The role of integrin-linked kinase in platelet-derived growth factor stimulated vascular smooth muscle cell migration

by

SAHAR ABDOLI YAZDI

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ABSTRACT

Background: Vascular smooth muscle cell migration and accumulation in response to growth factors extensively contribute to the development of intimal thickening within the vessel wall. Cumulative evidence has shown that actin cytoskeleton polymerization and rearrangement are critical steps during cellular spreading and migration. Integrin-linked kinase, an intracellular serine/threonine kinase, is a cytoplasmic interactor of integrin beta-1 and beta-3 receptors and has been reported to regulate cell-cell and/or cell-extracellular matrix interaction, cell contraction, extracellular matrix modification, and cell spreading and migration in response to various stimuli. However, the regulatory role of ILK during vascular smooth muscle cell migration and the importance of integrin signaling in occlusive vascular diseases have not yet been elucidated.

Results: In the present study, we report that integrin-linked kinase controls mouse aortic smooth muscle cell migration in response to platelet-derived growth factor. We have also identified p38 mitogen activated protein kinase as a downstream signaling pathway of the integrin-linked kinase that regulates platelet-derived growth factor- induced actin polymerization and smooth muscle cell migration.

Conclusion: This study provides new insight into the potential therapeutic value of modulating integrin signaling in an attempt to block or delay smooth muscle cell migration and the progression of vascular diseases.

PREFACE

This thesis contains material that has been published in BMC cell biology.

The published paper is due to efforts of Sahar Abdoli Yazdi, Dr. Mitra Esfandiarei and research supervisor Dr. Cornelis Van Breemen. The scientific idea and experimental design were developed by Sahar Abdoli Yazdi, Dr.Mitra Esfandiarei and Dr. Cornelis Van Breemen. Cell culture, western blot, transient transfection of cultured cells, wound healing assay, migration assay and actin cytoskeleton staining were carried out by Sahar Abdoli Yazdi .Cell viability experments were carried out by Dr. Mitra Esfandiarei. ILK kinase assay was performed by Virginia Gray. Data analysis for all experiments except for the kinase assay and the cell viability assay were done by Sahar Abdoli Yazdi. Data analysis of kinase assay and cell viability assay were done by Dr. Mitra Esfandiarei. Manuscript was written by Sahar Abdoli Yazdi and Dr. Mitra Esfandiarei and was revised by Dr. Cornelis Van Breemen and Dr.Shoukat Dedhar. All authors read and approved the final manuscript.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AngII	Angiotensin II
ApoE $^{-/-}$	Apolipoprotein E knockout
ATP	Adenosine-5'-triphosphate
Ca^{+2}	Calcium ion
CAD	Coronary artery disease
CP-I17	Protein-kinase-C-dependent phosphatase inhibitor of 17 kDa
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extra cellular matrix
EGF	Epidermal growth factor
Erk	Extracellular signal regulator kinase
ET-1	Endothelin-1

FA	Focal adhesion
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
fl	Floxed
GEF	Guanine nucleotide exchange factor p115 RhoGEF
GFs	Growth factors
GPCRs	G protein-coupled receptors
GSK3	Glycogen synthase kinase 3
Grb2	Growth factor receptor-bound protein 2
Grb7	Growth factor receptor-bound protein 7
HGF	Hepatocyte growth factor
IL-1	Interleukin-1
ILK	Integrin-linked kinase

ILKAP	LK-associated phosphatase
IP_3	Inositol 1,4,5-trisphosphate
JNK	c-Jun N terminal kinase
MAPK	Mitogen activated protein kinases
MKK3/6	MAPK kinase 3/6
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MYPT1	Myosin phosphatase target subunit 1
MSMC	Mouse smooth muscle cells
Nck	Non-catalytic region of tyrosine kinase adaptor protein1
oxLDL	Oxidized low density lipoprotein
p38 MAPK	p38 mitogen activated protein kinase
PDGF	Platelet-derived growth factor

PDGFR	Platelet-derived growth factor receptors
PH	Pleckstrin homology
PH-I1	Phosphatase-holoenzyme inhibitor-1
PI3K	Phsophatidylinositol-3 kinase
PINCH1	Particularly interesting new cysteine-histidine rich protein 1
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositole-3,4,5-triphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PLC-γ	Phospholipase C-γ
PM	Plasma membrane
Ras-GAP	Ras GTPase activating protein
ROCK	Rho Kinase
RTK	Receptor Tyrosine Kinase

SH2	Src Homology 2
Shp-2	Tyrosine phosphatase Shp-2
siRNA	Small interfering RNA
SM-A	Smooth muscle -actin
SMCs	Smooth muscle cells
SM-MHC	Smooth muscle myosin heavy chain
SOS	Guanylnucleotide exchange factor
SR	Sarcoplasmic reticulum
TIMPs	Tissue inhibitors of metalloproteinases
TNF-α	Tumor necrosis factor
VEGF	Vascular endothelial cell growth factor
VLDL	Very low density lipo-proteins
VSMC	Vascular smooth muscle cell

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Chapter 1. Introduction

1.1. Atherosclerosis

Atherosclerosis or the hardening of the arteries is a proliferative and inflammatory disease of the arterial wall. It is a disease of muscular arteries, starting with endothelial dysfunction followed by vascular inflammation, buildup of cholesterol, lipids, cellular debris and calcium within the intima of the vessel wall. This accumulation results in plaque formation, vascular remodeling, acute and chronic luminal obstruction, abnormalities of blood flow and diminished oxygen supply to target organs.

Atherosclerosis is the underlying cause of potentially fatal diseases such as coronary artery disease (CAD), abdominal aortic aneurysms, heart failure, and stroke. It is the primary cause of mortality in both men and women in Unites States and the secondary cause of mortality in Canada following cancer. The prevalence of the disease is rapidly increasing in the developing countries, and as people in developed countries live longer it is expected to be the leading cause of death worldwide by the next decade.

1.1.1. Plaque formation

Injury to the intimal layer of the artery caused by trauma, surgery, infection (by Chlamydia or viruses), toxins (such as tar produced by the burning of tobacco and other plant material) or turbulent flow at bifurcations leads to the formation of plaques, which are regions of thickening on the inner lining of the artery. How then do the plaques form? Injured endothelial cells have increased low density lipoprotein (LDL) receptor expression as well as increased adherence to monocytes, macrophages and T cells. At sites of turbulence, where there may be relative anoxia, increased quantities of LDL cross the endothelium of an artery into its wall and are modified by oxidation. When LDL level is high and the endothelium is damaged (by smoking, hypertension, oxidation, glycation, etc) circulating monocytes and T-cells are attracted to the site of injury crossing the endothelial layer to enter the sub-intimal space. Here the monocytes take up oxLDL and become macrophages. The macrophages ingest the oxLDL from the tissue fluid in large quantities and load their cytoplasm with cholesterol droplets and become foam cells making them less mobile. Accumulation of foam cells leads to the formation of fatty streaks seen in the arterial wall, the earliest visible atherosclerotic lesion. T cells are also recruited to the lesion, propagating inflammation.

On the other hand, release of growth factors such as platelet derived growth factors (PDGF) in the injured area causes migration of smooth muscle cells (SMCs) from the media into the fatty streak region in the sub-endothelial space. In the intima, migrated vascular SMCs proliferate and synthesize connective tissue; these processes of migration, division, and synthesis, which collectively are referred to as intimal proliferation (buildup), cause thickening of the intima. This response, which is clearly part of an inappropriate activated tissue repair process, leads to the development of the mature atheromatous lesion. The collagen produced by fibroblasts is laid over the foam cells, which undergo either necrosis or apoptosis. This result in the formation of a pool of extracellular cholesterol trapped beneath a fibrous cap. As these plaques grow, they accumulate scar (fibrous) tissue and abundant calcium. Hence, the plaques are often hard, which is why atherosclerosis is sometimes referred to as "hardening of the arteries”. The shoulder of the atheromatous lesion where the fibrous cap joins the normal arterial wall continues to be active, and it is here that active foam cell formation continues as the lesion advances across the inner surface of the artery. Progression of atherosclerosis leads extensive plaques and narrows the blood vessel lumen, endangering the blood supply to target organs. The fibrous plaque is vulnerable to rupture and therefore capable of producing acute coronary syndrome.

1.2. Vascular structure

The artery consists of three structural components:

- Tunica adventitia is the outermost layer of arterial wall and consists of collagen fibers and elastic tissue it carries the blood and nerve supply to the artery itself.

- Tunica media is the middle layer of arterial wall and the thickest. It is comprised of smooth muscle (oriented around the vascular wall in charge of controlling vascular tone), connective tissue and elastic tissue.

- Tunica intima is the inner layer of the arterial wall, it is in direct contact with blood and regulates homeostasis, thrombosis, vascular tone and permeability and it too consists of three layers. The innermost is the layer of endothelial cells. In the middle, the tunica intima contains a thin sub-endothelial layer of fine areolar tissue (connective tissue made up largely of interlacing fibers), and the outermost layer is a thin membrane of connective tissue.

Understanding the mechanism that governs smooth muscle migration under disease conditions is important to cardiovascular medicine. Our objective was to study how smooth muscle cells build up along blood vessel walls, a process that represents a crucial step in the development of atherosclerotic plaques.

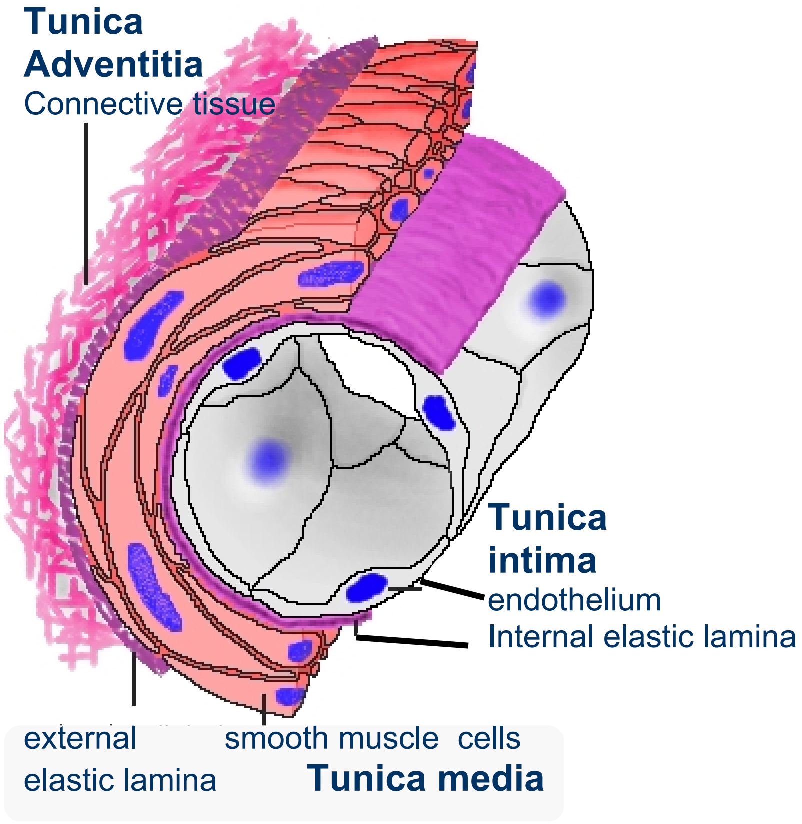
Tunica Adventitia
Connective tissue
Tunica intima
endothelium
Internal elastic lamina
external elastic lamina
smooth muscle cells
Tunica media

Figure 1.1. Vascular structure. Schematic diagram showing the different layers of an artery: Tunica intima, tunica media and tunica adventitia. (Image Copyright Blue Histology, Lutz Slomianka 1998-2009).

1.2.1.Vascular smooth muscle cells (VSMC) in disease

VSMC are located in the tunica media of arteries. They are highly elongated, and aligned circularly in and act as a functional unit to maintain vascular tone and thus are responsible for controlling regional and general blood flow and blood pressure. VSMCs respond to signals from the autonomic nervous system (e.g Norepinephrine) and are responsive to autacoids and hormones (e.g Nitric oxide; Angiotensin II, estrogen) as well as physical stimuli (i.e. pressure or stretch).

During the progression of vascular disease VSMC undergo phenotypic changes from a stable contractile state to a more proliferative and invasive state. SMCs in developing blood vessels proliferates rapidly and express low levels of smooth muscle contractile proteins and synthesize a variety of extracellular matrix (ECM) proteins including elastin and collagen. Fully differentiated SMCs in mature blood vessels are geared almost exclusively to contraction; they synthesize ECM components at much reduced levels but retain the ability to increase this synthesis following vascular injury.

In response to a variety of atherogenic stimuli including extracellular matrix, cytokines, shear stress, reactive oxygen species, and lipids, SMCs can switch between the “contractile” and “synthetic” phenotypic states. VSMCs predominantly express proteins involved in the contractile function such as smooth muscle myosin heavy chain (SM-MHC) or smooth muscle -actin (SM-A). However, SMCs found in the intima express lower levels of these proteins; have a higher proliferative index and a greater synthetic capacity for extracellular matrix, proteases, and cytokines [50-51]. This phenotypic modulation involves loss of contractile function, most likely due to a reduction and disorganization of myosin-containing thick filaments, and is associated with an increase in synthetic organelles responsible for increased production and secretion of extracellular matrix proteins. These “synthetic” SMCs migrate and proliferate more readily than “contractile” SMCs and can synthesize up to 25 to 46 times more collagen [50]. In addition, they express a greater proportion of VLDL, LDL, and scavenger receptors allowing more efficient lipid uptake and foam cell formation. Therefore, transition to the “synthetic” state facilitates many of the pathogenic roles of SMCs. Plaque formation is initiated largely due to migration and subsequent proliferation of VSMC in the sub-intimal space in response to circulating growth factors and inflammatory cytokines. Under these conditions calcium homeostasis is altered and the frequency of apoptotic cell death is increased. This together with the accumulation of foam cells results in vessel wall hardening, thickening, and remodeling. Therapeutic approaches targeting any of the above mechanisms in VSMCs could be effective in the treatment of vascular disease.

1.3. Actin polymerization and vascular smooth muscle cell migration

Smooth muscle cell migration occurs during development, in response to injury and during atherogenesis. Vascular injury occurs clinically after angioplasty, vascular stent implantation, or organ transplantation. In diseased or injured tissues, there are more cells in the intima mainly due to cell migration.

Cellular migration is regulated via a complex interaction between growth factors that attach to their cognate receptors, trans-membrane integrins that bind to the components of extracellular matrix, and mechanical stress, all of which cooperatively induce polymerization and reorganization of the actin cytoskeleton. These events are coordinated by mechanisms involved in assembly or disassembly of local adhesion sites, transient changes in actin filaments dynamics, and formation of discrete structures such as stress actin fibers, membrane ruffles, lamellipodia, and filopodia [11-13].

In order to migrate from the media to the sub-intima, VSMCs need to extend lamellipodia toward the stimulus via actin polymerization, detach the trailing edge by degrading focal contacts, and generate force by myosin II in the body of the cell to propel the cell forward [49]. Actin polymerization is a key step in migration. Regulation of actin polymerization allows cells to control their shape, to move, divide, secrete, and phagocytose. In addition, actin filaments provide strength, connections to neighboring cells and the extracellular matrix, paths for intracellular transport, and a scaffold for generating force.

Briefly, the actin network is continuously regenerated by new polymerization at the leading edge and depolymerization at the back of the lamellipodia. Cofilin is a member of the ADF (actindepolymerizing factor) protein family. Cofilin plays an essential role in the rapid turnover of actin filaments and actin-based cytoskeletal reorganization by stimulating depolymerization and severance of actin filaments. The LIM kinase (LIMK) family, which includes LIMK 1 and 2, are serine protein kinases involved in the regulation of actin polymerization and microtubule disassembly. Activation of LIM kinase leads to the phosphorylation and inhibition of cofilin, which prevents depolymerization of actin.

1.3.1. Platelet derived growth factor (PDGF)

The initiation and progression of intimal thickening in arterial walls is largely due to migration and subsequent proliferation of VSMCs in the sub-intimal space in response to various stimuli including oxidized low density lipoprotein (oxLDL), circulating growth factors such as PDGF , and inflammatory cytokines, tumor necrosis factor (TNF)-α, and interleukin-1 (IL-1) [1, 2]. Of these, PDGF, a growth factor released by VSMCs, endothelial cells, and platelets, has been reported as the most potent inducer of SMC migration within the injured area of the vascular wall. PDGF is a heparin-binding growth factor composed of polypeptide chains that can be assembled into homodimers (PDGF-AA, -BB) or heterodimer (PDGF-AB) structures and bind to two related cell-surface receptors with tyrosine kinase activity, PDGF receptor α (alpha) and β (beta) [3-5]. In the last few years two additional homodimers PDGF-CC and PDGF-DD were also discovered [6-9]. The PDGFs are inactive as monomers. The synthesis of PDGF isoforms is carefully regulated, and their action on receptors is modulated by interaction with components in the matrix as well as with soluble binding proteins.

PDGF induces reorganization of the actin filament system and stimulates chemotaxis, i.e., a directed cell movement within a gradient of PDGF [10]. PDGF binding to its cognate receptors results in dimerization, and subsequent auto-phosphorylation of specific tyrosine residues outside the kinase domain, creating a docking site for Src Homology 2 (SH2) domain-containing signaling proteins. All three isoforms of PDGF have shown to induce contraction in a variety of isolated blood vessels with PDGF-BB having the greatest efficacy [52]. This contraction is associated with a rise in intracellular calcium and is blocked by the inhibitors of tyrosine kinases. PDGF binding to its cognate receptor results in activation of multiple signaling pathways in vascular SMCs, including phosphatidylinositol 3-kinase (PI3K / Akts), Protein Kinase A (PKA), Src , Ras and mitogen-activated protein kinases (MAPK) . These signaling cascades are involved in cell growth, proliferation, survival, and migration. MAPKs play the major role in stress-induced cellular responses, including cell proliferation, survival, or apoptosis. Three members of MAPKs family, p38 MAPK, c-Jun NH2-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) 1/2 are known to participate in vascular diseases via differential molecular mechanisms and are new therapeutic targets for treatment of vascular diseases [23].

1.3.1.1. PDGF receptor: a class of receptor tyrosine kinase

The PDGF receptors (PDGFR) are classified as receptor tyrosine kinases (RTK). RTKs are high affinity cell surface receptors for many polypeptide growth factors, cytokines, and hormones. The intracellular C-terminal region displays the highest level of conservation and comprises catalytic domains responsible for the kinase activity of these receptors, which catalyses receptor auto-phosphorylation and tyrosine phosphorylation of RTK substrates. Kinase enzymes that specifically phosphorylate tyrosine amino acids are termed tyrosine kinases. Two types of PDGFRs have been identified: α-type and β-type PDGFRs. The α-type binds to PDGF-AA, PDGF-BB, PDGF-AB, and PDGF-CC whereas the β-type

PDGFR binds with high affinity to PDGF-BB, PDGF-DD, PDGF-CC and PDGF-AB. The most significant direct role (i.e. cell-autonomous) of β-type PDGFR is to mediate responses that involve cell migration rather than proliferation [28]. Activation of the β isoform of PDGFR (PDGFR- ββ) is coupled via phosphatidylinositol 3-kinase (PI3K) and phospholipase C (PLC) to changes in myoplasmic calcium, hydrolysis of Phosphatidylinositol 4,5-bisphosphate (PIP2), and activation of MAPK [54].

PDGF binds to PDGFRs ligand binding pocket located within the second and third immunoglobulin domains. Upon activation by PDGF, these receptors dimerize, and are "switched on" by auto-phosphorylation of several sites on their cytosolic domains, which serve to mediate binding of cofactors and subsequently activate signal transduction. A large number of Src Homology-2 (SH2) domains containing proteins including PI3K, PLC-γ, the tyrosine phosphatase SHP-2, Ras GTPase activating protein (Ras-GAP), Grb2, Grb7, Nck, and the Src family of tyrosine kinases have been shown to bind to cytoplasmic tails of PDGF receptors activating various downstream signaling proteins involved in cell growth, proliferation, survival, and migration [10].

1.3.2. Integrins

In blood vessels shear stress and stretch are important activators of integrins and signal transduction pathways. Integrins are transmembrane protein receptors that link the cytoskeleton to the extracellular matrix (ECM) and have signaling as well as adhesive roles. Integrins mediate cell-cell and cell-matrix interaction and communication, providing intracellular (‘outside-in’) and extracellular (‘inside-out’) signaling essential for VSMC migration in atherosclerosis. They transduce information from the ECM to the cell as well as reveal the status of the cell to the outside, allowing rapid and flexible responses to changes in the environment. These tranduction functions of integrins require interaction of integrin cytoplasmic domains with cellular proteins.

Cell-matrix interaction via integrins is essential for embryonic development, survival, adhesion, proliferation, differentiation and migration of cells. Integrins are a group of non- covalently associated heterodimers containing two distinct chains, called α and β subunits. The cytoplasmic tail of the β subunit binds directly to cytoskeletal proteins (actin filament) that associate with signaling molecules (***figure 1.5.***). The extracellular domains of the integrins mediate cell adhesion to extracellular matrix and basement membrane components. Ligand binding to the extracellular integrin domain induces conformational changes and integrin clustering which leads to the activation of signaling cascades and recruitment of multi-protein complexes to focal adhesions [55].

Integrins have been classified according to their β subunits, which may associate with different α subunits. β1 and β3 integrins are expressed in mouse aortic SMCs. Members of the β1 -integrin family mainly mediate binding of VSMCs to the extracellular matrix proteins collagen I (α1 β1 and α2 β1 integrin) and fibronectin (α5 β1 integrin). VSMC spreading and differentiation is impaired in mice when the β1 integrin gene is inactivated, additionally, VSMC proliferation is increased leading to postnatal lethality [57].

Cell adhesion through integrins leads to ligand-independent phosphorylation of several different growth-factor receptors including those for PDGF, fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and epidermal growth factor (EGF). Integrins lack enzymatic activity, therefore, in order to transduce signals they interact with cellular proteins that have kinase activity. Hence, integrins transmit signals through a variety of intracellular protein kinases and adaptor molecules such as integrin linked kinase (ILK),focal adhesion kinase (FAK), particularly interesting new cysteine-histidine rich protein (PINCH) and non-catalytic (region of) tyrosine kinase adaptor protein-2 (Nck2) [53]. Through these molecules, integrin signaling interacts with RTKs signaling to regulate cell survival, proliferation, adhesion, migration, cell shape and differentiation.

1.3.2.1. “outside-in” and “inside out” signals

Integrins integrate the extracellular and intracellular environment of the cell through bidirectional signaling in specialized focal contact regions. The inactive integrin has a bent over conformation, while in the fully activated state the globular ligand binding domains extend out maximally from the cell surface. Binding of ligands by extracellular domains may generate conformational changes that affect interaction of integrins with intracellular cytoskeletal and signal proteins. VSM cell migration depends on integration of both soluble signals acting from the outside in and on the changes in matrix attachment via activation of integrins from inside out [55]. The affinity of integrins for extracellular ligands is regulated by cell signals.

Outside-in signaling is induced by extracellular ligand binding that produces intracellular signaling responsible for regulating various cell function such as proliferation, survival and migration. In contrast, inside-out signaling typically involves the activation of signaling pathways via growth factor receptor activation, consequently affecting integrin affinity /clustering, which enables rapid changes in cytoskeletal reorganization that critically influence cell shape, cell adhesion and migration, growth and survival.

1.4. Focal adhesion complex

In blood vessels shear stress and stretch are important activators of integrins and signal transduction pathways. Upon integrin-mediated cell adhesion to ECM, a massive reorganization of the actin cytoskeleton occurs, resulting in the formation of focal adhesions. Many proteins, including catalytic proteins such as ILK and focal adhesion kinase (FAK) and structural proteins are recruited to these focal adhesions in response to cell adhesion, which leads to morphological changes that contribute to cell spreading, migration, and cell signaling.

The primary sub-cellular structures that mediate the regulatory effects of ECM adhesion on cell behavior are the focal adhesions (FAs). These macromolecular complexes are large, elongated, flat structures that mediate cell anchorage to ECM by physically coupling integrins to the contractile actin cytoskeleton. Mechanical load applied to integrin ligands causes the assembly and growth of focal contacts and activation of FAK and MAP kinases. FA complex also serves as an important transducer of extracellular signals to control many aspects of cell morphology and behavior ranging from migration and proliferation to apoptosis.

FAs are limited to clearly defined ranges of the cell, at which the plasma membrane closes to within 15nm of the ECM substrate. FAs are in a state of constant flux: proteins associate and disassociate with it continually as signals are transmitted to other parts of the cell. FA is comprised of cytoskeletal proteins (e.g. tensin, vinculin, paxillin, a-actinin, parvin/actopaxin and talin), tyrosine kinases (e.g. Src, FAK, PYK2, Csk and Abl), serine/threonine kinases (e.g. ILK, PKC and PAK), modulators of small GTPases (e.g. ASAP1, Graf and PSGAP), tyrosine phosphatases (e.g. SHP-2 and LAR PTP) and other enzymes (e.g. PI 3-kinase and the protease calpain II). These macromolecules can contain over a 100 different proteins. Under normal conditions, FA remains stationary with respect to the extracellular matrix, and the cell uses this as an anchor on which it can push or pull itself over the ECM. In moving cells however, FA stability is diminished [60].

The dynamic assembly and disassembly of focal adhesions plays a central role in cell migration. The composition and the morphology of the FA changes during cell migration; In motile cells, focal adhesions are being constantly assembled and disassembled as the cell establishes new contacts at the leading edge, and breaks old contacts at the trailing edge of the cell.

Integrin-mediated adhesions can undergo dynamic changes in structure and molecular properties from dot like focal complexes to stress-fiber-associated focal contacts, which are capable of maturing to form fibronectin, bound fibrillar adhesions. These changes are driven by mechanical force generated by the actin- and myosin containing contractile machinery of the cells, or by external forces applied to the cells, and regulated by matrix rigidity [59].

1.4.1. Integrin linked kinase (ILK)

Integrin-linked kinase (ILK) is a serine-threonine protein kinase and scaffolding protein mainly looked at in cancer models. It is involved in cell growth and survival, proliferation, migration and invasion, motility and contraction, vascular development and tumor angiogenesis. ILK is a key signaling molecule acting downstream of integrin receptors in response to extra cellular matrix signals, transducing inside-out and outside-in signals by directly connecting to the cytoplasmic domain of β1 or β3 integrins in the plasma membrane (PM), as well as directly connecting to the actin cytoskeleton. Genetic studies have revealed that the adaptor molecule ILK is essential for integrin function in vivo. ILK is also an important regulator of actin organization and it plays a critical role in cell migration. ILK gene knockouts in mice, flies, and worms result in early embryonic lethality because of cell adhesion defects and cytoskeletal disorganization. ILK interacts with growth factor receptors, according to various researches however; the effects of PDGF treatment on ILK signaling appear to be cell specific.

ILK is comprised of three structurally distinct domains: a putative kinase domain at C terminus, a central pleckstrin homology (PH)-like domain, and five ankyrin-like repeats at the N terminus. The particularly interesting new cysteine-histidine rich protein 1 (PINCH1), binds to the Ankyrin repeats, which, in turn is required for localization of ILK to focal adhesion. In addition, it connects ILK to other proteins, including Nck-2, a SH2-containing adaptor protein, involved in the growth factor receptor kinase signaling pathways. The ILK- associated phosphatase (ILKAP) also binds to the Ankyrin repeats. Phosphatidylinositole- 3,4,5-triphosphate (PIP3) has been shown to interact with the PH sequence, this domain participates in the regulation of the kinase activity of ILK. The binding sites of β integrins, α / β -Parvins and Paxillin lie within the C-terminal kinase domain .ILK is a downstream substrate of Phosphoinositide 3-kinase (PI3K) and has been demonstrated to induce the phosphorylation and activation of protein kinase B (PKB/Akt at Ser-473), and the phosphorylation and inhibition of glycogen synthase kinase 3 (GSK3 at Ser 21/9). Survival signal mediated by various growth factors and cytokines are dependent on the PI 3- kinase/Akt signal transduction pathway.

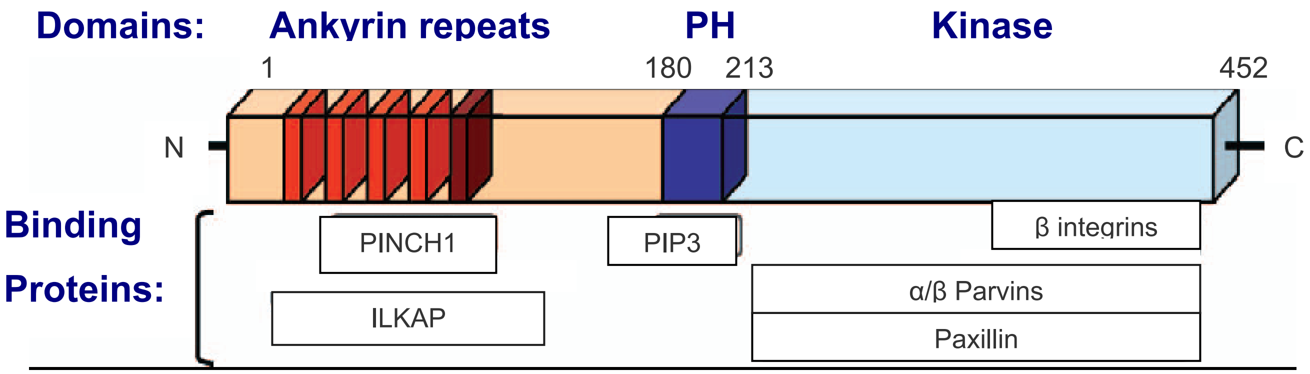

Figure 1.2. Structure of integrin-linked kinase Schematic diagram showing the different domains and binding proteins of Integrin-Linked Kinase. Numbers indicate the amino acid residues from the N- to C-terminus. *Eke et al.,2009.*

1.4.2. Integrin linked kinase in vascular smooth muscle cells

SMCs are electrically coupled by numerous gap junctions in small resistance arteries, which contract rhythmically. Larger blood vessels appear to lack this coupling and their VSMC display asynchronous Ca^{2+} waves and tonic contractions. The degree of SMC contraction determines the diameter (tone) of the vessel. Injury and vascular disease results in vascular wall remodeling which in turn decreases elasticity and hence vascular tone. Disorganization of the smooth muscle layers and loss of VSMC contractility are features associated with atherosclerosis and other vascular diseases. In addition to being in the cell periphery in order to maintain a stable cell adhesion to matrix, in the quiescent differentiated SMC, ILK functions to mediate contraction and aid the cell in exerting force on surrounding ECM fibers. ILK is localized to myofilaments in SMCs, and it promotes contraction by directly phosphorylating myosin light chain (MLC) or myosin light chain phosphatase (MLCP) [46-48]. ILK may also be able to induce contraction of SMCs indirectly by phosphorylating and hence activating MLCP inhibitors including CPI-17 and PH-I [47]. ILK is required to organize VSMCs into the unitary smooth muscle layer and regulates VSMC contractility and focal adhesion assembly during cell migration. Presence of ILK limits vascular constriction, thereby facilitating normal vessel wall development. Interestingly enough, it has been shown that ILK is upstream of Rho/Rock and is negatively regulating Rho/ROCK signaling in a variety of cell types. The RhoA effector ROCK can phosphorylate myosin phosphatase (MLCP) target subunit 1 (MYPT1) at Thr853 inhibiting dissociation of the phosphatase (Pi) complex from myosin light-chain (MLC), and therefore inhibiting relaxation. Loss of ILK causes increased VSMC contractility due to elevated MLC phosphorylation through activation of Rho/ROCK signaling [58].

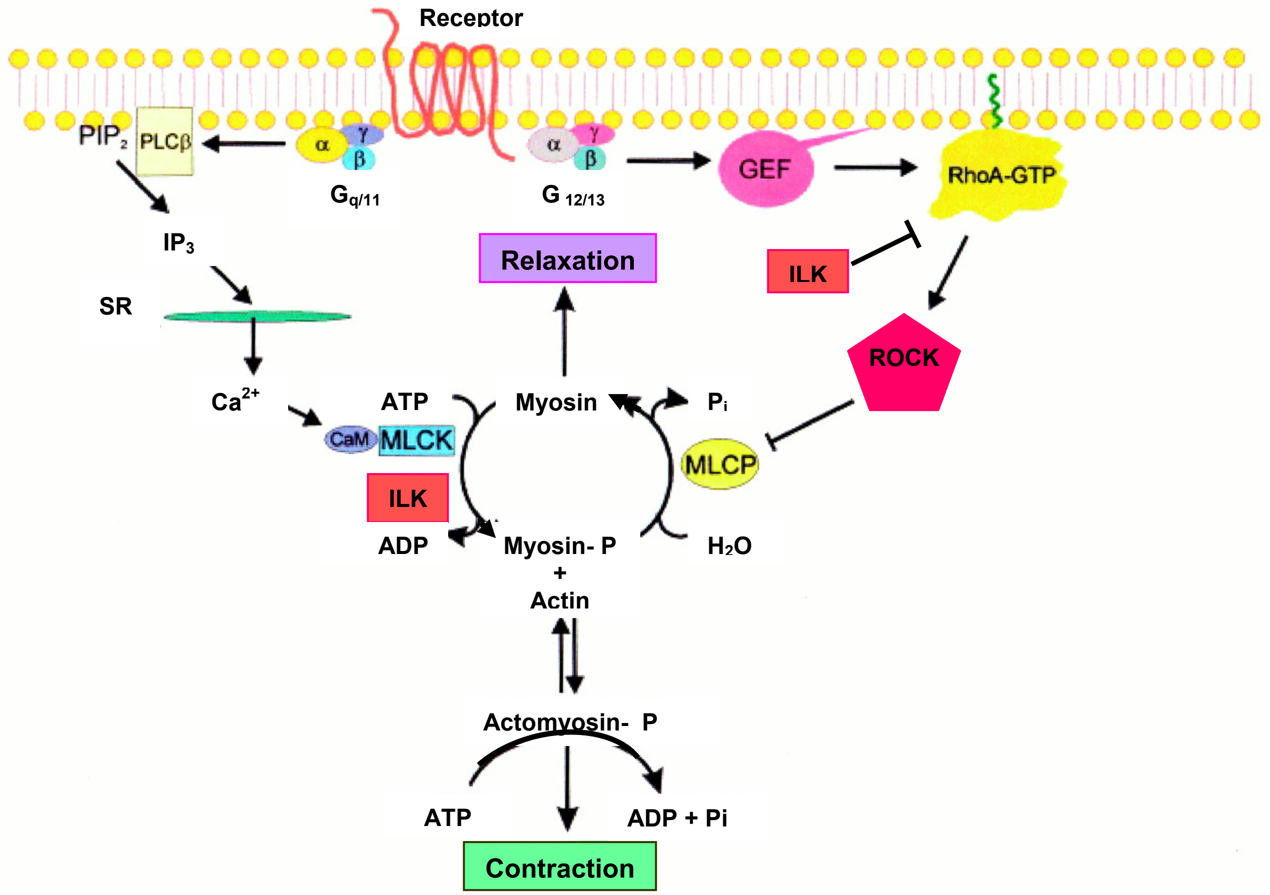
Receptor
PIP2
PLCβ
α
γ
β
Gq/11
α
γ
β
G 12/13
GEF
RhoA-GTP
IP3
SR
Relaxation
ILK
ROCK
Ca2+
ATP
CaM
MLCK
ILK
ADP
Myosin
Pi
MLCP
Myosin- P
+
Actin
H2O
Actomyosin- P
ATP
ADP + Pi
Contraction

Figure 1.3. Regulation of smooth muscle contraction. Deng J T et al. J. Biol. Chem.2001;276:16365-16373.

Integrins and RTKs can exchange or amplify their signaling pathways via both "Outside-in" and “Inside-out" signaling. Integrin-ECM interactions induce the phosphorylation of key tyrosine residues of integrin subunits such as β3which results in recruitment of signaling adaptor molecules such as ILK. These adaptor molecules not only transmit integrin- dependent signals, but also contribute to crosstalk with other signaling receptors including RTKs [34]. Integrin-ECM interactions significantly amplify growth factor-mediated signaling events, which suggests that synergy between integrin and RTK signaling could maximize biochemical responses. It is possible that multiple mechanisms may regulate crosstalk between integrins and RTKs. Signaling cooperation between integrins and RTKs may be the result of receptor co-clustering upon cell adhesion or growth factor stimulation. Growth factor stimulation of RTKs or ECM-integrin interactions induces an increase in the local concentration of integrins and RTKs at focal adhesions and at leading edges of migrating VSMCs, such that crosstalk could occur by alteration of the intracellular localization of integrins.

ILK regulates fibroblast migration through the phosphatidylinositol-3 kinase (PI3K) [15], osteosarcoma cell spreading and motility via Rho-associated kinase (ROCK) [16], and mammary epithelial cell migration through the guanine nucleotide exchange factor α-PIX [17]. There is also growing evidence on the cooperation between PDGF receptor and integrins in regulating cellular survival and adhesion [18-20]. However, little is known about the existence of cross-talk between ILK and PDGF signaling in vascular smooth muscle cells and the probable regulatory role of ILK during PDGF-induced SMC migration. In the present study, we have examined the potential function for ILK as an upstream protein regulating the migratory response to PDGF in a primary mouse aortic SMC culture. We have also characterized one downstream pathway that mediates the regulatory role of ILK in modulating actin polymerization and SMC migration.

1.5. Mitogen activated protein kinase (MAPK) signaling

Intracellular mitogen-activated protein kinase (MAPK) signaling cascades are serine/threonine-specific protein kinases that are activated by diverse stimuli ranging from mitogens , cytokines, growth factors, neurotransmitters, hormones, cellular stress, and cell adherence. They regulate various cellular activities, such as gene expression, mitosis, differentiation, proliferation, migration and cell survival/apoptosis. There are three main families of MAPKs that exist in mammalian species, grouped by their structures and functions: the extracellular signal-regulated protein kinases (ERKs), the p38 MAPK, and the c-Jun NH2-terminal kinases (JNKs).

ERK signaling is activated by growth factors and cytokines and it is generally involved in stimulating cell growth. The JNK and p38 signaling pathways are activated by growth factors, pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β or in response to cellular stresses such as genotoxic, osmotic, hypoxic, or oxidative stress. These two pathways can be responsible for both cell growth and cell death, depending on the activating stimuli and cellular context.

In response to arterial injury there is a local release of ligands such as endothelin-1 (ET-1), angiotensin II (AngII) and thrombin, growth factors (GFs), and cytokines that bind to transmembrane receptors. These receptors include G protein-coupled receptors (GPCRs), RTKs and cytokine receptors that activate intracellular signaling proteins such as G proteins (Gq/11) and the Grb2/SOS complex. Intracellular MAPK cascades are in turn activated and ultimately leads to VSMC migration into the intima and proliferation, resulting in neointima formation.

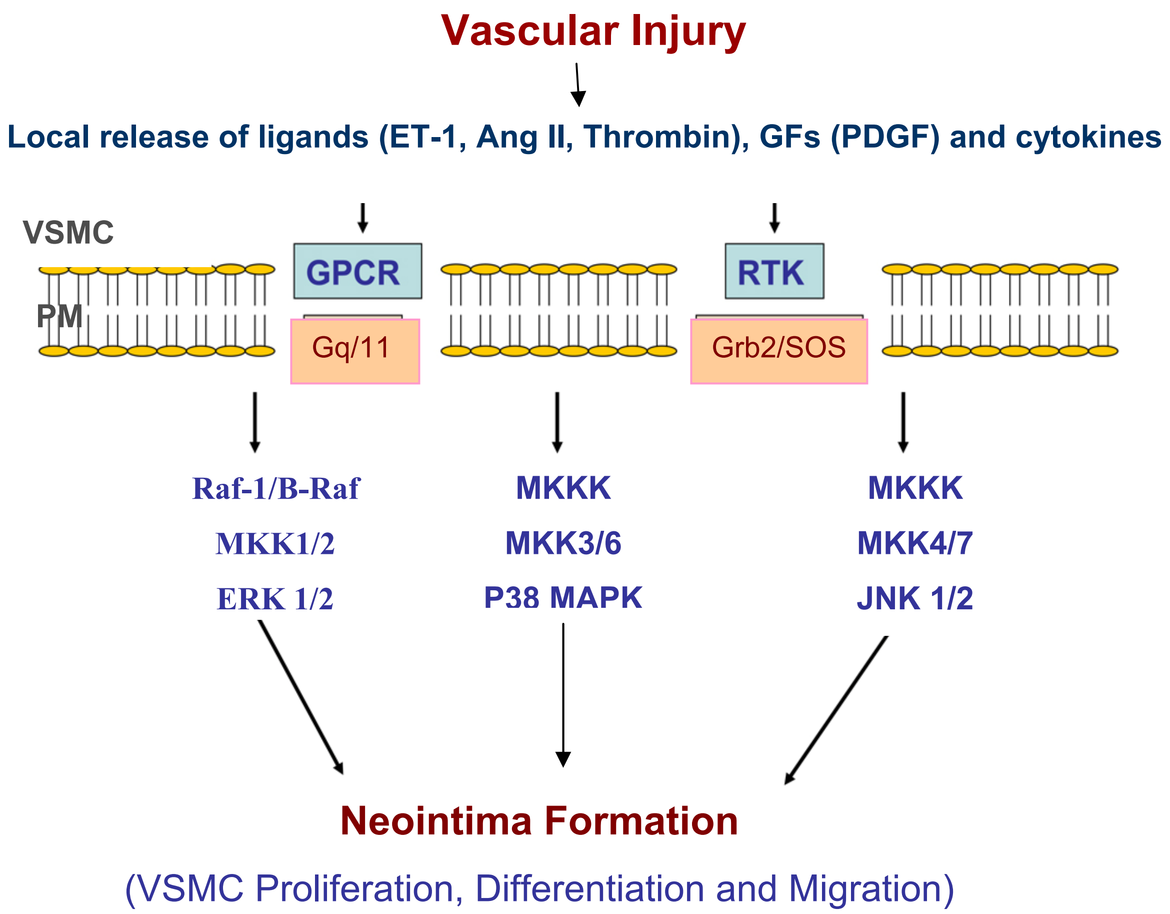
Vascular Injury
Local release of ligands (ET-1, Ang II, Thrombin), GFs (PDGF) and cytokines
VSMC
PM
GPCR
Gq/11
RTK
Grb2/SOS
Raf-1/B-Raf
MKK1/2
ERK 1/2
MKKK
MKK3/6
P38 MAPK
MKKK
MKK4/7
JNK 1/2
Neointima Formation
(VSMC Proliferation, Differentiation and Migration)

Figure 1.4. Model of smooth muscle cell migration and proliferation in neointima formation after vascular injury. Muslin J A. 2008.

The cross talk between PDGF and integrin pathways

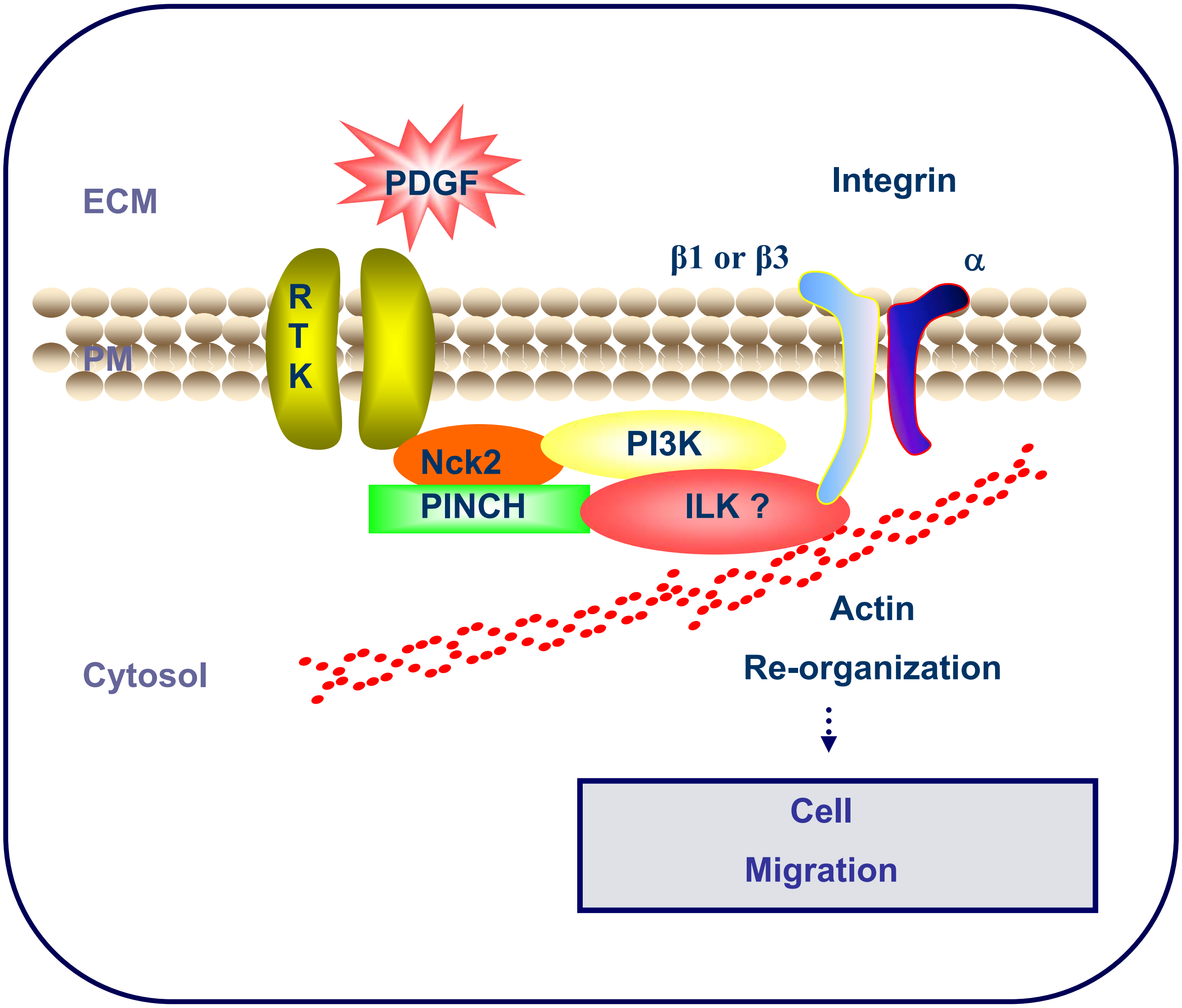
ECM
PDGF
Integrin
β1 or β3
α
R
T
K
PM
PI3K
Nck2
PINCH
ILK ?
Actin
Re-organization
Cytosol
Cell
Migration

Figure 1.5. **Cross talk between PDGF receptor and integrin receptor.** Following injury, SMC migratory response to various stimuli begins with the activation of cell surface receptors followed by remodeling events that involve focal adhesion complex and filament actin remodeling. **Q.** What is the role of ILK in PDGF induced VSMC migration?

1.6. Hypothesis and aims

1.6.1. Research rationale

The two main questions to be addressed in this thesis are:

1. What is the intermediary role of ILK, if any, in PDGF induced vascular smooth muscle migration?

2. Is there any cross talk between the ILK pathway and MAPK signaling pathway.

1.6.2. Hypothesis and objectives

Our hypothesis is:

Integrin-link kinase functions as a downstream mediator of PDGF to control vascular smooth muscle cell migration.

Our objectives are:

1. To characterize the potential regulatory role of integrin-linked kinase in growth factor-induced smooth muscle cell migration.

2. To determine if ILK regulates anyone of the three members of MAPK family; JNK, P38 MAPK and Erk 1/2.

Experimental design

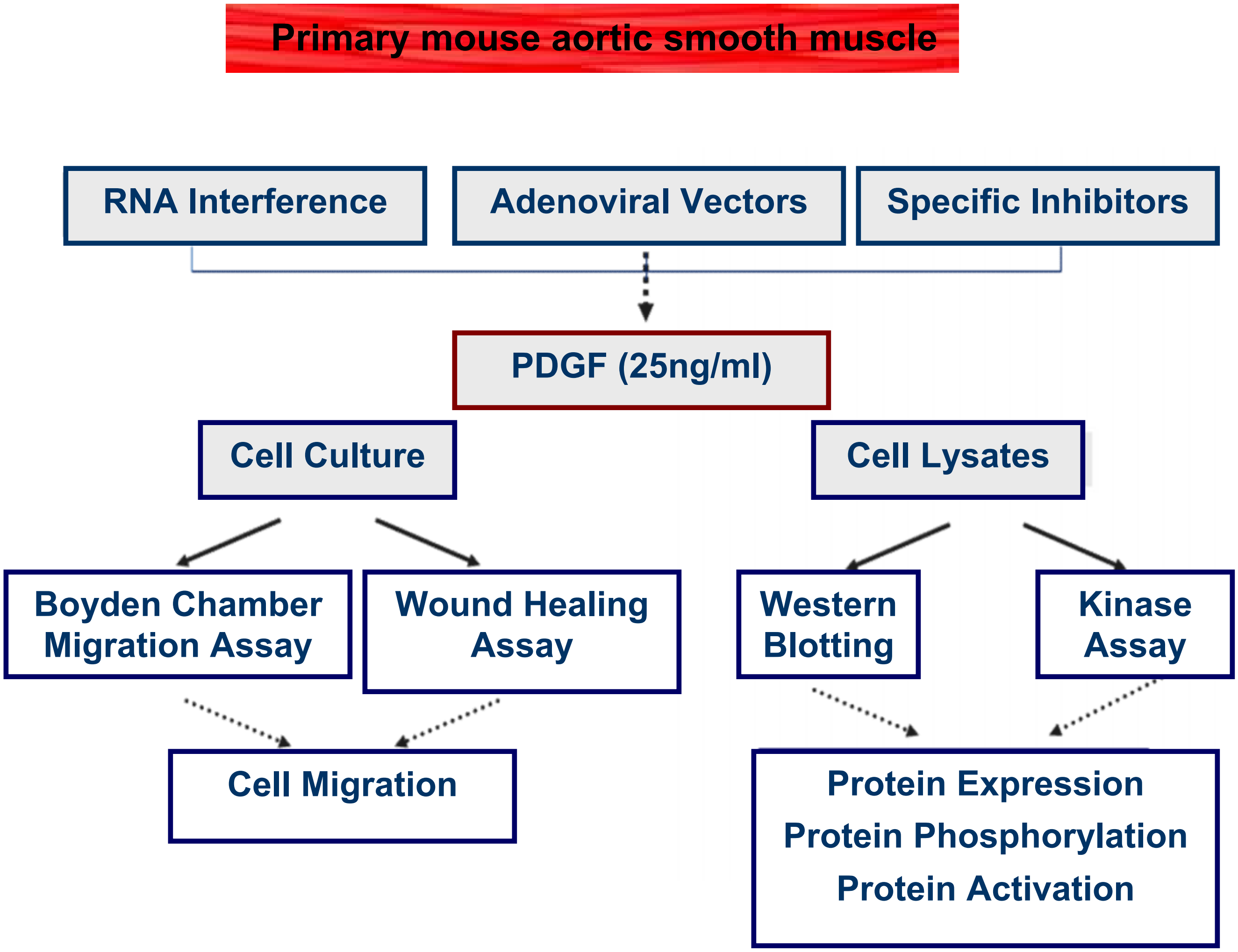
Primary mouse aortic smooth muscle
RNA Interference
Adenoviral Vectors
Specific Inhibitors
PDGF (25ng/ml)
Cell Culture
Cell Lysates
Boyden Chamber Migration Assay
Wound Healing Assay
Western Blotting
Kinase Assay
Cell Migration
Protein Expression
Protein Phosphorylation
Protein Activation

Figure1. 6. Experimental design. For this study primary mouse aortic SMCs were used largely because techniques for genetic manipulation are more fully developed in the mouse than in any other mammalian species**.** RNA interference, adenoviral vectors and inhibitors were used to modulate the signaling pathways. ILK expression was inhibited by ILK-siRNA and increased by wild type-ILK. Inhibition of ILK kinase activity was done using E395K mutant of ILK. Following the above manipulations, the non control cells cultures were later treated with PDGF. VSMC cultures were used to measure cell migration using two methods: (1) transwell Boyden chamber migration assay or (2) wound healing assay.

In addition, cell lysates were used in western blotting and kinase assay to measure protein expression and protein phosphorylation or activation. Cell viability was measured using a non-radioactive cell proliferation assay (MTS)- a colorimetric assay that measures mitochondrial activity.

Actin cytoskeleton was stained using phallodin to view actin polymerization in presence and absence of p38 MAPK inhibitor, ILK siRNA and PDGF-BB treatment.

Chapter 2. Materials and methods

2.1. Isolation and preparation of primary mouse vascular SMC culture

A vascular smooth muscle cell culture was established from the aortae of ILK fl/fl (floxed/floxed) mice by a modification of an enzymatic dispersion technique described previously (61). Adult male mice were euthanized in accordance with ethical guidelines set out by the University of British Columbia animal care committee. The thoracic aorta was removed, washed in MCDB-131 medium (Sigma-Aldrich, Oakville, ON, Canada) containing 10% serum and penicillin /streptomycin, and cleaned of excess adventitial connective tissue. The tissue was then minced and placed in 0.5 mg/mL collagenase II (Worthington Biochemical Corp., Lakewood, NJ). The tissue was incubated at 37°C for 2 to 3 hours with regular pipetting to disperse cells. Liberated cells were pelleted, resuspended in 1 mL MCDB-131 containing 10% FBS, and seeded into a 60 mm tissue culture dish. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated newborn calf serum (Invitrogen, Burlington, ON) at 37°C, in a humidified incubator in 5% CO2. Penicillin G (100 µg/ml) and streptomycin (100 µg/ml) (Invitrogen) were added to all culture media. Passage 8-13 of SMCs was used for all experiments. SMCs were grown to confluence, trypsinized, and sub-cultured at a density of 1.0×10^4 cells/cm^2. Sub-confluent (60-80%) cultures were used for all experiments. ILK recombinant adenoviral constructs were kind gifts from Dr. Hyo-Soo Kim (Seoul National University Hospital, Korea). Adenoviral constructs for recombinant p38 MAPK and constitutively active forms of MKK3/6 were kindly provided by Dr. Donald R. Menick (Medical University of South Carolina, USA).

2.2. Transient transfection

For transient transfection experiments, cells were infected with adenoviruses encoding dominant negative p38 MAPK (Ad-Dn-p38), wild type of p38 MAPK (Ad-Wt-p38), constitutively active MKK3/6 (Ad-Ca-MKK3/6), kinase-deficient ILK (Ad-Kd-ILK), or wild type ILK (Ad-Wt-ILK) with multiplicity of infection of 100 (MOI = 100). Following overnight incubation at 37°C, SMCs were replenished with fresh medium. Transfection efficiency was measured by fluorescence microscopy and Western blot analysis at 36 hours post transfection. For all experiments, transfected SMCs were used at 36 hours post transfection.

RNA interference is a type of posttranscriptional gene silencing, and gene therapy using small interfering RNA (siRNA). This approach is more specific in decreasing the expression of genes than traditional inhibitors is proposed to be a novel treatment strategy in clinic for cancer treatment. Thus for ILK RNA inhibition experiments, SMCs were transfected with 20 nM of a 21-base pair double-stranded small interfering RNA molecule targeting the PH domain of ILK where by selectively silencing ILK gene expression in the mouse SMC . Transfection of the cell culture with nonspecific siRNA was done as internal control. ILK siRNA was introduced to cells using 2.5 µl of siLentFect Transfection Reagent according to the manufacturer's protocol (Bio-Rad Laboratories, Mississauga, ON, Canada). Twenty four hours post-transfection cells were replenished with fresh serum-containing medium, incubated for 96 hours, and then used for various experiments. ILK silencing was determined by Western blot of transfected lysates with an anti-ILK antibody.

2.3. Western blot analysis

Cells either untreated or treated with various experimental reagents were washed twice with ice-cold PBS, and kept on ice for 15 min in lysis buffer containing 50 mM pyrophosphate, 50 mM NaF, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 100 μM Na_3VO_4, 10 mM HEPES (pH 7.4), 0.1% Triton X-100, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were collected by scraping and protein concentration was determined using Bradford assay. Extracted protein (40-80 μg) was fractionated by electrophoresis in 7% to 9% sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose membranes, and blocked with PBS containing 0.1% Tween-20 and 5% non-fat dry milk for 1 hour. Afterward, the membrane was incubated with specific primary antibody overnight at 4°C, followed by secondary antibody for one hour at room temperature. Immunoblots were visualized with an enhanced chemiluminescence detection system according to the protocol of the manufacturer (Pierce Biotechnology, Rockford, IL, USA). Densitometry analysis was performed by using the National Institutes of Health ImageJ software (version 1.27z). Density values for proteins were normalized to the level for control groups (arbitrarily set to 1.0-fold). Beta-actin is a house keeping gene shown as proof of equal loading within each lane. Experiments were performed in triplicates and repeated three times.

2.4. ILK kinase assay

Kinase assay was performed using 250μg of protein lysates immunoprecipitated with 5 μg ILK antibody and protein A Sepharose beads overnight at 4°C, while shaking. The immunocomplex was washed twice with high salt NP-40 lysis buffer (containing 750 mM NaCl), and then three times with wash buffer containing 50 mM HEPES pH 7.5, 85 mM KCl, 10 mM ethyleneglycol tetraacetate

(EGTA), 0.1% Tween 80, 1 mM Na_3VO_4, 10 mM Mg_2Cl. The kinase assay was performed in the reaction buffer containing 0.5 µg ATP (250 µM ATP and 5 µCi [γ -^{32}P] ATP) and 5 µg of myelin basic protein (MBP) as substrate at 30°C for 20 minutes in a shaker-incubator. Samples were fractionated by electrophoresis in a 12% sodium dodecyl sulfate-polyacrylamide gel, and phosphorylation of substrate by ILK was measured by a scintillation counter autoradiography.

2.5. Cell migration assay

Cell migration was measured using QCM™ Transwell Colorimetric Cell Migration Assay according to the manufacturer's protocol (Chemicon International, Temecula, CA, USA). Briefly, 1×10^5 serum-starved SMCs were loaded onto the upper well of the modified boyden chamber (with pore size of 8.0um) in the presence or absence of inhibitors while lower wells were filled with culture medium with or without PDGF-BB (25 ng/ml). Following 12 hours incubation, non-migrating cells on the upper side of membranes were removed by wiping with a cotton tip and rinsing, and migrated cells were counted using colorimetric assay. Data are represented as fold change in cell migration where migratory rates for control groups are arbitrarily set to 1.0 fold.

2.6. Wound healing assay

Sub-confluent transfected or non-transfected SMCs were grown on glass cover slips. Following overnight serum starvation, cell cultures were scratched with a sterile pipette tip to form a wound, washed with pre-warmed sterile PBS, and incubated with medium in the presence or absence of inhibitors for 18-24 hours. At desired time points post injury cells were fixed and subjected to imaging using Nikon inverted microscope and Spot digital camera.

2.7. Cell viability assay

Sub-confluent SMCs were plated in a 24-well culture plate and treated with various doses of PDGF for 24 hours. The CellTiter 96® AQ$_{\text{ueous}}$ Non-Radioactive Cell Proliferation Assay (MTS) was used to measure cell viability by measuring the metabolic capacity of the cells according to the manufacturer's protocol (Promega, Madison, WI). The CellTiter 96® AQ$_{\text{ueous}}$ Assay is composed of solutions of a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine methosulfate) PMS. The conversion of MTS (a pale yellow chemical) into the aqueous soluble formazan (dark blue) product is accomplished by dehydrogenase enzymes found in metabolically active cells.

Metabolism in viable cells produces "reducing equivalents" such as NADH or NADPH. These reducing compounds pass their electrons to an intermediate electron transfer reagent that can reduce the tetrazolium product, MTS, into an aqueous, soluble formazan product. At death, cells rapidly lose the ability to reduce tetrazolium products. The production of the colored formazan product, therefore, is proportional to the number of viable cells in culture. This process requires active mitochondria. The colorimetric assay can be used for either proliferation or complement-mediated cytotoxicity assays.

2.8. Actin cytoskeleton staining

Cells were grown on glass slides and subjected to various treatments. At desired timepoints cells were fixed (3.7% formaldehyde for 15 min and 70% ethanol for 2 min). Following permeabilization in

0.1% Triton X-100, cells were incubated with AlexaFluor 488-labelled phalloidin (Molecular Probes, Invitrogen Detection Technologies) for 1 hour in room temperature. Glass slides were mounted and sealed using ProLong® Gold anti-fade reagent (Molecular Probes, Invitrogen Detection Technologies), and then imaged with Olmpus inverted microscope and Spot digital camera.

2.9. Antibodies and inhibitors

All primary antibodies used in this study were purchased from Cell Signaling. Specific MEK/ERK1/2 inhibitor U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene) were purchased from Cell Signaling. Specific JNK inhibitor SP600125 (Anthra[1,9-*cd*]pyrazol-6(2*H*)-one), p38 MAPK inhibitor SB202190 (4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole), and platelet-derived growth factor (PDGF) were from Calbiochem. Anti-rabbit and anti-mouse immunoglobulin G conjugated with horseradish peroxidase were purchased from Santa Cruz Biotechnology. Concentrations used for MEK/ERK1/2 inhibitor U0126 (10 µM) was as per manufacturer's recommendation. Concentrations inhibitors of JNK (SP600125) and p38 MAPK (SB202190) was 50 µM and 10 µM respectively [63].

2.10. Statistical analysis

Two-way analysis of variance (ANOVA) with multiple comparisons and paired Student's t tests were performed. Values shown are the mean ± standard deviation. A value of $P < 0.05$ was considered to be statistically significant. Experiments were done in triplicates and repeated three time ($n = 9$) unless otherwise stated.

Chapter 3. Results

Q1: What is the effect of PDGF on ILK in SMC culture?

3.1. PDGF activates integrin-linked kinase in mouse aortic SMCs

To study the effect of PDGF treatment on ILK activation, primary mouse aortic SMCs were serum-starved overnight and then treated with 25 ng/ml of PDGF-BB for various time periods. ILK kinase activity was measured at 0, 5, 15, 30, 60 and 120 minutes following treatment. Cell lysates were collected and subjected to the kinase activity assay. As shown in ***figure 3.1.***, PDGF treatment increased ILK kinase activity showing a two fold increase at 30 minutes post PDGF treatment in the SMC culture. However, no significant effect was observed on the total ILK protein expression in the mouse SMCs following PDGF treatment. PDGF-induced ILK kinase activity declined 60 minutes after treatment (***figure 3.1***). SMC culture transfected with ILK siRNA were used as negative control for the Kinase assay and western blot assay. To ensure the specificity of the test and as a negative control, a group of SMCs culture was transfected with specific ILK siRNA for 96 hours. This treatment completely abolished ILK protein expression in SMCs (***figure 3.2.***).As seen in the first lane of the blot ***figure 3.1*** ILK expression was abolished in the ILK-siRNA group (negative control) that led to a significant decrease in ILK kinase activity in this group.

It is noteworthy that we visually monitored the morphology of SMCs transfected with ILK siRNA for the incubation period prior to PDGF treatment (96 hours) to assure that siRNA treatment had no cytotoxic effect on mouse SMC.

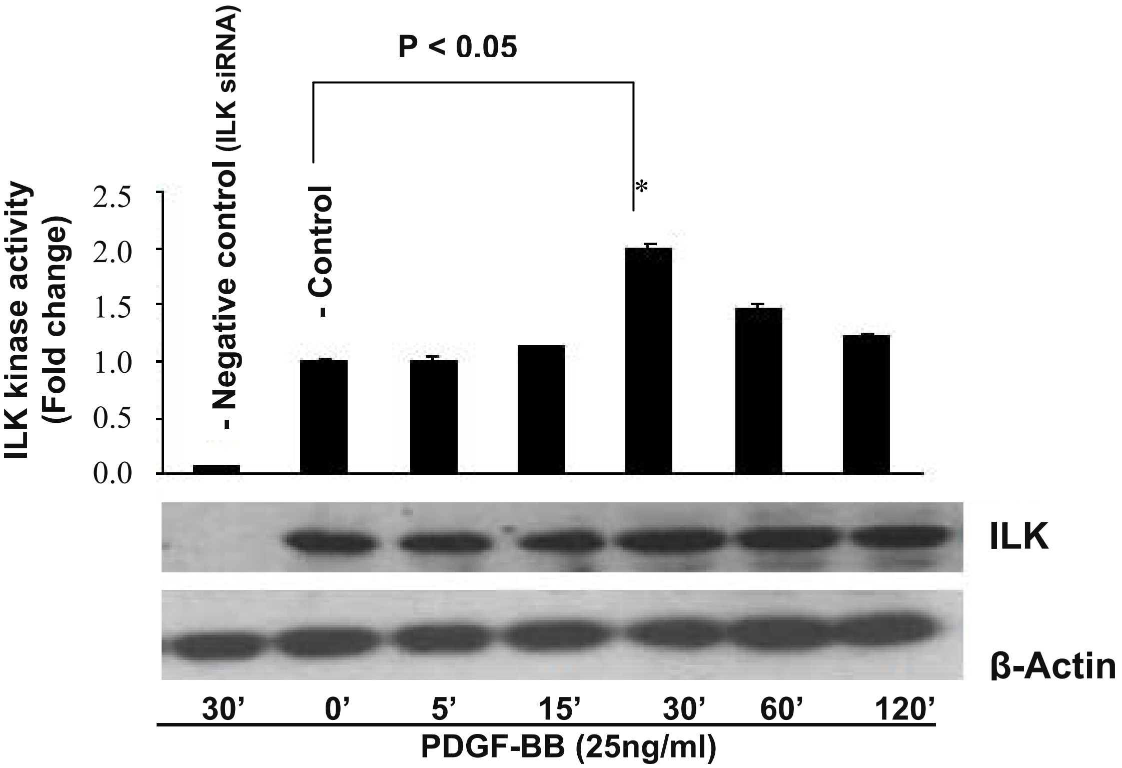

Figure 3.1. PDGF increases ILK kinase activity in mouse aortic SMCs. Western blot showing ILK protein expression and fold change bar graphs showing ILK Kinase activity at different time points following PDGF-BB treatment. PDGF treatment causes an increase in the kinase activity of ILK, having a peak at 30 min (30') of treatment. While the ILK protein expression stays the same throughout. Note the first column shown the results from cells that were treated with ILK siRNA to inhibit ILK protein expression. *Kinase activity is significantly enhanced as compared to control (absence of PDGF) ($P<0.05$).

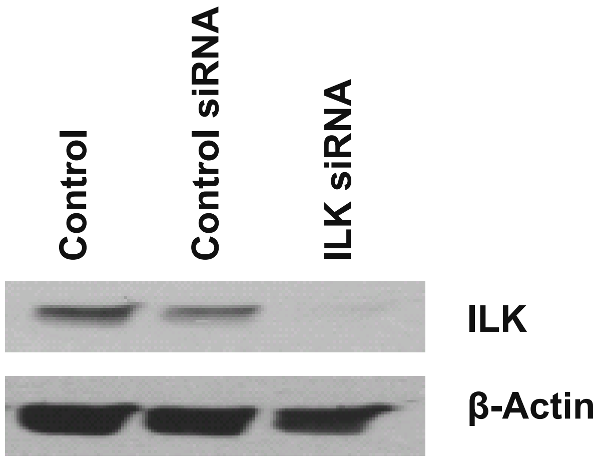

Figure 3.2. Transfection of mouse aortic SMCs with ILK siRNA for 96 hours completely blocked ILK protein expression. Data presents three independent experiments.

Q2: What is the role of Ilk during PDGF-induced migration in VSM cells?

3.1.1. PDGF induces SMC migration through an ILK-dependent pathway

To investigate the potential function of ILK in regulation of PDGF-induced SMC migration, cells were transfected with specific ILK siRNA, and then treated with 25 ng/ml of PDGF- BB. The migratory response was measured using both modified Transwell Boyden chamber and wound healing assays. It is important to keep in mind that the conditions for these two assays are different, even though they are both used to measure cells migration. In the wound healing assay we caused injury which triggers other cellular responses including the production of inflammatory cytokines and growth factors. Inhibition of ILK protein expression markedly decreased SMC migration indicating that the presence of ILK was required for transducing the migratory signal stimulated by PDGF-BB (*figures 3.3 and 3.4*).

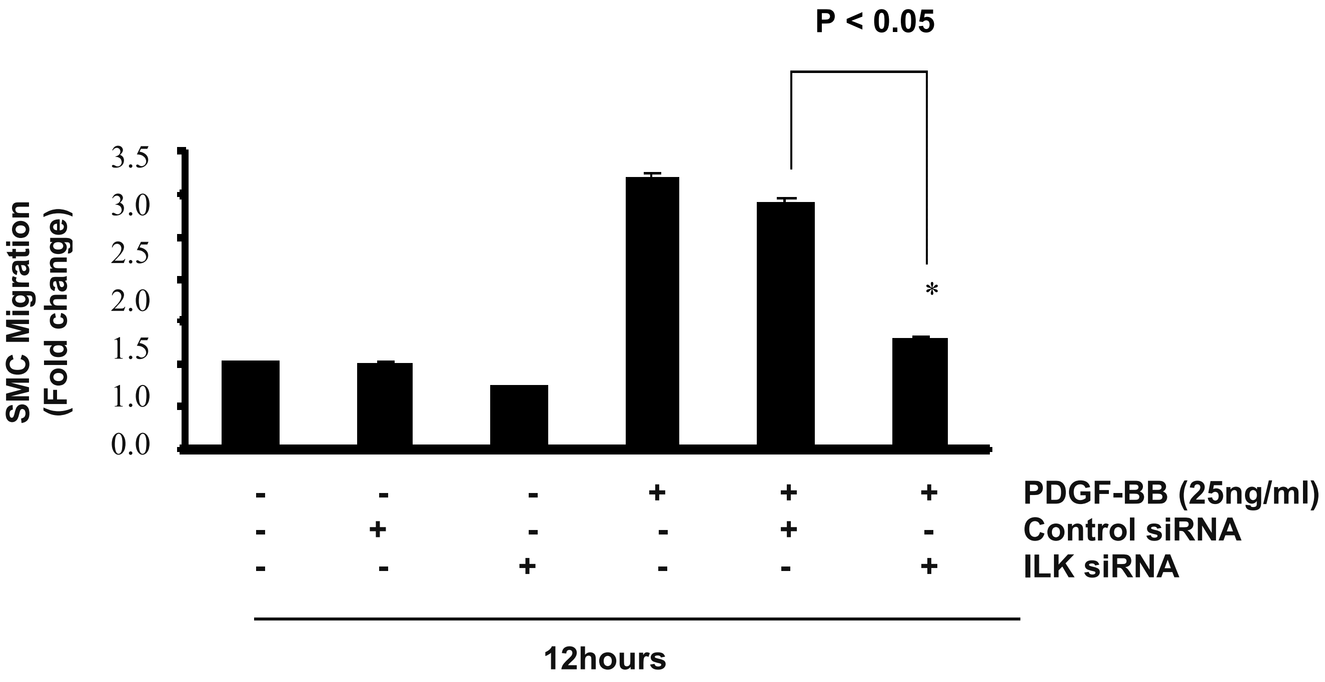

Figure 3.3. PDGF induces migration through an ILK dependent manner. Bar graph representing SMC migration detected using modified Transwell Boyden chamber. Inhibition of ILK protein expression with 20 nM ILK siRNA markedly decreased SMC migration in response to PDGF-BB.

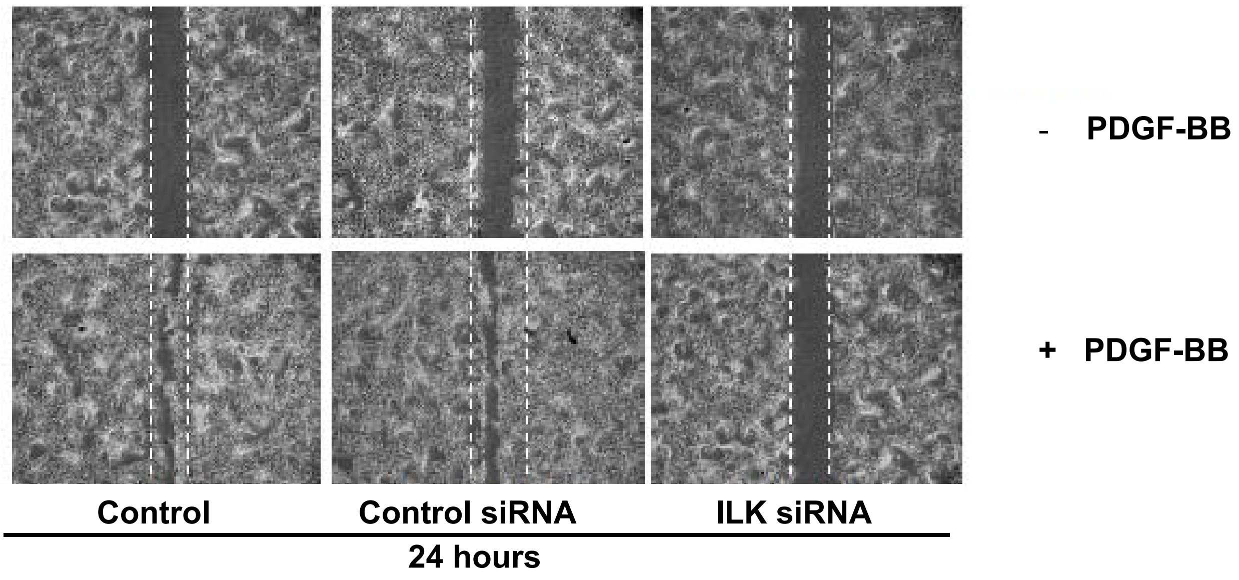

Figure 3.4. PDGF induces migration through an ILK dependent manner during injury. Wound healing assay showing migration of SMC in presence or absence of ILK, 24 hours post PDFG-BB treatment. ILK inhibition decreased the number of migrated SMCs into the site of injury in response to PDGF-BB treatment as compared to the control group (contorl siRNA). Original magnification, ×40. Data present one of three independent experiments.

PDGF is considered as a very potent mitogen. The observed effect of PDGF-BB on wound closure in figure 2B could be due to the effect of this growth factor on SMC proliferation and/or migration. Thus, to determine whether the proliferatory effect of PDGF-BB had contributed to the observed increase in cell migration, cells were treated with increasing doses of PDGF-BB for 24 hours (the maximum time required for wound closure), and cell proliferation and migration were measured using the modified Transwell Boyden chamber assay and MTS cell proliferation assay, respectively. Treatment of primary mouse SMCs with 25 ng/ml of PDGF-BB significantly increased cell migration without having any noticeable effect on SMC proliferation. To assure the accuracy of both assays and as a positive control, mouse SMC culture were also treated with DMEM that contains 10% serum, Serum provides growth factors, hormones, attachment and spreading factors, binding proteins, lipids, and minerals. These findings emphasized that the observed increase in cell migration in both assays used in this study (24 hours post injury or following loading onto Transwell Boyden Chambers) was due to the pro- migratory, not proliferatory, effect of PDGF-BB on mouse aortic SMC (***figure 3.5***).

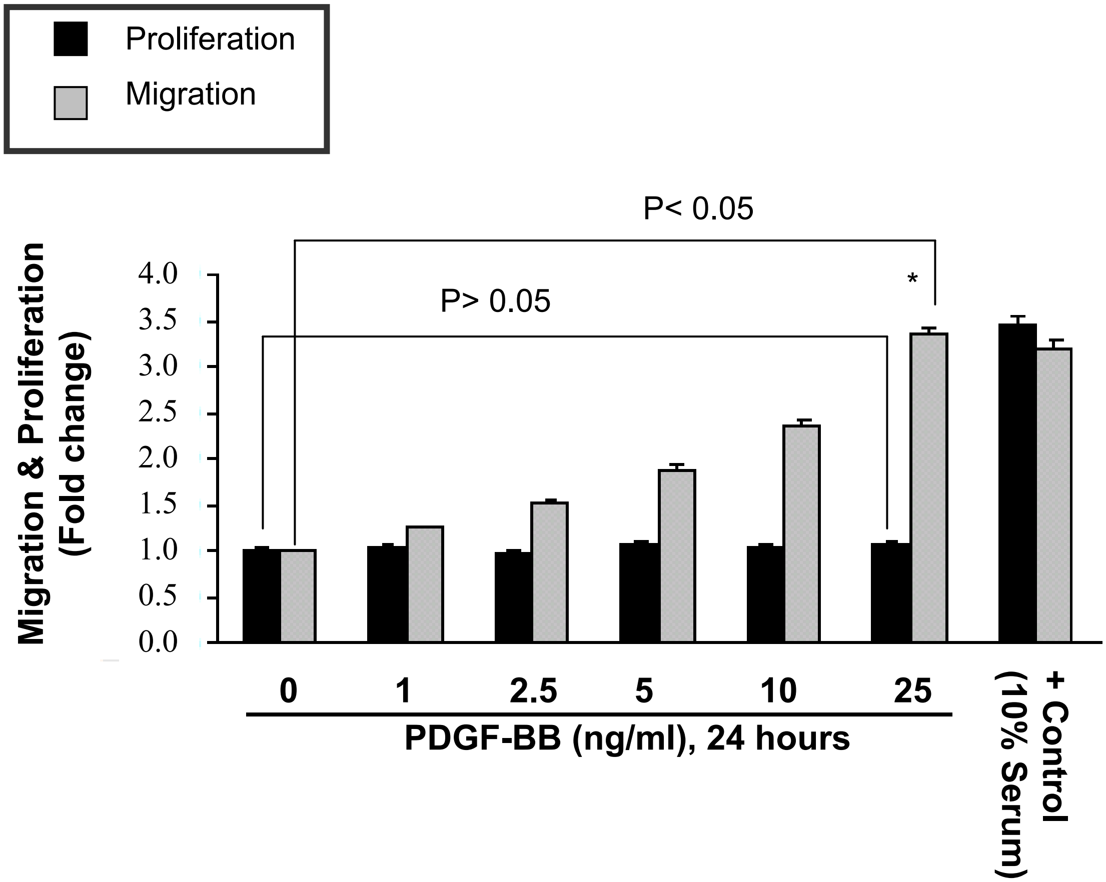

Figure 3.5. Effect of PDGF-BB treatment on mouse aortic SMCs migration and proliferation. Mouse SMCs were treated with increasing doses of PDGF-BB for 24 hours and cell migration and proliferation were measured. Cells treated with 10% serum were used as the positive control. As shown, 25 ng/ml of PDGF-BB significantly increased SMCs migration (3 fold increase) with no effect on cell proliferation indicating that the observed increase in cell migration is not due to the proliferatory effect of PDGF-BB in SMC culture.

3.1.2. ILK inhibition abolishes PDGF-induced p38 MAPK activation in mouse aortic SMC culture

Studies in various *in vitro* models have shown that members of the family of the mitogen activated protein kinases (MAPKs) become activated and play a crucial role in regulating cell migration in response to PDGF treatment [21-24]. However, the actual mechanisms underlying this activation and the identity of the upstream kinases involved are not fully elucidated. Here, we first characterized the dynamics of MAPKs family activation in response to PDGF-BB treatment in our primary mouse aortic SMC model. In agreement with previous reports in various cell models, PDGF-BB treatment resulted in the phosphorylation of all three members of MAPKs family, extracellular signal-regulated protein kinase 1/2 (Erk1/2), Jun N-terminus kinase (JNK), and p38 MAPK starting around 5 minutes and with a peak activation at 20-30 minutes post-treatment (***figure 3.6.***).

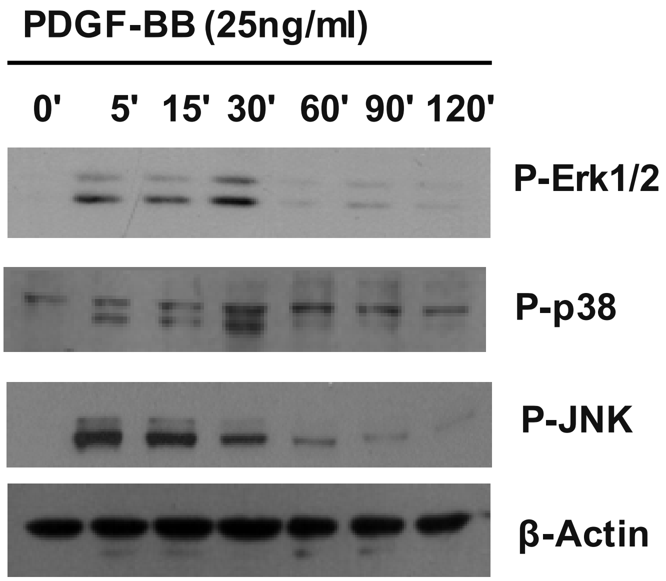

Figure 3.6. Kinetics of MAPKs activation in mouse aortic SMC culture. Western blot showing

protein expression of the activated form of three members of MAPKs (P-Erk 1/2, P-p38, P-JNK) at various time points following PDGF-BB treatment. Beta-Actin is a house protein used as proof of equal loading for each lane. PDGF-BB treatment resulted in phosphorylation and activation of all three members of MAPKs family in mouse aortic SMCs, with peak activation starting at 5 minutes to 30 minutes post PDGF-BB treatment for P-Erk 1/2 and P-JNK. However p38 gets phosphorylated in the presence of PDGF-BB especially at 30 minutes post treatment. Data represents three independent experiments.

To investigate the role of each member of MAPKs family in regulating PDGF-induced cell migration, specific inhibitors were used to block the activation of each one at a time. SMCs were treated with DMSO as internal control, specific MEK/Erk1/2 inhibitor (20 µM U0126), JNK inhibitor (50 µM SP600125), and p38 MAPK inhibitor (10 µM SB202190) for two hours prior to PDGF-BB treatment and cell migration was measured using the modified Transwell Boyden Chamber assay. Inhibition of all members of MAPKs family markedly reduced mouse SMC migration in response to PDGF-BB treatment (***figure 3.7***).

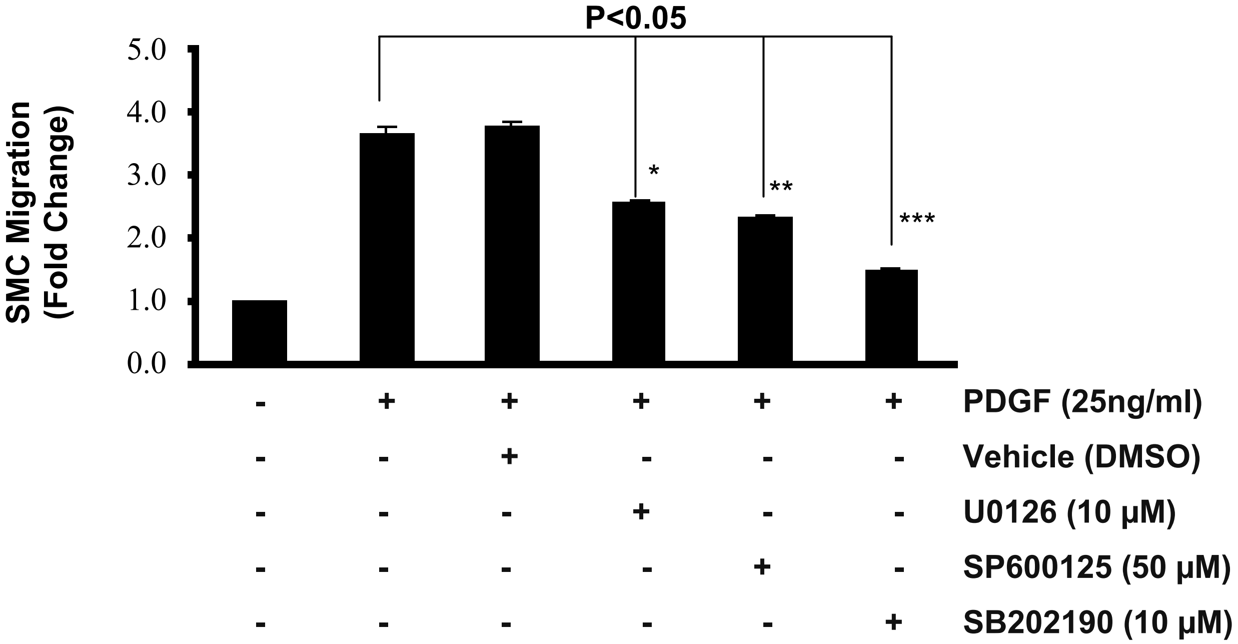

Figure 3.7. PDGF regulates mouse SMC migration through activation of mitogen activated protein kinases (MAPKs). Mouse aortic SMCs were treated with specific MAPKs inhibitors at specified concentrations for 2 hours prior to seeding onto Transwell Boyden Chambers and PDGF-BB treatment (25 ng/ml), as well as during the incubation period (12 hours). Cell migration was measured at 12 hours post culture. As shown, inhibition of Erk1/2, JNK and p38 MAPK activation significantly reduced SMC migration in response to PDGF-BB. *Significantly different from the internal control (*, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$).

Q3: Can ILK regulate the activation of members of MAPKs in response to PDGF?

Various components of focal adhesion complex have been shown to regulate or cross talk with members of MAPKs family [24]. ILK, as a key constituent of the focal adhesion complex, also plays an important function in transducing inside-out and outside-in signals [25]. To investigate the role of ILK in regulating MAPKs activation in response to PDGF, SMCs were transfected with specific ILK siRNA prior to PDGF-BB treatment. Inhibition of ILK expression with specific siRNA resulted in significant decrease in PDGF-induced p38 MAPK phosphorylation (***figure 3.8***), while having no effect on Erk1/2 and JNK phosphorylation (***figure 3.9***). This observation suggests that ILK presence is necessary for PDGF-induced phosphorylation of p38 MAPK in mouse SMC. Expression of P-ERK 1/2 and P-JNK is not affected by the absence of ILK.

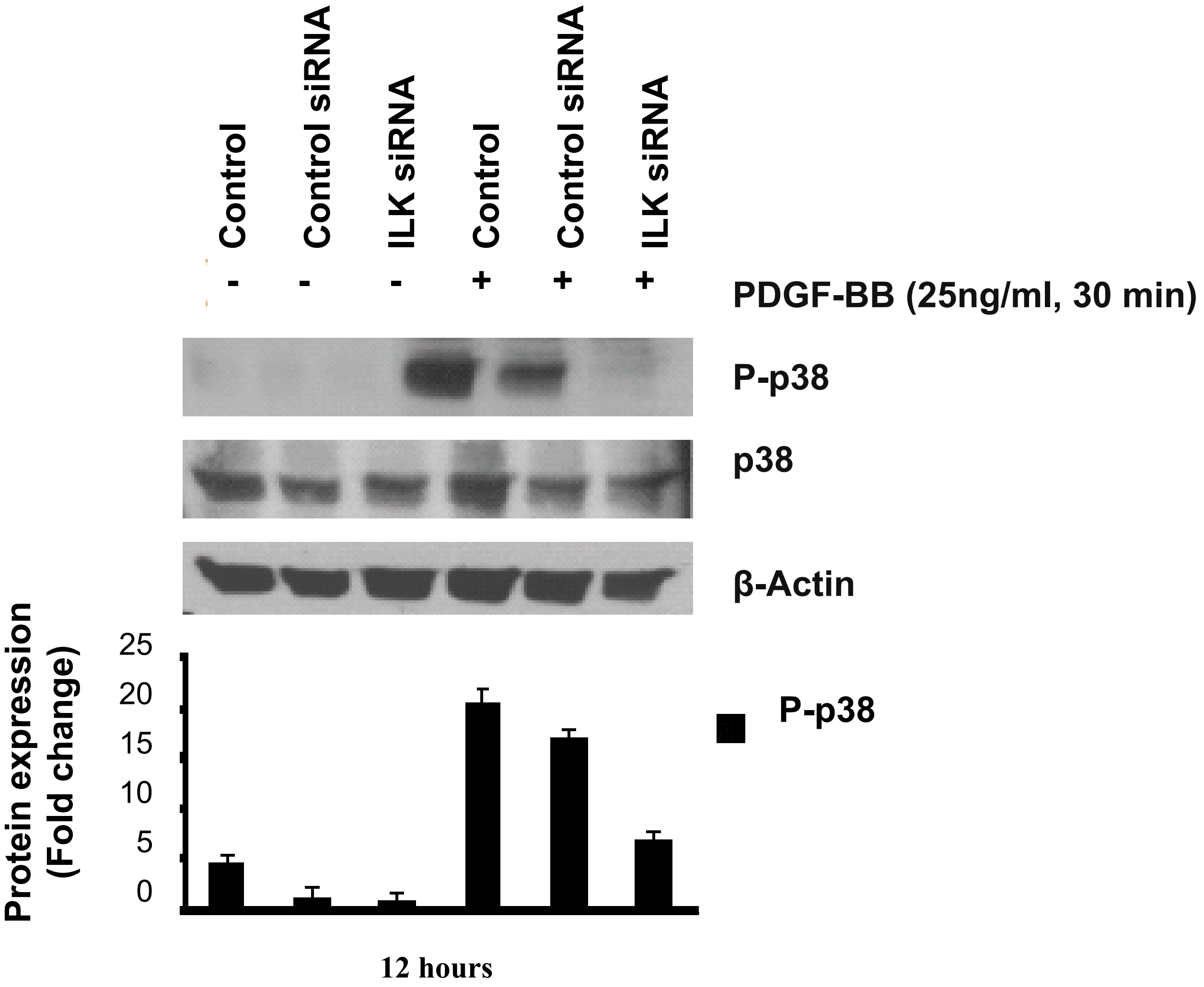

Figure 3.8. ILK inhibition abolishes PDGF-induced p38 MAPK activation in mouse aortic SMCs. Mouse aortic SMCs were transfected with 20 nM of specific ILK siRNA and then treated with 25 ng/ml of PDGF-BB for specified time points. Cell lysates were collected to measure phosphorylation of p38 MAPK. Inhibition of ILK protein expression markedly reduced PDGF-induced p38 MAPK phosphorylation in mouse aortic SMCs, indicating that ILK is a regulator of PDGF-induced p38 MAPK activation. The blot presents one of three independent experiments. The value of P-p38 in the graph has been normalized to β-Actin (house keeping protein).

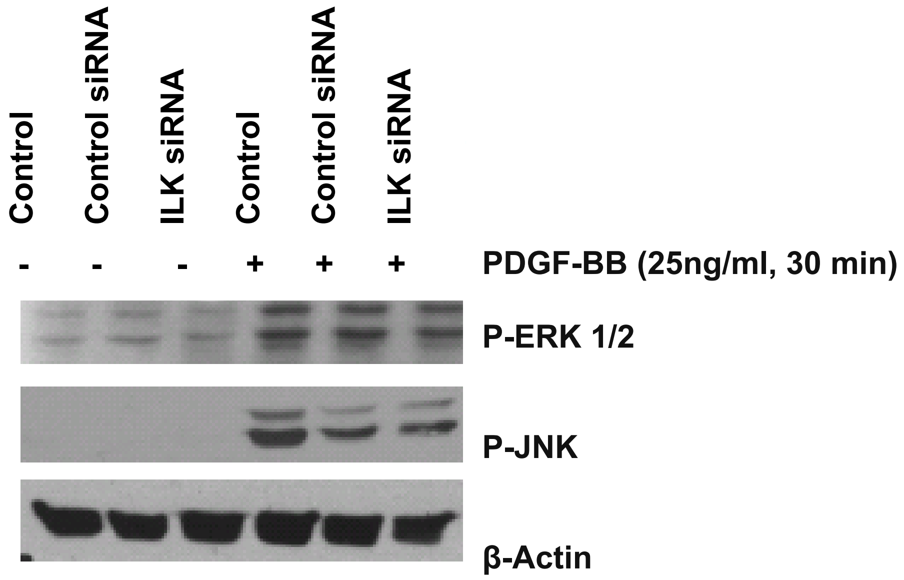
Control
Control siRNA
ILK siRNA
Control
Control siRNA
ILK siRNA
- - - + + +
PDGF-BB (25ng/ml, 30 min)
P-ERK 1/2
P-JNK
β-Actin

Figure 3.9. PDGF activates ERK 1/2 and JNK through an ILK-independent pathway. Mouse SMCs were transfected with 20nm ILK siRNA for 96 hours prior to PDGF treatment. Cell lysates were collected to measure phosphorylation of ERK 1/2 and JNK.ILK inhibition by specific siRNA had no detectable effect on Erk1/2 and JNK phosphorylation in response to PDGF-BB treatment in mouse aortic SMC culture. Data presents one of three independent experiments.

3.2. ILK mediates PDGF-induced mouse aortic SMC migration through a p38 MAPK- dependent pathway

Taking into account that PDGF-BB activates both ILK and p38 MAPK in mouse aortic SMCs (***figures 3.1. and figure 3.5.),*** and the observation that p38 MAPK has a crucial role in PDGF-induced cell migration (***figure3.7.***); we sought to determine whether ILK can also modulate the migratory effect of p38 MAPK activation in response to PDGF-BB. To examine this hypothesis, we utilized a gain and loss of function approach using adenoviral constructs of ILK and/or p38 MAPK. In order to revalidate the important role of p38 MAPK activation during the migratory response, and also to exploit a more target-specific approach we used adenoviral vectors carrying dominant negative and constitutively active forms of ILK and p38 MAPKs. We first infected primary mouse SMCs with adenoviral constructs expressing either a dominant negative form of p38 MAPK (Ad-Dn-p38), a wild type form of p38 MAPK (Ad-Wt-p38), or a GFP (Ad-GFP) protein. Western blot analysis (measuring protein expression) and fluorescent microscopy (visualizing GFP expression) were used to evaluate the transfection efficiency at 36 hours post transfection (***figures 3.10 and 3.11***).

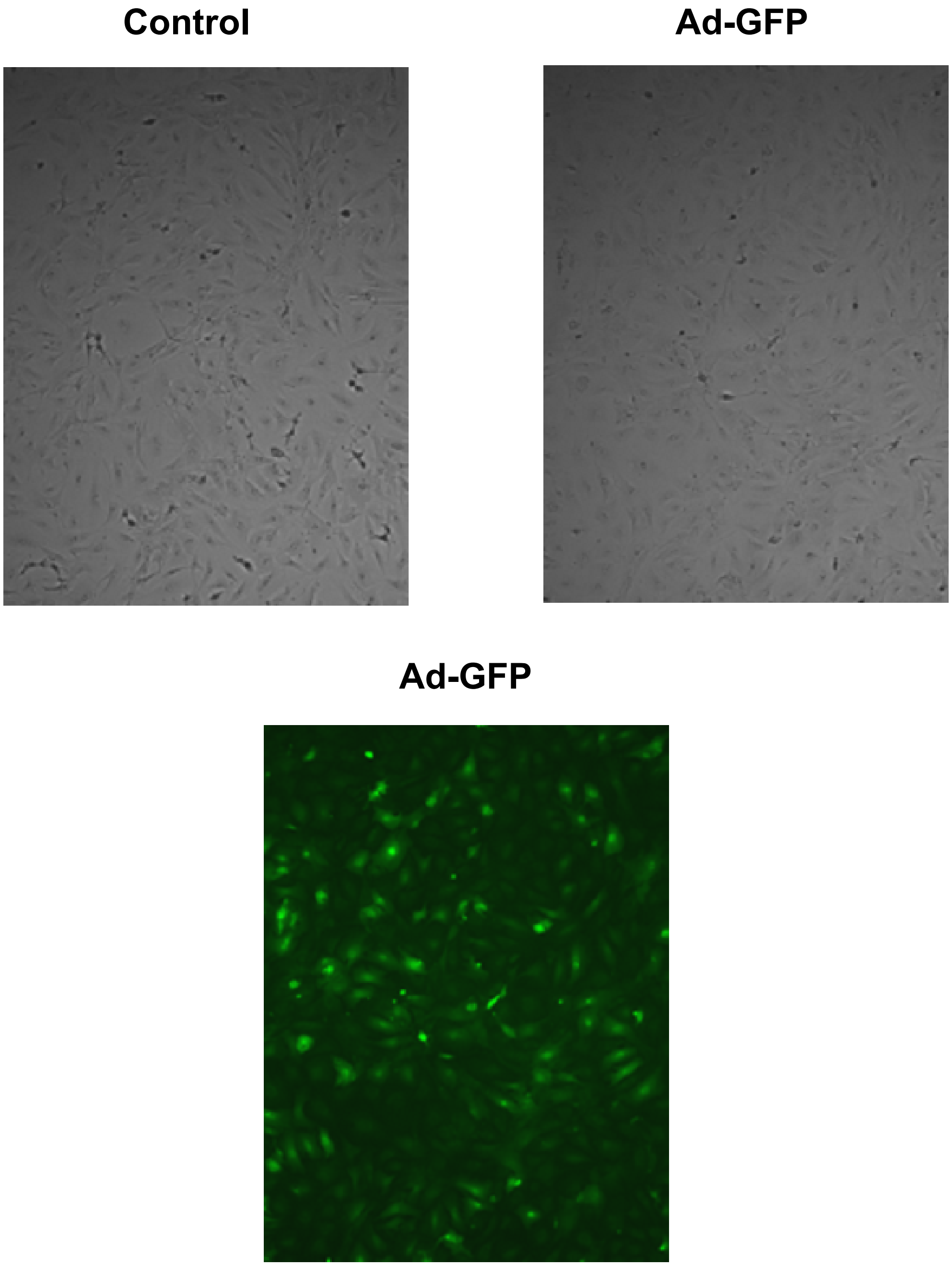

Figure 3.10. Mouse aortic SMC morphology and transfection efficiency. Photomicrograph of mouse aortic SMCs showing the morphology and GFP expression at 36 hours following transfection. Sub-confluent SMCs were infected with adenoviral vector at the multiplicity of infection of 100. Note the high level of GFP expression as well as the normal phenotype of Ad-GFP-transfected SMCs. Original magnification, x200.

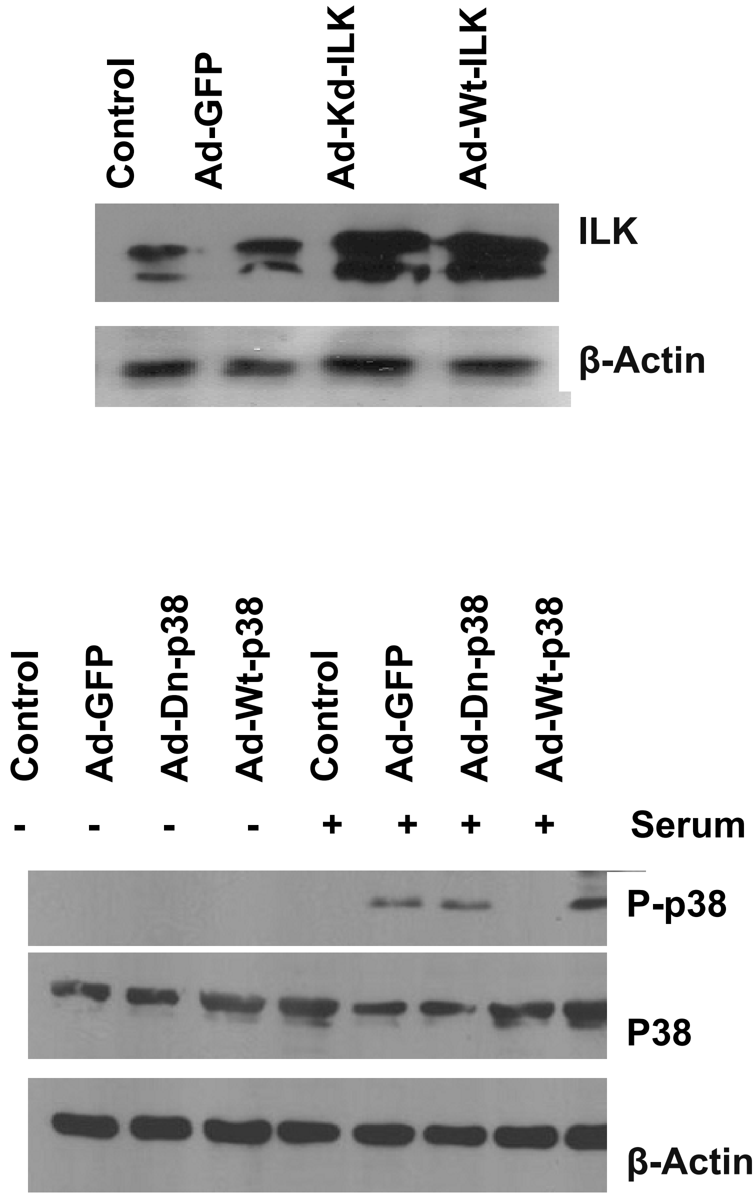

Figure 3.11. Specificity of adenoviral vectors used shown in western blot. Over- expression of a dominant negative form of p38 MAPK markedly increased total p38 MAPK expression while blocking p38 MAPK 28 phosphorylation in response to 10 % fetal calf serum (FCS) treatment in SMCs (10% serum is used as a strong activator for p38 MAPK). Also, note the considerable

increase in ILK expression level in SMCs transfected with adenoviral ILK constructs.

In concurrence with our previous observations, over-expression of a dominant negative mutant of p38 MAPK significantly reduced PDGF-induced SMC migration (***figure 3.12***). Furthermore, over-expression of a wild type form of p38 rescued SMC migration in response to PDGF-BB, corroborating the specificity of p38 MAPK inhibition in mouse SMC culture transfected with the Ad-Dn-p38 MAPK construct (***figure 3.12***). This again confirms that inhibition of p38 MAPK pathway blocks SMC migration.

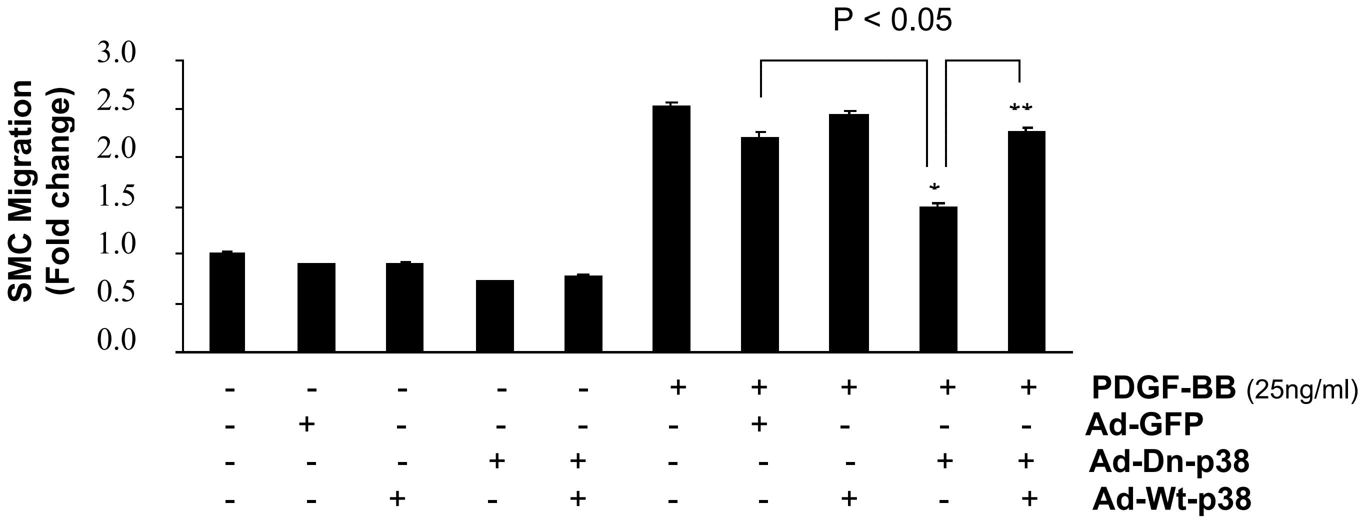

Figure 3.12. Dominant negative form of p38 MAPK significantly decreases SMC migration in response to PDGF. For migration assay, transfected SMCs were cultured in Transwell Boyden Chambers in the presence or absence of 25ng/ml of PDGF-BB and cell migration was measured 12 hours post culture. Dominant negative p38 MAPK significantly decreases SMC migration in response to PDGF-BB treatment while expression of a wild type form of p38 MAPK increases SMC migration in response to PDGF-BB. In addition, over- expression of a wild type mutant of p38 MAPK subverted the inhibitory effect of p38 inhibition on SMC migration. Moreover, a kinase-

deficient form of ILK (previously referred to as dominant negative ILK) which has a point mutation (E359K – Glutamic acid to Lysine at amino acid 359) in subdomain VIII of the ILK kinase domain which is also incapable of binding to paxillin and parvin and therefore unable to participate in formation of the focal adhesion complex, resulted in a significant decline in PDGF-induced SMC migration, an event that can be reversed by a wild type form of ILK (***figure 3.13***).

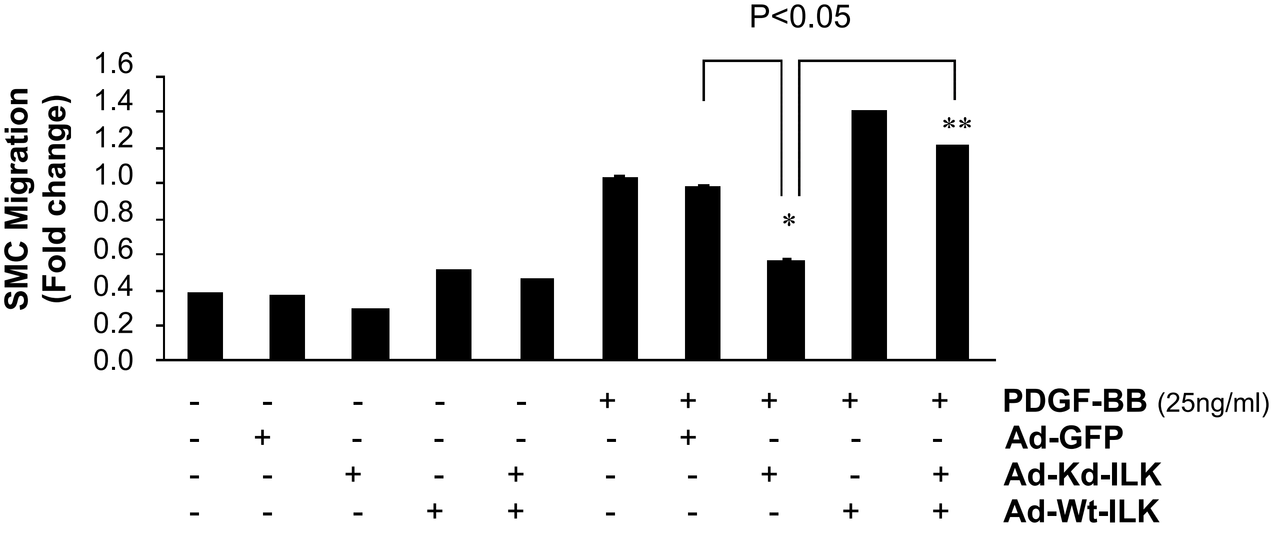

Figure 3.13. Kinase deficient form of ILK significantly decreases SMC migration in response to PDGF. Kinase deficient mutant of ILK significantly reduced PDGF-induced SMC migration in a Traswell Boyden Chamber migration assay. As shown, expression of a wild type form of ILK rescued SMC migratory response indicating the specificity of ILK inhibition by the adenoviral construct. Finally, to establish a cause and effect association between inhibition of ILK, a downstream decrease in p38 MAPK phosphorylation, and the subsequent decline in cell migration, we co- transfected cells with a kinase deficient form of ILK (E359K) along with an active form of MKK3/6 (the specific upstream activator of p38 MAPK) or a wild type form of p38 MAPK.

As shown in figure 3.14, expression of Ad-Ca-MKK3/6 significantly reversed the inhibitory effect of

ILK inhibition (Ad-Kd-ILK) in PDGF-treated SMCs. In contrast, over-expression of the wild type p38 MAPK in SMCs already transfected with Ad-Kd-ILK, did not rescue cell migration, confirming our previous observation that the existence of an active form of ILK was required to facilitate the p38 MAPK-mediated migratory response to PDGF-BB (***figure 3.14***).

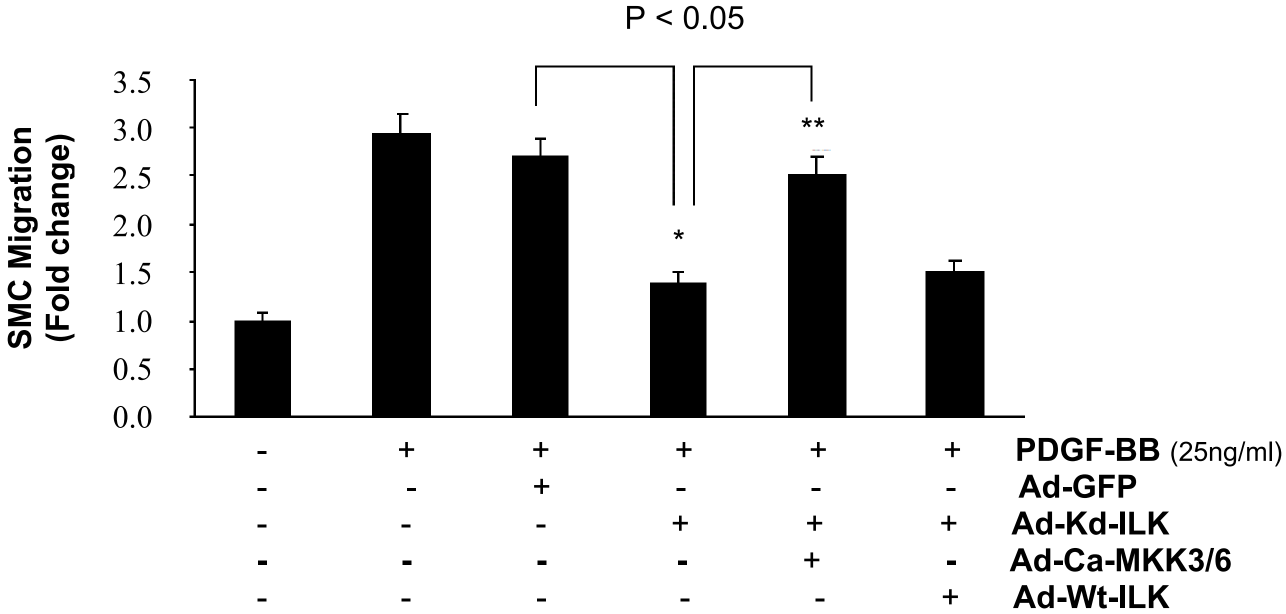

Figure 3.14. ILK regulates PDGF-induced SMC migration through a p38 MAPK- dependent pathway. Over-expression of an active form of MKK3/MKK6 (specific upstream activators of p38 MAPK), but not a wild type form of p38 MAPK, rescued SMC migration in response to PDGF-BB indicating that an active form of ILK is required for the initiation of a p38 MAPK-dependent migratory response to PDGF-BB in mouse aortic SMCs.

3.2.1. ILK and p38 MAPK regulate PDGF-induced actin cytoskeleton polymerization in vascular SMC

Actin polymerization and re-organization is a critical step in cell motility and migration. To characterize the role of ILK and p38 MAPK in regulating actin polymerization in response to PDGF, cells were transfected with specific ILK siRNA (20 nM) or treated with specific p38 MAPK inhibitor (10 µM SB202190), prior to PDGF treatment. Inhibition of ILK expression and p38 MAPK activity significantly blocked PDGF-induced actin polymerization and re- organization in mouse SMC, indicating a crucial role for ILK and p38 MAPK in regulation actin-mediated cell migration (***figures 3.15 and 3.16).***

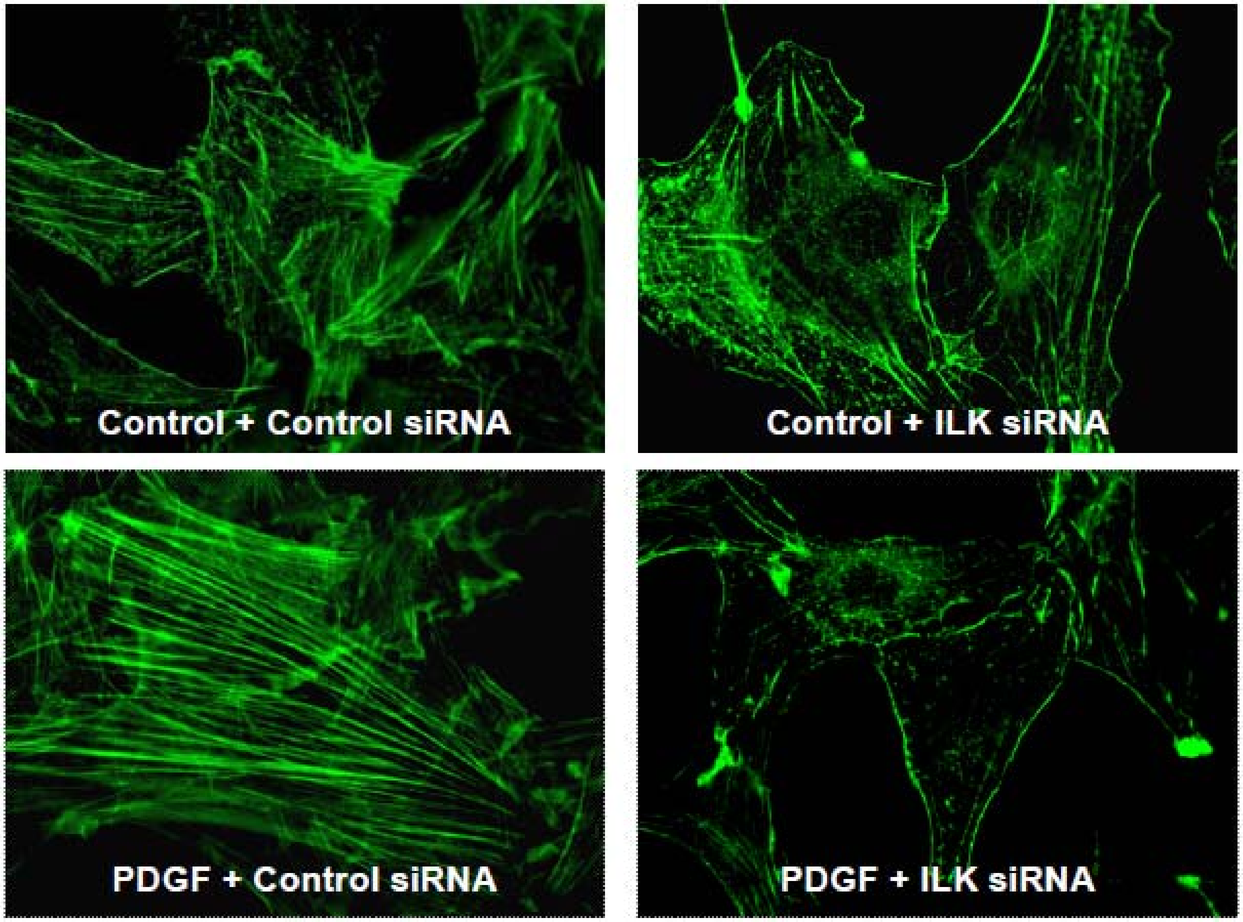
Control + Control siRNA
Control + ILK siRNA
PDGF + Control siRNA
PDGF + ILK siRNA

Figure 3.15. Inhibition of ILK expression disrupts PDGF-induced actin re-organization in mouse SMC. Mouse SMCs were treated with either vehicle (DMSO) or with 10 µM of p38 MAPK specific inhibitor SB202190 for 1 hour prior to PDGF treatment. Following treatment, cells were washed and fixed and then stained for actin filaments. Inhibition of p38 MAPK markedly blocks PDGF-induced actin polymerization and reorganization (original magnification x400).

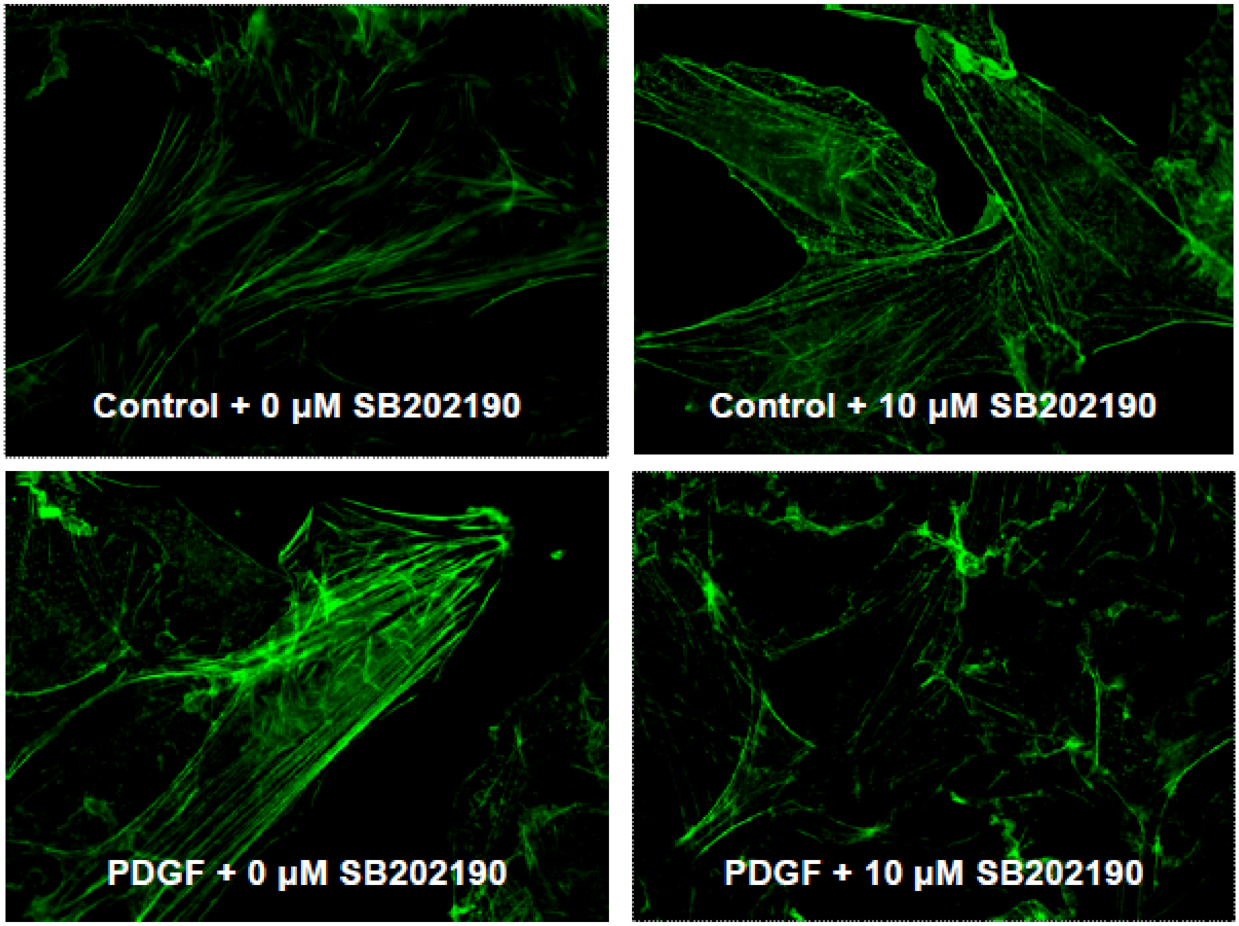
Control + 0 µM SB202190
Control + 10 µM SB202190
PDGF + 0 µM SB202190
PDGF + 10 µM SB202190

Figure 3.16. Inhibition of p38 MAPK disrupts PDGF-induced actin re-organization in mouse SMC. Mouse aortic SMCs were transfected with 20 nM of specific ILK siRNA and then treated with 25 ng/ml of PDGF-BB for 20 minutes. Cells were then fixed and stained for actin filaments. Inhibition of ILK expression in SMCs significantly reduced PDGF-induced actin polymerization and reorganization in mouse aortic SMCs. Images are representative image of three independent experiments (original magnification x400).

3.2.2. PDGF increases phosphorylation of LIMK and cofilin-induced actin cytoskeleton polymerization in vascular SMC

Migration of SMCs depends directly on the regulated dynamics of the actin cytoskeleton. Cofilin is essential for effective depolymerization of actin filaments. LIMK-1 inhibits the effects of cofilin on the actin cytoskeleton by reducing the pool of active cofilin. Phosphorylation of Cofilin by LIMK inactivates cofilin, leading to accumulation of actin filaments. LIMK-1 is involved in cytoskeletal regulation, possibly by reducing depolymerization of actin filaments.

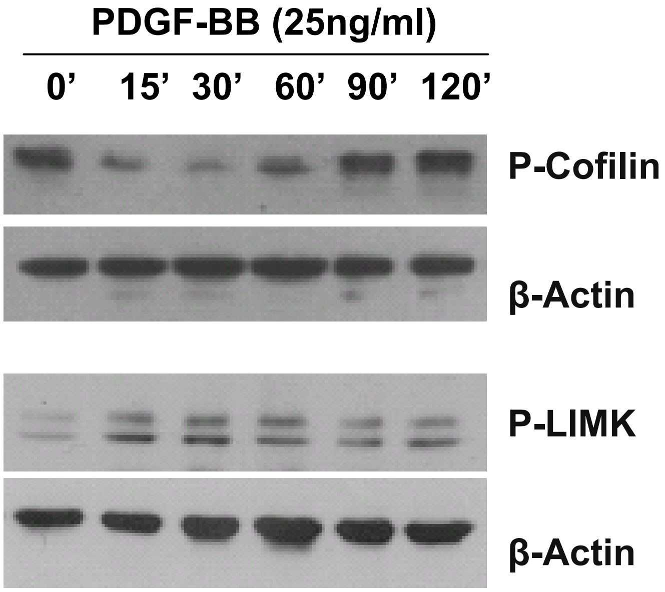

Figure 3.17. PDGF induces LIMK and cofilin phosphorylation in mouse aortic SMCs. Aortic SMCs were serum starved overnight and then treated with 25 ng/ml of PDGF- BB. Cell lysates were collected in various time points and phosphorylation of LIMK and cofilin was measured. Data represents three independent experiments. Treatment of mouse aortic SMCs with PDGF-BB resulted in phosphorylation and activation of LIMK, 15 minutes post treatment; And phosphorylation and inactivation of Cofilin starting 60 minutes post treatment (***figures 3.17***).

3.2.3. PDGF increases expression of β1and β3 integrins in mouse aortic SMCs

Integrins are present in different isoforms and are involved in cardiovascular physiopathology. We have already shown that PDGF can increase ILK activity (***figure 3.1***). We examined whether PDGF treatment had any effect on β1 and β3 protein expressions in our mouse aortic SMC cultures. SMCs were treated with PDGF-BB, and cell lysates were collected at various time-points following treatment. Western blot analysis of samples revealed (***figure 3.18***) that while PDGF-BB treatment increases the expression of both these integrins especially from 15 minutes to 1 hour post PDGF treatment; β3 integrin is expressed at a much higher level in our system. This increase in PDGF induced integrin expression is associated with considerable raise in ILK kinase activity and slight increase in ILK protein expression.

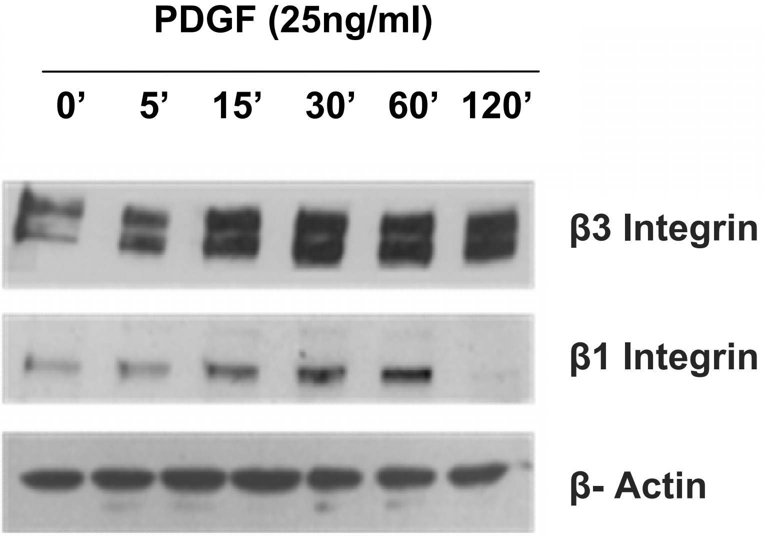

Figure 3.18. PDGF increases expression of β1 and β3 integrin. PDGF-BB treatment increased the expression of both β1 and β3 integrin in mouse aortic SMC cultures, starting around 15 minutes post-treatment.

To test the ‘inside-out’ response to PDGF-BB treatment and determine if ILK plays any regulatory part in PDGF-induced increase in β1 and β3 integrins; we inhibited expression of ILK protein by transfecting our cells with 20nM ILK siRNA and then treating the cell culture with 25ng PDGF-BB. β1 and β3 integrin expressions were observed in western immunoblot, 30 minutes post PDGF-BB treatment. Inhibiton of ILK protein expression significantly decreased PDGF-BB induced β1 and β3 integrin expression in aortic mouse SMCs (***figure 3.19***). This observation confirms that ILK has an important function in PDGF-induced increase in β1 and β3 integrins, as a molecular scaffold.

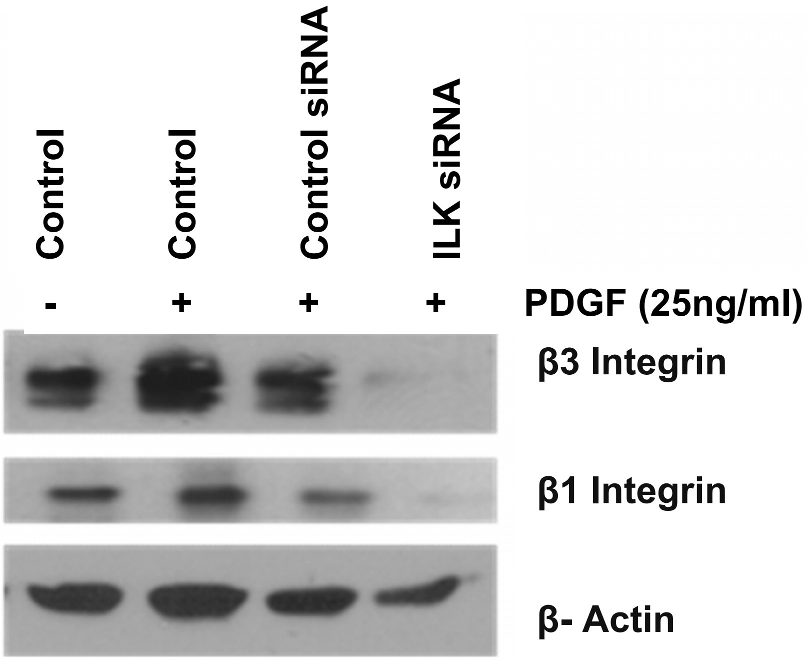

Figure 3.19. PDGF regulates β1 and β3 integrin expression through ILK in SMCs. Transfected or non-transfected cells were treated with PDGF-BB and cell lysis was collected at various time-points. Inhibition of ILK expression using specific ILK siRNA resulted in significant decrease in β1 and β3 integrin expression in SMCs.

Chapter 4. Discussion

Under physiological conditions mechanical stretch and shear stress are important in regulating the function of the circulatory system. Under disease conditions these same mechanosensors (which include integrins and RTKs) may detect pathological insult and consequently activate signal transduction pathways involving Rho GTPases (RhoA, Rac1 and Cdc42), MAPK (ERK1/2, JNK and p38) and subsequent transcription factors that regulate the function of VSMC, cardiac myocytes and platelets and are involved in VSMC migration, intimal thickening and occlusive vascular diseases. Various cytokines, peptide growth factors, and components of the extracellular matrix (ECM) have been characterized as pro-migratory molecules. PDGF is a very potent stimulant for SMC migration through activation of several signaling pathways [30-32]. The signaling pathways used by PDGF receptor stimulation In VSMC involve the stimulation of the MAPK pathway (other signaling pathways activated by PDGF include the PI3K/Akt, protein kinase A (PKA), Src and Ras).The MAPKs have been divided into three major subfamilies of protein kinase: p38 MAPK, JNK, and Erk1/2, all of which have been shown to be involved in vascular diseases and remodeling [28]. Of these P38 MAPK and JNK cascades respond to stress stimuli.

Intracellular signals generated by growth factors and their receptor tyrosine kinases (RTKs) are generated independently from those produced by interaction between the ECM and integrin. Many of the interactions between integrins and growth factor receptors are unique to specific cell types and integrins expressed, in addition; the synergy between these signals plays an important role in VSMC migration in disease condition. Defining the mechanism by which these two signaling pathways cooperate is essential for understanding VSMC behavior after injury and aid in finding a possible therapeutic target that can stop or decrease the narrowing of vascular lumen due to VSMC migration.

In the present study, we have shown that treatment of mouse aortic SMCs with PDGF-BB increased their migration by 2 fold (***figure 3.3***). PDGF-BB treatment also resulted in an increase in ILK kinase activity without affecting ILK protein expression (***figure 3.1***) in mouse aortic SMC culture. ***(figures 3.18 and 3.19)***. It is well known that the adhesive function of integrin receptors affects cytoskeleton rearrangement and cell motility. Therefore, it is plausable that an increase in β1 and β3 integrins expression contributes to the pro-migratory effect of PDGF-BB in SMC culture. However, further investigation is required to evaluate the potential cross-talk between β1 and β3 integrins and PDGF receptors and the significance of such events in vascular SMC migration.

Numerous studies have provided a wealth of information on the crucial regulatory role for ILK as a protein kinase and also as a molecular scaffold and adaptor protein during cell spreading and migration in a variety of cancer cells [13, 24]. Moreover, a recent study has shown that an increase in ILK expression coincides with higher rate of SMC migration within the atherosclerotic plaque in ApoE -/- mice, a reliable model that mimics the progression of atherosclerosis in humans [38].

In this study, we examined the role of ILK in cell migration, by inhibiting ILK expression (using siRNA) or increasing ILK expression (using an adenoviral vector expressing wild type ILK). Inhibition of ILK either by RNA interference or adenoviral vectors, significantly decreased cell migration in response to PDGF-BB, demonstrating that ILK plays an important role in regulating PDGF-induced vascular SMC migration in mouse aortic SMCs.

Expression of a wild type form of ILK increased SMC migration even in the absence of growth factor.

It is noteworthy that the effect of wild type ILK in increasing SMC migration is more evident when SMCs were treated with PDGF-BB (***figure 3.13***).These observations are in agreement with a previous report by Dwivedi et al [40] that over-expression of a dominant negative form of ILK markedly reduced intimal thickening in human saphenous vein organ culture.

The ‘kinase-deficient’ ILK mutant E359K, formally known as dominant-negative ILK , has a mutation within the catalytic domain. Kd-ILK exhibits reduced kinase activity comparable with the wild-type protein. In addition, this mutation (changing glutamic acid at position 359 to lysine) disrupts the interaction and binding of ILK with two important components of focal adhesion complex, β-parvin and paxillin (focal adhesion adapter protein involved in integrin signaling). This interaction is required for the assembly of a functional focal adhesion complex and subsequent actin polymerization and reorganization, and thus normal smooth muscle function [41, 42]. Over-expression of the E395K mutant of ILK resulted in significant reduction in PDGF-induced SMC migration. The effect was fully reversed by a wild type form of ILK (***figure 3.13***). These findings accentuate the crucial function of ILK as molecular scaffold during SMC migration.

In an effort to understand how ILK mediates the pro-migratory effect of PDGF-BB in primary mouse SMC using RNA silencing technique, we were able to show that ILK inhibition could lead to a considerable decrease in p38 MAPK phosphorylation, while having no visible effect on Erk1/2 and JNK phosphorylation in response to PDGF-BB treatment. Rac1 is an essential downstream protein in ILK-induced actin filament rearrangements and cell migration [15]; furthermore, Rac 1 is thought to regulate p38 MAPK [53]. We also demonstrated that over-expression of an active form of MKK3/6 (specific upstream activator of p38 MAPK) dramatically reversed the inhibitory effect of ILK inhibition on SMC migration. Expression of a wild type form of p38 MAPK markedly increased SMC migration in the presence of PDGF-BB; however, it could not restore migratory response in cells over-expressing a kinase deficient form of ILK. It is of importance that the latter observation supports the view that the presence of a functional ILK is required for the initiation of a p38 MAPK-dependent migratory response to PDGF-BB in mouse aortic SMCs (***figure 5.1***). Further studies are required to characterize the precise mechanisms underlying the ILK-mediated regulation of p38 MAPK in SMCs.

Actin polymerization and reorganization is a crucial step in cell migration. We have shown that PDGF increases actin polymerization and filament reorganization in SMCs. This event was significantly blocked by both ILK siRNA and p38 MAPK inhibitor, confirming the importance of this pathway in regulating SMC migration. The actual mechanism by which p38 MAPK may regulate cell migration is not well understood. Actin polymerization and reorganization is under rigorous regulation by a group of protein kinases and/or phosphatases that coordinate the constant turnover of actin filaments in a stimulated cell. Among these, cofilin is a conserved actin-binding protein that enhances actin filament reorganization through de-polymerization and severing of preexisting actin filaments [43]. It is well known that phosphorylation of cofilin (serine 3) by LIM kinase and testicular protein kinase 2 (TESK-2) results in deactivation of cofilin and subsequent inhibition of actin reorganization [44, 45]. In mouse aortic SMCs, PDGF treatment resulted in rapid de-phosphorylation and activation of cofilin around 5-15 minutes post-treatment (***figure 3.17).*** PDGF also increased LIMK phosphorylation leading to phosphorylation and deactivation of cofilin (***figure 3.17***). This feedback mechanism provides a strict control system for cell responses to migratory stimuli such as PDGF. Our preliminary observation suggests that p38 MAPK may control actin reorganization partly via regulating the de-phosphorylation and consequent activation of cofilin. Further detailed studies are ongoing in our laboratory to characterize the signaling network and downstream pathways mediating such regulatory events.

Chapter 5. Conclusions

We were able to show that short-term treatment of mouse aortic SMCs with PDGF-BB enhances ILK kinase activity without any effect on ILK protein expression. PDGF-BB addition also increased Beta 1 and Beta 3 integrin expression in mouse aortic SMCs. We showed that inhibition of ILK kinase activity and protein expression results in a significant decrease in SMC migration in response to PDGF. Furthermore, we were able to show that inhibition of p38 MAPK markedly decreases migration of mouse aortic SMC in response to PDGF. And finally, we showed PDGF induces p38 MAPK activation in mouse SMC through an ILK-dependent pathway.

In summary, we have provided evidence that ILK is an important regulator of mouse vascular smooth muscle cell migration in response to PDGF-BB by modulating p38 MAPK phosphorylation. To our knowledge, this is the first report of the regulation of p38 MAPK by integrin signaling in mediating platelet-derived growth factor-induced migration in vascular smooth muscles. A goal for future studies might be to incorporate fluorescent markers in to ILK and other key proteins and track redistribution within the cell in 3 dimensions during migration. For example trafficking of critical signal transduction proteins could be followed by covalently pulse labeling epitope tags with fluorescent substrates. These approaches may provide information highly relevant to behavior of cells in vivo. In addition, future in vivo studies using specific small molecule inhibitors for ILK and/or p38 MAPK as well as developing transgenic animal models would provide valuable information on the potential therapeutic value of integrin signaling, in general, and the integrin-linked kinase, in particular, in controlling vascular smooth muscle cell migration during the progression of occlusive vascular diseases.

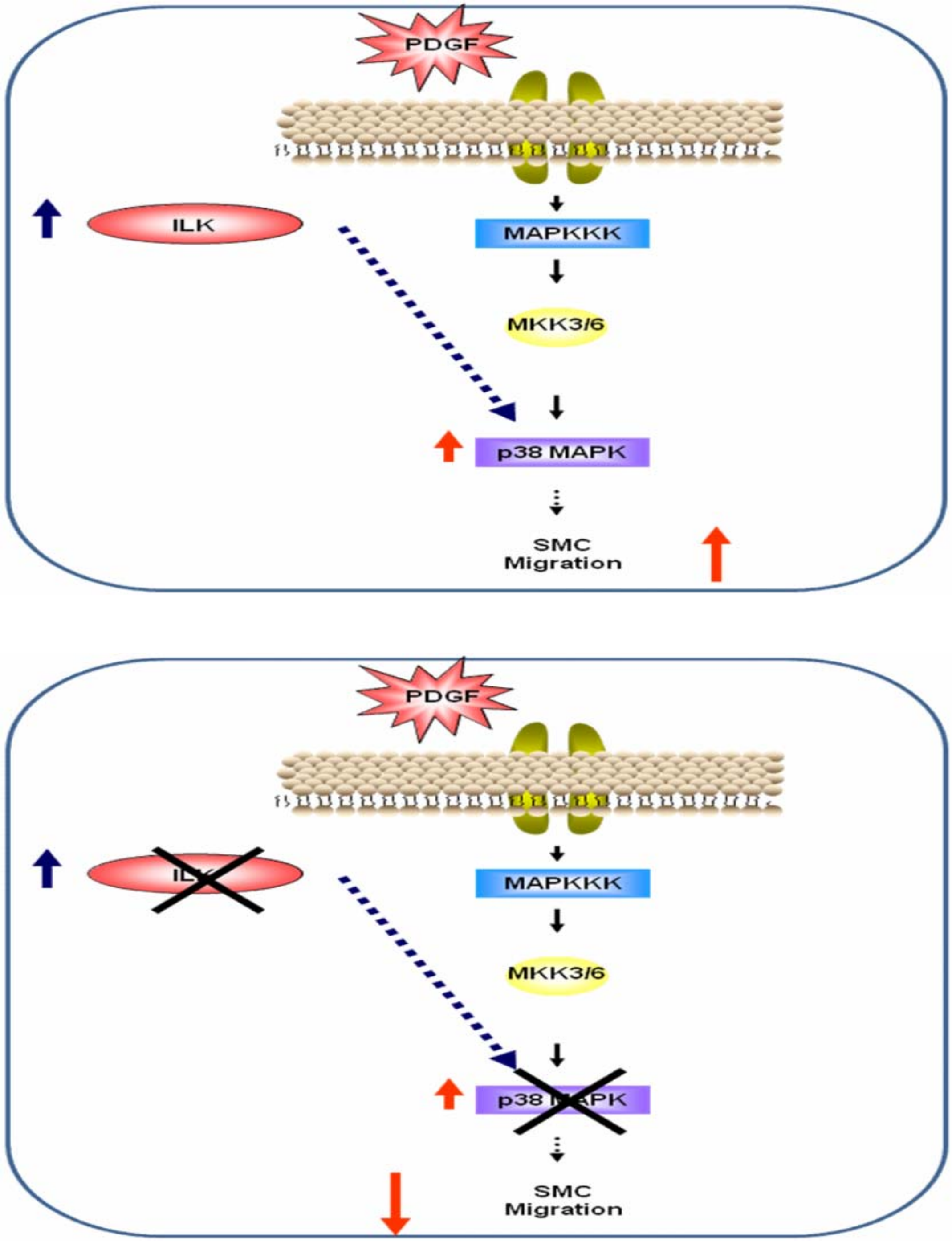
PDGF
ILK
MAPKKK
MKK3/6
p38 MAPK
SMC Migration
PDGF
ILK
MAPKKK
MKK3/6
p38 MAPK
SMC Migration

Figure 5.1. Summary: ILK regulates mouse aortic SMC migration in response to PDGF, through modulating p38 MAPK phosphorylation. In the presence of PDGF-BB, the expression of activated form of JNK, p38 MAPK and Erk 1/2 goes up, resulting in increased VSMC migration.

PDGF also increases the kinase activity of ILK, which is an upstream regulator of p38 MAPK. Inhibition of ILK expression or inhibition of ILK kinase activity inhibits the expression of P-p38, the active form of p38 MAPK. This decrease in P- p38 causes a decrease in SMC migration. Our experiments have shown that PDGF induces p38 MAPK activation in mouse SMC through an ILK-dependent pathway.

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Appendix A: List of publications and abstracts

This thesis contains work that has been published in:

- Esfandiarie M, **Yazdi SA**,Gray V, Dedhar S, Van Breemen C.**Integrin-linked kinase functions as a downstream signal of platelet-derived growth factor to regulate actin polymerization and vascular smooth muscle cell migration.** BMC Cell Bio. 2010:**11**:1**6**

Published Abstracts:

- **Abdoli Yazdi S,** Esfandiarei M and Cornelis van Breemen. *Integrin-Linked Kinase as a regulator of vascular Smooth Muscle cell migration.* UBC Department of Pharmacology and Therapeutics annual research day. June 2010, Vancouver, Canada.
- Esfandiarei M, **Abdoli Yazdi S**, and Cornelis van Breemen. Mapping the pathway regulating vascular smooth muscle cells migration: From PDGF to actin cytoskeleton. 7th International Conference of Pathways, Networks, and Systems Medicine. June 2009, Corfu, Greece.
- Esfandiarei M, **Abdoli Yazdi S**, and Cornelis van Breemen. *Integrin-linked kinase regulates aortic smooth muscle cell migration in response to platelet-derived gwoth factor through a p38 MAPK-dependent pathway.* UBC Department of Pharmacology and Therapeutics annual research day. June 2008, Vancouver, Canada.

- Esfandiarei M, **Abdoli Yazdi S**, and Cornelis van Breemen. *Platelet-derived growth factor regulates smooth muscle cell migration through a integrin-linked kinase dependent mechanism.* American Society for Pharmacology and Experimental Therapeutics, April 2008, San Diego, USA.

Appendix B: UBC biohazard approval certificate

The University of
British Columbia

Biohazard Approval Certificate

PROTOCOL NUMBER: **B09-0250**

INVESTIGATOR OR COURSE DIRECTOR: **Cornelis Van Breemen**

DEPARTMENT: **Anesthesiology, Pharmacology & Therapeutics**

PROJECT OR COURSE TITLE: **Calcium Oscillation in Vascular Smooth Muscle Cells**

APPROVAL DATE: **January 28, 2010** START DATE: **January 1, 2010**

APPROVED CONTAINMENT LEVEL: **1**

FUNDING TITLE: **Calcium oscillations in vascular smooth muscle**
FUNDING AGENCY: **Canadian Institutes of Health Research (CIHR)**

UNFUNDED TITLE: **N/A**

The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the University of British Columbia Policies and Procedures, Biosafety Practices and Public Health Agency of Canada guidelines.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there are no changes. Annual review is required.

A copy of this certificate must be displayed in your facility.

Office of Research Services
102, 6190 Agronomy Road,
Vancouver, V6T 1Z3
Phone:604-827-5111
FAX: 604-822-5093