSS. If DAPI was used, it was added at a final concentration of 300nM to each coverslip and was incubated at 37°C for 10 minutes in the dark. Coverslips were then washed 3 times for 10 minutes each with HBSS prior to mounting on glass slides. Fluorescence was visualized using an Olympus AX70 fluorescent microscope. The fields shown are representative data collected from three independent experiments.

Reverse transcription and semi quantitative PCR

PAC-1 cells were cultured in 12-well tissue culture plates at a starting density of 2.0×10^4 cells/mL in media 199 supplemented with 10% FBS plus gentamicin. 24 hours post-plating, cells were pre-treated with HA1077 (20μM), Y-27632 (10μM) or vehicle control (water) for 3 hours prior to stimulation with TGF-β1 (2.5ng/mL diluted in 4mM HCl + 0.1% BSA) or vehicle control (4mM HCl + 0.1% BSA) for 48 hours. As a positive control, cells were stimulated to differentiate by serum withdrawal (media 199 supplemented with 0.5% FBS) for 48 hours. Following treatment, media was removed and cells were washed once with phosphate buffered saline (PBS). Total RNA was isolated with TRIzol® reagent (Invitrogen, Carlsbad, CA) and resuspended in

DNase/RNase-free water (Ambion, Austin, TX). First-strand cDNA was synthesized from 2 μg of total RNA using random decamer or oligo-dT primers and SuperScript™III RT (Invitrogen, Carlsbad, CA) per manufacturers instructions. Semi-quantitative PCR was then performed using gene specific primers for SM-specific marker genes, GAPDH (Integrated DNA Technologies, Coralville, IA) or a commercially available 18S rRNA primer set (Ambion, Austin, TX). Sequences of gene specific primers are shown in Table 4. To analyze products within the linear range of PCR, optimal cycle number was determined. 20 cycles of PCR was used for SM α-actin, SM22α, 18S rRNA or GAPDH and 30 cycles of PCR was used for SM-MHC. The first cycle was preceded by 2 minutes of incubation at 95°C. Each cycle thereafter consisted of 30 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72°C. The last cycle was followed by 2 minutes of incubation at 72°C. Following the completion of PCR, products were separated on a 2% agarose gel. Gels were stained with ethidium bromide to visualize PCR products.

Cell proliferation assay

PAC-1 cells were cultured in 6-well tissue culture plates at a starting density of 4 x 10^4 cells per well in media 199 supplemented with 10% FBS plus gentamicin. 6 hours post-plating, cells were pre-treated with HA1077 (20μM), Y-27632 (10μM) or vehicle control (water) for three hours. Following pre-treatment, TGF-β1 (2.5ng/mL diluted in 4mM HCl + 0.1% BSA) or vehicle control (4mM HCl + 0.01% BSA) was added. At this time, total cell number from one group of treated cells was counted and this number was considered the 0 time point. Total cell number was then determined following 12, 24, 36

and 48 hours of TGF-β1 stimulation using a Coulter cell counter (Beckman Coulter, Miami, FL). As a control, cells were stimulated to differentiate by serum withdrawal (media 199 supplemented with 0.5% FBS) and were also counted at 12, 24, 36 and 48 hours. Each group was tested in triplicate for each time point per experiment. Three independent experiments were performed to confirm the results.

Transient transfection and promoter-reporter assays

For TGF-β1 experiments, PAC-1 cells were cultured in 12-well tissue culture plates at a starting density of 2.33 x 10^4 cells/mL in media 199 supplemented with 10% FBS plus gentamicin. 40 hours post-plating, cells (approximately 50% confluent) were transfected with 30 ng/well of the SM α-actin-, SM-MHC- or SM22α-luciferase promoter reporter plasmids using LipofectAMINE™ Plus reagent per manufacturer's instructions (Invitrogen, Carlsbad, CA). Three hours post transfection, transfection reagent was removed and cells were re-fed in medium 199 supplemented with 10% FBS. Transfected cells were then pre-treated with HA1077 (20μM), Y-27632 (10μM) or vehicle control (water) for three hours prior to the addition of TGF-β1 (2.5ng/mL diluted in 4mM HCl + 0.1% BSA) or vehicle control (4mM HCl + 0.1% BSA). 48 hours later, the cells were lysed with Reporter Lysis Buffer (Promega, Madison, WI) and luciferase activity was determined by luminometry (Turner Designs Luminometer Model 20, Turner Designs, Sunnyvale, CA) using a commercially available substrate kit (Promega, Madison, WI). Each data set was independently replicated a minimum of three times with each experimental group tested in triplicate. Results are expressed as the mean ±

SEM. Data was analyzed with GraphPad Prism software (version 4.0, GraphPad Software Inc., San Diego, CA) using one-way ANOVA with Bonferroni's post-hoc test for inter-group comparisons.

For PKN experiments, PAC-1 cells were cultured in 12-well tissue culture plates at a starting density of 2.33 x 10^4 cells/mL in media 199 supplemented with 10% FBS plus gentamicin. 48 hours post-plating, cells (approximately 70% confluent) were co-transfected with empty vector control (pSG5 or pcDNA3.1$^+$), PKN-AF3, PKN-AF3(K644E), MKK6(E) or p38α_{AF} plus one reporter (SM α-actin-, SM-MHC- or SM22α- luciferase promoter-reporter or 4 x SRF-, 6 x GATA- or 3 x MEF2-luciferase enhancer-reporter) using LipofectAMINE™ reagent per manufacturer's instructions (Invitrogen, Carlsbad, CA). Table 5 lists the amount of each plasmid used per well for each experiment. Total plasmid DNA/well was maintained constant by inclusion of empty vector plasmid. 10-12 hours post transfection, transfection reagent was removed and cells were re-fed in medium 199 supplemented with 10% FBS. 24 hours post-re-feeding, the cells were lysed with Reporter Lysis Buffer (Promega, Madison, WI) and luciferase activity was determined by luminometry (Turner Designs Luminometer Model 20, Turner Designs, Sunnyvale, CA) using a commercially available substrate kit (Promega, Madison, WI). Each data set was independently replicated a minimum of three times with each experimental group tested in triplicate. Results are expressed as the mean ± SEM. Data was analyzed with GraphPad Prism software (version 4.0, GraphPad Software Inc.) using one-way ANOVA with Bonferroni's post-hoc test for inter-group comparisons.

RhoA activity assay

PAC-1 cells were cultured in 100mm tissue culture dishes at a starting density of 2.8 x 10^5 cells/dish in media 199 supplemented with 10% FBS plus gentamicin. 48 hours post plating, cells (approximately 80% confluent) were treated with vehicle control (4mM HCl + 0.1% BSA) or treated with 2.5ng/mL TGF-β1 (diluted in 4mM HCl + 0.1% BSA) for 2, 5, 10, 30 or 60 minutes. Following treatment, RhoA activity was determined using the EZ-Detect™ Rho activity kit (Pierce Biotechnology, Rockford, IL) per manufacturer's instructions. This system uses a GST-Rhotekin Rho-binding domain (RBD) fusion protein, which selectively binds and pulls down only GTP-bound (active) RhoA. First, the media was removed and cells were washed once with ice-cold PBS. The cells were lysed with 0.5 mL of Lysis/Binding/Wash Buffer (25mM Tris-HCl pH7.5, 150mM NaCl, 5 mM $MgCl_2$, 1%NP-40, 1mM DTT and 5% glycerol) (Pierce Biotechnology, Rockford, IL) and collected by scraping. Lysates were then centrifuged for 15 minutes at 16,000 x g at 4°C. Supernatants were transferred to a sterile 1.5 mL microfuge tube. Active RhoA was immunoprecipitated for 1 hour at 4°C using GST-Rhotekin-RBD (Pierce Biotechnology, Rockford, IL). Active RhoA was released from the spin column by the addition of 50µl of 2 x SDS sample buffer (125mM Tris-HCl pH 6.8, 2 % glycerol, 4% SDS (w/v) and 0.05% bromphenol blue) supplemented with 2.5µl of β-mercaptoethanol followed by boiling the sample at 95°C for 5 minutes. Active RhoA was collected by centrifugation at 7,200 x g for 2 minutes. The amount of active RhoA was then analyzed by western blotting using the XCell *SureLock*™ Mini-Cell (Invitrogen, Carlsbad, CA). Proteins were transferred to nitrocellulose and blocked for 2

hours at room temperature in TBST plus 3% BSA. Mouse anti-Rho was diluted in TBST plus 3% BSA plus 0.1% NaN_3 and incubated with the membrane over night at 4°C with gentle rocking. Protein was detected using an HRP conjugated secondary antibody and LumiGLO reagent (Cell Signaling Technology, Beverly, MA) per manufacturer's instructions.

Assessment of PKN phosphorylation

PAC-1 cells were cultured in 100mm tissue culture dishes at a starting density of 2.8 x 10^5 cells per dish in media 199 supplemented with 10% FBS plus gentamicin. 48 hours post-plating, cells (approximately 80% confluent) were treated with vehicle control (4mM HCl + 0.1% BSA) or TGF-β1 (2.5ng/mL diluted in 4mM HCl + 0.1% BSA) for 2, 5, 10, 30 or 60 minutes prior to lysis with 1 x SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS (w/v), 10% glycerol, 50mM DTT, 0.1% (w/v) bromphenol blue). Cell lysate was collected by scraping and transferred to a sterile 1.5 mL microfuge tube. Genomic DNA was sheared by passing the lysate through a 22-gauge syringe (5 times). The lysate was centrifuged at 12,000 x g and the supernatant was transferred to a sterile 1.5 mL microfuge tube. To detect the phosphorylation status of PKN (indicative of its activity), lysates were subjected to western blot analysis using an primary antibody that recognizes the phosphorylated (active) form of PKN and PRK2 (rabbit anti-phospho-PKN(Thr778)/PRK2(Thr816) (Cell Signaling Technology, Beverly, MA). In addition, total PKN was detected using an antibody specific for PKN (rabbit anti-PKN) (Santa Cruz Biotechnology, Santa Cruz, CA) to detect any changes in PKN expression with

treatment. Results were independently replicated a minimum of three times.

Assessment of MAP kinase phosphorylation

PAC-1 cells were cultured in 100mm tissue culture dishes at a starting density of 2.8 x 10^5 cells per dish in media 199 supplemented with 10% FBS plus gentamicin. 48 hours post-plating, cells (approximately 80% confluent) were treated with vehicle control (4mM HCl + 0.1% BSA) or TGF-β1 (2.5ng/mL diluted in 4mM HCl + 0.1% BSA) for 2, 5, 10, 30 or 60 minutes prior to lysis with 1 x SDS sample buffer. Cell lysate was collected by scraping and transferred to a sterile 1.5 mL microfuge tube. Genomic DNA was sheared by passing the lysate through a 22-gauge syringe (5 times). The lysate was centrifuged at 12,000 x g and the supernatant was transferred to a sterile 1.5 mL microfuge tube. To detect the phosphorylation status of ERK1/2, JNK1/2, p38 MAP kinase and MKK3/6 (indicative of their activity), lysates were subjected to western blot analysis using primary antibodies that recognize the phosphorylated (active) forms of these proteins: mouse anti-phospho-p44/p42(Thr202/Tyr204) (p-ERK1/2), rabbit anti-phospho-SAPK/JNK(Thr183/Tyr185) (p-JNK1/2), rabbit anti-phospho-p38 MAP kinase(Thr180/Tyr182) or rabbit anti-phospho-MKK3/6 (Ser189/207) (p-MKK3/6) (Cell Signaling Technology, Beverly, MA). In addition, the total amount of these proteins was analyzed using rabbit antibodies specific for ERK 1/2 (anti-p44/p42), JNK1/2 (anti-SAPK/JNK) and p38 MAP kinase (anti-p38 MAPK) (Cell Signaling Technology, Beverly, MA) or MKK3/6 (Santa Cruz Biotechnology, Santa Cruz, CA) to detect any changes in the expression of these following treatment. Results were independently

replicated a minimum of three times.

p38 MAP kinase assay

The kinase activity of p38 MAPK was examined using a non-radioactive p38 MAP Kinase Assay Kit (Cell Signaling Technology, Beverly, MA) per the manufacturer's instructions. First, PAC-1 cells were cultured in 100mm tissue culture dishes seeded at a starting density of 3.0 x 10^5 cells per dish in media 199 supplemented with 10% FBS plus gentamicin. 48 hours post plating, cells were treated with vehicle control (4mM HCl + 0.1% BSA) or TGF-β1 (2.5ng/mL diluted in 4mM HCl + 0.1% BSA) for 2, 5, 10, 30 or 60 minutes prior to lysis with 1 X Cell Lysis Buffer (20mM Tris pH7.5, 150mM NaCl, 1 mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM sodium pyrophosphate, 1mM β-glycerolphosphate, 1mM Na_3VO_4, 1μg/mL Leupeptin) (Cell Signaling Technology, Beverly, MA). Whole cell lysate was sonicated 4 times for 5 seconds each. Lysate was then centrifuged and the supernatant was transferred to a sterile 1.5 mL microfuge tube. From each sample, active p38 MAP kinase was immunoprecipitated from 200μL of cleared whole cell lysate using phospho-specific antibody conjugated to agarose beads (immobilized mouse anti-phospho-p38 MAP kinase (Thr180/Tyr182) monoclonal antibody) (Cell Signaling Technology, Beverly, MA). Immunoprecipitation was carried out overnight at 4°C with gentle rocking. The beads were then washed two times with 1 x Cell Lysis Buffer and 2 times with 1 x Kinase Buffer (25mM Tris pH 7.5, 5mM β-glycerolphosphate, 2mM DTT, 0.1mM Na_3VO_4, 10mM $MgCl_2$) (Cell Signaling Technology, Beverly, MA) and were resuspended in a

final volume of 50 μL of 1 x Kinase Buffer. The immunoprecipitated p38 MAP kinase (bound to beads) was then used in an *in vitro* kinase assay by adding 200μM ATP and 2μg of ATF-2 fusion protein as the substrate (Cell Signaling Technology, Beverly, MA). The kinase assay was performed for 30 minutes at 30°C. In addition, duplicate samples were tested for the specificity of kinase activity by the inclusion of SB203580 (a p38 MAP kinase inhibitor) (Calbiochem®, EMD Biosciences, San Diego, CA) during the kinase assay. The kinase reaction was terminated by the addition of 3 x SDS sample buffer. Samples were boiled at 95°C for 5 minutes prior to a 2 minute centrifugation. Samples were analyzed by western blotting as described above. The phosphorylation of ATF-2 was detected with an antibody specific for phospho-ATF2 (rabbit anti-phospho-ATF-2(Thr71)) (Cell Signaling Technology, Beverly, MA). The kinase assay was independently replicated a minimum of three time to confirm results.

Table 4. Sequence of primers used for PCR amplification

Primers were designed using Integrated DNA Technologies PrimerQuest^{SM} (Steve Rozen, Helen J. Skaletsky (1996,1997,1998) Primer3. Code available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html.) Primers were chosen to have a length of 20-24 basepairs (bp), a T_m of 60°C, %GC content of 50% or less and minimal secondary structure. Once designed, primers were generated by Integrated DNA Technologies and resuspended in sterile DNase/RNase free water at a concentration of 10μM.

Gene amplified	Primers	PCR product size
SM α-actin		
Forward	5'ACTGGGACGACATGGAAAAG3'	168 bp
Reverse	5'CATACATGGCAGGGACATTG3'	
SM-MHC		
Forward	5'GAAAGCCAAGAGTCTGGAGG3'	190 bp
Reverse	5'CACTCATGGCCTCCATGTTG3'	
SM22α		
Forward	5'TGAGCAAGTTGGTGAACAGC3'	221 bp
Reverse	5'ACTGCCCAAAGCCATTACAG3'	
GAPDH		
Forward	5'GTGTGAACGGATTTGGCCGTATGG3'	746 bp
Reverse	5'TCATACTTGGCAGGTTTCTCCAGG3'	

Table 5. Amount of each plasmid used for transient transfections

Transient transfections were performed using LipofectAMINE™ Plus or standard LipofectAMINE™ reagent (see materials and methods). The amount of PKN-AF3 used was determined by dose-response (see results) and was optimized for each promoter or enhancer. The same amount of PKN-AF3(K644E) was used to show loss of function.

Plasmid	**Amount used (ng/well)**
4 x SRF-luc	100ng
6 x GATA-luc	100ng
3 x MEF2-luc	100ng
SM α-actin-luc	30ng
SM-MHC-luc	30ng
SM22α-luc	30ng
PKN-AF3	
4 x SRF-luc	1ng
6 x GATA-luc	1ng
3 x MEF2-luc	10ng
SMα-actin-luc	10ng
SM-MHC-luc	10ng
SM22α-luc	10ng
PKN-AF3(K644E)	
4 x SRF-luc	1ng
6 x GATA-luc	1ng
3 x MEF2-luc	10ng
SMα-actin-luc	10ng
SM-MHC-luc	10ng
SM22α-luc	10ng
MKK6(E)	25ng
$p38\alpha_{AF}$	100ng
pSG5 or pcDNA3.1^{+}	1 to 300ng

CHAPTER III

RESULTS

Preface

The following studies were completed using the rat pulmonary arterial smooth muscle cell line (PAC-1) (152). PAC-1 cells can be maintained in the proliferative phenotype when cultured in media supplemented with 10% fetal bovine serum (FBS), thus providing a model to study whether a specific stimulus, such as TGF-β1, induces SMC differentiation. Moreover, these cells are known to differentiate in response to serum withdrawal, which serves as a positive control for the characteristics of the differentiated phenotype.

As previously discussed, it is well documented that TGF-β1 is capable of inducing differentiation of SMCs both *in vitro* and *in vivo* (13, 42). Although this has been tested in multiple cell lines such as rat and porcine aortic SMC (41, 63) and multipotential cell lines such as 10T1/2, neural crest stem cells and Monc-1 cells (24, 25, 57, 145, 162), no studies to date have examined the effects of TGF-β1 on the rat pulmonary arterial smooth muscle cell line, PAC-1. Moreover, because TGF-β1 has been reported to elicit different effects depending upon cell type and culture conditions, we first tested whether TGF-β1could stimulate differentiation of PAC-1 cells in culture. The ability of TGF-β1 to induce differentiation of PAC-1 cells was assessed using the following four criteria, i.) SM α-actin re-organization (stress fiber formation), ii.)

expression SM-specific marker genes (SM α-actin, SM-MHC and SM22α), iii.) relative rate of proliferation and iv.) promoter activity of the SM-specific marker genes. Once it was established that TGF-β1 does indeed promote differentiation of PAC-1 cells, the intracellular signaling mechanisms through which TGF-β1-mediated its effects were examined. Specifically, we were interested in examining whether RhoA and its downstream target, PKN, contributed to TGF-β1-mediated SMC differentiation and, if so, what signal transduction pathway(s) downstream of PKN were required for its function.

TGF-β1 stimulates actin re-organization in PAC-1 cells

To demonstrate that TGF-β1 induces differentiation of PAC-1 cells, morphological changes in the actin organization of proliferating cells cultured in the absence or presence of TGF-β1 for 48 hours was examined. Actin morphology was visualized by immunofluorescence using an antibody specific for the smooth muscle isoform of actin (SM α-actin). As shown in Figure 10A, PAC-1 cells cultured in media containing 10% FBS without TGF-β1 (10% serum) were "fibroblast-like" in shape indicative of the proliferative phenotype. Moreover, the actin present in these cells was mostly found around the periphery of the cell, not organized into contractile stress fibers. In contrast, PAC-1 cells treated with TGF-β1 displayed increased stress fiber formation (actin re-organization) compared to untreated proliferating cells (Figure 10B compared to Figure 10A). Moreover, these cells were "spindle-like" in shape, indicative of the differentiated phenotype. Similarly, PAC-1 cells that had been stimulated to differentiate

by serum withdrawal (0.5% serum) also displayed actin re-organization into stress fibers, much like the TGF-β1 treated group (Figure 10C). This demonstrates that TGF-β1 can induce actin re-organization of PAC-1 cells, indicative of their differentiation.

TGF-β1 increases expression of SMC-specific marker genes in PAC-1 cells

To further demonstrate that TGF-β1 promotes differentiation of PAC-1 cells, changes in mRNA expression of several SM-specific marker genes in response to TGF-β1 was examined. Actively proliferating PAC-1 cells were cultured in the absence or presence of TGF-β1 or were stimulated to differentiate by serum withdrawal (0.5% serum) as a positive control for differentiation. The PCR primers used for these experiments were designed to specifically amplify the smooth muscle specific isoforms of actin (SM α-actin) and myosin (SM-MHC) as well as the smooth muscle specific marker, SM22α. The relative mRNA expression for these genes was measured using semi-quantitative RT-PCR. As shown in Figure 11, untreated proliferating PAC-1 cells expressed only a very small amount of the three definitive SMC-specific marker genes, SM α-actin, SM-MHC and SM22α, consistent with the proliferative phenotype. In contrast, PAC-1 cells treated with TGF-β1 displayed increased expression of all three SMC-specific marker genes in a manner similar to PAC-1 cells that were stimulated to differentiate by serum withdrawal (Figure 11). Lastly, the expression of 18S rRNA (a housekeeping gene) was unchanged, regardless of culture conditions. This demonstrates that the increased expression of the SM-specific marker genes in response to treatment with TGF-β1 or serum withdrawal is not due to a global change in gene expression, nor is

it due to unequal amounts of total starting template. This further establishes that TGF-β1 promotes differentiation of PAC-1 cells.

TGF-β1 attenuates the proliferation of PAC-1 cells

Differentiation of SMC is associated with cell cycle arrest and a subsequent reduction in proliferation. Therefore, the ability of TGF-β1 to inhibit serum-induced proliferation of PAC-1 cells was tested. To do this, PAC-1 cells were plated at an equal starting density in media containing 10% FBS and allowed to proliferate for 12, 24, 36 or 48 hours in the absence or presence of TGF-β1. In addition, PAC-1 cells stimulated to differentiate by serum withdrawal (0.5% serum) were used as a positive control for the differentiated phenotype. Following treatment, total cell number per group was counted for each time point. As seen in Figure 12, cells that were cultured in 10% serum in the absence of TGF-β1 proliferated rapidly, in a relatively linear manner. In contrast, PAC-1 cells cultured in 10% serum in the presence of TGF-β1 displayed a reduced rate of proliferation compared to untreated cells. Interestingly, cells that were stimulated to differentiate by serum withdrawal showed very little proliferation over the 48 hour time period (Figure 12). This argues that TGF-β1 does attenuate the relative rate of proliferation of PAC-1 cells in response to serum. However, TGF-β1 alone is clearly not sufficient to overcome all of the mitogenic signals present in serum.

TGF-β1 activates the promoters of the SM-specific marker genes

The promoters that control the expression of the SM-specific genes SM α-actin, SM-MHC, and SM22α have been isolated and well characterized. Data shown in Figure 11 demonstrates that TGF-β1 increases the expression of mRNA for these SM-specific genes. Next, the ability of TGF-β1 to stimulate transcription driven by the promoters that control the expression of these genes was tested. This was accomplished by monitoring the expression of a luciferase reporter under the transcriptional control of truncated promoters for either SM α-actin, SM-MHC or SM22α (herein referred to as the SM-specific promoter-reporters). Although these promoters are truncated, they contain the known regulatory regions that have been shown to govern the expression of these genes, including the critical CArG box elements (15, 122, 165, 197). PAC-1 cells were transiently transfected with one of the three SM-specific promoter-reporters. Following transfection, cells were cultured in the absence or presence of TGF-β1 for 48 hours prior to determination of luciferase expression as a measure for promoter activity. As shown in Figure 13, TGF-β1 treated PAC-1 cells displayed between a 3 to 5-fold increase in the activity of all three SM-specific promoters compared to untreated control cells. These data demonstrate that TGF-β1-mediated up-regulation of SM-specific marker gene expression is mediated through activation of the transcription of these genes.

TGF-β1-induced actin re-organization depends on RhoA signaling

The mechanism(s) through which TGF-β1 elicits its effects on SMC differentiation have not been well characterized. Specifically, the signaling pathways

that are required for TGF-β1 function have not been well examined. Several recent studies have demonstrated that TGF-β1-mediated up-regulation of SM α-actin expression in epithelial cells undergoing epithelial to mesechymal transition (EMT) requires the activity of the small GTPase RhoA (12, 104). It has also been well documented that RhoA activates stress fiber formation in multiple cell types including SMCs (59, 91, 96). However, TGF-β1 has not been demonstrated to require the activity of RhoA or its downstream signal transduction targets in SMCs. To examine whether TGF-β1-mediated changes in actin morphology of PAC-1 cells depend on signaling through the small GTPase RhoA, two chemical inhibitors, HA1077 and Y-27632, were utilized. Both of these inhibitors have been reported to inhibit the function of several downstream targets of RhoA, including Rho kinase (ROCK) and PKN/PRK2 (4, 27, 65). These inhibitors compete with ATP for binding to the ATP binding site within the catalytic domain of these enzymes, thereby blocking their kinase activity (65). Thus, the ability of TGF-β1 to induce actin re-organization of PAC-1 cells in the presence of either of these inhibitors was examined. Pre-treatment of PAC-1 cells with either HA1077 or Y-27632 prior to stimulation with TGF-β1 attenuated TGF-β1-mediated actin re-organization (Figure 14). Moreover, whereas the TGF-β1 treated cells displayed the characteristic "spindle-like" shape indicative of the differentiated phenotype, cells that had been pre-treated with either HA1077 or Y-27632 were more similar in shape to the control untreated cells, displaying the characteristic "fibroblast-like" phenotype (Figure 14). These data demonstrate that the downstream targets of RhoA are required for TGF-β1-induced changes in actin re-organization in PAC-1 cells.

TGF-β1-induced up-regulation of SM-specific gene expression depends on RhoA signaling

To further test whether the downstream kinases activated by RhoA play a role in TGF-β1-mediated differentiation of PAC-1 smooth muscle cells, the effect of HA1077 and Y-27632 on TGF-β1-mediated up-regulation of SM-specific marker gene expression was examined. Again, PAC-1 cells were pre-treated with either HA1077 or Y-27632 prior to stimulation with TGF-β1. Following stimulation, the relative expression of SM α-actin, SM-MHC and SM22α was determined by semi-quantitative RT-PCR. As previously demonstrated, the expression of all three of these SM-specific marker genes was increased in PAC-1 cells treated with TGF-β1. However, pre-treatment of cells with either HA1077 or Y-27632 attenuated the increase in expression of these genes caused by TGF-β1 treatment (Figure 15). This argues that the kinases downstream of RhoA are required for TGF-β1-mediated up-regulation of SM-specific marker gene expression.

RhoA signaling is required for TGF-β1-induced inhibition of PAC-1 proliferation

Next, the requirement of RhoA signaling for TGF-β1-mediated inhibition of serum-stimulated proliferation was tested. PAC-1 cells were pre-treated with HA1077 and Y-27632 prior to stimulation with TGF-β1 for 0, 12, 24, 36 or 48 hours. At each time point, total cell number per treatment group was analyzed. Again, TGF-β1 treatment of PAC-1 cells resulted in a decrease in the rate of proliferation compared to control, untreated cells. However, this effect could be almost completely blocked by pretreatment of the cells with HA1077 and Y-27632 prior to TGF-β1 stimulation (Figure

16). Thus, the activities of the kinases downstream of RhoA are required for TGF-β1-mediated effects on PAC-1 cell proliferation.

RhoA signaling is required for TGF-β1-mediated activation of the SM-specific promoters

Lastly, the effects of HA1077 and Y-27632 on TGF-β1-mediated activation of the SM-specific promoters were tested. Again, PAC-1 cells were transiently transfected with the SM α-actin, SM-MHC or SM22α promoter-reporter plasmid. Following transfection, the cells were pre-treated with either HA1077 or Y-27632 prior to stimulation with TGF-β1. 48 hours after the addition of TGF-β1, the activity of these promoters was determined based on the amount of luciferase expressed. As shown in Figure 17, TGF-β1 stimulation of transfected PAC-1 cells in the absence of HA1077 or Y-27632 resulted in an increase in the activity of all three SM-specific promoters, as previously demonstrated. However, pre-treatment of the cells with either inhibitor significantly reduced the ability to TGF-β1 to increase the activity of these promoters. This demonstrates that TGF-β1-mediated effects on the transcriptional activation of the SM-specific marker gene promoters are dependent upon the downstream targets of RhoA.

TGF-β1 activates the small GTPase RhoA

When taken together, the preceding data strongly argue that: i.) TGF-β1 stimulates differentiation of the PAC-1 smooth muscle cell line and ii.) TGF-β1-mediated effects on SMC differentiation require the activity of one or more of the kinases

16). Thus, the activities of the kinases downstream of RhoA are required for TGF-β1-mediated effects on PAC-1 cell proliferation.

RhoA signaling is required for TGF-β1-mediated activation of the SM-specific promoters

Lastly, the effects of HA1077 and Y-27632 on TGF-β1-mediated activation of the SM-specific promoters were tested. Again, PAC-1 cells were transiently transfected with the SM α-actin, SM-MHC or SM22α promoter-reporter plasmid. Following transfection, the cells were pre-treated with either HA1077 or Y-27632 prior to stimulation with TGF-β1. 48 hours after the addition of TGF-β1, the activity of these promoters was determined based on the amount of luciferase expressed. As shown in Figure 17, TGF-β1 stimulation of transfected PAC-1 cells in the absence of HA1077 or Y-27632 resulted in an increase in the activity of all three SM-specific promoters, as previously demonstrated. However, pre-treatment of the cells with either inhibitor significantly reduced the ability to TGF-β1 to increase the activity of these promoters. This demonstrates that TGF-β1-mediated effects on the transcriptional activation of the SM-specific marker gene promoters are dependent upon the downstream targets of RhoA.

TGF-β1 activates the small GTPase RhoA

When taken together, the preceding data strongly argue that: i.) TGF-β1 stimulates differentiation of the PAC-1 smooth muscle cell line and ii.) TGF-β1-mediated effects on SMC differentiation require the activity of one or more of the kinases

downstream of RhoA. As discussed in the introduction, RhoA requires GTP exchange in order to bind and activate its downstream targets (30, 157). Accordingly, the amount of active, GTP-bound RhoA would be expected to increase following TGF-β1 stimulation if these downstream kinases are required for the changes in SMC phenotype elicited by TGF-β1. In order to demonstrate that RhoA is activated in response to TGF-β1 stimulation, PAC-1 cells were treated with TGF-β1. Changes in GTP-bound RhoA were monitored following 2, 5, 10, 30 and 60 minutes of TGF-β1 stimulation, as the GTP exchange on RhoA occurs very rapidly. Following stimulation, active, GTP-bound RhoA was isolated from whole cell lysate using a GST-rhotekin Rho binding domain (RBD) fusion protein, which binds to RhoA in a GTP-dependent manner. The relative amount of active RhoA at each time point was then analyzed by western blot. As shown in Figure 18, RhoA activity was increased following 2 minutes of TGF-β1 stimulation compared to control untreated cells. RhoA activity remained elevated through 5 minutes of TGF-β1 stimulation, and then quickly declined. This demonstrates that TGF-β1 stimulation of PAC-1 cells does involve the activation of RhoA. Moreover, this activation occurs in a very rapid and transient manner.

TGF-β1 activates the downstream RhoA kinases PKN and PRK2

PKN and PRK2 have not been extensively studied in general, much less with respect to their effects on SMC phenotype. However, several functions attributed to these downstream RhoA kinases make them an interesting potential target for TGF-β1 signaling. First, they both have been shown to regulate SRF-dependent transcription (43,

114). Secondly, PKN can directly bind and interact with cytoskeletal proteins such as α-actinin (119). Lastly, the mouse homologue of PKN maps to the same chromosomal region as the mouse mutation myodystrophy (myd), which causes skeletal muscle dystrophy in mice with this mutation (10). Taken together, these previous studies argue that PKN and PRK2 may have an important role in maintaining normal muscle phenotype.

To examine whether TGF-β1-induced differentiation of PAC-1 cells involves the activation of PKN and PRK2, phosphorylation of the activation loop of these proteins was monitored following TGF-β1 stimulation. Again, because phosphorylation of these sites occurs fairly rapidly, PAC-1 cells were treated with TGF-β1 for 2, 5, 10, 30 or 60 minutes. Following treatment, PKN activity was measured by Western blot analysis using an antibody that recognizes the active form of PKN (phospho-Thr778) and its closely related family member, PRK2 (phospho-Thr816). Both proteins were phosphorylated after 5 minutes of stimulation and remained elevated through 60 minutes compared to control untreated cells (Figure 19). Moreover, the total quantity of PKN was not altered by TGF-β1 treatment, demonstrating a true increase in phosphorylation status. These results demonstrate that TGF-β1 stimulation increases the amount of phosphorylated (active) PKN and PRK2 in PAC-1 cells.

PKN regulates transcriptional activation of smooth muscle-specific genes

Data from Figures 18 and 19 demonstrate that TGF-β1 is capable of activating both RhoA and its downstream targets PKN and PRK2. Moreover, data from Figures 14,

15, 16 and 17 demonstrate that Rho kinase and/or PKN/PRK2 are important for TGF-β1-meditated effects on SMC phenotype. However, since these inhibitors cannot distinguish between Rho kinase and PKN/PRK2, they provide no direct evidence that PKN, *independently* of other signaling molecules, plays a role in mediating any of the resulting effects of TGF-β1 on SMC differentiation. To determine whether PKN alone was sufficient to stimulate transcription of SMC-specific marker genes, PAC-1 cells were co-transfected with one of three SMC-specific promoter-reporter constructs and increasing amounts of an expression vector encoding a constitutively active form of PKN (PKN-AF3, which lacks the regulatory domain). Active PKN was able to increase the activity of the SM α-actin, SM-MHC and SM22α promoters in a dose dependent manner, compared to an empty vector control plasmid (Figure 20). Furthermore, to test whether the kinase activity of PKN is required for its ability to stimulate SM-specific promoter activity, a kinase-dead form of PKN (K644E) containing a point mutation in the ATP binding domain was transfected into PAC-1 cells and the activity of the SM-specific promoter reporters was tested. Expression of active, but not kinase-dead PKN, stimulated transcriptional activity of the SM α-actin, SM-MHC and SM22α promoters approximately 3-fold over empty vector control, arguing that the kinase activity of PKN is required for its ability to stimulate promoter activity of the SM-specific marker genes (Figure 21).

PKN activates SRF, GATA and MEF2 transcription factors

SRF is by far the most important single transcription factor characterized to date to control the expression of SM-specific marker gene expression. However, proper expression of SMC-specific marker genes requires the activation of multiple transcription factors that cooperate and interact in concert to drive tissue specific expression of these genes (78, 137). SRF, GATA and MEF2 are three transcription factors that have each been characterized to play a role in the proper expression of the SM-specific marker genes (71, 95, 101, 165, 197). In order for PKN to drive the expression of these genes *in vivo*, it would need to regulate the activity of these key transcription factors. To examine whether PKN can stimulate the transcriptional activity of SRF, GATA and/or MEF2, PAC-1 cells were co-transfected with an increasing amount of active PKN along with either an SRF-dependent enhancer-reporter (containing 4 tandem CArG box elements), a GATA-dependent enhancer-reporter (containing 6 tandem GATA consensus binding sites) or a MEF2-dependent enhancer-reporter (containing 3 tandem MEF2 consensus binding sites). Expression of active PKN induced all three enhancer-reporters in a dose-dependent manner with a maximal response of approximately 3-fold compared to an empty vector control (Figure 22).

To determine whether this effect was dependent upon the kinase activity of PKN, PAC-1 cells were co-transfected with the SRF, GATA or MEF2 enhancer-reporter plasmids along with either constitutively active PKN or the kinase-dead PKN mutant. Expression of active PKN increased the activity of all three enhancers approximately 3-fold over empty vector control however, kinase-dead PKN was ineffective at activating

these enhancers (Figure 23). When taken together, these data show that PKN activates the transcription factors SRF, GATA and MEF2 in a dose and kinase dependent manner.

TGF-β1 alters MAP kinase phosphorylation

MAP kinases play an integral role in mediating intracellular signaling. They are involved in numerous cellular processes such as growth, proliferation, differentiation and apoptosis (139). The three distinct families of MAP kinases (ERK, JNK and p38 MAP kinase) are each regulated by phosphorylation through specific upstream kinases (139). MAP kinase activity is increased in a number of different vascular injury models. In particular, ERK and JNK activities are increased following balloon angioplasty and in response to blood pressure elevation (60, 188). Inhibition of ERK and JNK activities using dominant negative mutants or by antisense oligonucleotides reduces neointimal formation *in vivo* and inhibits proliferation of cultured SMCs (34, 66). These studies argue that ERK and JNK may be associated with proliferative of SMCs. In contrast, p38 MAP kinase promotes differentiation of skeletal muscle and stimulates expression of SM α-actin in SMCs (38, 84, 195). p38 MAP kinase also directly phosphorylates and activates members of the GATA and MEF2 family of transcription factors in other systems (14, 23). In addition, p38 MAP kinase has been shown to modulate the activity of SRF (38, 53, 172). Thus, p38 MAP kinase may function in the opposite manner as the ERK and JNK families with respect to smooth muscle phenotype.

PKN has been previously shown to alter gene expression by activating members of the p38 MAPK family in other systems (102). Moreover, it was recently shown that

PKN directly binds and activates both p38 MAP kinase and MKK6, one of the upstream kinases responsible for activation of the p38 MAP kinase family (169). Based on these previous reports, it was hypothesized that TGF-β1/PKN-mediated activation of SMC-specific marker gene expression may require p38 MAPKs. As previously mentioned, each of the MAP kinase family members must be phosphorylated on specific threonine and tyrosine residues to be active. Thus, the phosphorylation status of ERK, JNK, p38 MAP kinase and MKK3/6 following TGF-β1 stimulation was examined. To do this, PAC-1 cells were treated with TGF-β1 for 2, 5, 10, 30 or 60 minutes. Following treatment, the phosphorylation of these proteins was analyzed by Western blot using phospho-specific antibodies designed to detect only the active forms of these kinases. Interestingly the phosphorylation of ERK1/2 (p44/p42) and JNK1/2 (p46/p54) was highest in the control, untreated PAC-1 cells (Figure 24 and Figure 25, respectively, top panel). Moreover, the phosphorylation of these enzymes decreased in a time dependent manner following TGF-β1 stimulation. The total amount of ERK1/2 and JNK1/2 was unchanged with TGF-β1 treatment (Figure 24 and Figure 25, respectively, bottom panel). In contrast, the phosphorylation of p38 MAPK increased in a time dependent manner following TGF-β1 stimulation (Figure 26, top panel). Again, there was no change in the total amount of p38 MAP kinase with TGF-β1 treatment (Figure 26, bottom panel).

Lastly, the phosphorylation of MKK3 and MKK6, the up-stream kinases responsible for activating p38 MAP kinase, was also tested using an antibody that recognized both phospho-MKK3 and phospho-MKK6. Following TGF-β1 treatment phosphorylation of MKK3/6 increased in a time dependent manner following TGF-β1

stimulation (Figure 27, top panel), although its maximal activity occurred slightly later compared to p38 MAP kinase (see discussion). Again, there was no significant change in the total amount of MKK3/6 (Figure 27, bottom panel). Taken together, these results imply that TGF-β1-mediated differentiation of PAC-1 SMCs involves a decrease in the activity of ERK1/2 and JNK1/2 with a concomitant increase in the activities of p38 MAPK and one or both of its upstream kinases.

TGF-β1 increases the kinase activity of p38 MAP kinase

Examination of the phosphorylation status of p38 MAP kinase provides insight into the activity of this protein at specific times following treatment. However, this method is an indirect way of examining its activity and does not provide direct evidence for its ability to phosphorylate its downstream targets. Thus, to directly test the activity of p38 MAP kinase following TGF-β1 stimulation, an *in vitro* kinase assay was performed. To do this, PAC-1 cells were again treated with TGF-β1 for 2, 5, 10, 30 or 60 minutes. Following treatment, phospho-p38 MAP kinase was immunoprecipitated using an antibody (conjugated to agarose beads) that specifically binds only the active, phosphorylated form of this enzyme. The isolated phospho-38 MAP kinase was then used in an *in vitro* kinase assay using a purified ATF-2 peptide as the substrate. The kinase activity of the isolated p38 MAP kinase was then directly assessed by examining the phosphorylation status of ATF-2 by western blotting. As shown in Figure 28, the kinase activity of p38 MAP kinase was increased following 5 to 10 minutes of TGF-β1 stimulation compared to control, untreated cells. Moreover, the *in vitro* kinase assay was

completely dependent upon p38 MAP kinase activity as the inclusion of SB203580, a specific p38 MAP kinase inhibitor, completely blocked the phosphorylation of ATF-2. These results demonstrate that the kinase activity of p38 MAP kinase is indeed increased in response to TGF-β1 stimulation, arguing that it might play a role in mediating the downstream effects of TGF-β1 and potentially PKN.

MKK6 regulates transcriptional activation of smooth muscle-specific genes

Data from Figures 26, 27 and 28 suggest that MKK6 and p38 MAP kinase may play a role in mediating the downstream effects of TGF-β1 on SM differentiation. To test whether MKK6 and p38 MAP kinase play a role in regulating the promoter activity of the SM-specific marker genes, PAC-1 cells were transiently transfected with an empty vector control or a constitutively active form of MKK6 (MKK6(E)) in the absence or presence of a dominant negative form of p38 MAP kinase ($p38\alpha_{AF}$). The activity of each the three SM-promoter-reporter plasmids (SM α-actin, SM-MHC or SM22α) was examined. As seen in Figure 29, expression of active MKK6 was sufficient to stimulate the promoter activity of all three SM-specific promoters compared to an empty vector control in the absence of dominant negative p38 MAP kinase ($-p38\alpha_{AF}$). However, in the presence of dominant negative p38 MAP kinase ($+p38\alpha_{AF}$), the effects of active MKK6 were completely abolished. These data demonstrate that MKK6 (like PKN) can activate SM-specific marker gene expression. Moreover, this effect requires p38 MAP kinase activity.

MKK6 activates the transcription factors SRF, GATA and MEF2

To further examine whether MKK6 and p38 MAP kinase play a role in TGF-β1-mediated activation of SM-specific marker gene expression, the ability of MKK6 to activate the three previously described transcription factors (SRF, GATA and MEF2) was tested. PAC-1 cells were transiently transfected with empty vector control or constitutively active MKK6 in the absence or presence of dominant negative p38 MAP kinase. The activity of each the three enhancer-reporter plasmids (4 x SRF-, 6 x GATA- or 3 x MEF2-luc) was examined. As seen in Figure 30, active MKK6 increased the transcriptional activity of SRF and GATA in the absence of dominant negative p38 MAP kinase (-p38α_{AF}). Surprisingly, MEF2 was not affected by the expression of active MKK6. In the presence of dominant negative p38 MAP kinase (+p38α_{AF}), the effects of MKK6 on SRF and GATA were completely abolished. These data demonstrate that MKK6 can stimulate SRF- and GATA-dependent transcription, but not MEF2. Moreover, the effect of MKK6 on SRF and GATA requires the activity of p38 MAP kinase.

Dominant negative p38 MAP kinase blocks PKN-mediated effects

The previous data demonstrate that PKN and p38 MAP kinase are activated in response to TGF-β1 stimulation (Figures 19, 26 and 28). Since PKN has been shown to active p38 MAP kinase in other cell types (102, 169), it is possible that these enzymes may work together as part of a signal transduction cascade to modulate the expression of SM-specific marker genes. Thus, the requirement of p38 MAP kinase for PKN-mediated

effects on the SM-promoter-reporters and the transcription factor enhancer-reporters was tested. To do this PAC-1 cells were transfected with active PKN in the absence or presence of dominant negative p38 MAP kinase and the activity of the SMC-specific promoters or the SRF, GATA, or MEF2-enhancers was measured by luciferase assay. PKN enhanced the activity of each promoter or enhancer, as expected. However, co-expression of dominant negative p38 MAP kinase abolished the ability of PKN to enhance transcription from either the SM-specific promoters (Figure 31) or the transcription factor-enhancers (Figure 32). This demonstrates the necessity of p38 MAP kinase for PKN-mediated activation of both the SMC-specific promoters as well as the SRF, GATA and MEF2 enhancers.

TGF-β1 may stimulate hypertrophy of a subpopulation of PAC-1 cells

Thus far, TGF-β1 stimulation of PAC-1 smooth muscle cells has been discussed from the standpoint of differentiation. Interestingly, in addition to the numerous reports describing TGF-β1's function as a pro-differentiation factor for SMCs, it has also been shown to stimulate hypertrophy of rat aortic SMCs in culture (130). Indeed, hypertrophy of a subpopulation of PAC-1 cells was seen following 48 hours of TGF-β1 treatment. These cells, on average, more than doubled in size and were often multinucleated as seen by the DAPI staining in Figure 33. In addition, the SM α-actin in these cells was highly reorganized into stress fibers as seen by indirect immunofluorescence. Thus, TGF-β1 may also contribute to the production of a hypertrophied phenotype in PAC-1 smooth muscle cells.

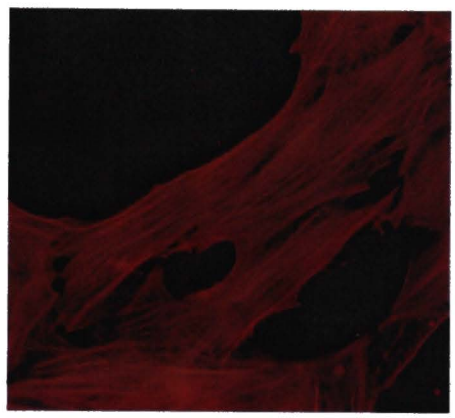
Figure 10. TGF-β1 stimulates actin re-organization of PAC-1 cells

PAC-1 cells were cultured on gelatin-coated cover slips in medium containing (A.) 10% FBS + vehicle control, (B.) 10% FBS + TGF-β1 (2.5ng/mL) or (C.) 0.5% FBS for 48 hours. Following treatment, actin was visualized by indirect immunocytochemistry using an antibody that specifically binds to smooth muscle alpha actin.

A.

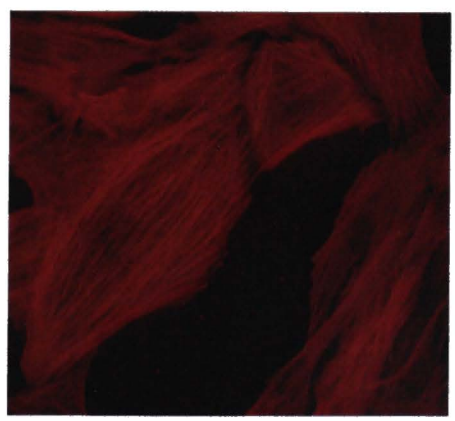
10% serum

B.



10% serum
+ TGF-β1

C.



0.5 % serum

Figure 11. TGF-β1 up-regulates the expression of SM-specific marker genes

PAC-1 cells were cultured in medium containing 10% FBS in the absence or presence of TGF-β1 (2.5ng/mL) for 48 hours or were stimulated to differentiate by serum withdrawal (0.5% FBS). Total RNA from each group was isolated and 2μg was used for reverse transcription. The relative expression of SM α-actin, SM-MHC, SM22α or 18S rRNA was analyzed by semi-quantitative PCR using gene specific primers. PCR products were visualized by ethidium bromide staining following separation on a 2% agarose gel.

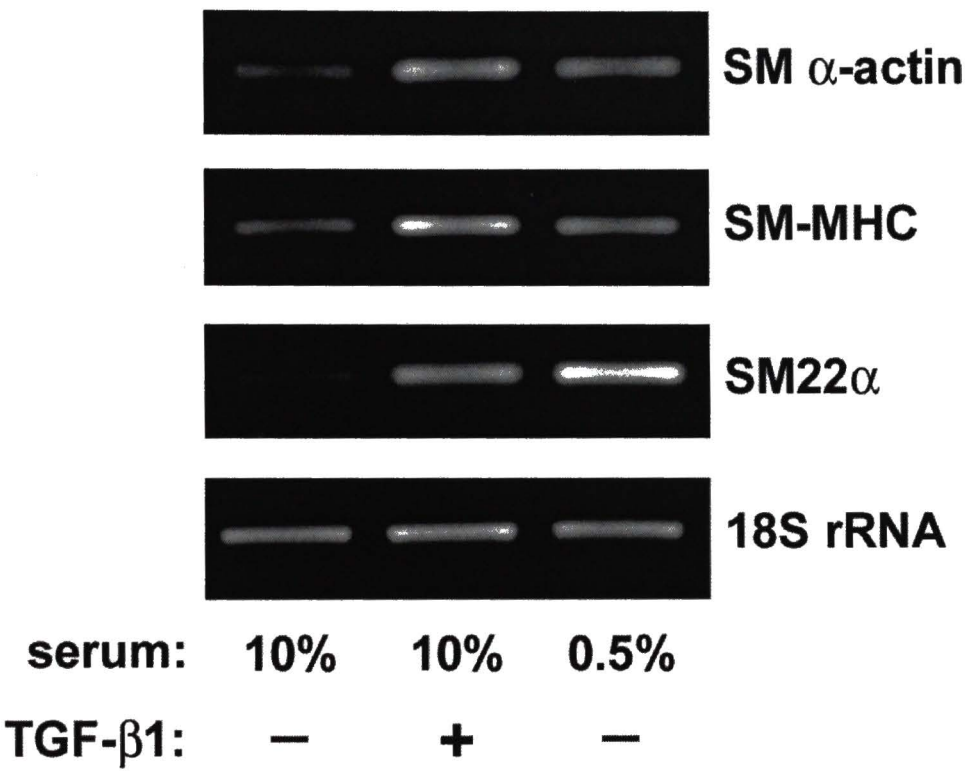

SM α-actin
SM-MHC
SM22α
18S rRNA
serum: 10% 10% 0.5%
TGF-β1: — + —

Figure 12. TGF-β1 inhibits serum-induced proliferation of PAC-1 cells

PAC-1 cells were plated at an equal starting density and cultured in media supplemented with 10% FBS + vehicle control (closed squares), 10% FBS + 2.5ng/mL TGF-β1 (open circles) or 0.5% FBS (closed triangles). Total cell number per group was counted following 12, 24, 36 and 48 hours of treatment.

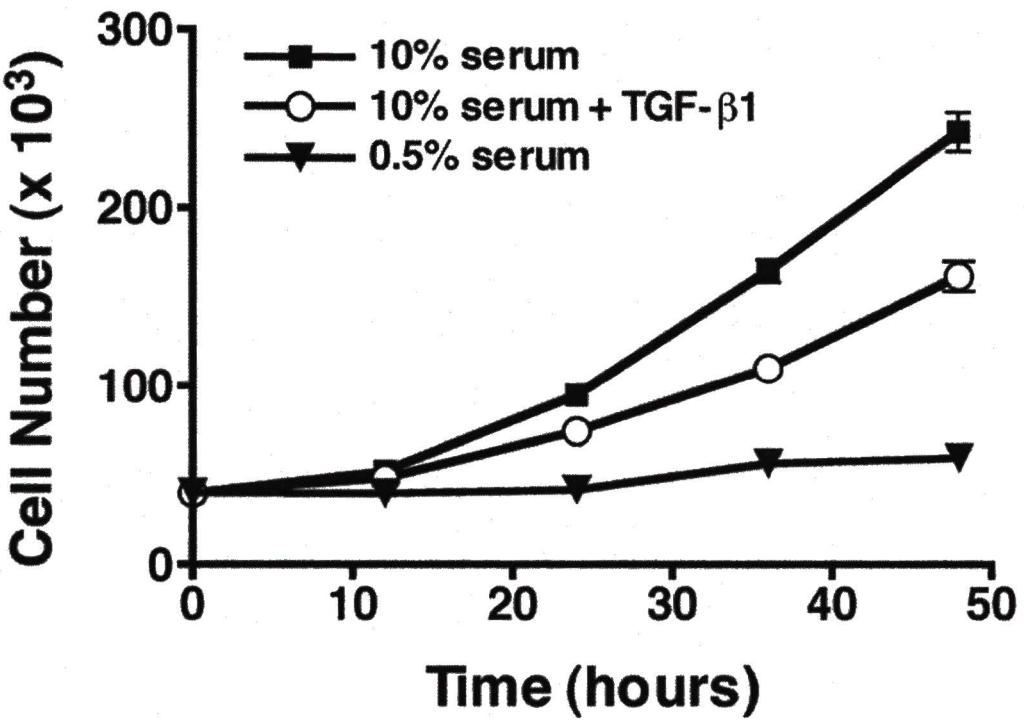
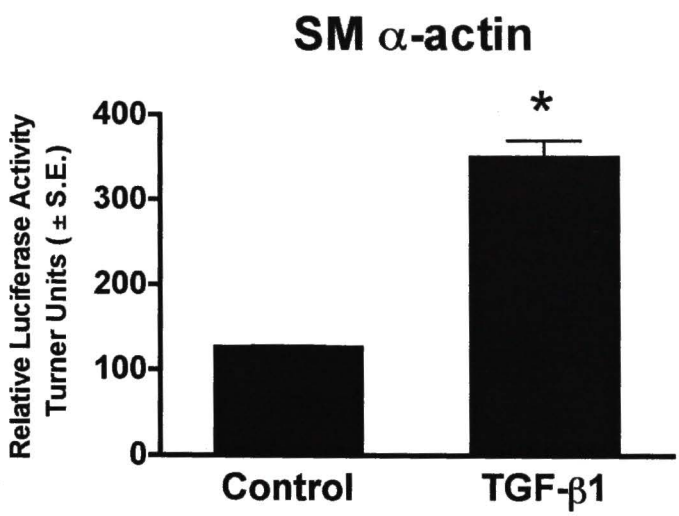
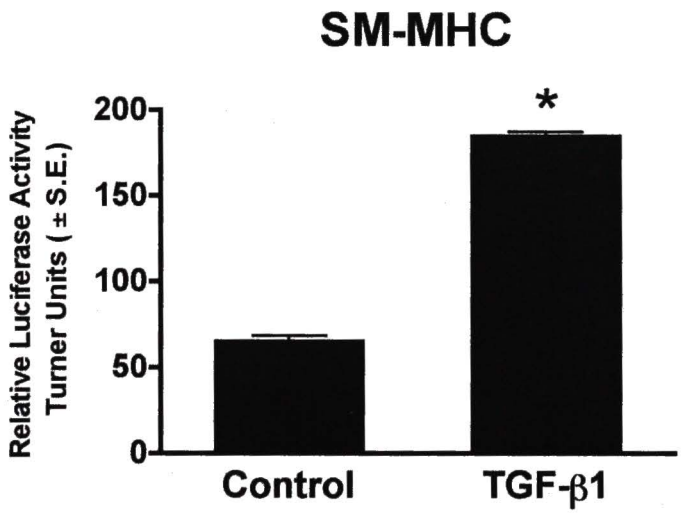

300
200
100
0
Cell Number (x 10³)
10% serum
10% serum + TGF-β1
0.5% serum
0
10
20
30
40
50
Time (hours)

Figure 13. TGF-β1 increases the transcriptional activity of the SM α-actin, SM-MHC and SM22α promoters

PAC-1 cells were transiently transfected with 30ng of either the SM α-actin-, SM-MHC- or SM22α-luciferase promoter-reporter plasmids (described in methods) using LipofectAMINE™ Plus reagent. Three hours post transfection, cells were re-fed in medium supplemented with 10% FBS. Cells were treated with either vehicle control (Control) or 2.5 ng/mL TGF-β1 (TGF-β1) for 48 hours. Following treatment, luciferase activity was determined by luminometry. (* $p < 0.001$)


SM α-actin
Relative Luciferase Activity
Turner Units (± S.E.)
400
300
200
100
0
*
Control
TGF-β1


SM-MHC
Relative Luciferase Activity
Turner Units (± S.E.)
200
150
100
50
0
*
Control
TGF-β1

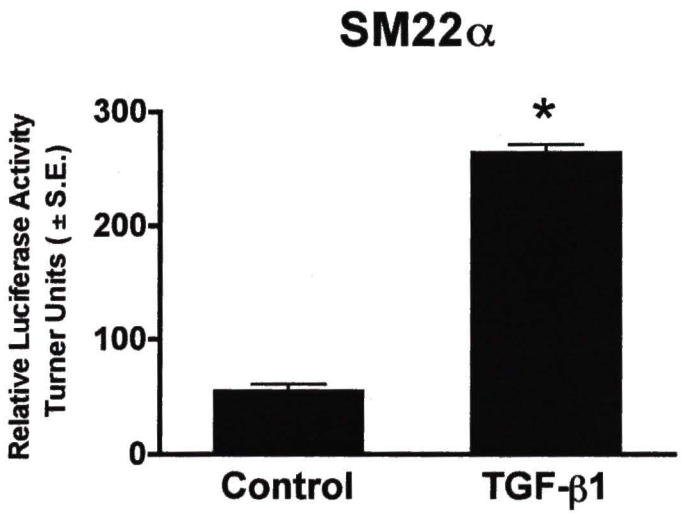
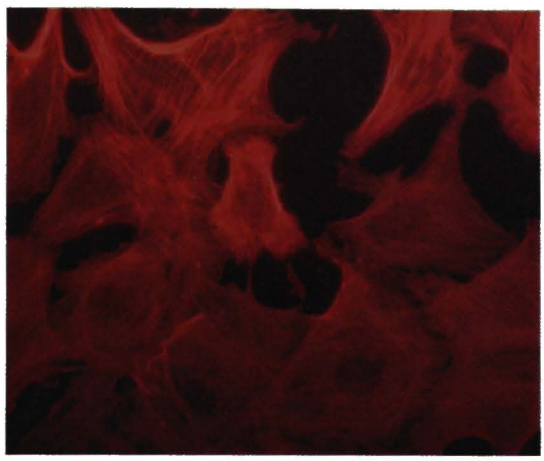
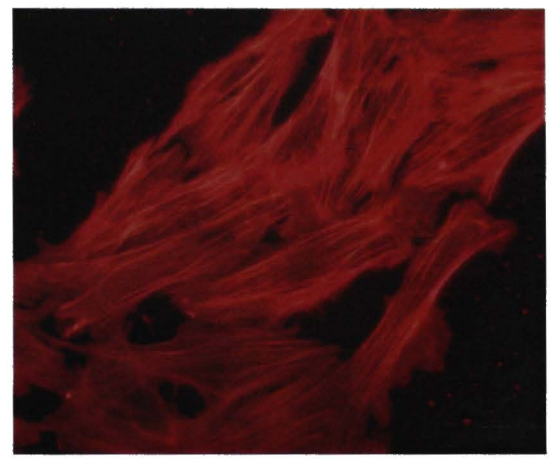

SM22α
Relative Luciferase Activity
Turner Units (± S.E.)
300
200
100
0
*
Control
TGF-β1

Figure 14. TGF-β1-induced actin re-organization is blocked by HA1077 and Y-27632

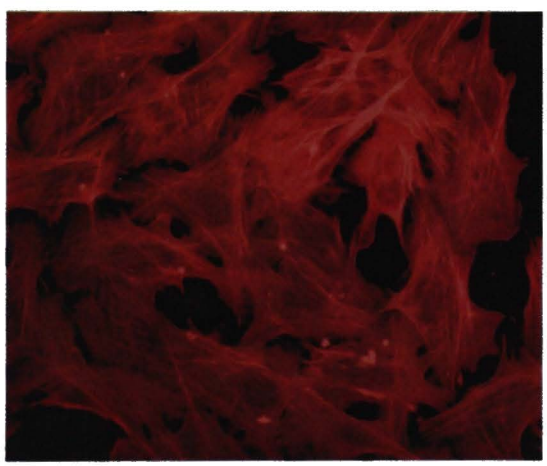
PAC-1 cells were cultured on gelatin-coated cover slips in media supplemented with 10% FBS. 24 hours post-plating, cells were pre-treated with HA1077 (20μM), Y-27632 (10μM) or vehicle control (water) for three hours. Cells were then treated with TGF-β1 (2.5 ng/mL) or vehicle control (4mM HCl + 0.1% BSA) for 48 hours. Following treatment, actin was visualized by indirect immunocytochemistry using an antibody that specifically binds to smooth muscle alpha actin.



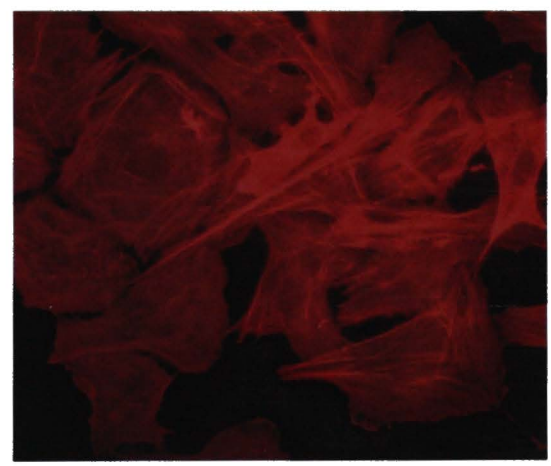
Control



TGF-β1



TGF-β1 + HA1077



TGF-β1 + Y-27632

Figure 15. TGF-β1-induced up-regulation of SM-specific gene expression is blocked by HA1077 or Y-27632

PAC-1 cells were cultured in media supplemented with 10% FBS. 24 hours post-plating, cells were pre-treated with HA1077 (20μM), Y-27632 (10μM) or vehicle control (water), for 3 hours prior to stimulation with TGF-β1 (2.5ng/mL) or vehicle control (4mM HCl + 0.1% BSA) for 48 hours. Total RNA from each group was isolated and 2μg was used for reverse transcription. The relative expression of SM α-actin, SM-MHC, SM22α or GAPDH was analyzed by semi-quantitative PCR using gene specific primers. PCR products were visualized by ethidium bromide staining following separation on a 2% agarose gel.

Figure 15. TGF-β1-induced up-regulation of SM-specific gene expression is blocked by HA1077 or Y-27632

PAC-1 cells were cultured in media supplemented with 10% FBS. 24 hours post-plating, cells were pre-treated with HA1077 (20μM), Y-27632 (10μM) or vehicle control (water), for 3 hours prior to stimulation with TGF-β1 (2.5ng/mL) or vehicle control (4mM HCl + 0.1% BSA) for 48 hours. Total RNA from each group was isolated and 2μg was used for reverse transcription. The relative expression of SM α-actin, SM-MHC, SM22α or GAPDH was analyzed by semi-quantitative PCR using gene specific primers. PCR products were visualized by ethidium bromide staining following separation on a 2% agarose gel.

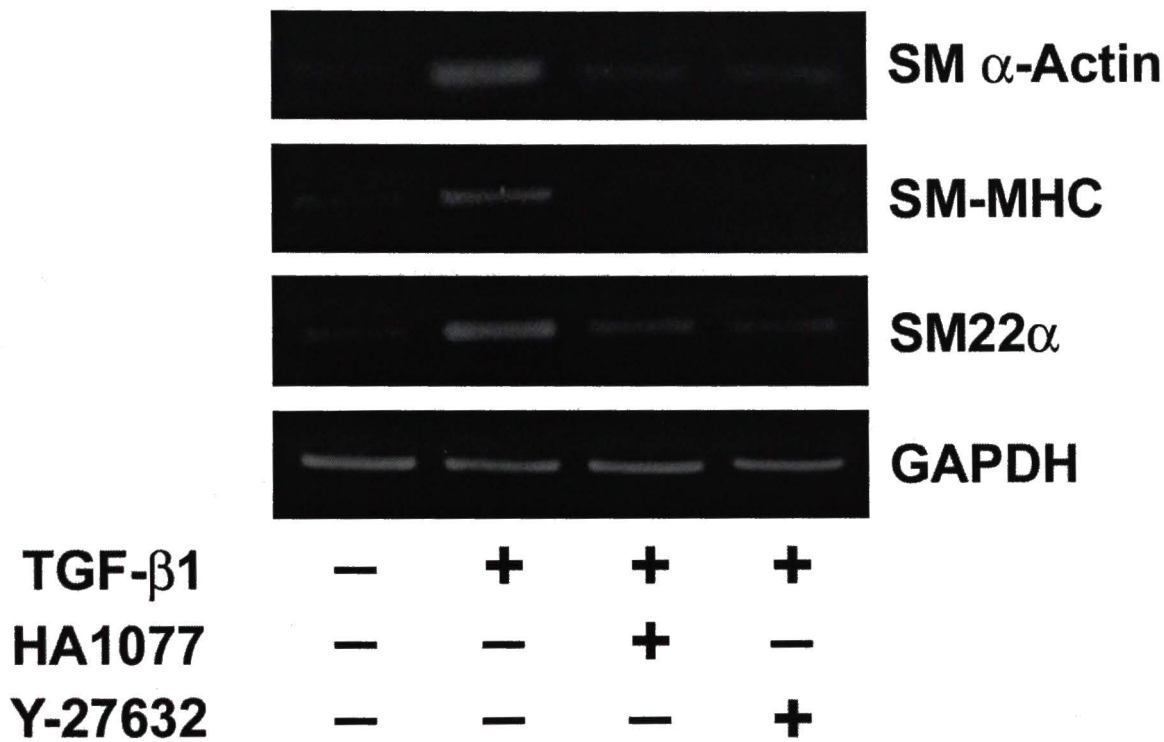

SM α-Actin
SM-MHC
SM22α
GAPDH
TGF-β1 — + + +
HA1077 — — + —
Y-27632 — — — +

Figure 16. TGF-β1-induced inhibition of SMC proliferation is attenuated by HA1077 or Y-27632

PAC-1 cells were plated at an equal starting density and cultured in media supplemented with 10% FBS plus vehicle control (open circles), TGF-β1 alone (closed circles), TGF-β1 + HA1077 (closed triangles) or TGF-β1 + Y-27632 (closed diamonds). Total cell number per group was counted following 12, 24, 36 and 48 hours of treatment.

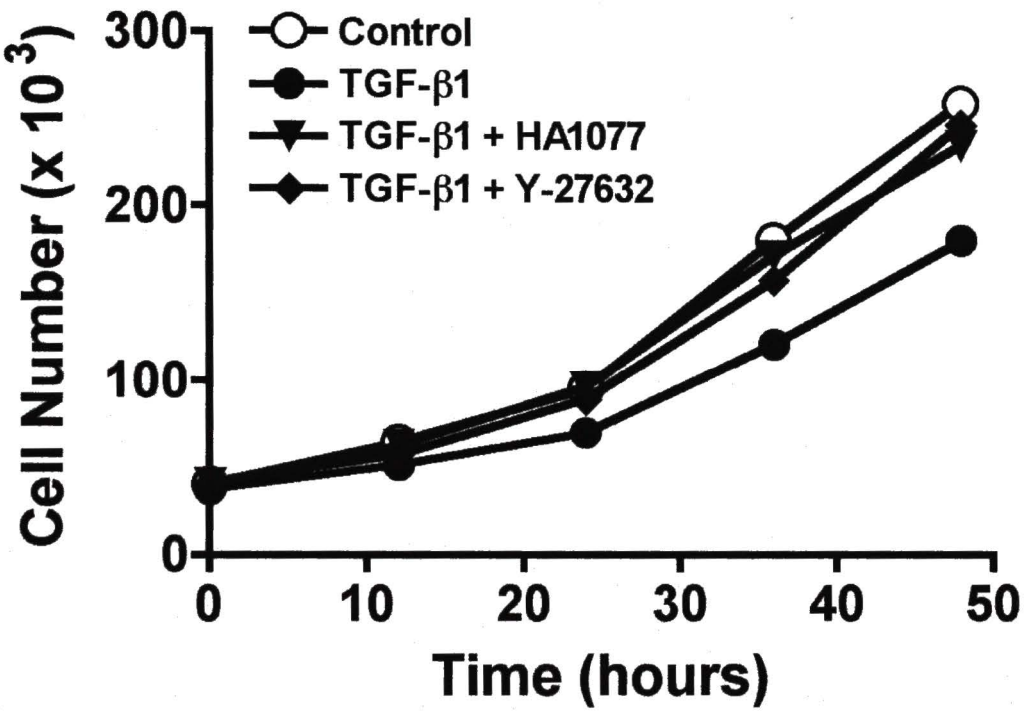

Cell Number (x 10^3)
300
200
100
0
0
10
20
30
40
50
Time (hours)
Control
TGF-β1
TGF-β1 + HA1077
TGF-β1 + Y-27632

Figure 17. TGF-β1-induced activation of the SM α-actin, SM-MHC and SM22α promoters is blocked by HA1077 or Y-27632

PAC-1 cells were transiently transfected with 30ng of either the SM α-actin-, SM-MHC- or SM22α-luciferase promoter-reporter plasmids (described in methods) using LipofectAMINE™ Plus reagent. Three hours post transfection, cells were re-fed in media supplemented with 10% FBS. Transfected cells were pre-treated with HA1077 (20μM), Y-27632 (10μM) or vehicle control (water) for three hours prior to treatment with TGF-β1 (2.5 ng/mL) or vehicle control (4mM HCl + 0.1% BSA) for 48 hours. Following treatment, luciferase activity was determined by luminometry. (* $p < 0.01$ compared to control, † $p < 0.01$ compared to TGF-β1 alone)

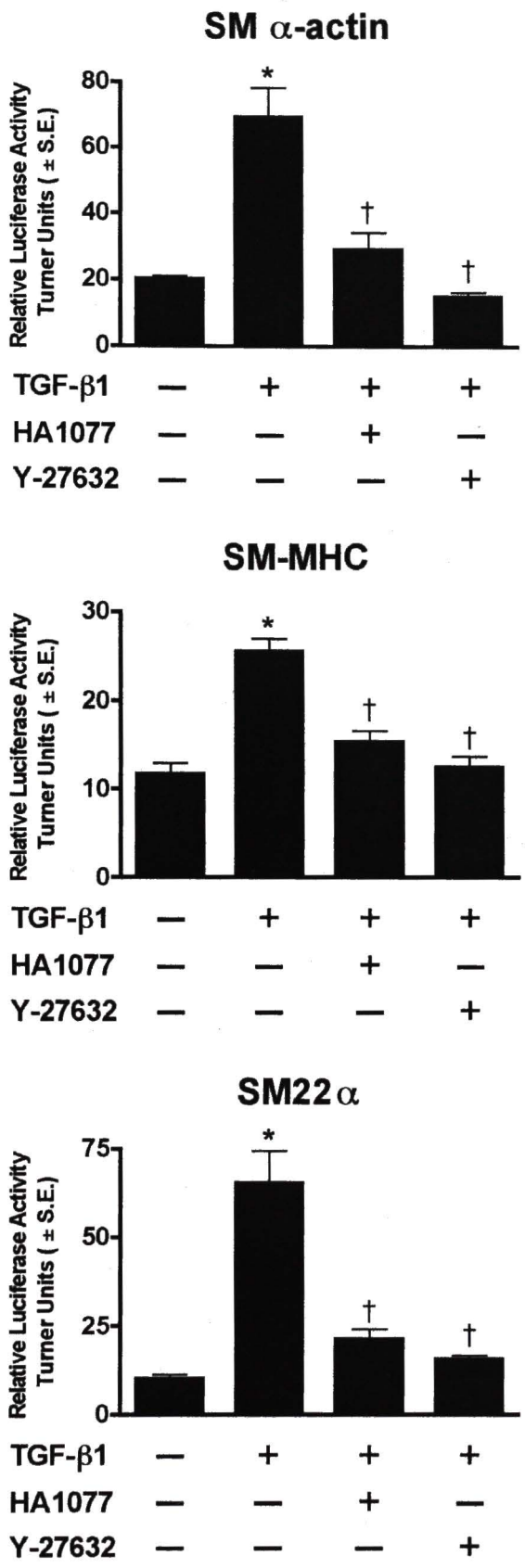

SM α-actin
Relative Luciferase Activity
Turner Units (± S.E.)
80
60
40
20
0
*
†
†
TGF-β1 — + + +
HA1077 — — + —
Y-27632 — — — +
SM-MHC
Relative Luciferase Activity
Turner Units (± S.E.)
30
20
10
0
*
†
†
TGF-β1 — + + +
HA1077 — — + —
Y-27632 — — — +
SM22α
Relative Luciferase Activity
Turner Units (± S.E.)
75
50
25
0
*
†
†
TGF-β1 — + + +
HA1077 — — + —
Y-27632 — — — +

Figure 18. TGF-β1 activates the small GTPase RhoA

PAC-1 cells were cultured in media supplemented with 10% FBS. 48 hours post-plating, cells were untreated (control) or treated with TGF-β1 (2.5 ng/mL) for 2, 5, 10, 30 or 60 minutes. Following treatment, cells were lysed and GTP-bound (active) RhoA was immunoprecipitated using a GST-Rhotekin RBD fusion protein. The relative amount of active RhoA present in each group was then analyzed by western blotting.

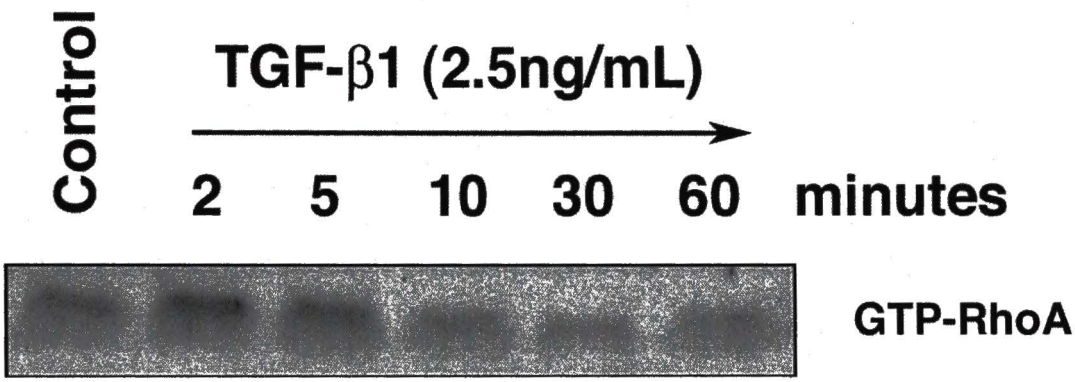

Control
TGF-β1 (2.5ng/mL)
2
5
10
30
60
minutes
GTP-RhoA

Figure 19. TGF-β1 increases the phosphorylation of PKN and PRK2

PAC-1 cells were cultured in media supplemented with 10% FBS. 48 hours post-plating, cells were untreated (control) or treated with TGF-β1 (2.5 ng/mL) for 2, 5, 10, 30 or 60 minutes. The phosphorylation status of PKN/PRK2 following TGF-β1 stimulation was analyzed by western blot using a primary antibody that recognizes phospho-PKN(Thr778) and phospho-PRK2(Thr816). In addition, total PKN was analyzed by western blot using an antibody specific for PKN to detect any changes in expression of this protein following TGF-β1 treatment.

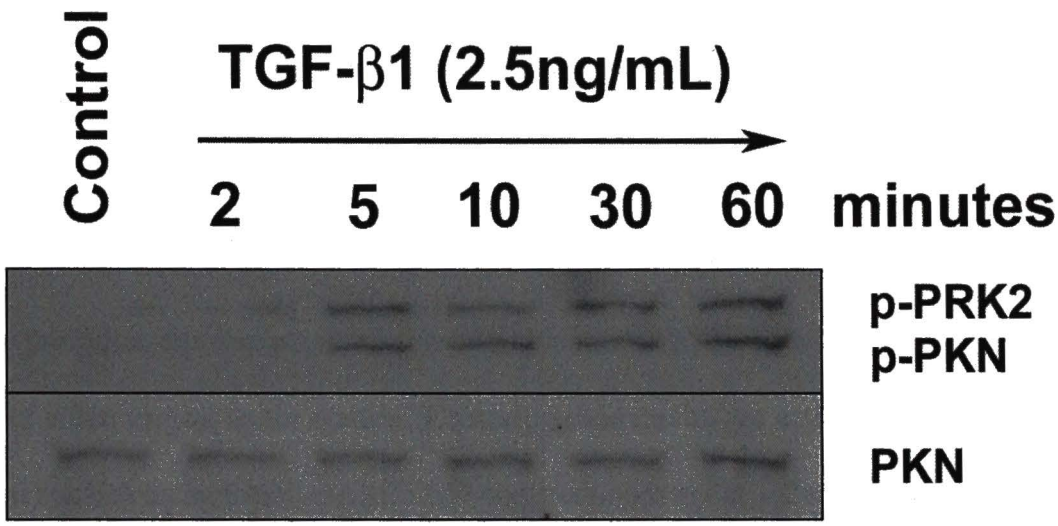
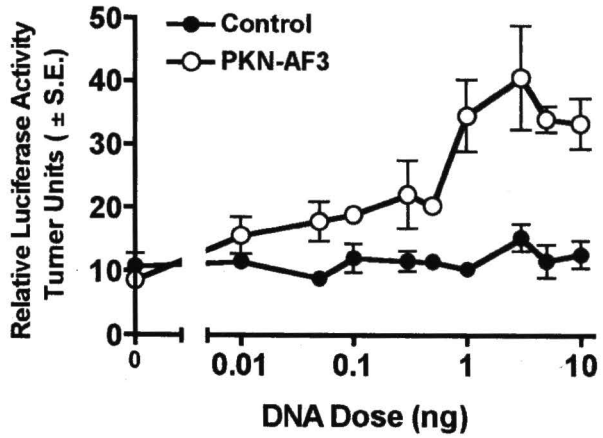

Control
TGF-β1 (2.5ng/mL)
2
5
10
30
60
minutes
p-PRK2
p-PKN
PKN

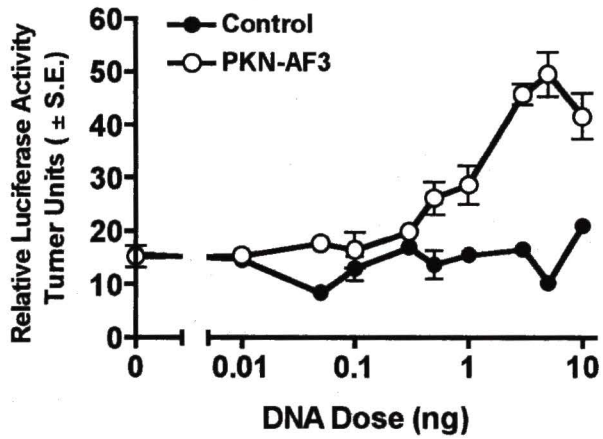
Figure 20. PKN increases the promoter activity of SM α-actin, SM-MHC and SM22α in a dose dependent manner

PAC-1 cells were transfected with 30ng of the SM α-actin-, SM-MHC- or SM22α-luciferase promoter-reporter plasmids (described in methods) along with increasing amounts of either empty vector control (Control, closed circles) or active PKN (PKN-AF3, open circles) using LipofectAMINE™ transfection reagent. Cells were refed with media supplemented with 10% FBS following 12 hours of transfection. Luciferase activity was measured 24 hours after re-feeding.

SM α-actin



SM-MHC



SM22α

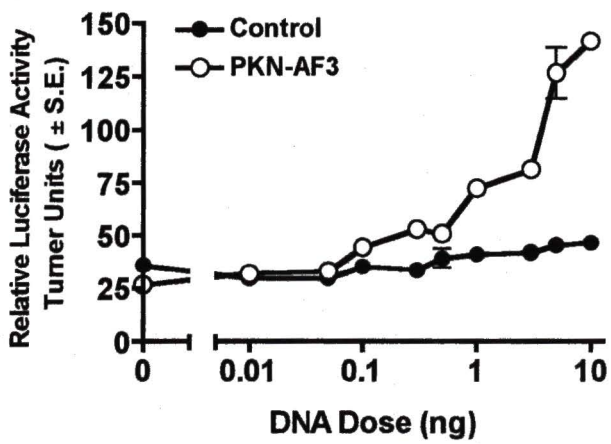


Figure 21. PKN increases the promoter activity of SM α-actin, SM-MHC and SM22α in a kinase dependent manner

PAC-1 cells were transfected with 30ng of the SM α-actin-, SM-MHC- or SM22α-luciferase promoter-reporter plasmids (described in methods) along with either empty vector control (Control), active PKN (PKN-AF3) or kinase-dead PKN (K644E) using LipofectAMINE™ transfection reagent. Cells were refed with media supplemented with 10% FBS following 12 hours of transfection. Luciferase activity was measured 24 hours after re-feeding. (* $p < 0.01$ compared to control, † $p < 0.01$ compared to PKN-AF3)

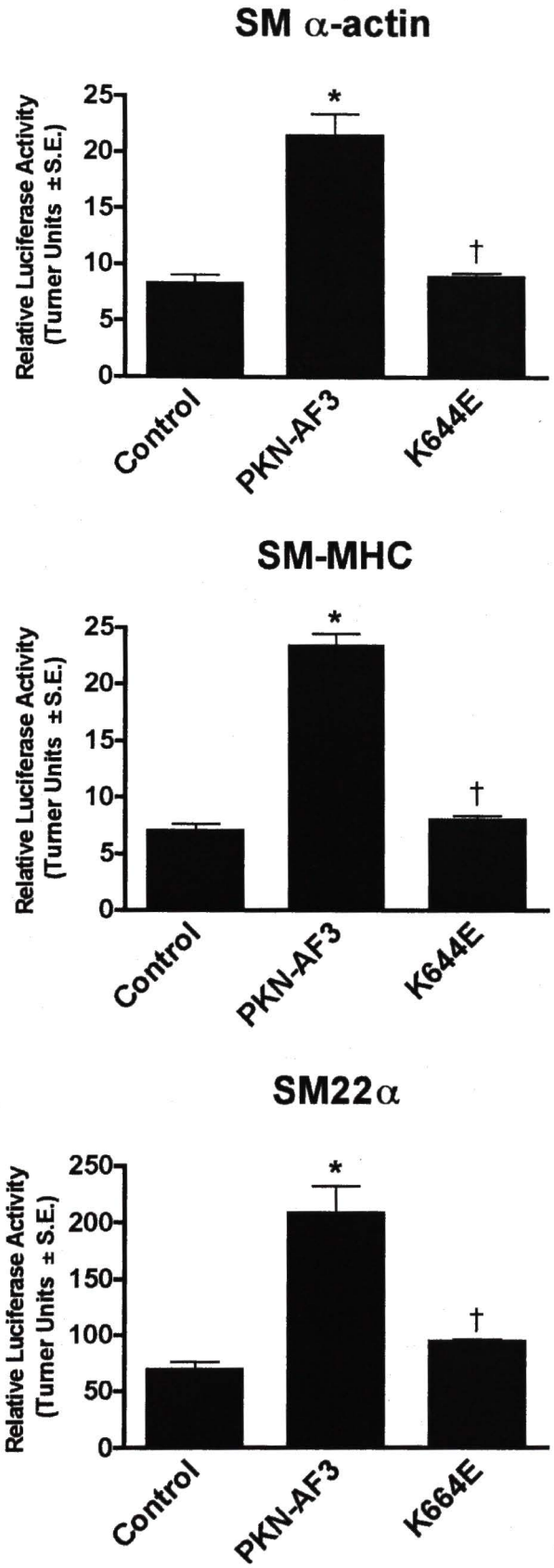
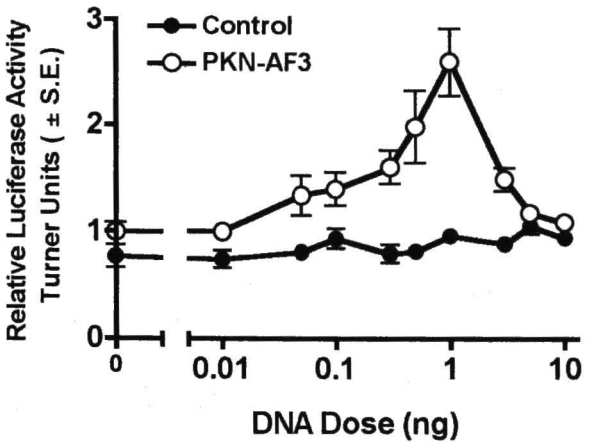

SM α-actin
Relative Luciferase Activity (Tumer Units ±S.E.)
25
20
15
10
5
0
*
†
Control
PKN-AF3
K644E
SM-MHC
Relative Luciferase Activity (Tumer Units ±S.E.)
25
20
15
10
5
0
*
†
Control
PKN-AF3
K644E
SM22α
Relative Luciferase Activity (Tumer Units ± S.E.)
250
200
150
100
50
0
*
†
Control
PKN-AF3
K664E

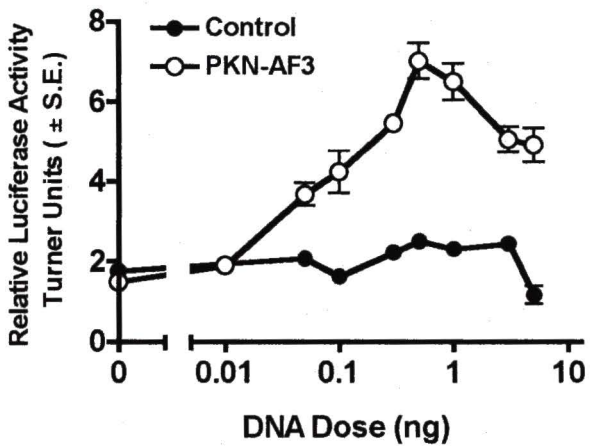
Figure 22. PKN increases the activity of the transcription factors SRF, GATA and MEF2 in a dose dependent manner

PAC-1 cells were transfected with 100ng of the 4 x SRF-, 6 x GATA- or 3 x MEF2-luciferase enhancer-reporter plasmids (described in methods) along with increasing amounts of either empty vector control (Control, closed circles) or active PKN (PKN-AF3, open circles) using LipofectAMINE™ transfection reagent. Cells were refed with media supplemented with 10% FBS following 12 hours of transfection. Luciferase activity was measured 24 hours after re-feeding.

4 x SRF



6 x GATA



3 x MEF2

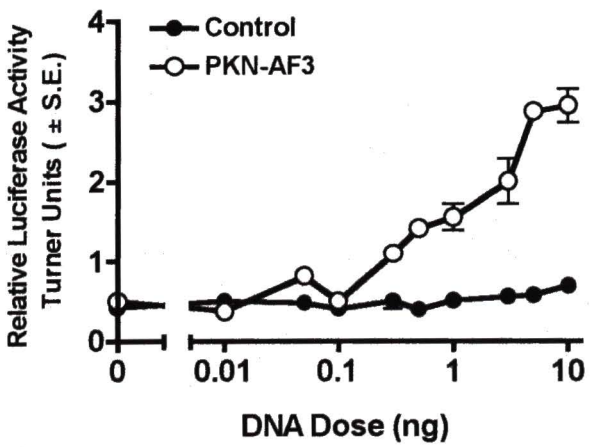
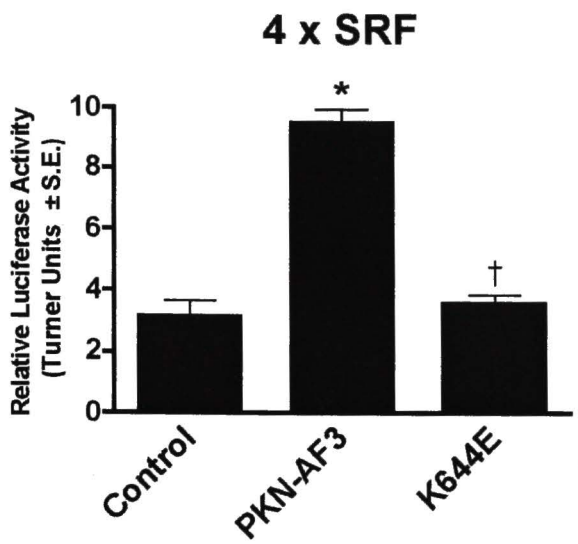
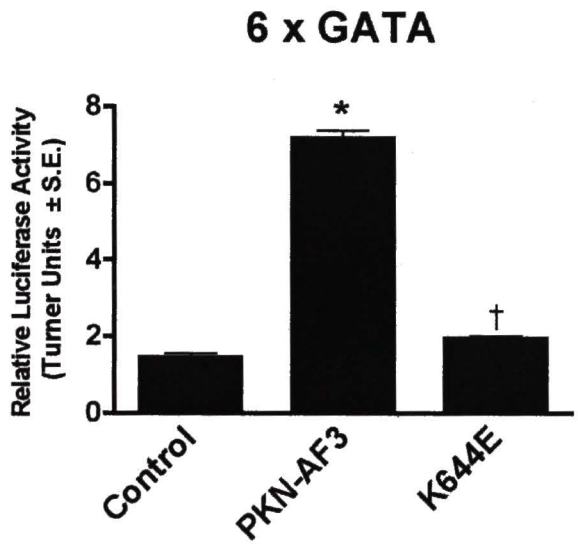


Figure 23. PKN increases the activity of the transcription factors SRF, GATA and MEF2 in a kinase dependent manner

PAC-1 cells were transfected with 100ng of the 4 x SRF-, 6 x GATA- or 3 x MEF2-luciferase enhancer-reporter -reporter plasmids (described in methods) along with either empty vector control (Control), active PKN (PKN-AF3) or kinase-dead PKN (K644E) using LipofectAMINE™ transfection reagent. Cells were refed with media supplemented with 10% FBS following 12 hours of transfection. Luciferase activity was measured 24 hours after re-feeding. (* $p < 0.001$ compared to control, † $p < 0.001$ compared to PKN-AF3)


4 x SRF
Relative Luciferase Activity
(Turner Units ± S.E.)
10
8
6
4
2
0
*
†
Control
PKN-AF3
K644E


6 x GATA
Relative Luciferase Activity
(Turner Units ± S.E.)
8
6
4
2
0
*
†
Control
PKN-AF3
K644E

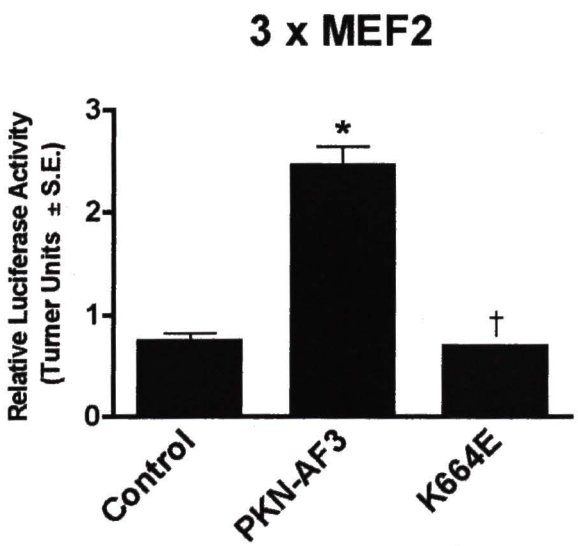

3 x MEF2
Relative Luciferase Activity
(Turner Units ± S.E.)
3
2
1
0
*
†
Control
PKN-AF3
K664E

Figure 24. TGF-β1 decreases ERK1/2 phosphorylation

PAC-1 cells were cultured in media supplemented with 10% FBS. 48 hours post-plating, cells were untreated (control) or treated with TGF-β1 (2.5 ng/mL) for 2, 5, 10, 30 or 60 minutes. The phosphorylation status of ERK1/2 following TGF-β1 stimulation was analyzed by western blot using a primary antibody that specifically recognizes phospho-ERK1/2 (p-ERK1/2). In addition, total ERK1/2 was analyzed by western blot using an antibody specific for ERK1/2 to detect any changes in the expression of these proteins following TGF-β1 treatment.

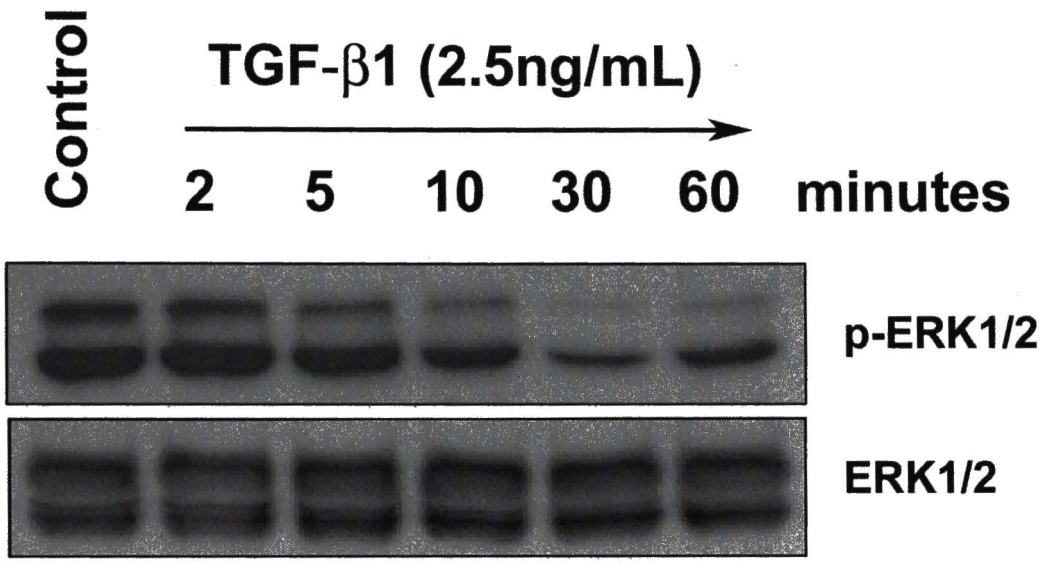

Control
TGF-β1 (2.5ng/mL)
2
5
10
30
60
minutes
p-ERK1/2
ERK1/2

Figure 25. TGF-β1 decreases JNK1/2 phosphorylation

PAC-1 cells were cultured in media supplemented with 10% FBS. 48 hours post-plating, cells were untreated (control) or treated with TGF-β1 (2.5 ng/mL) for 2, 5, 10, 30 or 60 minutes. The phosphorylation status of JNK1/2 following TGF-β1 stimulation was analyzed by western blot using a primary antibody that specifically recognizes phospho-JNK1/2 (p-JNK1/2). In addition, total JNK1/2 was analyzed by western blot using an antibody specific for JNK1/2 to detect any changes in the expression of these proteins following TGF-β1 treatment.

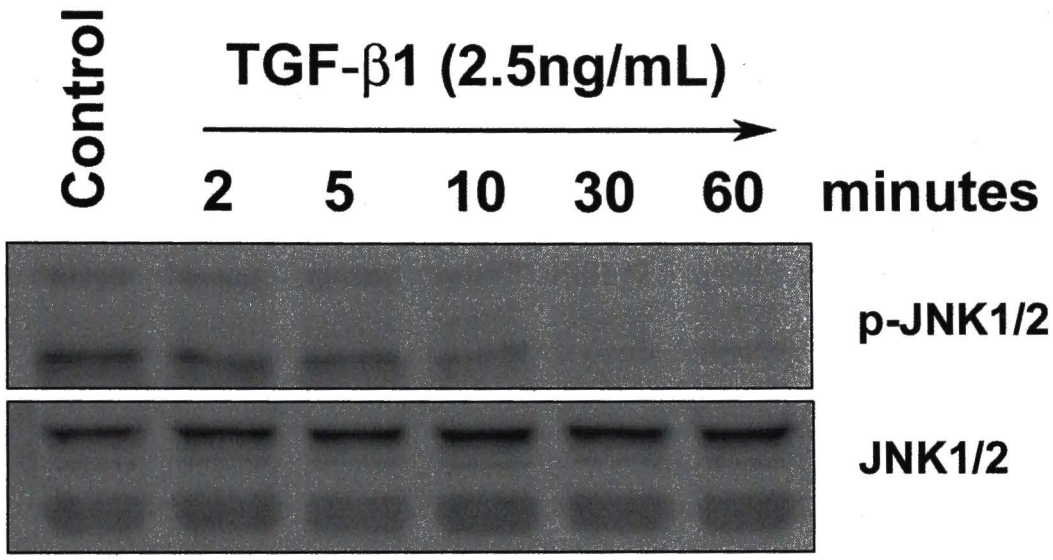

Control
TGF-β1 (2.5ng/mL)
2
5
10
30
60
minutes
p-JNK1/2
JNK1/2

Figure 26. TGF-β1 increases p38 MAP kinase phosphorylation

PAC-1 cells were cultured in media supplemented with 10% FBS. 48 hours post-plating, cells were untreated (control) or treated with TGF-β1 (2.5 ng/mL) for 2, 5, 10, 30 or 60 minutes. The phosphorylation status of p38 MAP kinase following TGF-β1 stimulation was analyzed by western blot using a primary antibody that specifically recognizes phospho-p38 MAP kinase (p-p38 MAPK). In addition, total p38 MAP kinase was analyzed by western blot using an antibody specific for p38 MAP kinase (p38 MAPK) to detect any changes in the expression of this protein following TGF-β1 treatment.

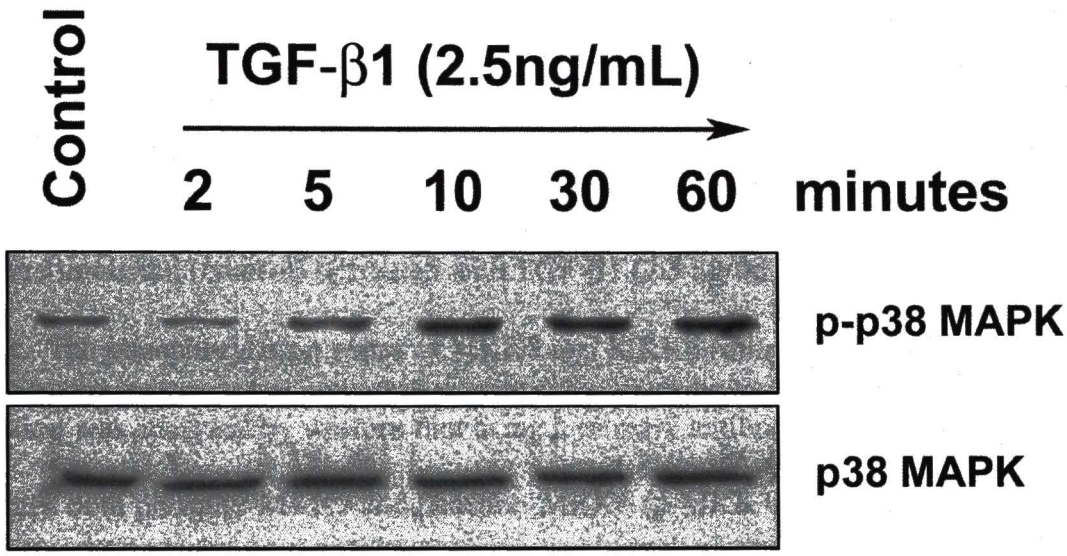

Control
TGF-β1 (2.5ng/mL)
2
5
10
30
60
minutes
p-p38 MAPK
p38 MAPK

Figure 27. TGF-β1 increases MKK3/6 phosphorylation

PAC-1 cells were cultured in media supplemented with 10% FBS. 48 hours post-plating, cells were untreated (control) or treated with TGF-β1 (2.5 ng/mL) for 2, 5, 10, 30 or 60 minutes. The phosphorylation status of MKK3 and MKK6 following TGF-β1 stimulation was analyzed by western blot using a primary antibody that specifically recognizes both phospho-MKK3 and phospho-MKK6 (p-MKK3/6). In addition, total MKK3/6 was analyzed by western blot using an antibody that binds both MKK3 and MKK6 to detect any changes in the expression of these proteins following TGF-β1 treatment.

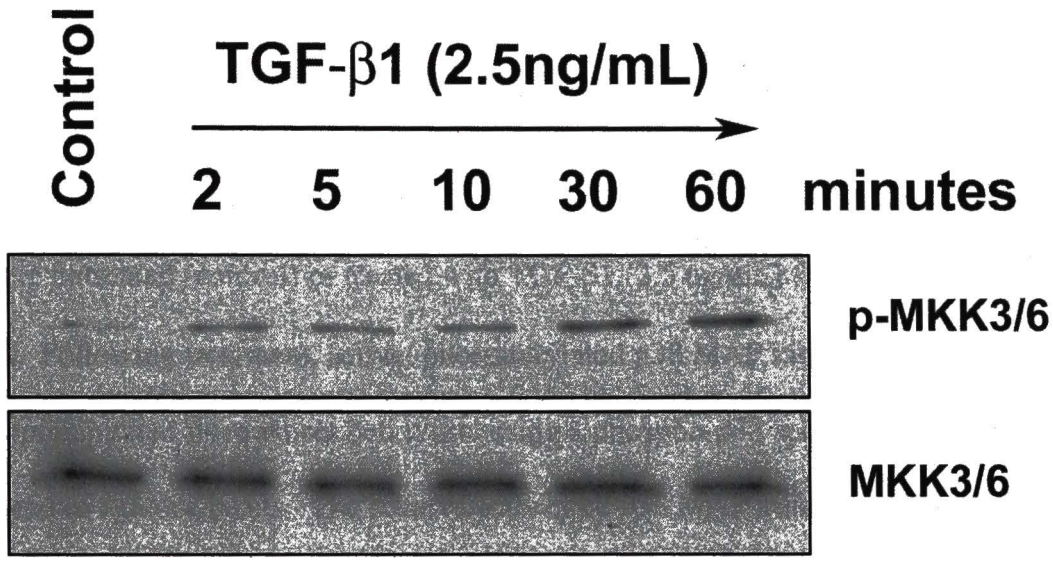

Control
TGF-β1 (2.5ng/mL)
2
5
10
30
60
minutes
p-MKK3/6
MKK3/6

Figure 28. p38 MAP kinase activity is increased following TGF-β1 stimulation

PAC-1 cells were cultured in media supplemented with 10% FBS. 48 hours post-plating, cells were untreated (control) or treated with TGF-β1 (2.5 ng/mL) for 2, 5, 10, 30 or 60 minutes. Following treatment, active, phosphorylated p38 MAP kinase was immunoprecipitated from whole cell lysate using a phospho-p38 specific antibody conjugated to agarose beads. The relative activity of p38 MAP kinase following TGF-β1 stimulation was then tested by subjecting the isolated phospho-p38 MAP kinase to an *in vitro* kinase assay using an ATF-2 peptide as the substrate. Phosphorylated ATF-2 was analyzed by western blot using an antibody that specifically recognizes ATF-2 when phosphorylated on threonine 71. SB203580 (10μM) was used to inhibit p38 MAP kinase activity during the *in vitro* kinase assay.

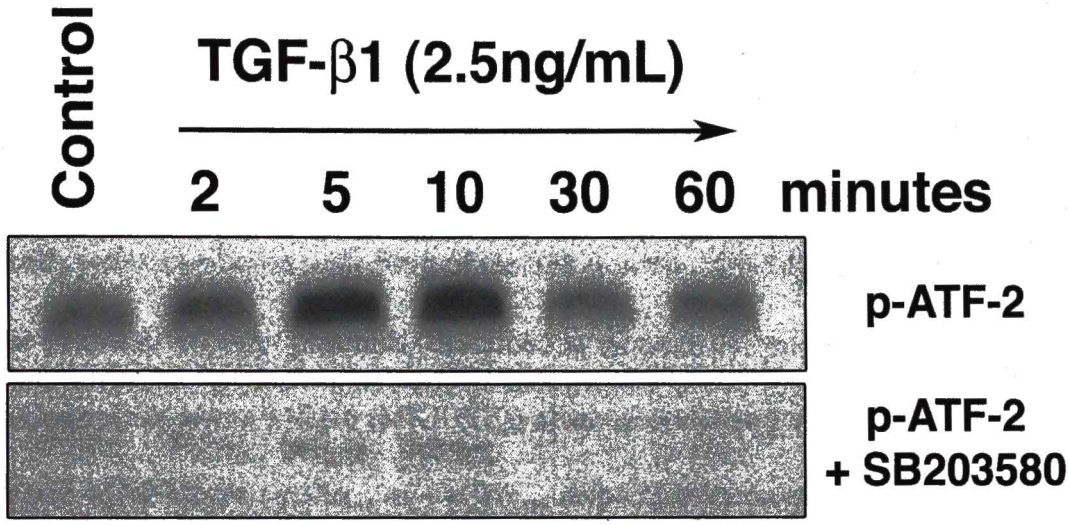

Control
TGF-β1 (2.5ng/mL)
2
5
10
30
60
minutes
p-ATF-2
p-ATF-2
+ SB203580

Figure 29. MKK6 increases the promoter activity of SM α-actin, SM-MHC and SM22α in a p38 MAP kinase dependent manner

PAC-1 cells were transfected with 30ng of the SM α-actin-, SM-MHC- or SM22α-luciferase promoter-reporter plasmids (described in methods) along with empty vector control (Control) or active MKK6 (MKK6(E)) alone or combined with dominant negative p38 MAP kinase (-p38α_{AF} or + p38α_{AF}, respectively) using LipofectAMINE™ transfection reagent. Cells were refed with media supplemented with 10% FBS following 12 hours of transfection. Luciferase activity was measured 24 hours after re-feeding. (* $p < 0.001$ compared to control -p38α_{AF}, † $p < 0.01$ compared to MKK6 -p38α_{AF})

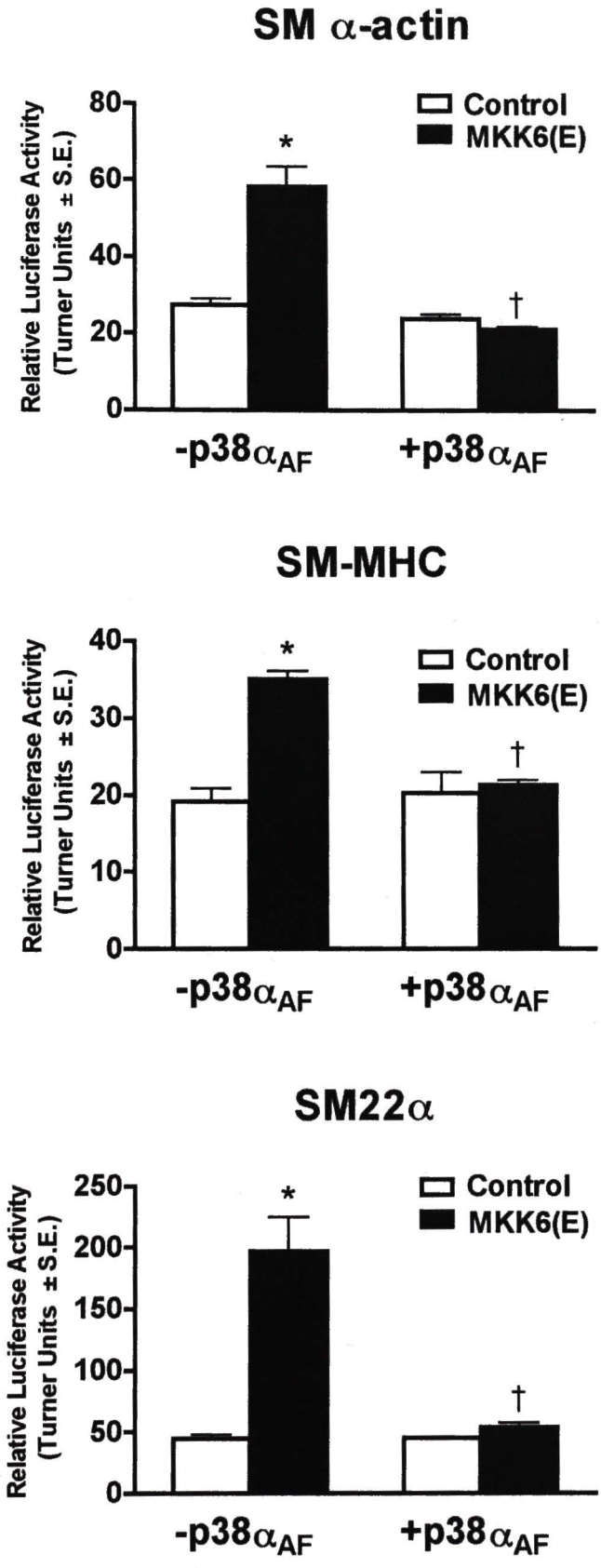
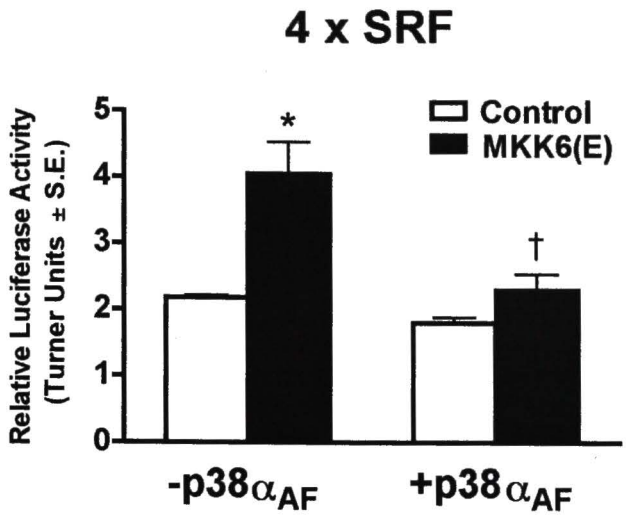
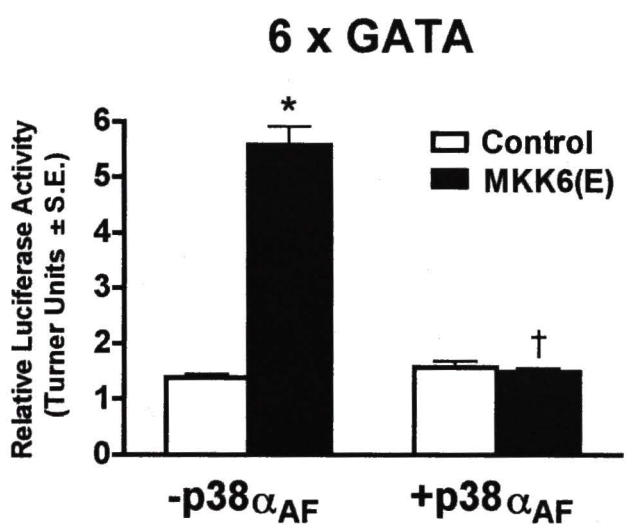

SM α-actin
Relative Luciferase Activity (Turner Units ± S.E.)
80
60
40
20
0
Control
MKK6(E)
*
†
-p38αAF
+p38αAF
SM-MHC
Relative Luciferase Activity (Turner Units ± S.E.)
40
30
20
10
0
Control
MKK6(E)
*
†
-p38αAF
+p38αAF
SM22α
Relative Luciferase Activity (Turner Units ± S.E.)
250
200
150
100
50
0
Control
MKK6(E)
*
†
-p38αAF
+p38αAF

Figure 30. MKK6 increases the activity of the transcription factors SRF and GATA in a p38 MAP kinase dependent manner

PAC-1 cells were transfected with 100ng of the 4 x SRF-, 6 x GATA- or 3 x MEF2-luciferase enhancer-reporter plasmids (described in methods) along with empty vector control (Control) or active MKK6 (MKK6(E)) alone or combined with dominant negative p38 MAP kinase (-p38α_{AF} or + p38α_{AF}, respectively) using LipofectAMINE™ transfection reagent. Cells were refed with media supplemented with 10% FBS following 12 hours of transfection. Luciferase activity was measured 24 hours after re-feeding. (* $p < 0.01$ compared to control -p38α_{AF}, † $p < 0.05$ compared to MKK6 -p38α_{AF})


4 x SRF
Relative Luciferase Activity
(Turner Units ± S.E.)
0
1
2
3
4
5
*
†
Control
MKK6(E)
-p38αAF
+p38αAF


6 x GATA
Relative Luciferase Activity
(Turner Units ± S.E.)
0
1
2
3
4
5
6
*
†
Control
MKK6(E)
-p38αAF
+p38αAF

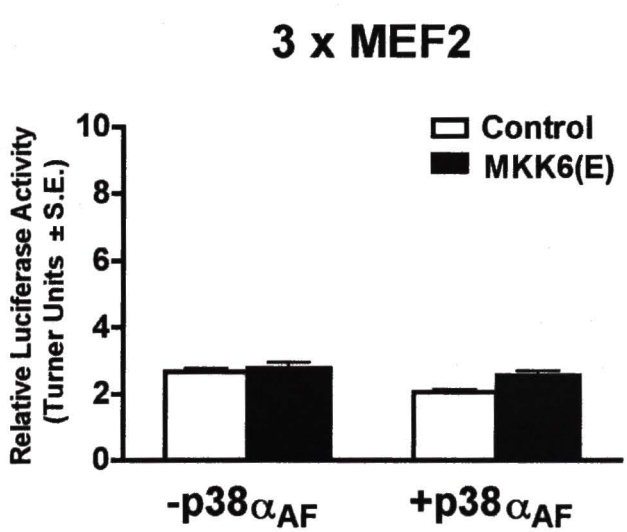
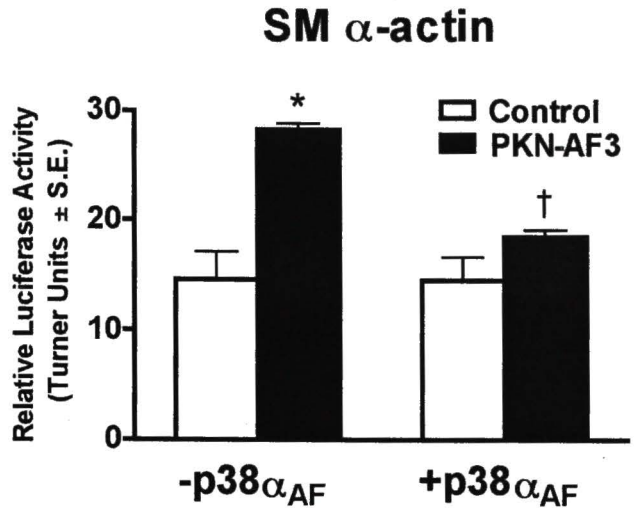
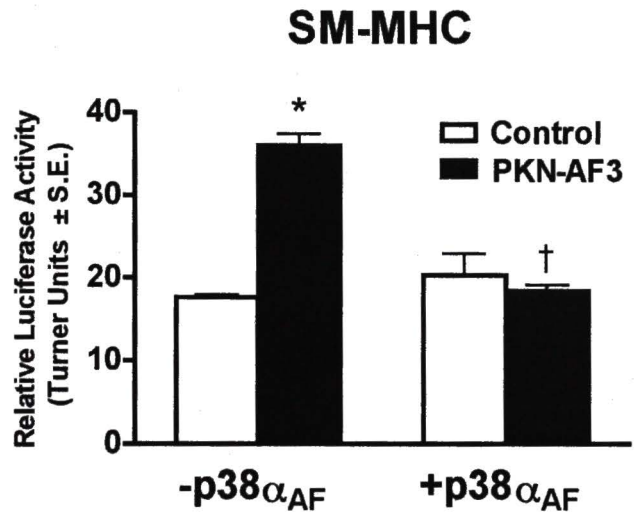

3 x MEF2
Relative Luciferase Activity
(Turner Units ± S.E.)
0
2
4
6
8
10
Control
MKK6(E)
-p38αAF
+p38αAF

Figure 31. PKN increases the promoter activity of SM α-actin, SM-MHC and SM22α in a p38 MAP kinase dependent manner

PAC-1 cells were transfected with 30ng of the SM α-actin-, SM-MHC- or SM22α-luciferase promoter-reporter plasmids (described in methods) along with empty vector control (Control) or active PKN (PKN-AF3) alone or combined with dominant negative p38 MAP kinase (-p38α_{AF} or + p38α_{AF}, respectively) using LipofectAMINE™ transfection reagent. Cells were refed with media supplemented with 10% FBS following 12 hours of transfection. Luciferase activity was measured 24 hours after re-feeding. (* $p < 0.01$ compared to control -p38α_{AF}, † $p < 0.05$ compared to PKN-AF3 -p38α_{AF})


SM α-actin
Relative Luciferase Activity (Turner Units ± S.E.)
30
20
10
0
*
†
Control
PKN-AF3
-p38αAF
+p38αAF


SM-MHC
Relative Luciferase Activity (Turner Units ± S.E.)
40
30
20
10
0
*
†
Control
PKN-AF3
-p38αAF
+p38αAF

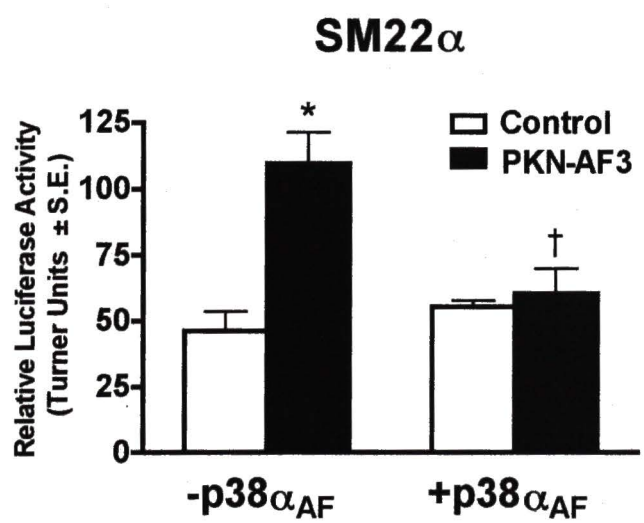
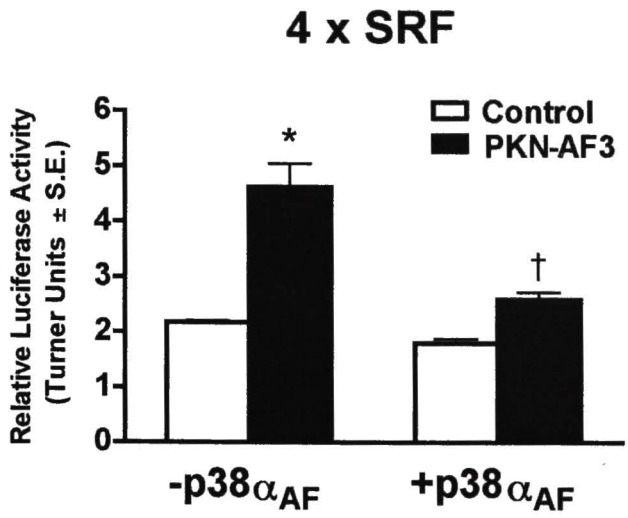
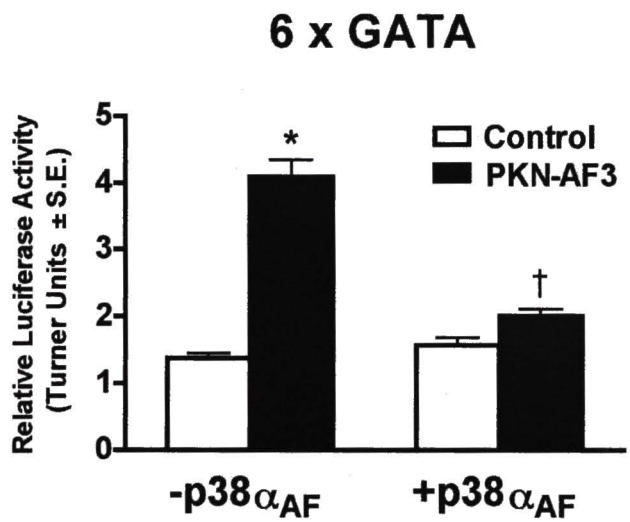

SM22α
Relative Luciferase Activity (Turner Units ± S.E.)
125
100
75
50
25
0
*
†
Control
PKN-AF3
-p38αAF
+p38αAF

Figure 32. PKN increases the activity of the transcription factors SRF, GATA and MEF2 in a p38 MAP kinase dependent manner

PAC-1 cells were transfected with 100ng of the 4 x SRF-, 6 x GATA- or 3 x MEF2-luciferase enhancer-reporter plasmids (described in methods) along with empty vector control (Control) or active PKN (PKN-AF3) alone or combined with dominant negative p38 MAP kinase (-p38α_{AF} or + p38α_{AF}, respectively) using LipofectAMINE™ transfection reagent. Cells were refed with media supplemented with 10% FBS following 12 hours of transfection. Luciferase activity was measured 24 hours after re-feeding. (* $p < 0.001$ compared to control -p38α_{AF}, † $p < 0.01$ compared to PKN-AF3 -p38α_{AF})


4 x SRF
Relative Luciferase Activity
(Turner Units ± S.E.)
Control
PKN-AF3
*
†
-p38αAF
+p38αAF


6 x GATA
Relative Luciferase Activity
(Turner Units ± S.E.)
Control
PKN-AF3
*
†
-p38αAF
+p38αAF

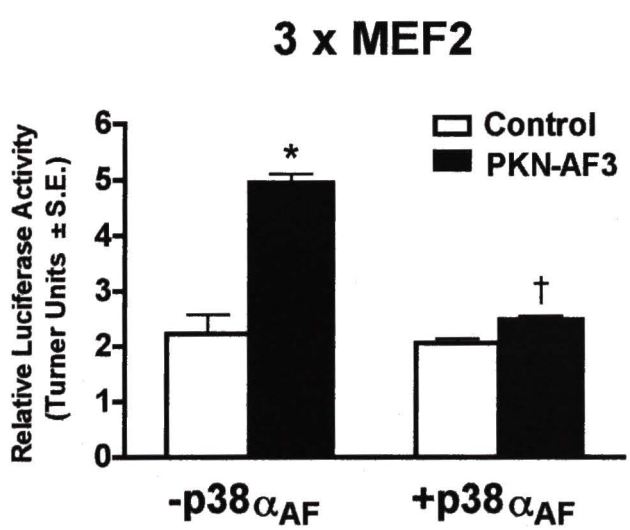
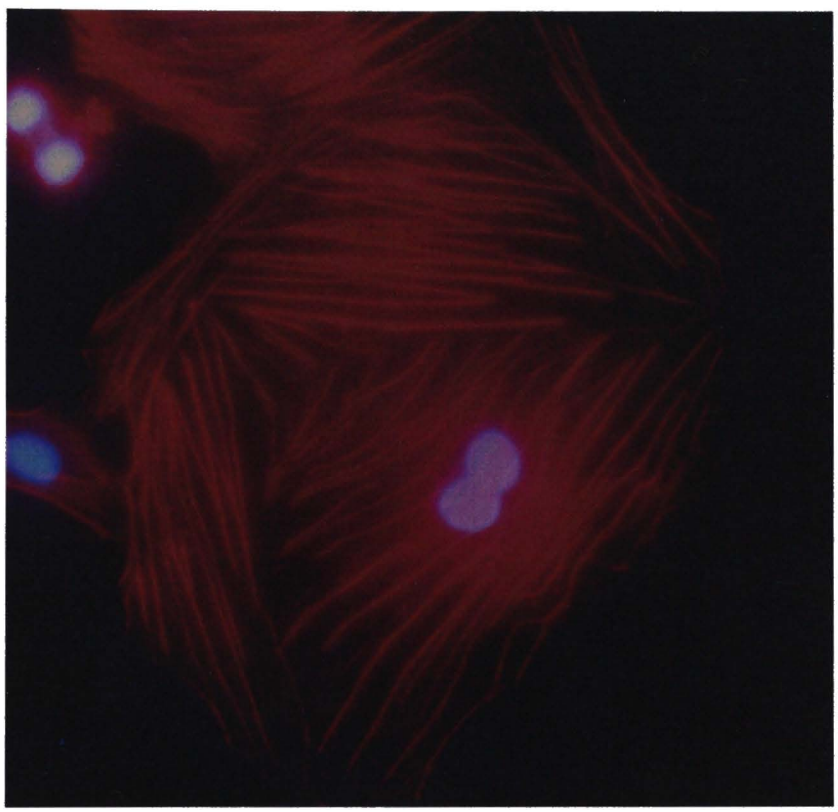

3 x MEF2
Relative Luciferase Activity
(Turner Units ± S.E.)
Control
PKN-AF3
*
†
-p38αAF
+p38αAF

Figure 33. TGF-β1 induces hypertrophy of a subset of PAC-1 cells

PAC-1 cells were cultured on gelatin-coated cover slips in media containing 10% FBS + TGF-β1 (2.5ng/mL). Following treatment, actin was visualized by indirect immunocytochemistry using an antibody that specifically binds to smooth muscle alpha actin. Nuclei (blue) were visualized by DAPI staining.



CHAPTER IV

DISCUSSION

In the healthy adult animal, differentiated smooth muscle cells play an important role in the regulation of arterial tone, blood flow and blood pressure. However, during vascular disease, SMCs undergo a phenotypic switch from the differentiated phenotype to a synthetic, proliferative phenotype. In the proliferative phenotype, SMCs regain the ability to migrate from the medial layer of the vessel into the intima, where their aberrant proliferation contributes to the formation of atherosclerotic and restenotic lesions. Thus, understanding the molecular mechanisms that promote and maintain SMCs in the differentiated phenotype will ultimately contribute to the development of novel therapeutics to block the contribution of SMC proliferation to vascular disease.

A role for TGF-β1 in the differentiation of SMCs has been demonstrated both *in vitro* and *in vivo* (42, 83, 103) (13). However, despite the importance of TGF-β1 on SMC phenotype, very little is known regarding the molecular mechanisms through which TGF-β1 elicits its downstream effects. Of interest, it has been recently demonstrated that TGF-β1 can activate the small GTPase RhoA in epithelial cells undergoing EMT, which results in a myofibroblast-like phenotype due to the up-regulation of SM α-actin expression in these cells (12, 104). Moreover, TGF-β1 and RhoA are known to produce similar phenotype changes in SMC (55, 96). When taken together, these data support the hypothesis that TGF-β1 may mediate its downstream effects on SMC differentiation

through the activation of a RhoA-driven signaling cascade. The goals of the present study were to i.) examine the ability of TGF-β1 to stimulate the differentiation of PAC-1 smooth muscle cell line, ii.) determine whether RhoA signaling was required for TGF-β1-mediated effects, iii.) determine the specific role of PKN in SM-specific gene expression and iv.) elucidate the important signaling pathways through which TGF-β1 and PKN elicit their downstream effects.

Data from the present study demonstrate that TGF-β1 does indeed cause differentiation of PAC-1 smooth muscle cells. TGF-β1 stimulated SM α-actin re-organization as well as other morphological changes indicative of differentiation. Importantly, TGF-β1 up-regulated the expression of SM-specific marker genes in PAC-1 cells, presumably by increasing the activity of the promoters that regulate their expression. Lastly, TGF-β1 inhibited serum-induced proliferation of PAC-1 cells. Interestingly, despite the fact that TGF-β1 could reduce the rate of proliferation of PAC-1 cells, TGF-β1 was not sufficient to completely block all of the mitogenic signals present in serum, as evidenced by the higher rate of proliferation of TGF-β1 treated PAC-1 cells compared to cells simulated to differentiate by serum withdrawal. This is not surprising as serum contains numerous growth factors that are mitogenic to SMCs in culture. TGF-β1 may inhibit the mitogenic effects of a discreet subset of these factors, but may have no effect on others. In particular, TGF-β1 is known to block PDGF-induced proliferation of SMC (13). Since serum undoubtedly contains PDGF, the inhibitory effects of TGF-β1 on SMC proliferation may be mediated though a similar effect in PAC-1 cells, although no direct evidence for the existence of this mechanism is provided in the present study.

In addition, SMCs have been shown to be heterogeneous both *in vivo* and *in vitro* (35, 47, 48). Indeed, the study by Frid *et al.* specifically highlights the heterogeneity of pulmonary smooth muscle cells (35). Although PAC-1 cells have not been characterized to be heterogeneous in nature, the possibility exists that they do contain distinct subpopulations of cells. Therefore, it is possible that different subpopulations of SMCs would respond differently to TGF-β1, which would explain why some cellular proliferation persists following TGF-β1 treatment.

Through the use of two chemical inhibitors, HA1077 and Y-27632, TGF-β1-induced differentiation of PAC-1 cells was shown to require the activity of the main downstream targets of RhoA. These inhibitors, while marketed as Rho kinase-specific inhibitors, have been shown to inhibit the kinase activity of Rho kinase and PKN/PRK2 (4, 27, 65). Thus, although these inhibitors cannot distinguish between these proteins, their use can provide direct evidence that one or more of these kinases are important for mediating the downstream functions of TGF-β1. Indeed, HA1077 and Y-27632 inhibited the ability of TGF-β1 to stimulate actin re-organization of PAC-1 cells. In addition, both inhibitors effectively blocked the up-regulation of SM-specific gene expression stimulated by TGF-β1 and attenuated the ability of TGF-β1 to activate the promoters that control the expression of these genes. Lastly, HA1077 and Y-27632 also reversed the inhibitory effect of TGF-β1 on the rate of proliferation of PAC-1 cells. When combined, the data generated from the use of HA1077 and Y-27632 strongly argue that RhoA and its downstream signaling targets play an important role mediating the downstream responses of TGF-β1 on SMC differentiation.

TGF-β1 has been recently shown to stimulate RhoA activity in multiple cell types, although it has not been demonstrated to do so in SMCs (12, 29, 104). Supporting the data collected from the use of HA1077 and Y-27632, TGF-β1 stimulation of PAC-1 cells was associated with an increase in the amount of active, GTP-bound RhoA. This occurred in a time dependent manner and was transient in nature, occurring following 2-5 minutes of TGF-β1 stimulation. Additionally, phosphorylation of the activation loop of PKN and PRK2, which indicates the activation state of these proteins, was also increased in a time dependent manner by TGF-β1. PKN/PRK2 phosphorylation was increased within 5 minutes of TGF-β1 stimulation and remained elevated throughout the full 60 minutes of treatment. Since PKN/PRK2 require the binding of GTP-bound RhoA for their activation, it correlates well that the phosphorylation of PKN/PRK2 appears following 5 minutes of TGF-β1 stimulation, just after the increase in GTP-bound RhoA occurs. While the remainder of data presented here focused on the downstream effects of PKN, it is likely that PKN and PRK2 may have overlapping roles in this system since both are activated in a similar manner in response to TGF-β1. Further studies will be needed to determine the relative similarities and differences between PKN and PRK2 with respect to SMC differentiation.

Corresponding with its increased activity following TGF-β1 stimulation, the specific activation of PKN alone was capable of regulating SMC-specific marker gene expression. Constitutively active PKN trans-activated the SM α-actin, SM-MHC and SM22α promoters in a dose dependent manner. Moreover, this effect was dependent upon the kinase activity of PKN, as a kinase dead form of PKN (K644E) was unable to

stimulate the activity of any of the SMC-specific promoters tested. Because the kinase activity of PKN was required for its ability to increase promoter activity, this argues that PKN elicits its effects through the activation of other downstream targets (such as other signaling molecules or transcription factors). Accordingly, PKN was also capable of stimulating the activity of the transcription factors SRF, GATA and MEF2 in a dose- and kinase-dependent manner. While there are no SMC-specific transcription factors, most SMC-specific marker genes contain one or more consensus SRF binding sites (CArG box elements) within their promoters, and it is well accepted that these sites are required for proper expression of these genes in SMCs (85, 95, 100, 165). In addition, expression and activity of GATA-6, the prevalent isoform of GATA in SMCs, is regulated by SMC phenotype. Suzuki *et al.* demonstrated that expression of GATA-6 is rapidly down-regulated when SMCs are stimulated to proliferate (168). Mano *et al.* further expanded these results by demonstrating that restoration of GATA-6 expression following balloon-injury of rat carotid artery lessened neointimal formation and restored proper SMC-specific marker gene expression (101). Finally, Lin *et al.* demonstrated that MEF2 is required for normal SMC differentiation during development and Katoh *et al.* reported that MEF2 is important for SM-MHC expression, implying that MEF2 may also participate in SMC differentiation (71, 89). It is also interesting to note that RhoA signaling has been reported to regulate the activity and/or expression of each of these transcription factors in all three muscle cell types (23, 96, 171). Interestingly, Mack *et al.* reported that the effect of RhoA on SRF activity could be blocked by the use of Y-27632, which they conclude to be due to the "specific" inhibition of Rho kinase (96). However,

based on the data presented here, the effects of RhoA could have also been caused by the activation of PKN. When taken together, the ability of PKN to trans-activate the SMC-specific marker gene promoters as well as increase the activity of these key transcription factors further supports our hypothesis that this signaling pathway is important for promoting expression of SMC-specific marker genes in differentiated SMCs.

Virtually nothing is known regarding the signal transduction pathway(s) through which PKN elicits its downstream effects on transcription factor activation and gene expression. Marinissen *et al.* demonstrated that PKN alters gene expression though activation of p38 MAP kinases in NIH-3T3 and HEK 293 cells (102). Moreover, it was recently shown that PKN directly binds to both p38 MAP kinase and MKK6, one of the kinases responsible for the activation of p38 MAP kinase (169). This strongly argues that p38 MAP kinase is an important downstream target of PKN. Accordingly, in PAC-1 cells, the phosphorylation and kinase activity of p38 MAP kinase were increased following 5-10 minutes of TGF-β1 stimulation. The appearance of p38 MAP kinase activity following 5-10 minutes of TGF-β1 stimulation correlates with it being a downstream target of PKN, as the phosphorylation of PKN/PRK2 increased following 5 minutes of TGF-β1 stimulation. The phosphorylation of MKK3 and MKK6 were also increased following TGF-β1 stimulation. Interestingly, while the phosphorylation of MKK3/6 increased slightly upon the onset of TGF-β1 stimulation, it did not reach peak activity until 30 to 60 minutes of treatment, which is later than the time of p38 MAP kinase activity. These results raise the possibility that an MKK-independent mechanism may be responsible for the initial early activation of p38 MAP kinase. Although the

classic activation of p38 MAP kinase involves phosphorylation by MKKs, an MKK-independent mechanism to activate p38 has been reported by Ge *et al.* Of particular interest, this mechanism involves the interaction of p38 MAP kinase with a protein called TAB1 (transforming growth factor-beta-activated protein kinase 1-binding protein) resulting in the autophosphorylation and activation of p38 MAP kinase (39). Whether this mechanism is operative in PAC-1 cells and how PKN fits into this novel signaling mechanism will be an interesting area for future research.

Unfortunately, there is no known way to make p38 MAP kinase constitutively active. Thus, to test whether p38 MAP kinase is important for the regulation of the SM-specific promoter-reporters and the transcription factor enhancer-reporters, a constitutively active form of MKK6, which results in the activation of p38 MAP kinase, was used. Despite the discrepancy in the time of activation of MKK3/6 and p38 MAP kinase following TGF-β1 stimulation, over-expression of active MKK6 was capable of stimulating the SM-specific promoter-reporters in a p38 MAP kinase dependent manner. Interestingly, MKK6 also stimulated SRF- and GATA-dependent transcription (which was dependent on p38 MAP kinase), but had no effect on the activation of MEF2-dependent transcription in PAC-1 cells. This could be due to one of three reasons: i.) activation of MEF2-dependent transcription by p38 MAP kinase requires an MKK other than MKK6, ii.) activation of MEF2-dependent transcription is regulated by p38 MAP kinase, but in an MKK-independent manner or iii.) MEF2-dependent transcription is not regulated by p38 MAP kinase in PAC-1 cells. Since MEF2 is known to be regulated by p38 MAP kinase in other cell types, it is highly unlikely that the activation of MEF2 in

PAC-1 cells is independent of p38 MAP kinase (14, 195). Indeed, PKN-mediated activation of the three SMC-specific marker gene promoters (SM α-actin, SM-MHC and SM22) and the three key transcription factors (SRF, GATA and MEF2) could be almost completely abolished by co-expression of dominant negative p38 MAPK, demonstrating its requirement in mediating the downstream effects of PKN on SM-specific gene expression. It will be of great interest to discern the mechanism through which PKN regulates p38 MAP kinase activity, especially with respects to MEF2.

While p38 MAPK has not been previously characterized to play a role in SMC differentiation, it is known to be critical for skeletal muscle differentiation through activation of MEF2-dependent gene transcription (195). Moreover, p38 MAP kinase has been shown to directly phosphorylate and activate GATA in cardiomyocytes and is associated with an increase in SRF-dependent transcription in SMCs corresponding to up-regulation of SM α-actin gene expression (23, 38). Based on these reports and data presented here, it is easy to speculate that p38 MAP kinase may play a similar role in the regulation of muscle-specific genes in all muscle types. Whether PKN-mediated activation of p38 MAPK is critical for the up-regulation of muscle-specific genes in other cell types will be an interesting area for future studies.

The p38 MAPK family of enzymes represents only one branch of the MAP kinase signaling cascade. Activation of the ERK and JNK branches of MAP kinase signaling has been shown to be associated with growth and proliferation in multiple cell types including SMCs. Multiple researchers have reported that ERK and JNK proteins are rapidly activated following balloon-injury (60, 74). Moreover, Izumi *et al.* found that

inhibition of ERK and JNK blocked neointimal formation resulting from balloon-injury by reducing proliferation of the underlying SMCs (66). In addition, they found that inhibiting ERK and JNK also attenuated serum induced SMC proliferation *in vitro*. Thus, the effect of TGF-β1 on the activity of the other MAP kinase pathways, ERK and JNK, was also examined. In PAC-1 cells, the activities of ERK1/2 and JNK1/2 were quite robust in proliferating (control) cells, which is consistent with data from these previous studies. Furthermore, treatment of these cells with TGF-β1 reduced the phosphorylation of ERK1/2 and JNK1/2 indicative of a decrease in their activities. These results are consistent with our model of TGF-β1 induced differentiation of SMCs. It is not clear whether TGF-β1-induced activation of p38 MAPK plays a direct role in inhibiting ERK and JNK activity in SMCs, or whether other signaling pathways downstream of TGF-β1 mediate this effect. However, it is interesting to note that p38 MAPK has been shown to negatively regulate angiotensin II- and PDGF-induced activation of ERK as well as increases in expression of cyclin D1 and DNA synthesis in SMCs (75, 133).

The findings of these studies delineate a novel signaling pathway through which TGF-β1 activates the small GTPase RhoA and its downstream target PKN to induce SMC differentiation. Conversely, activation of RhoA and Rho kinase has also been shown to regulate processes involved in cell growth and proliferation, including increasing expression of immediate early genes such as c-*fos*. Seasholtz *et al.* found that thrombin stimulated an increase in SMC migration and DNA synthesis, which could be attenuated using inhibitors of RhoA and Rho kinase (158). However, they also found that

constitutively active RhoA could not produce a change in DNA synthesis unless combined with active Ras, indicating that the effects of thrombin on SMC proliferation are not solely dependent upon RhoA-mediated signaling. Moreover, Mack *et al.* demonstrated that constitutively active RhoA had no effect on c-*fos* promoter activity in SMCs but could robustly activate the SM α-actin and SM22 promoters (96). Other studies have demonstrated that RhoA expression and activity is elevated in several different models of hypertension (159). Angiotensin II (AngII), which is known to be elevated in most hypertensive animal models, was recently shown to increase the activity of RhoA in SMCs (190). AngII treatment of cultured SMCs also resulted in increased protein synthesis, activation of ERK signaling and expression of c-*fos*. Interestingly, inhibition of RhoA signaling blocked AngII-induced protein synthesis, but had little to no effect on ERK activation or c-*fos* expression. These findings argue that RhoA signaling may be more involved in mediating a hypertrophic response (perhaps by increasing expression and synthesis of contractile apparatus-associated proteins), versus a hyperplastic response during hypertension. Medial SMC hypertrophy is known to develop during hypertension (130, 131). In addition, it has been proposed that TGF-β1 may stimulate SMC hypertrophy. Owens *et al.* demonstrated that TGF-β1-induced growth arrest of SMCs was associated with an increase in the overall cell size and protein content and that there was a slight increase in G_2-arrested cells (130). Furthermore, PKN activation has been previously shown to induce G_2 cell cycle arrest in other cell types (109). Interestingly, treatment of PAC-1 smooth muscle cells with TGF-β1 also increased the number of hypertrophied cells in culture, although only a small

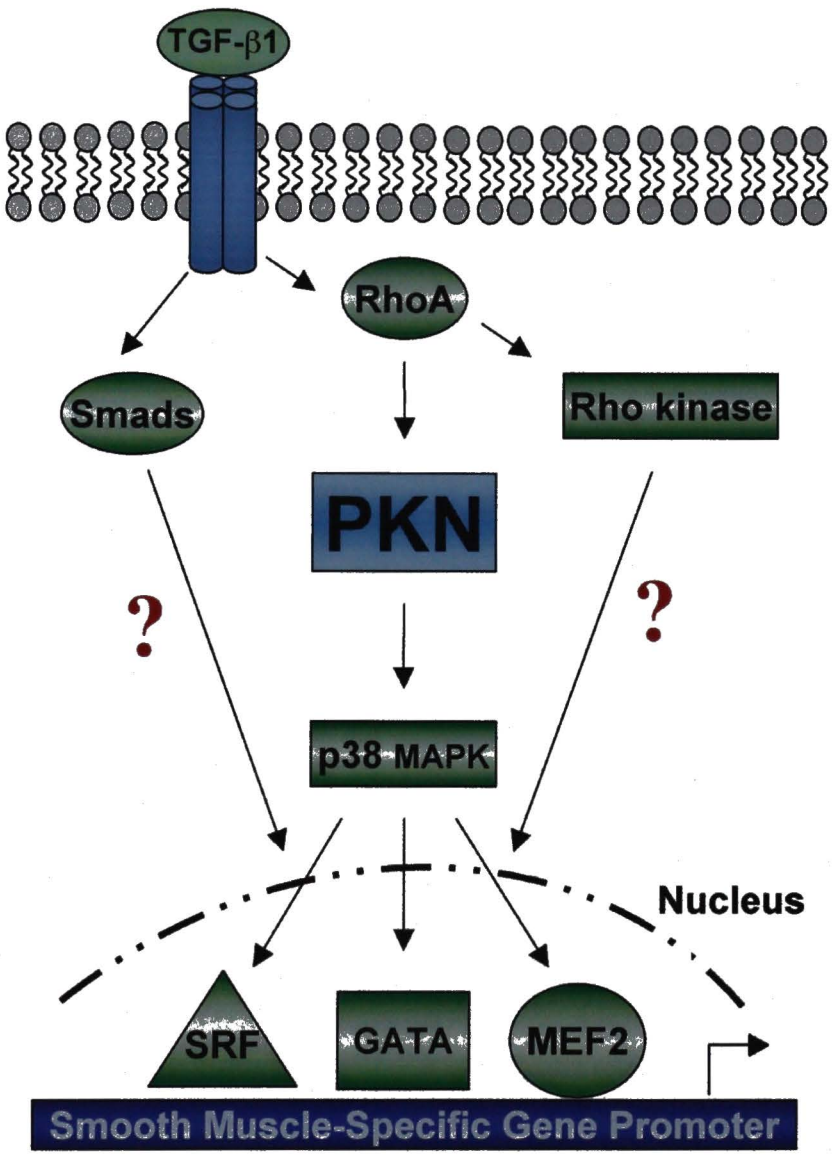
subpopulation of cells seemed to respond in this way. These cells were 2-3 times the size of a normal PAC-1 cell and were usually (although not always) multinucleated. Again, the heterogeneity of SMCs in culture may also play a role in whether the cell responds by differentiating (from a synthetic to contractile state) or hypertrophying (from a normal contractile to a hypertrophied state). Thus, it is plausible that TGF-β1/PKN-mediated increases in SMC-specific marker gene expression may result in either differentiation or cellular hypertrophy depending on the SMC phenotype at time of stimulus.

In summary, the present study demonstrates that PKN plays a pivotal role in the up-regulation of SMC-specific marker gene expression. TGF-β1 up-regulates expression of SMC-specific marker genes, stimulates actin re-organization and reduces proliferation of PAC-1 smooth muscle cells. These changes are indicative of SMC differentiation and the onset of a working, contractile phenotype. We show that these changes correlate with a concomitant increase in the activities of RhoA and PKN. Furthermore, our data clearly demonstrate that PKN trans-activates three SMC-specific promoters, whose gene products are contractile apparatus-associated proteins expressed only in differentiated SMCs. Although the exact mechanisms through which PKN elicits these changes in promoter activity have not been completely elucidated, we show that it involves activation of the transcription factors SRF, GATA and MEF2. We also demonstrate that PKN-mediated activation of these transcription factors and SMC-specific promoters is dependent upon p38 MAP kinase signaling. It is easy to envision a linear signaling pathway involving RhoA/PKN-mediated activation of p38 MAPK, which in turn activates SRF, GATA and MEF2 leading to up-regulation of SMC-specific marker gene

expression. However, other downstream targets of TGF-β1 (such as Smads) as well as other downstream targets of RhoA (such as Rho kinase) may also play a role in the up-regulation of SM-specific gene expression required for the differentiated, work phenotype of SMCs (Figure 34). We have identified PKN as an important component of this complex signaling pathway. Understanding how PKN regulates SMC phenotype (differentiation and/or potentially hypertrophy) in cooperation with other downstream targets of RhoA will provide insight into potential therapeutic targets designed to prevent the aberrant proliferation of SMCs that contributes to the pathophysiology of vascular disease.

Figure 34. Schematic pathway illustrating the downstream targets of TGF-β1 that may cooperate to control SMC differentiation, including the recently identified PKN

A role for PKN in the expression of SM-specific marker genes has been demonstrated. However, the downstream effects most likely require multiple signaling pathways including cooperation between Smads and RhoA activated kinases such as PKN and Rho kinase.


TGF-β1
RhoA
Smads
Rho kinase
PKN
?
?
p38 MAPK
Nucleus
SRF
GATA
MEF2
Smooth Muscle-Specific Gene Promoter

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Data described in this dissertation clearly demonstrate an important role for TGF-β1 in the regulation of vascular smooth muscle cell differentiation. TGF-β1 stimulates differentiation of PAC-1 smooth muscle cells based on 3 independent criteria: actin re-organization and change in cell morphology, up-regulation of SM-specific marker gene expression and inhibition of cellular proliferation. These three criteria, when put together, strongly argue that PAC-1 cells treated with TGF-β1 adopt a differentiated, contractile phenotype. Based on these studies, PAC-1 cells can be used as a model system to study the molecular processes involved in both differentiation (based on treatment with TGF-β1 or serum withdrawal) was well as processes involved in proliferation (by culturing cells in media supplemented with serum or specific growth factors). Findings from studies done using PAC-1 cells, including the data collected for this dissertation, must be confirmed using other smooth muscle cell culture systems, such as primary SMC cultures, to ensure that the effects seen are not specific to PAC-1 cells. Moreover, these studies should be re-examined using *in vivo* models, such as transgenic mice, to confirm that the results are not an artifact of SMCs in culture. As previously mentioned, genetic knockout of TGF-β1 or the TGF-β type I receptor results in embryonic lethality in mice (83, 103). Interestingly, however, targeted over-expression of TGF-β1in the vasculature is also embryonic lethal (2). Because TGF-β1 can produce

a wide range of effects on different tissue types, conditional expression of TGF-β1 or its signaling components may allow for better study of their functions *in vivo* by bypassing any lethal effects during development. Moreover, because TGF-β1 can have a multitude of different effects *in vivo*, individually studying the downstream targets of TGF-β1 (such as PKN) may provide better insight to that specific aspect of TGF-β1 signaling. Of interest, if PKN is indeed involved in maintaining the differentiated phenotype of SMCs, transgenic mice over-expressing PKN might be resistant to the development of restenotic lesions following balloon angioplasty. Moreover, crossing PKN transgenic mice with ApoE-/- deficient mice would provide insight on whether targeted over-expression of PKN can lessen or prevent the development of atherosclerosis in this animal model. The generation and development of these mice will make for interesting future studies.

The data presented here show that treatment of PAC-1 smooth muscle cells with TGF-β1 is associated with an increase in the activity of the small GTPase RhoA. As previously mentioned, TGF-β1 has been shown to increase the activity of RhoA in epithelial cells undergoing EMT (12, 104). In these studies, the authors did not provide any data as to how TGF-β1 results in the activation of RhoA. The TGF-β1 receptor has not been linked to changes in RhoA activity in previous studies. However, a recent study in Swiss 3T3 cells demonstrated that TGF-β1 treatment led to an increase in expression of the Rho-GEF, NET1, which was required for stress fiber formation (164). They also reported that this mechanism required Smad-dependent up-regulation of NET1 gene expression. Data presented here show that in PAC-1 cells, RhoA is activated within 2-5 minutes of TGF-β1 stimulation. Because of the very quick and transient time frame, the

mechanism of TGF-β1 activation of RhoA in PAC-1 cells must not be dependent upon new transcription of a Rho-GEF as was reported by Shen *et al* (164). However, it remains possible that TGF-β1 can increase the activity of a Rho-GEF in PAC-1 cells, such as NET1 or others, which would be consistent with the rapid activation of RhoA. The mechanism of TGF-β1-mediated activation of RhoA thus also provides an interesting area for future research.

In addition to increasing the activity of RhoA, TGF-β1 stimulation of PAC-1 cells also results in an increase in the phosphorylation of PKN and its closely related family member, PRK2, indicating an increase in the activity of these proteins. The specific activity of PKN was further examined by over-expressing a constitutively active form of this protein. From these studies, it was shown that PKN plays an important role in up-regulating the expression of SM α-actin, SM-MHC and SM22α in PAC-1 cells. Because TGF-β1 activates both PKN and PRK2, it is likely that these enzymes might both contribute to the up-regulation of SM-specific marker gene expression in PAC-1 cells. Based on previous studies, PKN and PRK2 have been shown to have overlapping functions. Specifically, PRK2, much like PKN stimulates SRF-dependent transcription (43). Moreover, PRK2 has also been previously shown to participate in actin re-organization, although this effect has not been tested in SMC (179). In addition to PRK2, other downstream targets of RhoA, including Rho kinase, may also mediate some of the downstream effects of TGF-β1 on SMC differentiation. The data presented in this dissertation show that the inhibitors HA1077 and Y-27632 both attenuate actin re-organization, SM-specific marker gene expression and inhibition of serum-induced

proliferation induced by TGF-β1 treatment of PAC-1 cells. Because these inhibitors block the function of PKN, PRK2 and Rho kinase, it cannot be determined which of these enzymes is important for different aspects of TGF-β1-induced differentiation (4, 27, 65). This can be accomplished through the use of RNA interference (RNAi) by designing specific small interfering RNA (siRNA) to individually inhibit expression of each of these enzymes. It is unlikely that all three of these enzymes have completely redundant roles in SMCs. Thus, it will be extremely important to determine the specific contribution of each of these enzymes to the differentiation of SMCs.

In addition to stimulating the activity of the promoters that control the expression of SM-specific marker genes, data presented here show that PKN activates the transcription factors SRF, GATA and MEF2 in PAC-1 cells. As previously discussed, the activity of these transcription factors is required for the proper expression of SM-specific marker genes in differentiated SMCs (71, 85, 89, 95, 100, 101, 165, 168). Accordingly, the ability of PKN to activate these transcription factors provides a mechanism through which PKN increases SM-specific marker gene expression in PAC-1 cells. In addition, data presented here demonstrate that the ability of PKN to regulate the SM-specific promoters as well as the transcription factor enhancers is dependent upon the activity of p38 MAP kinase. p38 MAP kinase is a known downstream target of PKN (102, 169). Exactly how PKN and p38 MAP kinase regulate the transcriptional activity of these three transcription factors will also be an interesting area for future research. Potential mechanisms include: i.) post-translational modification, such as phosphorylation, ii.) increased expression of these transcription factors or iii.) enhanced

binding of these transcription factors to their consensus DNA binding elements. Indeed, SRF, GATA and MEF2 can all be phosphorylated in other systems, although the relative contribution of this modification to the activity of these proteins in is not exactly clear (14, 23, 150). Moreover, the expression of SRF and GATA-6 is regulated by SMC phenotype (18, 101, 168).

In addition to activating proteins that positively regulate the differentiation of PAC-1 cells, TGF-β1 may also decrease the activity of proteins that are involved in stimulating proliferation of these smooth muscle cells. Data presented in this dissertation show that TGF-β1 stimulation of PAC-1 cells decreases the phosphorylation status of both ERK1/2 and JNK1/2. These enzymes have been traditionally characterized to be associated with growth and proliferation of most cell types including SMCs (60, 66, 74). It is interesting to speculate that the ability of TGF-β1 to block enzymes that promote proliferation may also contribute its effect on SMC differentiation. Future research in this area should examine whether ERK and JNK can regulate the transcription of genes known to be expressed in proliferating SMCs, such as the non-muscle isoform of myosin (SMemb) and growth related genes such as cyclins and PCNA. Moreover, whether TGF-β1 can down-regulate the expression of these growth associated genes will also be an important area for future studies.

The data presented here have focused primarily on the effects of TGF-β1 and PKN with respect to morphological changes (actin re-organization) and gene expression (up-regulation of SM-specific marker genes) associated with the induction of SMC differentiation. However, in addition to changes in cytoskeletal structure and gene

expression, the differentiation of SMCs also involves the exit of the cells from the cell cycle. Interestingly, TGF-β1, PKN and Rho kinase have all been reported to cause cell cycle arrest in multiple cell types. Specifically, TGF-β1 has been documented to cause both G_1 and G_2 arrest in SMCs. Owens *et al.* previously reported that TGF-β1 stimulation of rat aortic SMCs causes an increase in G_2-arrest (130). Furthermore, it was recently reported that inhibition of TGF-β1 in SMCs (through the use of antisense plasmid DNA) leads to an increase in cellular proliferation, associated with a decrease in the cyclin-dependent kinase inhibitor p21 and an increase in the expression of c-*myc* (an early response gene involved in cell growth) (72). Lastly, a recent study by Bhowmick *et al.* demonstrated that TGF-β1 activation of Rho kinase results in the phosphorylation and inhibition of Cdc25A, a phosphatase required for progression through G_1 into S-phase (11). Interestingly, it has also been reported that PKN can cause G_2-arrest of cycling *Xenopus* embryos through the direct phosphorylation and inhibition of Cdc25C, a phosphatase required for G_2 to M-phase progression (109). Based on this previously reported data and the data generated from the present studies, it is easy to speculate that TGF-β1-induced differentiation of PAC-1 smooth muscle cells might also involve cell cycle arrest through one or more of the following mechanisms: i.) PKN-mediated inhibition of Cdc25C, ii.) Rho kinase mediated-inhibition of Cdc25A or iii.) TGF-β1 up-regulation of cyclin-dependent kinase inhibitors such as p21 and p27.

Lastly, a role for TGF-β1 in stimulating the hypertrophied phenotype of SMCs must also be addressed. During the course of the present studies, it was noted that a small fraction of PAC-1 cells treated with TGF-β1 adopt a hypertrophied phenotype.

This was determined by the large increase in cell size accompanied by gross actin re-organization and the presence of multiple nuclei in these cells. Further studies are needed to truly dissect the hypertrophy response of these cells, including: i.) quantification of the change in cell size, ii.) measurement of changes in protein synthesis by leucine incorporation, iii.) measurement of changes in DNA synthesis by thymidine incorporation, iv.) analysis of changes in cell cycle events by flow cytometry, v.) analysis of changes in DNA content (ploidy) of the nuclei and vi.) a detailed quantification of the number of cells per cm^2 that respond to TGF-β1 by hypertrophy. It is unknown why only a fraction of the cell population responds in this manner, although this may possibly be explained by the known heterogeneity of SMCs in culture. This is not the first time that TGF-β1 has been reported to stimulate hypertrophy of SMCs in culture. Indeed, Owens *et al.* reported that TGF-β1 stimulation of rat aortic SMCs in culture causes an increase in hypertrophy as measured by increased protein content and cell size (130). Moreover, the authors demonstrate that TGF-β1-induced hypertrophy of SMCs is associated with an accumulation of cells in the G_2 phase of the cell cycle. As discussed above, PKN has been reported to cause G_2-arrest in other cell types through inhibition of Cdc25C (109). As such, the activation of PKN by TGF-β1 reported in the present studies provides a potential mechanism through which TGF-β1 may produce G_2-arrest in SMCs, although further studies will be needed to demonstrate that this mechanism occurs in PAC-1 cells and other SMCs in culture. As discussed in the introduction, the hypertrophied phenotype of SMCs has been associated with several models of hypertension (128, 131, 132). In addition, the activities of RhoA and Rho kinase have

also recently been shown to be elevated in several animal models of hypertension (159). This has been primarily linked to the ability of Rho kinase to phosphorylate and inhibit myosin light chain phosphatase, which controls the relaxation of SMC contraction. Normally, myosin light chain (MLC) is phosphorylated by MLC kinase in a Ca^{2+}-dependent manner, which stimulates contraction of SMCs. MLC phosphatase dephosphorylates MLC to stop the contraction (166). Thus, inhibition of MLC phosphatase by Rho kinase results in a prolonged contraction of the SMCs, which contributes to a hypercontractile state of SMCs and thus increased peripheral vascular resistance and blood pressure (175). Interestingly, much like Rho kinase, PKN has also been shown to inhibit MLC phosphatase and increase contraction of SMCs. Based on these studies and the work presented in this dissertation, it is possible to envision a mechanism whereby TGF-β1 activation of RhoA and PKN leads to up-regulation of SM-specific maker genes (contractile proteins) and an increase in SMC contraction resulting in hypertrophy and increased blood pressure leading to hypertension. Confirmation of elevated PKN activity in hypertensive animal models would provide credibility to these speculations. Clarification of the role of TGF-β1 and PKN with respect to SMC hypertrophy will be required to determine whether these signaling pathways are beneficial or detrimental to SMC function *in vivo*.

In summary, data presented in this dissertation demonstrate an important role for TGF-β1 in promoting the contractile phenotype (either differentiated or hypertrophied) of SMCs based on studies using the PAC-1 smooth muscle cell line. Data generated and presented here provide strong evidence that the small GTPase RhoA and its downstream

target PKN play an important role in this process. Although PKN may have many other functions in SMCs that are not yet well established, it is clear that PKN is involved in TGF-β1-mediated up-regulation of the SM-specific marker genes, SM α-actin, SM-MHC and SM22α, presumably through activation of the SRF, GATA and MEF2 families of transcription factors. Lastly, p38 MAP kinase is required for mediating the downstream effects of PKN on the up-regulation of these genes. This data demonstrates, for the first time, the existence of a novel signaling cascade through which TGF-β1 modulates changes in SMC phenotype.

APPENDIX

LIST OF ABBREVIATIONS USED

ACC = antiparallel coiled-coil
AngII = Angiotensin II
ApoE = apolipoprotein E
ATF-2 = activating transcription factor-2
ATP = adenosine triphosphate
BHLH protein = basic helix-loop-helix protein
BMK or ERK5 = Big MAP kinase
BSA = Bovine Serum Albumin
BTEB2 = Basic Transcription Element-Binding Protein 2
Ca^{2+} = calcium
CAGA = Smads binding element
CArG box = SRF binding element $CC(A/T_6)GG$
CHF = congestive heart failure
CRP =cysteine-rich LIM-only protein
DNA = deoxyribonucleic acid
DTT = dithiothreitol
EMT = epithelial to mesenchymal transition
ER = endoplasmic reticulum
ERK = extracellular signal-regulated kinase
ET-1 = Endothelin 1
FBS = Fetal Bovine Serum
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
GDI = GDP dissociation inhibitor
GDP = guanosine 5'-diphosphate
GEF = Guanine nucleotide exchange factor
GPCR = G-protein coupled receptor
GST = Glutathione-S-Transferase
GTP = guanosine 5'-triphosphate
HBP = high blood pressure
HBSS = hank's balanced salt solution
HCl = hydrochloric acid
HRP = horse radish peroxidase
JNK = c-jun N-terminal kinase
MADS = MCM1, Agamous, Deficiens, SRF

MAP kinase = mitogen activated protein kinase
MEF2 = myocyte enhancer factor 2
MKK = MAP kinase kinase
MLC = myosin light chain
myd = myodystrophy mutation
NM-B MHC or SMemb = non-muscle myosin heavy chain
oxLDL = oxidized LDL
p38 MAPK = p38 mitogen activated protein kinase
PAC-1 = rat pulmonary arterial smooth muscle cell line
PCNA = proliferating cell nuclear antigen
PAGE = polyacrylamide gel electrophoresis
PCR = polymerase chain reaction
PDGF = platelet derived growth factor
PKC = protein kinase C
PRK2 = protein kinase C related kinase 2 (PKN family member)
PTCA = pericutaneous transluminal coronary angioplasty
PVDF = polyvinylidene difluoride
RBD = Rho binding domain
RNA = ribonucleic acid
RNAi = RNA interference
ROCK = Rho kinase
RT-PCR = reverse transcription-polymerase chain reaction
SDS = Sodium Dodecyl Sulfate
Ser = serine
siRNA = small interfering RNA
SM α-actin = smooth muscle α-actin
SM γ-actin = smooth muscle γ-actin
SMC = smooth muscle cell
SM-MHC = smooth muscle myosin heavy chain
SM-specific marker genes = smooth muscle specific marker genes
SRF = Serum Response Factor
Thr = threonine
TBS = Tris buffered saline
TBST = TBS + 1% Tween-20
TCE = TGF-β control element
TGF-β1 = transforming growth factor-beta 1
Tyr = tyrosine
WGATAR = GATA binding element (A/T)GATA(A/G)

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