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[Shareen Iqbal] Date

PDGF UPREGULATES MCL-1 THROUGH ACTIVATION OF β-CATENIN AND HIF-1α-DEPENDENT SIGNALING IN CANCER

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An abstract of
A dissertation submitted to the Faculty of the
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Abstract

PDGF UPREGULATES MCL-1 THROUGH ACTIVATION OF β-CATENIN AND HIF-1α-DEPENDENT SIGNALING IN CANCER

By
Shareen Iqbal

Background: Aberrant platelet derived growth factor (PDGF) signaling has been associated with cancer progression. However, its role in the regulation of cancer cell growth and survival has not been well characterized.

Methodology/Principal Findings: Prostate cancer (PCa) was employed as a model system. Using experimental models that closely mimic clinical pathophysiology of PCa progression, this study demonstrated that PDGF is a survival factor in PCa cells through upregulation of myeloid cell leukemia-1 (Mcl-1). PDGF treatment induced rapid nuclear translocation of β-catenin, presumably mediated by c-Abl and p68 signaling. Intriguingly, PDGF promoted formation of the nuclear transcriptional complex of β-catenin and hypoxia-inducible factor (HIF)-1α, and the binding of HIF-1α to Mcl-1 promoter. Deletion of a putative hypoxia response element (HRE) within the Mcl-1 promoter attenuated PDGF effects on Mcl-1 expression. Blockade of PDGF receptor (PDGFR) signaling with a pharmacological inhibitor AG-17 abrogated PDGF induction of Mcl-1, and induced apoptosis in metastatic PCa cells.

Conclusions/Significance: This study elucidated a crucial survival mechanism in PCa cells, indicating that interruption of the PDGF-Mcl-1 survival signal may provide a novel strategy for treating PCa metastasis. These results can be further applied to other model systems of tumorigenesis.

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Table of Contents

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Introduction

1.1. Growth Factors

Growth factors are locally acting polypeptides that stimulate cell proliferation by binding to specific high-affinity cell membrane receptors (1). The interaction of growth factors with their receptors triggers a cascade of intracellular biochemical signals, resulting in the activation and repression of various subsets of genes (1). Found in a number of embryonic and adult tissues, growth factors have also been identified in many cells in culture and in platelets (2-6). Similarly, growth factor membrane receptors are ubiquitously expressed in cells, often with a number of different receptors expressed within the same cell (7-9). However, receptor level expression differs, depending on the specific cell type (9).

To stimulate maximum cell proliferation, a number of growth factors are usually required (9-13). Multiple growth factors act on cell proliferation through several means. Exposure to one growth factor has been shown to reduce the threshold for mitogenicity for additional growth factors (14). Furthermore, growth factors operate at different points of the cell cycle (9,10). For example, transition through the G_1 phase required sustained growth factor stimulation. If growth factor stimulation is removed, the cell reverts to the G_0 state (10-12). Senescent cells can also undergo programmed cell death or apoptosis if there is an absence of growth factor stimulation (13).

1.2. Growth Factors and Cancer

Growth factors have been shown to play a role in cancer progression and metastasis. Loss of requirement for certain growth factors is also common among many cancer cell types (19,20). Tumorigenic cells require a decreased amount of growth factor to stimulate mitosis (16-18). The loss or reduced amount of growth factors for cancer cell proliferation is partly due to a gain in function of growth factor receptors, autocrine growth factor signaling and activation of a post-growth factor receptor signaling pathway. Growth factors produced by cancer cells can also stimulate proliferation of stromal cells, which have been shown to play a major role in tumor development (14). Similarly, stromal cells have been shown to produce growth factors, which in turn stimulate cancer cells (15). Furthermore, growth factor over expression has also been associated with tumor cell invasion, angiogenesis and metastasis initiation and spread (16-19).

Due to the ability of growth factors to induce cell proliferation, it is imperative to understand the involvement of growth factor-initiated pathways in the etiology of cancer.

1.3. Structural properties of PDGFs and PDGFRs

The platelet derived growth factor (PDGF) ligand family consists of four members PDGF-A, B, C and D. The A and B chains share 60% sequence homology and were first identified about four decades ago (20, 21). The newly identified PDGF-C and PDGF-D ligands (22, 23) contain the core PDGF domain shared by PDGF A and B, and in addition, an N-terminal CUB domain of approximately 110 amino acids. The PDGF A-, B-, C- and D-chain genes are localized to the chromosomes 7p22,

22q13, 4q31, and 11q22, respectively (24, 25). PDGF-A has two splice variants for a long and a short PDGF-A protein (196 and 211 amino acid residues) (20). The other PDGF mRNAs are not alternatively spliced. The PDGF-B, -C and -D proteins are 241, 345 and 370 amino acids in length, respectively (21).

The PDGF family is characterized by eight conserved cysteine residues, which allow for intra- and inter-chain disulfide bonds to create certain homo- and hetero-dimers. Intra-chain disulfide bonds are formed between the 1st and 6th, 3rd and 7th, and 5th and 8th cysteines, whereas the 2nd and 4th cysteines are responsible for the interchain disulfide bridges (26, 27). All PDGF ligands are synthesized and assembled into dimers in the endoplasmic reticulum in an inactive state. PDGF-A and –B are able to be activated intracellularly. In contrast, PDGF-C and –D are secreted by the cell as latent ligands and must be cleaved and activated extracellularly (23, 28).

1.4. PDGF Receptors

The PDGF ligands bind to and activate two structurally similar tyrosine kinase receptors, PDGFR-α and –β. PDGFRs can form either homodimers of the α– or β–receptors or heterodimers, depending on the ligand binding and receptor expression (23). As shown in Figure 1, PDGF-AA and –CC activate the homodimer PDGFR-α; PDGF-BB activates homodimers PDGFR-α and –β and the heterdimeric PDGFR-αβ; PDGF-AB can activate the homodimer PDGFR-α and the heterodimer PDGFR-αβ; PDGF-DD activates the homodimer PDGFR-β.

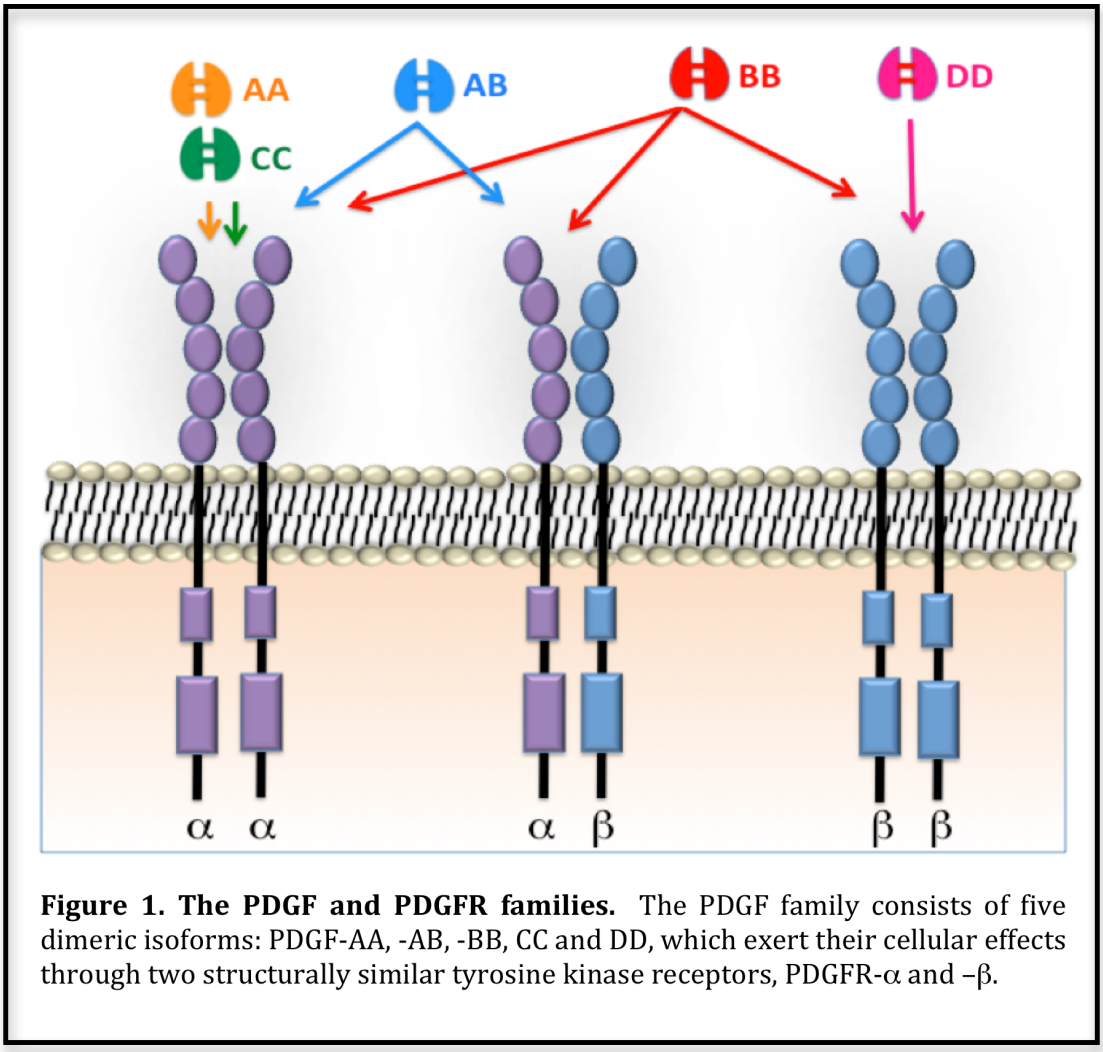

Figure 1. The PDGF and PDGFR families. The PDGF family consists of five dimeric isoforms: PDGF-AA, -AB, -BB, CC and DD, which exert their cellular effects through two structurally similar tyrosine kinase receptors, PDGFR-α and –β.

The chromosomal locations for the PDGFR-α and –β genes are 4q12 and 5q33, respectively. Both PDGFRs are class III receptor tyrosine kinases that consist of an extracellular ligand binding domain with five immunoglobulin-like domains, a transmembrane domain, a juxta-membrane domain, a tyrosine kinase 1 domain, a kinase insert domain, a tyrosine kinase 2 domain, and a C-terminal domain (29, 30).

1.5. PDGF signaling cascade

Ligand binding to PDGFRs results in the dimerization and autophosphorylation of the receptor kinases, subsequently recruiting certain Src

homology 2 (SH2) domain-containing adaptor proteins (e.g., Src, Grb2 and Shc) to specific phosphorylated tyrosine residues. Several signaling cascades, including Ras-mitogen-activated protein kinase (MAPK), phospholipase-γ and phos-phatidyl-inositol-3'-kinase (PI3K)/Akt, have been characterized as the major downstream pathways mediating PDGF functions (31). Other adaptor molecules (e.g., the Fer and Fes tyrosine kinase family) and transcriptional factors (e.g., β-catenin are also involved in PDGF signaling in certain cell types (32-34).

The PDGF signaling via PDGFRs is delicately regulated by diverse mechanisms, which contribute to the complexity of PDGF-PDGFR pathway. For example, SHP-2 not only transmits PDGFR activated cell signaling, but also exerts a negative effect on signaling by dephosphorylating active PDGFRs and PDGFRs substrates (31). It has also been observed that ligand engagement to PDGFRs results in the endocytotic internalization and lysosomal degradation of receptors (35).

1.6. PDGF Functions

PDGF was originally identified as a serum derived from platelets that promoted fibroblast cell growth and aids in blood clotting (36-38). Since then, PDGF and it the PDGFR families have been extensively characterized, particularly as potent mitogenic and chemostatic factors for mesenchymal cells, such as fibroblasts, smooth muscle cells, pericytes and hepatic stellate cells (39). PDGF and PDGFR have been primarily suggested as acting through paracrine signaling due to the non-overlapping expression of ligands and receptors (22). The PDGF-A ligand, mainly expressed in epithelial cells, muscle and neuronal progenitors, plays a key role in

several developmental processes that include the formation and development of lung alveoli, intestinal villi, mesenchymal dermis, hair follicles, central nervous system (oligodendrocytes and retinal astrocytes) and testis (39-42). The PDGF-B ligand, primarily expressed invascular endothelial cells, megakaryocytes and neurons, has been well documented for its role as a paracrinal-signaling factor from epithelial cells to pericytes (43-45). Similar to the PDGF-A ligand, the PDGF-C ligand has been found to be expressed in epithelial cells, muscle and neuronal progenitors (24). PDGF-D expression has been observed in fibroblasts and smooth muscle cells (25).

Genetic studies have revealed the essential roles for the different PDGF ligands and PDGF receptors. The loss of PDGF ligands and their receptors in knockout (KO) transgenic mice results in embryonic or perinatal death. The majority of PDGF-A knockout mice die before or at birth. Of the few that survive after birth, most demonstrate defective pulmonary alveoli development, reduced oligodentrocyte number, and a mesenchymal defect in the skin (41, 46). PDGFR-α KO mice exhibit embryonic lethality between E8 to E16 and display skeletal defects, cleft face, spina bifida, abnormally patterned somites, and vascular malformations (47). PDGF-B and PDGFR-β KO mice are embryonic lethal at E17-E19 and exhibit similar phenotypic abnormalities. Both have renal hematological and cardiovascular abnormalities and lack mesangial cells (48, 49). PDGF-C KO mice die shortly after birth due to complications from palate malformations (50). As of now, no PDGF-D KO mouse has been reported (31).

1.7. PDGF and Neuroscience

PDGF is a critical growth factor for the proliferation and migration of nervous cells in central nervous system (CNS). Oligodendrocyte progenitor (OP) cells have been shown to undergo proliferation and migration in response to PDGF (51, 52) via the PI3K and MAPK pathways (52, 53). Migration of cranial neural crest cells can be induced by PDGF through the activation of matrix metalloproteinase-2 (54). Animal studies showed that PDGF overexpression induces OP hyperproliferation (55).

Furthermore, PDGF also exerts neuroprotective effects. In spinal cord injury and chronic lesions, PDGF has been shown to play a role in recovery (56, 57). Genetically engineered PDGF deficient mice are severely hypomyelinated, with the loss of OP cells and myelin the greatest at the brain periphery and the spinal cord (46). PDGF can attenuate glutamate-induced calcium (Ca^{2+}) overload in neurons to reduce neuronal death due to excitotoxicity (58-61). PDGF also augments neuronal survival during oxidative stress and energy deprivation (62) and human immunodeficiency virus protein toxicity (63, 64). The PI3K/Akt and Bcl-2 family pathways have been implicated in PDGF-mediated neuroprotection (64).

1.8. PDGF Activation in Cancer

Several studies comparing malignant and normal tissues found that the expression of PDGF and PDGFR are commonly increased in a variety of human tumors. In clinical specimens, elevated PDGF and PDGFR expression have been correlated with poor patient outcome in breast, colorectal, glial, lung and osteosarcoma cancers. PDGF and PDGFR expression vary on individual (65) tumor

type (Table 1). Collectively, these findings demonstrate that PDGF activation is a common event in cancer and suggest that PDGF may play a role in tumorigenesis.

Table 1 PDGF activation in human cancer

Tumor type	**Increase in expression**	**PDGF and poor prognosis**
Bladder	PDGF-D (66, 67)	
Breast	PDGF (65, 68) PDGF-B (69) PDGF-D (70) PDGFR (71, 72) PDGFR-α (73) PDGFR-β (26)	PDGF (74) PDGFR (71)
Cardiac myxoma	PDGF (75) PDGFR (75)	
Colorectal	PDGF (27) PDGF-AB (76, 77) PDGF-B (77-79) PDGFR (71) PDGFR-β (28, 30)	PDGF-B (29)
Dermal	PDGF-A (80, 81) PDGF-B (81-83) PDGFR-α (84) PDGFR-β (80, 85)	
Esophageal	PDGF-A (86) PDGF-B (86) PDGFR (86)	
Gastric	PDGFR-α (32)	
Glial	PDGF (87, 88) PDGF-A (89-94) PDGF-B (91-95) PDGF-C (96-98) PDGF-D (70, 96) PDGFR (88, 92, 99) PDGFR-α (91, 94, 100) PDGFR–β (91, 94, 95, 100-103)	PDGFR-β (104)
Head and neck	PDGF (105, 106) PDGF-A (107-109) PDGF-B (107, 109) PDGF-C (109) PDGFR-α (34) PDGFR-β (107, 109)	

Hepatocellular	PDGFR-α (110)	
Kaposi's sarcoma	PDGF-A (111) PDGF-B (111) PDGFR-β (33)	
Lung	PDGF (31, 112, 113) PDGF-B (114) PDGF-D (70) PDGFR-β (113, 115, 116)	PDGF (112)
Myelomonocytic leukemia	PDGFR-β (35)	
Oral	PDGF (36)	
Osteoblastoma	PDGF-A (117) PDGFR-α (117)	
Osteosarcoma	PDGF (118) PDGF-A (119, 120) PDGF-B (120, 121) PDGFR-α (119, 120) PDGFR-β (120)	PDGF-A (120) PDGFR-α (120)
Ovarian	PDGF-A (38) PDGF-B (38) PDGF-D (70) PDGFR-α (38)	
Pancreatic	PDGF-D (122) PDGFR-β (39)	
Prostate	PDGF (123, 124) PDGF-A (125) PDGFR (71, 126) PDGFR-α (40-42, 125)	
Renal	PDGF-C (43) PDGF-D (70)	
Soft Tissue	PDGF (127, 128) PDGF-A (129) PDGF-B (129, 130) PDGFR (44) PDGFR-α (129, 131) PDGFR-β (129, 132, 133)	
Stomach	PDGF-B (45)	
Testicular	PDGF (134)	
Uterine	PDGF-B (135, 136) PDGFR-β (133, 135)	

Tumorigenesis involves a number of alterations in cell physiology that contribute to malignant growth (137). PDGF is involved in different stages of cancer development, including angiogenesis, proliferation, survival and metastasis. The PDGF/PDGFR pathway in tumorigenesis activates several signaling cascades including Ras-mitogen-activated protein kinase, NFκB, Abl kinases and phos-phatidyl-inositol-3'-kinase (PI3K)/Akt (33, 114, 122, 127, 138-147). As shown in Figure 2, downstream targets of PDGF/PDGFR signaling include some important genes that promote key aspects of tumorigenesis.

Angiogenesis	**Survival**	**Invasion/Metastasis**
ANG-1 (148) IL-8 (139) MMP-9 (122, 145, 148) VEGF (122, 127, 139, 140, 149)	Bcl-2 (147) Mcl-1(150)	Cadherins (33, 151) CD31/PECAM-1 (149) CXCR4 (142) Fibronectin (33) PLD1 (145) SDF-1 (142) Snail-2 (152) Vimentin (33, 151) ZEB-1 (152) ZEB-2 (147, 151, 152)

Table 2 List of PDGF-regulated genes that promote key aspects of tumorigenesis. ANG-1, angiopoietin 1; Bcl-2, B-cell lymphoma 2; CD31/PECAM-1, cluster of differentiation/platelet endothelial cell adhesion molecule 1; CXCR4, C-X-C chemokine receptor 4; IL-8, interleukin 8; Mcl-1, myeloid leukemia 1; MMP-9, matrix metallopeptidase 9; PLD1, phospholipase D1; SDF-1, stromal cell derived factor 1; VEGF, vascular endothelial growth factor; ZEB-1, zinc finger E-box-binding homeobox 1 ; ZEB-2, zinc finger E-box-binding homeobox2

1.8.1. Angiogenesis

Neovascularization, or the formation of newly formed blood vessels, is critical for tumor progression because the supply of oxygen and nutrients becomes limited in tumor cells that are located more than 100mm away from a blood vessel (46). Tumor cells are usually not angiogenic at first and must undergo as series of

steps to induce angiogenesis (153). This multistep process, termed the 'angiogenic switch' involves the disruption of positive and negative angiogenic regulators, which ultimately tips the balance toward pro-angiogenic factors (49). In tumor angiogenesis, the PDGF/PDGFR family primarily acts in vessel maturation through its role in recruitment of tumor vessel supporting vasculature. PDGF produced by the tumor endothelium and tumor cells is required for the recruitment of adequate numbers of pericytes, as well as for proper integration of pericytes in the vascular wall (154, 155). Pericytes in turn act to stabilize newly formed vasculature, regulate vascular tone and maintain local and tissue homeostasis (48). Impaired PDGF signaling results in impaired pericyte recruitment and increased tumor vessel diameter (50), or in the inhibition in invasion and angiogenesis (122).

Hypoxic conditions and other angiogenic factors serve to activate PDGF expression in angiogenesis. PDGF expression has been found to be induced in response to hypoxic conditions in cancer cell lines (127, 156). However, the size of the tumor is a variable for PDGF function in angiogenesis. In a melanoma mouse model, active PDGFR-β signaling helped in vascularization of tumors up to a size of 0.3 cm^3 (51).

PDGF also acts synergistically with other known angiogenic factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) to promote an interactive loop in tumor angiogenesis. In pulmonary metastasis, FGF-2 has been shown to trigger PDGF-BB responses in endothelial cells to enhance PDGF-BB induced tumor angiogenesis. Similarly, PDGF-BB can also augment the effect of FGF-2 (157) or stimulate the production of FGF-2 and FGF-7 by carcinoma

associated fibroblasts (158). PDGF has also been show to increase VEGF expression from pericytes to increase angiogenesis (159). Likewise, anti-angiogenic therapy is enhanced with inhibition to both VEGF and PDGF (160).

1.8.2. Proliferation

PDGF/R family plays an important role in promoting tumor growth. PDGF-A (52), PDGF-BB (78, 161)and PDGF-D (122, 162) isoforms have been shown to increase cell invasiveness with cancer cell over-expression. A similar study on the down-regulation of PDGF-D by siRNA demonstrated decreased tumor cell invasion (122).

Proliferation has been shown to act through the PDGFR-β to induce proliferation. A point mutation of the PDGFR-β revealed that the activation of the tyrosine residue at 857 promotes cell proliferation (53). The activated PDGFR-β can then enhance the expression of TGF-β signaling to increase cell proliferation (163).

1.8.3. Metastasis

A critical step in cancer pathogenesis is tumor metastasis. Metastasis involves the dissemination of cancer from the place where it first started to somewhere else in the body. Often the spread of cancer to vital organs is what results in human deaths. There are several factors involved in metastasis including tumor cell invasion (54), intravasation (56), extravasation (57) and proliferation in the new organ location (137). On an individual level, tumor cells that are involved in metastasis undergo a transition from an epithelial to a mesenchymal phenotype, or

commonly termed, epithelial to mesenchymal transition (EMT). EMT allows individual cells to go from their epithelial or polar and basement cell membrane orientation to a mesenchymal cell phenotype, which includes enhanced capacity for migration, invasiveness and apoptosis resistance (55).

Autocrine PDGF signaling has been associated with EMT in carcinoma cells from the breast, colon, prostate and liver, suggesting a causative role of autocrine PDGF signaling in metastasis (33, 72, 114, 147). As demonstrated in Table 2, several factors associated with EMT have been found to be upregulated upon PDGF overexpression.

1.9. Mcl-1

Myeloid cell leukemia 1 (Mcl-1) is a pro-survival member of the Bcl-2 family, regulators of both pro- and anti-apoptosis. All members of this family share a Bcl-2 homology (BH) domain. Pro-apoptotic members include Bax (Bcl-2 associated X protein) and Bak (Bcl-2 homologous antagonist/killer), which have multiple BH domains (BH1, BH2, and BH3). Other pro-apoptotic members, Bad (Bcl-2 associated death promoter), Bid (BH3 interacting domain death agonist), Bim (Bcl-2 interacting mediator of cell death), Bik (Bcl-2 interacting killer), NOXA and PUMA (p53 up-regulated modulator of apoptosis), contain only BH3 domains. The anti-apoptotic subgroup includes Bcl-2, Bcl-$_{XL,}$ Mcl-1, Bcl-w and A1. Bcl-2, Bcl-$_{XL}$, and Bcl-w contain four BH domains (BH1, BH2, BH3 and BH4), whereas Mcl-1 and A1 lack the BH4 domain. Cell fate is determined through the balance between the relative levels of the members of the Bcl-2 family (58, 59). Deregulation of pro- and anti-

apoptotic member of the Bcl-2 family can contribute to several diseases, such as tumorigenesis. Mcl-1 was originally identified as an immediate-early gene expressed in differentiating myeloid leukemia cells (60). Located on chromosome 1q21, the human *mcl-1* gene can result in to two isoforms, Mcl-1_L or Mcl-1_S. Mcl-1_S lacks an exon, as a result of alternative splicing (61). Mcl-1_L and Mcl-1_S have opposing functions, with the longer version being anti-apoptotic and the smaller one acting as a pro-apoptotic factor (164). Both versions of Mcl-1 contain two PEST (Proline (P), Glutamic Acid (E), Serine (S), Threonine (T)) sequences on their N-terminals, which are thought to be the main reasons for the short half-life of Mcl-1(63). The C-terminal serves to localize Mcl-1 to the outer mitochondrial membrane (64).

Similar to its pro-survival family members, Mcl-1 (Mcl-1_L is referred to as Mcl-1 from here on) uses the BH1, BH2 and BH3 domains to form a hydrophobic groove on its surface. This grove can then bind to α-helical BH3 domain of the pro-apoptotic factors Bak and Bax to neutralize their pro-apoptotic functions (96). If cells are experiencing apoptosis, the BH3-only proteins such as Bim, Bik, PUMA and NOXA can competitively bind against Bax and Bak for the hydrophobic grove on Mcl-1 (70, 87, 89, 97, 99). As shown in Figure 2, if Bax and Bak are not sequestered by Mcl-1, they are capable of forming pores in the mitochondrial membrane, causing the release of cytochrome c into the cytoplasm (95). Cytochrome c can then induce the activation of caspases, or proteases responsible for apoptotic cell degradation (101).

Mcl-1, similar to its Bcl-2 anti-apoptotic relatives, is widely expressed but has its unique tissue distribution and physiological roles (90, 100). Many different cell types rely on Mcl-1 for survival and development. Mcl-1 is essential for the development and maintenance of B and T-lymphocytes, neural development, survival of haematopoietic stem cells and synovial fibroblasts and regulation of macrophage and neutrophil apoptosis (88, 104, 165-168). Mcl-1 is also capable of protecting cells from growth factor withdrawal-induced apoptosis (85).

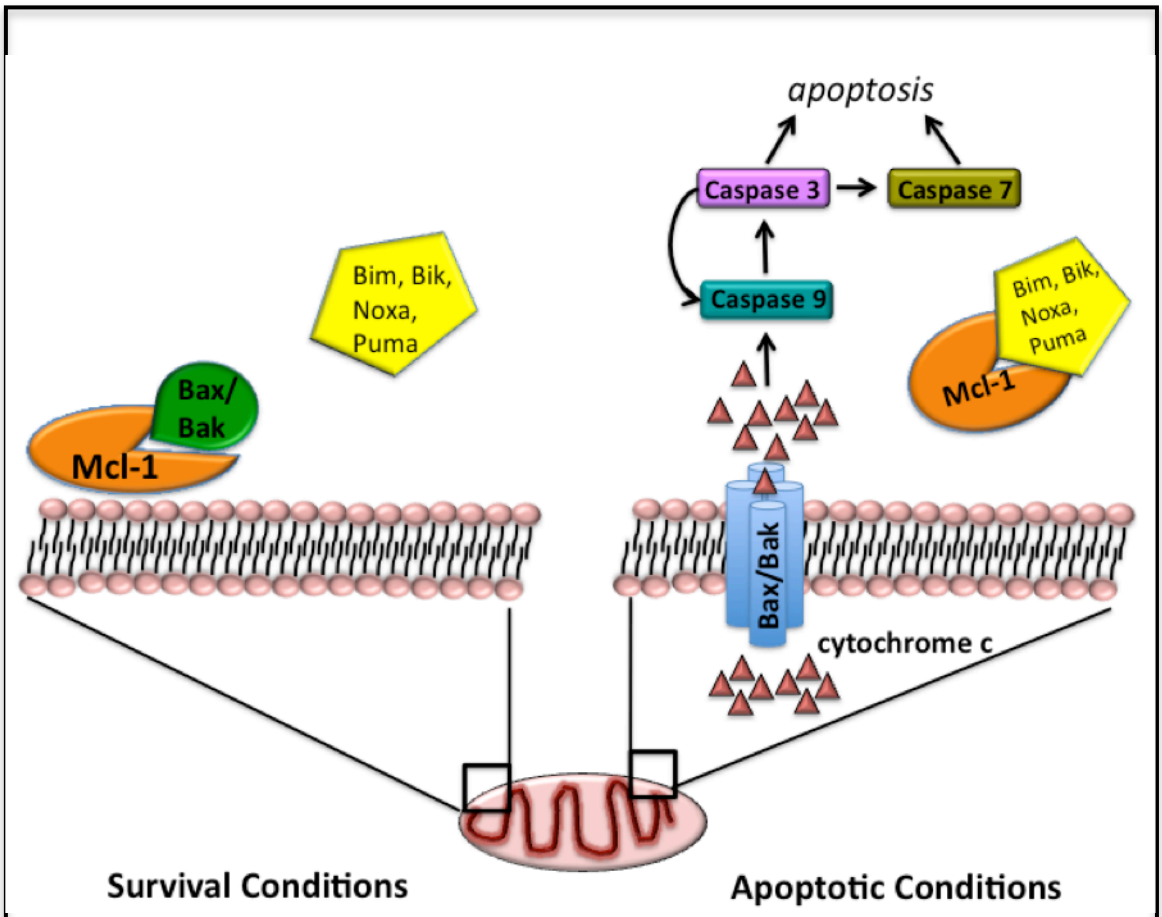

Figure 2. Mcl-1 has two main functions in cell apoptosis. In survival conditions, Mcl-1 can sequester Bax or Bak. If apoptotic signals are activated, BH3 only proteins can displace Bax or Bak from Mcl-1. Bax and Bak can then permeabilize the mitochondrial membrane for cytochrome c release. Cytochrome c can then activate the apoptotic cascade.

Mcl-1 expression can be activated by various growth factors. Transcriptional upregulation of Mcl-1 has been observed in the presence of cytokines interleukin (IL)-3, IL-5, and IL-6 (66, 67, 169). Furthermore, granulocyte-macrophage colony-stimulating factor (GM-CSF), as well as the growth factors epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF), have been shown to induce Mcl-1 expression (65, 68, 69).

Mcl-1 transcription is mediated by a number of factors. Characterization of the Mcl-1 promoter region demonstrated putative and confirmed transcription factor binding sites for STAT response elements, cAMP response elements (CRE), and SP1, PU.1, hypoxia-inducible factor (HIF) responsive element (HRE), and nuclear factor kappa B binding sites (71-74, 169). Confirmed transcription factor binding sites have been demonstrated for STAT3, with IL-6, VEGF and IL-3 treatment and STAT5 with Bcr-Abl signaling activation (75, 169, 170).

1.10. Mcl-1 and Neuroscience

The role of Mcl-1 in the nervous system has not been well studied, partly due to difficulties in detecting its expression in neuronal tissue (100). Several studies have suggested that Mcl-1 plays an important role in nervous system development. Mcl-1 upregulation was found to be essential for Notch-1 induced neural precursor cell survival (76). Neuronal progenitors expressing Nestin and bIIItubulin also require Mcl-1 expression (166). Furthermore, Mcl-1 has been shown to mediate the survival of granule neurons during the development of the cerebellar cortex (77).

In post-mitotic neurons, Mcl-1 acts to protect neurons against apoptosis. Mcl-1 was found to act as a cellular stress sensor, integrating several cellular clues. Its loss in post-mitotic neurons increases the number of autophagic neurons, and apoptosis with persistent autophagy (78). When Mcl-1 expression is depleted in post-mitotic neurons, the neurons become sensitized to apoptosis induced by DNA damage, and conversely, neurons with overexpressed Mcl-1 levels were protected against cell death (166).

1.11. Mcl-1 and Cancer

Although Mcl-1 has been found to be an essential factor for development and survival of normal cells, deregulation of the signaling pathways controlling Mcl-1 expression can result in a number of pathologies. Most notably, dysregulation leading to over expression of Mcl-1 has been reported in a number of cancers (Table 3) and in chemoresistance (79, 80).

Table 3 Mcl-1 activation in human cancer

Cancer type	**Reference**	**Poor Outcome**
Breast	(81-83, 86, 91-94)	(83, 86, 91)
Cervical	(98, 102, 103)	(103)
Colorectal	(81, 105, 106)	
Dermal	(31, 81, 108-111, 171)	(109)
Esophageal	(112)	
Gastric	(113, 115, 116, 172)	(113, 116)
Glial	(118-121)	(120)
Head and Neck	(122-124, 126, 127, 173-175)	
Hepatocellular	(128-130)	
Leukemia	(81, 131-133, 176)	(132)
Lung	(81, 177)	
Lymphoma	(81, 135, 136, 138-142, 178)	

Multiple Myeloma	(69, 143, 144, 146, 147)	(146)
Oral	(147)	(147)
Osteosarcoma	(148)	
Ovarian	(81, 149, 151)	(149, 151)
Pancreatic	(152, 179)	
Prostate	(81, 172, 180)	
Renal	(81, 154)	
Testicular	(155)	
Uterine	(156, 181)	

1.12. Mcl-1 as an attracting target for cancer therapy

There have been numerous approaches designed to target Mcl-1 in cancer therapy. Many drug treatments have been developed that result in Mcl-1 down regulation. Flavopiridol, a cyclinkinase inhibitor, has been shown to decrease Mcl-1 protein levels in B-cell chronic lymphocytic leukemia (CLL) cells (182). Another inhibitor involved in clinical trials, Sorafenib, a multi-kinase inhibitor, has been shown to induce apoptosis in human leukemia cells by downregulation of Mcl-1 (157).

The most promising therapeutic study for Mcl-1 expression in cancer has been with BH3 mimetics. BH3 mimetics are small molecules that have been developed that mimic the BH3 domain. These small molecules are able to fit into the hydrophobic pocket of anti-apoptotic proteins, such as Mcl-1. Through competitive binding, the BH3 mimetics can block the ability of anti-apoptotic proteins to bind pro-apoptotic proteins, such as Bax and Bak, which would prevent the induction of apoptosis. Several BH3 mimetic are currently in the pre-trial or clinical trial phase (158).

Mcl-1 has been shown to be an attractive and potential therapeutic target for cancer malignancies, and is the focus of a number of studies. In continuing efforts to develop better treatments, understanding the potential mechanisms of Mcl-1 activation and methods of resistance is critical. Chemoresistance is a significant problem in cancer therapy, and understanding the pathways that contribute to it, such as Mcl-1 upregulation and overexpression, is crucial in order to develop approaches for cancer therapy.

Chapter 2

Materials and Methods

2.1. Cell Culture

Human Prostate cancer (PCa) cell lines $ARCaP_E$, $ARCaP_M$ (159), LNCaP (American Type Culture Collection, ATCC, Manassas, VA), C4-2 (183) and PC3 (ATCC) were routinely maintained in T-medium (Invitrogen, Carlsbad, CA) with 5% fetal bovine serum (FBS). For the treatments with PDGF isoforms, PCa cells seeded in 96-well plates (3,000 cell/well) were serum-starved overnight, replaced with fresh serum-free T-medium, and incubated in the presence of varying concentrations of recombinant human PDGF-AA, -AB, -BB (R&D Systems, Minneapolis, MN), or phosphate-buffered saline (PBS) for indicated times. Recombinant human interleukin-6 (IL-6) was purchased from R&D Systems. For chemotherapy drug treatment, docetaxel (Sanofi Aventis, Bridgewater, NJ) or dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) was added to cells and incubated for 72 h. Cell proliferation was measured using the CellTiter 96 AQ proliferation assay according to the manufacturer's instructions (Promega, Madison, WI). Viable cells were counted in triplicate using a hemacytometer and trypan blue staining.

2.2. Plasmids and small interfering RNAs (siRNAs)

The full-length human Mcl-1 promoter region cloned into a firefly luciferase reporter vector pGL3-Basic (Promega, Madison, WI) was kindly provided by Dr. Steven W. Edwards (University of Liverpool, Liverpool, UK) (71). The hypoxia-

responsive element (HRE) (fragment -900 to -884)-truncated construct was obtained by digestion of the full-length promoter using KpnI (from position -3914 to -855) and then ligated using T4 DNA ligase (New England Biolabs, Ipswich, MA). Both plasmid constructs were confirmed by sequence analysis. The pHIF1-luc reporter was purchased from Panomics (Fremont, CA). TOPFlash and FOPFlash T-cell factor (TCF) reporters were obtained from Upstate (Billerica, MA), pTK-RL plasmid was purchased from Promega. Human Mcl-1 expression vector (pCMV-Mcl-1) was obtained from Origene, Inc. Human β-catenin expression plasmid was provided by Dr. Zhi-Ren Liu. ON-TARGET*plus*SMARTpoolsiRNAs against β-catenin, p68, PDGFR-α and PDGFR-β, and control siRNA were obtained from Dharmacon, Inc (Chicago, IL). HIF-1α and control siRNA were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Transient transfection of DNA constructs and siRNAs was performed using Lipofectamine 2000 or Oligofectamine reagents (Invitrogen), according to the manufacturer's protocols and our published procedures (161, 184).

2.3. Western Blot Analysis

Total cell lysates were prepared using radioimmunoprecipitation (RIPA) buffer (Santa Cruz Biotechnology, Inc.). Nuclear proteins were extracted using a Novagen kit (EMD Biosciences, San Diego, CA). Immunoblotting analysis followed standard procedures (184). ImageJ software (National Institutes of Health) was used to quantitate the relative protein expression as normalized to the loading controls. Information for the antibodies used in this study is shown in Table 4.

2.4. Immunoprecipitation

The Immunoprecipitation Starter Pack (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) was used according to the manufacturer's instructions. Total nuclear lysates (1 mg) were immunoprecipitated with 5 μg rabbit anti-HIF-1α antibody, mouse anti-β-catenin antibody, mouse anti-c-Abl and rabbit anti-p68 antibody (Table 4), or normal IgG (R&D Systems). Protein A/G Sepharose 4 Fast Flow beads were added to precipitate proteins, then washed and eluted. The samples were further processed for Western blot analysis.

Table 4 Antibodies used in study

Antibody	**Host Animal**	**Company**	**Cat. No.**	**Dilution**
β-actin	Mouse	Sigma	A 5441	1-1000
β–catenin	Mouse	BD Transduction	610154	1-500
β-catenin (H-102)	Rabbit	Santa Cruz	sc-7199	1-500
Bcl-2 (N-19)	Rabbit	Santa Cruz	sc-492	1-100
c-Abl (24-11)	Mouse	Santa Cruz	sc-23	1-200
E-cadherin (H-108)	Rabbit	Santa Cruz	sc-7870	1-500
HIF-1α	Rabbit	Millipore	07-628	1-200
HIF-1α	Mouse	BD Transduction	610958	1-200
Mcl-1	Rabbit	Santa Cruz	sc-819	1-500
p68 RNA Helicase (H-144)	Rabbit	Santa Cruz	sc-32858	1-200
PARP	Rabbit	Cell Signaling	9542	1-500
PDGFR-α (951)	Rabbit	Santa Cruz	sc-431	1-100
PDGFR-β	Rabbit	Santa Cruz	sc-339	1-100
TCF-4 (N-20)	Goat	Santa Cruz	sc-8631	1-100
TFIID (TBP) (N-12)	Rabbit	Santa Cruz	sc-204	1-200
Phospho-c-Abl (Tyr 245)	Rabbit	Cell Signaling	2861	1-500
Phospho-PDGFR-α (Tyr754)	Rabbit	Cell Signaling	2992	1-1000
Phospho-PDGFR-β (Tyr751)	Mouse	Cell Signaling	3166	1-1000
Phospho-Tyrosine (P-Tyr-100)	Mouse	Cell Signaling	9411	1-1000
Normal IgG	Rabbit	Cell Signaling	2729	
Normal IgG	Mouse	Millipore	12-371	

2.5. Immunofluorescence and Confocal Imaging

Immunofluorescence was performed as described previously (161) using mouse anti-β-catenin, rabbit anti-p68 and anti-HIF-1α antibodies (Table 4). Either Alexa 488 or 633 secondary antibodies (Invitrogen) were used at a dilution of 1:500 and were incubated for 1 h at room temperature. Nuclear staining was performed by incubating cells with 0.4 μmol/L 4',6-siamidino-2-phenylindole (DAPI) to mounting slides. Cells were imaged on a Zeiss LSM 510 META. In all cases, either a 63x or 100x Zeiss Plan-Apo oil objective was used (numerical aperture of 1.3 and 1.4, respectively). All images had contrast expansion performed in Adobe Photoshop.

2.6. Quantitative RT-PCR (qRT-PCR) and RT-PCR

Total RNA was prepared with Qiagen RNeasy Kit (Qiagen, Valencia, CA). The first-strand cDNA was synthesized using SuperScript®III One-Step RT-PCR System (Invitrogen). Quantitative PCR was performed by the LightCycler 480 system (Roche Applied Science) using Brilliant SYBR Green QPCR Master Mix (Stratagene) according to the manufacturer's instructions. The specific PCR primer pairs are described in Table 5. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified to normalize RNA inputs (184).

Table 5 Primers for PCR and RT-PCR

Gene	Sequence (5'-3')	Annealing Temp	Cycles
PDGFR-α (forward)	AAATGGGTGCTAAATTGATTGG	65	35
PDGFR-α (reverse)	GCACATCTTTAGCAGGAGCC		
PDGFR-β (forward)	GTGCTCACCATCATCTCCCT	55	40
PDGFR-β (reverse)	ACTCAATCACCTTCCATCGG		
PDGF-A (forward)	ACACGAGCAGTGTCAAGTGC	65	35
PDGF-A (reverse)	CCTGCAGTATTCCACCTTGG		

PDGF-B (forward)	AGATCGAGATTGTGCGGAAG	55	40
PDGF-B (reverse)	CAGCTGCCACTGTCTCACAC		
PDGF-C (forward)	GCCAGGTTGTCTCCTGGTTA	65	35
PDGF-C (reverse)	TGCTTGGGACACATTGACAT		
PDGF-D (forward)	CCCAGGAATTACTCGGTCAA	65	35
PDGF-D (reverse)	ACAGCCACAATTTCCTCCAC		
HRE (forward)	AGGTCACTTGAGGCCATGAG	59	40
HRE (reverse)	CACGTTCAGACGATTCGGTA		
GAPDH (forward)	GTCAGTGGTGGACCTGACCT	65	23
GAPDH (reverse)	AGGGTCTACATGGCAACTG		
Mcl-1 (forward)	GAGGAGGAGGAGGACGAGTT	65	23
Mcl-1 (reverse)	GTCCCGTTTTGTCCTTACGA		
p68 (forward)	CACCCCTTTTGCCCGCAGAGT	60	35
p68 (reverse)	TCTTGACAGGTCGTTCCAGGGG		

2.7. Chromatin Immunoprecipitation Assay (ChIP)

The sequential ChIP (ChIP-re-ChIP) experiments were performed using the Active Motif Re-ChIP-IT kit (Active Motif, Carlsbad, CA). Briefly, PCa cells were serum-starved overnight and replaced with fresh serum-free medium, incubated with PDGF-BB or PBS for indicated times. Cells were fixed 10 min at room temperature by 1% formaldehyde solution to cross-link DNA-protein interactions. Chromatin was sheared by enzymatic shearing for 8 min (184). A portion of chromatin was reversed and used as input DNA. For immunoprecipitation, 2 μg of anti-β-catenin antibody was added and incubated overnight, with normal IgG as the control. PCR primers for the HRE region in human Mcl-1 promoter are described in Table 5. PCRs were performed for 40 cycles, with primer concentration as 10 pmol/20 μl.

2.8. Statistical Analysis

All data represent three or more experiments. Errors are S.E. values of averaged results. For each assay, Student's *t-test* was used for statistical comparison with the control groups. Values of $p \leq 0.05$ were taken as a significant difference between means. Statistical analysis was performed using the Sigmaplot software 11.0 (Systat Software, Inc., Chicago, IL.

Chapter 3

Results

3.1. Mcl-1 is a survival factor in PCa cells

Previously our lab demonstrated that Mcl-1 overexpression is associated with *in vivo* bone metastatic propensity of human PCa cells, and importantly, correlated with clinical PCa bone metastasis (161). Consistently, using a human PCa ARCaP cell model that could closely mimic the pathophysiology of bone metastasis *in vivo* (159), it was found that Mcl-1 expression was significantly increased in highly bone metastatic $ARCaP_M$ cells when compared to that in the low-invasive counterpart $ARCaP_E$ cells (Figure 3A). It was hypothesized that upregulation of Mcl-1 may confer metastatic PCa cells survival advantages, allowing them to escape apoptotic fate during invasion and dissemination and successfully establish distant metastasis (162). Supporting this notion, ectopic expression of Mcl-1 enhanced PCa cell resistance to docetaxel (Figure 3B), a commonly used chemotherapeutic drug in hormone-refractory and metastatic PCa (185). These results indicated that upregulation of Mcl-1 may account for, at least in part, resistance to apoptosis in metastatic PCa cells.

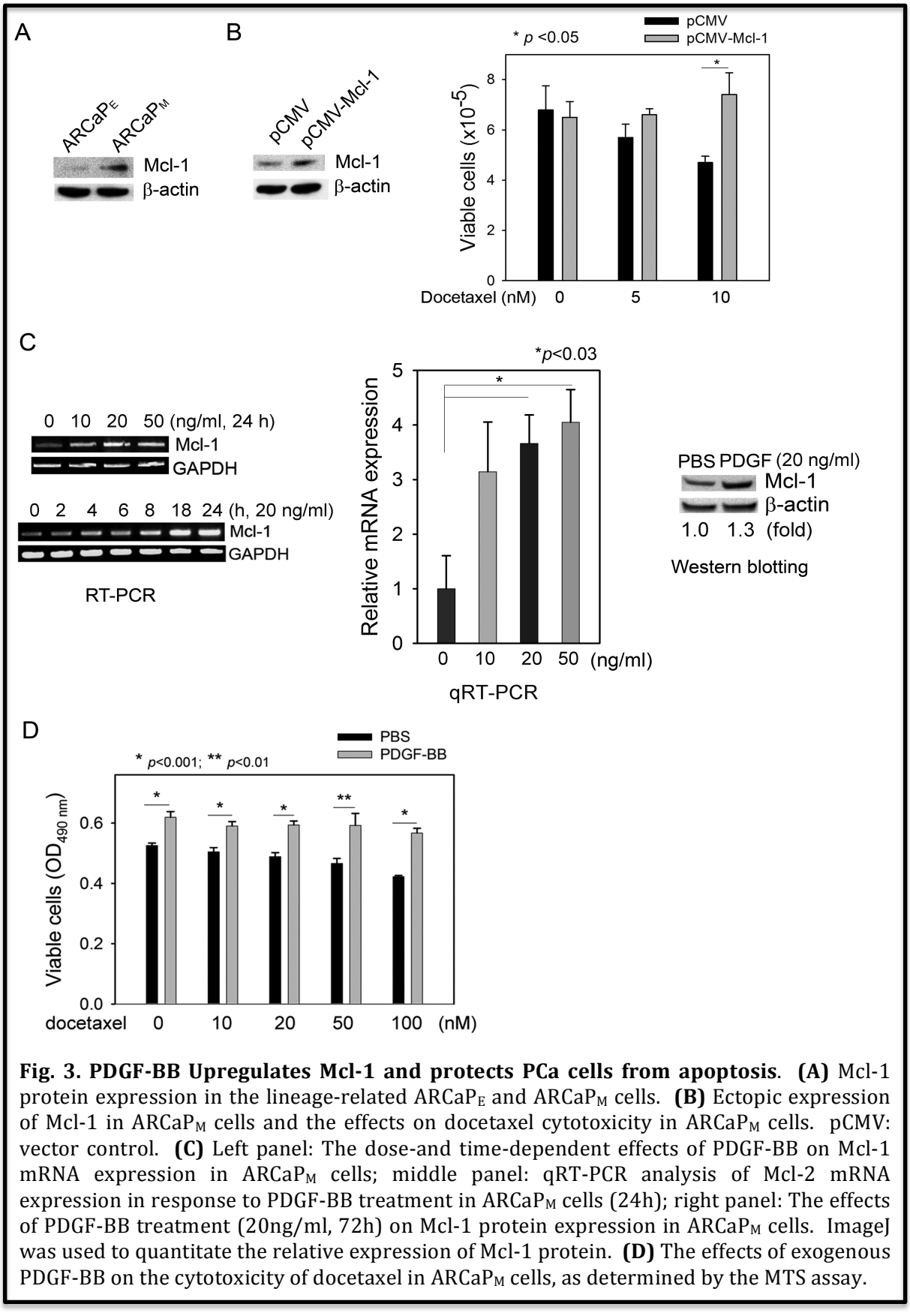

Fig. 3. PDGF-BB Upregulates Mcl-1 and protects PCa cells from apoptosis. **(A)** Mcl-1 protein expression in the lineage-related ARCaP$_E$ and ARCaP$_M$ cells. **(B)** Ectopic expression of Mcl-1 in ARCaP$_M$ cells and the effects on docetaxel cytotoxicity in ARCaP$_M$ cells. pCMV: vector control. **(C)** Left panel: The dose-and time-dependent effects of PDGF-BB on Mcl-1 mRNA expression in ARCaP$_M$ cells; middle panel: qRT-PCR analysis of Mcl-2 mRNA expression in response to PDGF-BB treatment in ARCaP$_M$ cells (24h); right panel: The effects of PDGF-BB treatment (20ng/ml, 72h) on Mcl-1 protein expression in ARCaP$_M$ cells. ImageJ was used to quantitate the relative expression of Mcl-1 protein. **(D)** The effects of exogenous PDGF-BB on the cytotoxicity of docetaxel in ARCaP$_M$ cells, as determined by the MTS assay.

3.2. PDGF-BB induces Mcl-1 expression and antagonizes apoptosis in PCa cells

Intriguingly, PDGF-BB was found to significantly induce Mcl-1 expression in PCa cells (Figure 3C, Figure 4). Treatment with recombinant human PDGF-BB increased Mcl-1 mRNA in a dose- and time-dependent manner, though the optimal conditions for the maximum accumulation of Mcl-1 mRNA varied in different PCa cell lines. Western blot analysis confirmed the inductive effects of PDGF-BB on Mcl-1 expression at protein level. These data identified PDGF-BB as a novel regulator of Mcl-1 expression, which could provide a survival mechanism to protect PCa cells from apoptosis. Indeed, addition of PDGF-BB in PCa cell cultures effectively antagonized the cytotoxicity of varying doses (0-100nM) of docetaxel (Figure 3D).

3.3. Expression profile of PDGF autocrine signaling components in PCa cells

This study examined the expression pattern of PDGFs and their receptors in PCa cells (Figure 5A). RT-PCR analyses showed that the PDGF isoforms were differentially expressed at mRNA level, and among them, increased PDGF-B and PDGF-D were observed in C4-2 and $ARCaP_M$ cells when compared to the parental LNCaP and $ARCaP_E$ cells, respectively. Consistent with previous studies (163), PC3 cells were found to express high levels of PDGF-D, PDGFR-α and -β. Interestingly, PDGFR-α mRNAs appeared to be substantially expressed in PCa cells, which was confirmed at protein level by Western blot analysis. In contrary, though PDGFR-β mRNAs were detected by RT-PCR in most PCa cell lines, immunoblotting analysis could only confirm protein expression in $ARCaP_E$ and $ARCaP_M$ cells (Figure 5A, right

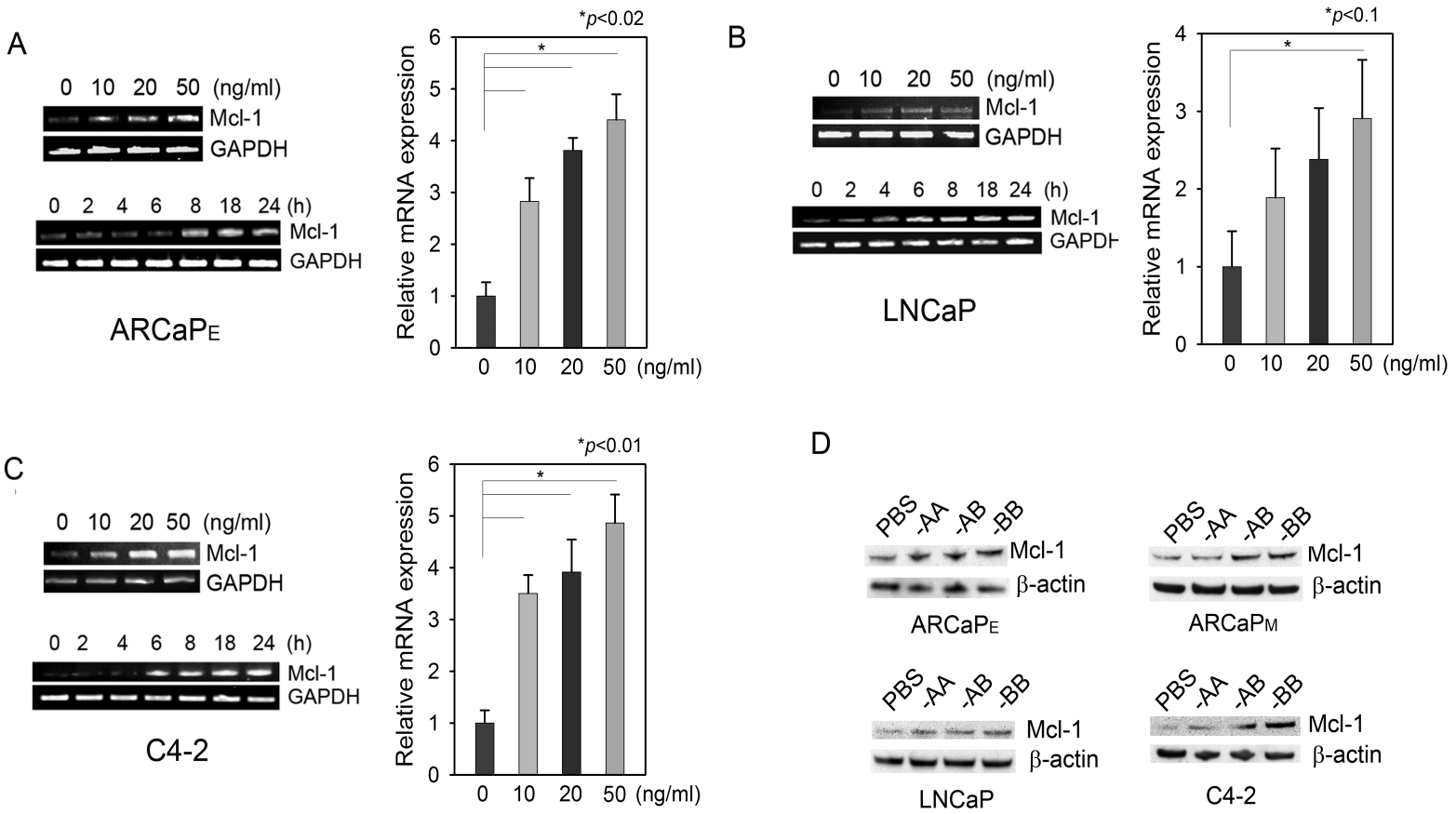

Fig. 4. The effects of PDGF-BB on the expression of Mcl-1 at mRNA and protein levels in PCa cells. (A-C) RT-PCR and qRT-PCR analyses of the time- and dose-dependent effects of PDGF-BB on Mcl-1 mRNA expression in $ARCaP_E$ **(A)**, LNCaP **(B)** and C4-2 **(C)** cells. The dose was 20 ng/ml in the time course experiments. **(D)** Western blot analysis of the effects of PDGF isoforms on the expression of Mcl-1 in several PCa cell lines. Treatment: 20 ng/ml, 72 h.

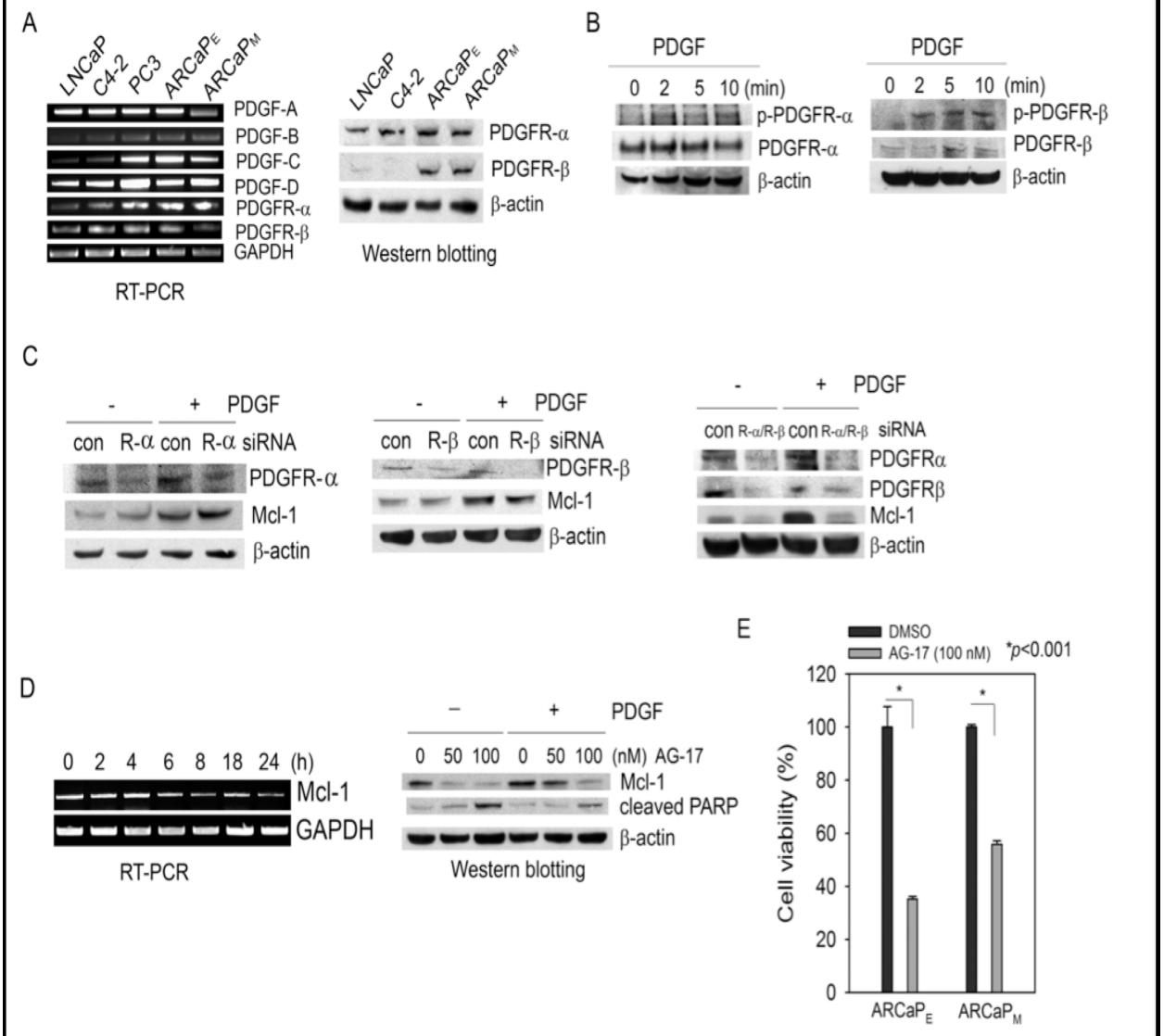

Fig. 5. Activation of the PDGFR signaling is required for Mcl-1 expression in PCa cells. (A) Expression profile of PDGFR signaling components in PCa cells, as analyzed by RT-PCR and Western blotting. **(B)** The effects of PDGF-BB (20 ng/ml) on the phosphorylation of PDGFR-α and -β in ARCaP$_M$ cells. **(C)** The effects of depleting PDGFR-α or/and -β on Mcl-1 protein expression in ARCaP$_M$ cells. The cells were transfected with either isotype-specific siRNAs targeting PDGFR-α (left panel, 30 nM) or PDGFR- β (central panel, 100 nM), or a mixture of PDGFR-α and -β siRNAs (right panel) for 48 h, serum-starved overnight, and incubated in the presence or absence of PDGF-BB (20 ng/ml) for 72 h. **(D)** Upper panel: The time-dependent effects of AG-17 (100 nM) on Mcl-1 mRNA expression in ARCaP$_M$ cells; bottom panel: The effects of AG-17 treatment on the expression of Mcl-1 and cleaved PARP in the presence (20 ng/ml) or absence of PDGF-BB (20 ng/ml) in ARCaP$_M$ cells. **(E)** The effects of AG-17 treatment (100 nM, 72 h) on the viability of ARCaP$_E$ and ARCaP$_M$ cells.

panel). Taken together, these data suggested a functional PDGF autocrine signaling in certain PCa cells.

3.4. An autocrine PDGFR signaling mediates PDGF-BB regulation of Mcl-1 in PCa cells

Both PDGFR-α and -β were highly expressed in bone metastatic $ARCaP_M$ cells, and rapidly phosphorylated in a time-dependent manner in response to the stimulation of exogenous PDGF-BB (Figure 5B). Interesting, depletion of either PDGFR-α or -β by isoform-specific siRNA did not block the inductive effect of PDGF-BB on Mcl-1 expression (Figure 5C, left and central panels), suggesting that activation of either receptors may be sufficient for the upregulation of Mcl-1. Supporting this hypothesis, transient transfection with a mixture of siRNAs targeting both PDGFR-α and -β inhibited the basal expression of Mcl-1, and abrogated PDGF-BB induction of Mcl-1 $ARCaP_M$ cells (Figure 5C, right panel). Alternatively, treatment with AG-17 (Tyrphostin), a selective pharmacological inhibitor of PDGFRs (186), reduced Mcl-1 expression at both mRNA and protein levels and markably increased cleavage of poly-ADP ribose polymerase (PARP), an indicator of apoptosis. These effects were attenuated by the presence of PDGF-BB in cultures (Figure 5D). Consistently, AG-17 treatment at low doses (such as 100 nM) effectively induced apoptosis in $ARCaP_E$ and $ARCaP_M$ cells (Figure 5E), indicating a pivotal role of PDGFR signaling in the survival of PCa cells.

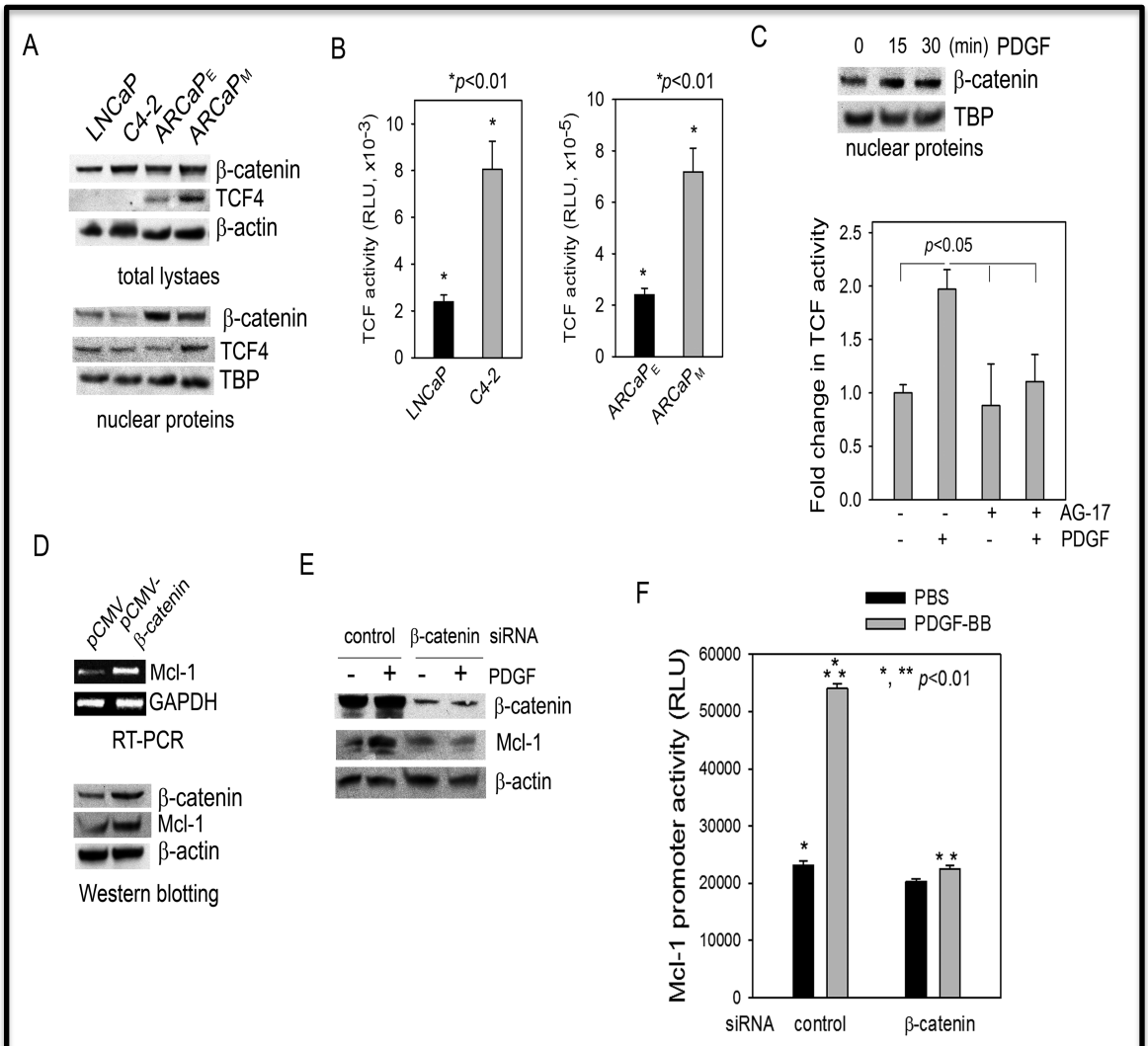

Fig. 6. β-catenin mediates PDGF regulation of Mcl-1 expression in PCa cells. (A) Expression profile of β-catenin-TCF signaling components in PCa cells. (B) TCF reporter activity in the LNCaP-C4-2 and ARCaP$_E$-ARCaP$_M$ cells. (C) Upper panel: The effects of PDGF-BB (20 ng/ml) on the nuclear translocation of β-catenin in ARCaP$_M$ cells; Bottom panel: The effects of PDGF-BB (20 ng/ml) on TCF reporter activity in the presence (100 nM) or absence of AG-17. (D) The effects of ectopic expression of β-catenin (72 h) on Mcl-1 expression at both mRNA and protein levels. (E) The effects of β-catenin depletion on PDGF-BB regulation of Mcl-1 expression in ARCaP$_M$ cells. The cells were transfected with β-catenin siRNA or control siRNA (30 nM) for 48 h, serum-starved overnight, and incubated in the presence or absence of PDGF-BB (20 ng/ml) for 72 h. (F) The effects of β-catenin depletion on Mcl-1 reporter activity in ARCaP$_M$ cells. The cells were transfected with β-catenin or control siRNA (30 nM) for 48 h, and further transfected with a human Mcl-1 reporter for 24 h. Following serum starvation overnight, the cells were incubated in the presence or absence of PDGF-BB (20 ng/ml) for 48 h.

3.5. β-catenin mediates PDGF regulation of Mcl-1 expression in PCa cells

Activation of the β-catenin pathway is a downstream event of PDGF signaling in certain epithelial cancer cells (187-189). Western blot analysis found that β-catenin and TCF4, a major β-catenin-interacting transcription factor (190), were differentially expressed in PCa cells (Figure 6A), suggesting a functional β-catenin-TCF4 signaling in these cells. In fact, an artificial TCF promoter was activated in both the LNCaP-C4-2 and $ARCaP_E$-$ARCaP_M$ cell lineages, and the reporter activities appeared to be associated with increased *in vivo* metastatic potential in C4-2 and $ARCaP_M$ cells (Figure 6B). It is worth noting that both β-catenin and TCF4 were substantially presented in the nucleus of $ARCaP_E$ and $ARCaP_M$ cells (Figure 6A, low panel), which exhibited markedly higher basal TCF activities than either LNCaP or C4-2 cells (by ~100-fold) (Figure 6B).

Upon PDGF-BB treatment, the nuclear presence of β-catenin was rapidly increased in $ARCaP_M$ cells (Figure 6C, upper panel). Consistently, TCF reporter activity was also significantly increased following PDGF-BB stimulation, which was attenuated by the pre-treatment with AG-17 (Figure 6C, bottom panel). These data indicated that PDGF-BB activated β-catenin signaling in a PDGFR-dependent manner.

To investigate the role of β-catenin in the regulation of Mcl-1 expression, $ARCaP_M$ cells were transiently transfected with a construct expressing wild-type β-catenin. RT-PCR and Western blot analyses showed that ectopic epression of β-catenin increseased Mcl-1 at both mRNA and protein levels (Figure 6D). In contrary, β-catenin depletion using a siRNA pool efficiently inhibited both the basal

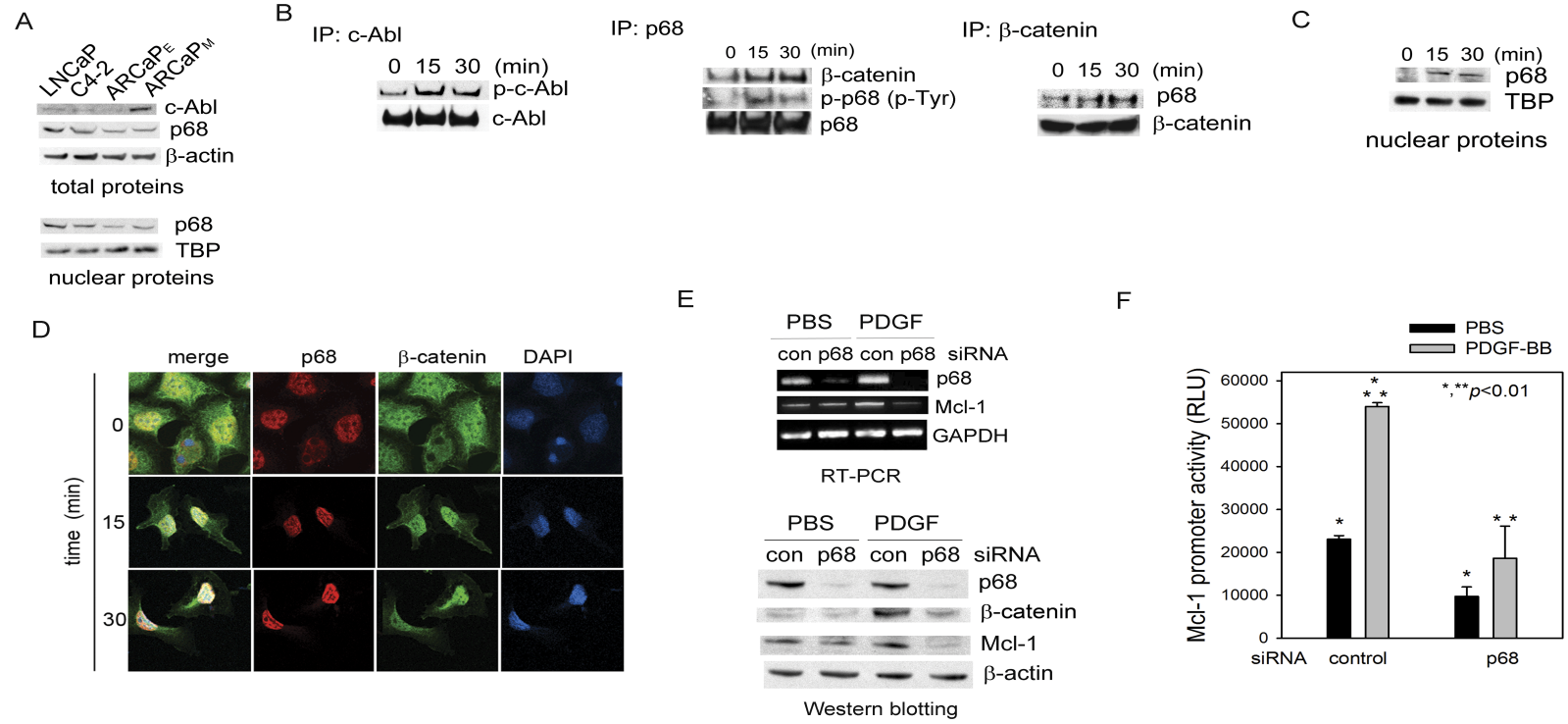

Fig. 7. PDGF-BB activates the c-Abl-p68–β-catenin signaling cascade in Pca cells. (A) Expression of c-Abl and p68 in Pca cells. **(B)** Left panel: The effects of PDGF-BB (20ng/ml) on the phosphorylation of c-Abl; middle panel: The effects of PDGF-BB (20ng/ml) on the phosphorylation of p68 and the expression of β-catenin in the p68 immunoprecipitates in ARCaP$_M$ cells; right panel: The effects of PDGF-BB (20ng/ml) on the expression of the p68 in the β-catenin immunoprecipitates in ARCaP$_M$ cells. **(C)** The effects of PDGF-BB (20 ng/ml) on the nuclear translocation of p68 in ARCaP$_M$ cells. **(D)** Confocal microscopy analysis of the effects of PDGF-BB on the co-localization of β-catenin and p68 in the nucleus in a time course experiment in ARCaP$_M$ cells. **(E)** The effects of p68 depletion on PDGF regulation of Mcl-1 in ARCaP$_M$ cells. The cells were transfected with p68 or control siRNA (30nM) for 48h, serum-starved overnight, and incubated in the presence or absence of PDGF-BB (20 ng/ml) for 24h (upper panel) or 72h (bottom panel). Upper panel: RT-PCR analysis of mRNA expression of p68 and Mcl-1; bottom panel: Western blot analysis of protein expression of p68, β-catenin and Mcl-1. **(F)** The effects of p68 depletion on Mcl-1 reporter activity in ARCaP$_M$ cells. The cells were transfected with p68 or control siRNA (30nM) for 48h, and further transfected with human Mcl-1 reporter for 24h. Following serum starvation overnight, the cells were incubated in the presence or absence of PDGF-BB (20ng/ml) for 48h.

expression of Mcl-1 and its induction by PDGF-BB (Figure 6E). Consistently, whereas PDGF-BB significantly induced the luciferase activity of a full-length human Mcl-1 promoter in ARCaP$_M$ cellstransfected with non-targeting control siRNAs, this effect was abrogated by transient depletion of endogeneous β-catenin (Figure 6F). These results suggested that activation of β-catenin signaling may be sufficient and required for Mcl-1 expression in PCa cells.

3.6. PDGF activates p68-β-catenin signaling in PCa cells

This study investigated whether a c-Abl-p68-dependent pathway is involved in the PDGF activation of β-catenin signaling in PCa cells (187). Western blot analyses found that c-Abl and p68 were differentially expressed in PCa cells (Figure 7A). Upon PDGF-BB treatment, tyrosine phosphorylation of c-Abl and p68 were rapidly activated, as evidenced by immunoprecipitation-immunoblotting assays (Figure 7B, left and middle panels). Importantly, the presence of β-catenin in p68 immunoprecipitates was also increased in a time-dependent manner, suggesting an enhanced physical association between β-catenin and p68 proteins (Figure 7B, middle panel), which was further confirmed by a reciprocal immunoprecipitation experiment (Figure 7B, right panel). In fact, PDGF-BB induced rapid nuclear translocation of p68 within 30 min (Figure 7C), which was associated with increased co-localization of p68 and β-catenin in the nucleus (Figure 7D). These data indicated that PDGF-BB could activate the c-Abl-p68 cascade and subsequent β-catenin signaling in PCa cells.

To examine whether p68 is required for the regulation of Mcl-1 expression, $ARCaP_M$ cells were transfected with p68 siRNA or control siRNA, and analyzed for the expression of Mcl-1 at the mRNA and protein levels. As shown in Figure 7E, depletion of p68 inhibited endogeneous β-catenin and effectively attenuated PDGF-BB induction of Mcl-1 protein. Consistently, Mcl-1 promoter activity was significantly inhibited by the treatment with p68 siRNA in $ARCaP_M$ cells, either with or without the presence of PDGF-BB in the cultures (Figure 7F). These data indicated an indispensible function of p68 in the regulation of Mcl-1 in PCa cells.

3.7. PDGF-BB promotes protein interaction between β-catenin and HIF-1α in PCa cells

Our previous studies demonstrated an important role of HIF-1α in bone metastatic PCa cells (184). Interestingly, transfection of a HIF-1α-specific siRNA significantly reduced Mcl-1 protein expression in $ARCaP_M$ cells (Figure 8A), suggesting that HIF-1α may be required for Mcl-1 regulation in PCa cells. To examine whether PDGF-BB could induce physical interaction between HIF-1α and β-catenin, nuclear proteins were prepared from $ARCaP_M$ cells treated with PDGF-BB for varying times. Western blot analysis found that both HIF-1α and β-catenin were rapidly increased in the nucleus (Figure 8B). A co-immunoprecipitation assay showed that in response to PDGF-BB stimulation, nuclear presence of β-catenin rapidly increased in the HIF-1α immunoprecipitates (Figure 8C, upper panel). Reciprocal co-immunoprecipitation with an anti-β-catenin antibody confirmed an increased association of nuclear HIF-1α with β-catenin following PDGF-BB

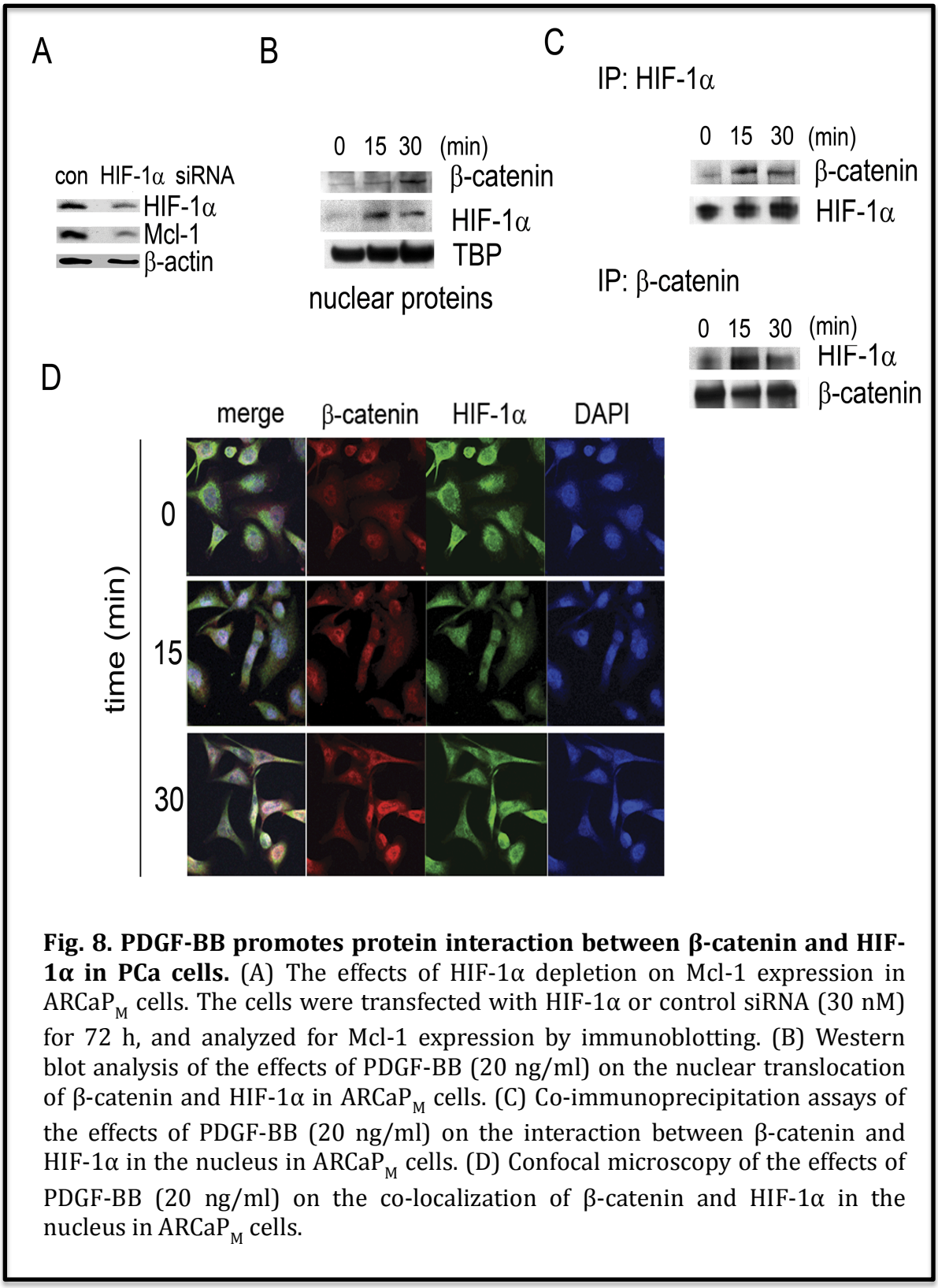

Fig. 8. PDGF-BB promotes protein interaction between β-catenin and HIF-1α in PCa cells. (A) The effects of HIF-1α depletion on Mcl-1 expression in $ARCaP_M$ cells. The cells were transfected with HIF-1α or control siRNA (30 nM) for 72 h, and analyzed for Mcl-1 expression by immunoblotting. (B) Western blot analysis of the effects of PDGF-BB (20 ng/ml) on the nuclear translocation of β-catenin and HIF-1α in $ARCaP_M$ cells. (C) Co-immunoprecipitation assays of the effects of PDGF-BB (20 ng/ml) on the interaction between β-catenin and HIF-1α in the nucleus in $ARCaP_M$ cells. (D) Confocal microscopy of the effects of PDGF-BB (20 ng/ml) on the co-localization of β-catenin and HIF-1α in the nucleus in $ARCaP_M$ cells.

treatment (Figure 8C, bottom panel). The enhanced co-localization of β-catenin and HIF-1α proteins was further demonstrated by confocal microscopy, which appeared to acheive the maximum intensity at 30 min upon PDGF-BB stimulation (Figure 8D) . These results indicated that in repsonse to PDGF-BB stimulation, β-catenin physically interacts with HIF-1α in the nucleus, which may lead to the activation of Mcl-1 transcription in PCa cells.

3.8. A putative HRE motif is required for PDGF-BB activation of Mcl-1 promoter

HIF-1α binds to the HRE *cis*-elements within the promoters of hypoxia-responsive genes and regulates their expression (71). This study examined whether PDGF-BB-induced nuclear accumulation of HIF-1α was associated with the activation of HRE-dependent transcription. In $ARCaP_M$ cells, PDGF-BB treatment significantly increased luciferase expression driven by an artificial HRE promoter (pHIF-luc) (Figure 9A). Interestingly, a putative HRE motif was identified within human Mcl-1 promoter region, which is located between -900 and -884 nucleotides at the 5'-upstream of transcription start site (71). To investigate the potential role of this *cis*-element in PDGF regulation of Mcl-1 transcription, the deletion mutant of the putative HRE motif using human Mcl-1 promoter region as the template was characterized (Figure 9B). The resulting reporter construct (p-Mcl-1-Luc: ΔHRE), or the luciferase reporter driven by the full-length Mcl-1 promoter (p-Mcl-1-Luc), was transiently expressed in $ARCaP_M$ cells respectively, and treated with PDGF-BB or PBS. IL-6, which has been shown to activate Mcl-1 transcription in PCa and

cholangiocarcinoma cells through a signal transducer and activator of transcription 3 (Stat3)-dependent mechanism (191, 192), was included as the positive control. Luciferase activity assay showed that PDGF-BB induced the activation of p-Mcl-1-Luc promoter to a greater degree than IL-6 in $ARCaP_M$ cells. Significantly, deletion of the HRE motif not only reduced the basal activity of Mcl-1 promoter, but also abrogated the inductive effects of PDGF-BB on reporter activity. In contrary, p-Mcl-1-Luc: ΔHRE, containing a Stat3-binding sequence at position between -92 and -83 (191), remained activated upon IL-6 treatment (Figure 9C). A similar effect of HRE deletion on the differential response of Mcl-1 promoter to PDGF-BB and IL-6 was also observed in C4-2 cells (Figure 10). These data indicated that the putative HRE *cis*-element is required for PDGF-BB activation of Mcl-1 expression in PCa cells.

3.9. PDGF-BB promotes HIF-α binding to Mcl-1 promoter region

To investigate whether PDGF-BB promoted specific binding of both β-catenin and Hif-1α to Mcl-1 promoter, a sequential ChIP assay was done. Fractionated chromatin from controls and PDGF-BB-treated $ARCaP_M$ cells was firstly immunoprecipitated with b-catenin antibody or control IgG. The precipitates were then subjected to a re-ChIP assay with an HIF-1α antibody or control IgG. From the isolated DNA, a 151-bp fragment containing the HRE region on the Mcl-1 promoter was amplified from the re-ChIP precipitates. Upon PDGF-BB stimulation, a considerable increase in the binding of both b-catenin and HIF-1α to the HRE region

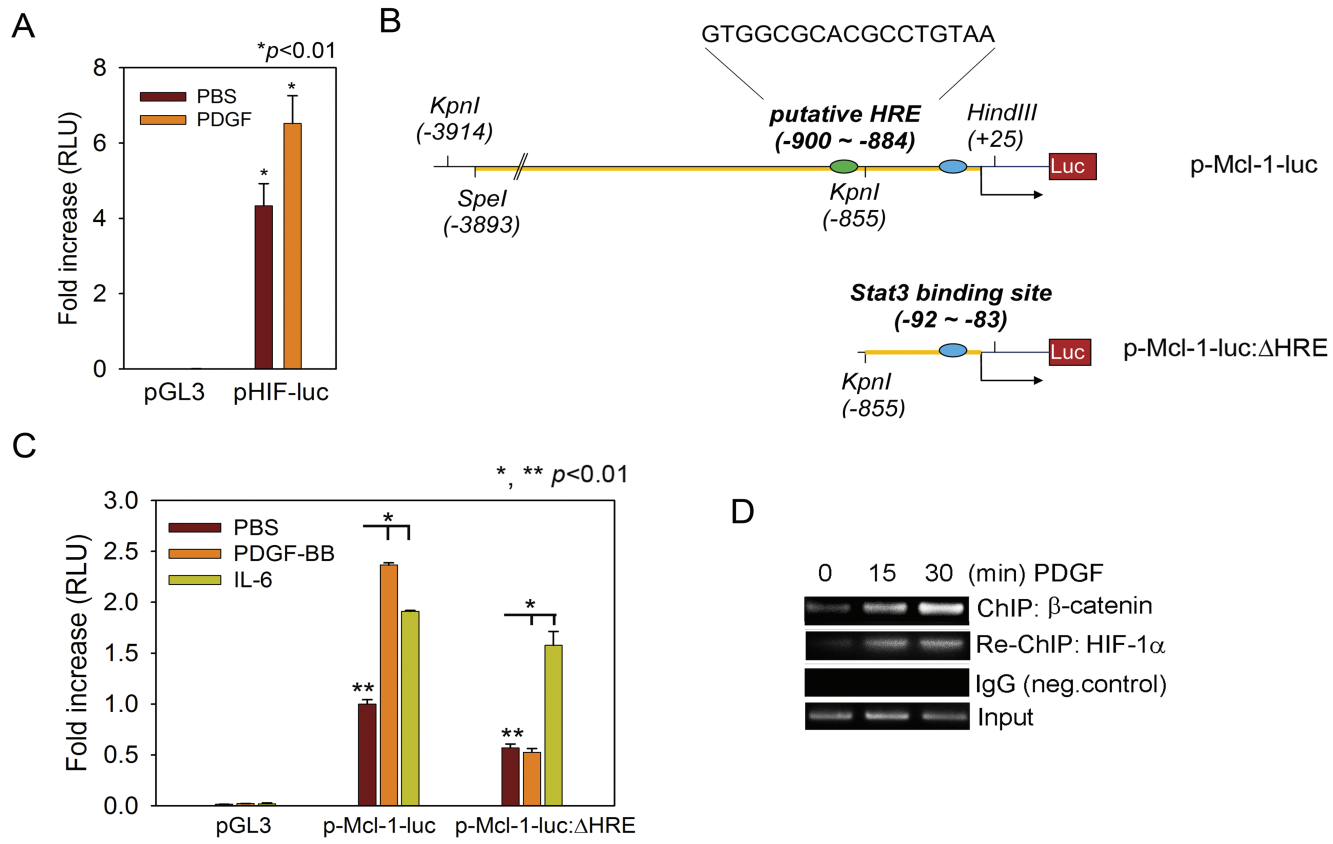

Fig. 9. A putative HRE site is required for PDGF-BB activation of Mcl-1 promoter. (A) The effects of PDGF-BB on the HIF-1 reporter activity in $ARCaP_M$ cells. The cells were transiently transfected with HIF-1 reporter or pGL3 for 24h, serum-starved and incubated in the presence or absence of PDGF-BB (20 ng/ml) for 48h. **(B)** Schematic diagram of human Mcl-1 promoter and its deletion mutation at the putative HRE site. **(C)** The effects of deleting the putative HRE site on PDGF regulation of Mcl-1 promoter activity in $ARCaP_M$ cells. IL-6 (200 ng/ml) was included as the positive control. **(D)** ChIP-Re-ChIP assay of the effects of PDGF-BB treatment (20 ng/ml) on the binding of β-catenin and HIF-1α to human Mcl-1 promoter region in $ARCaP_M$ cells.

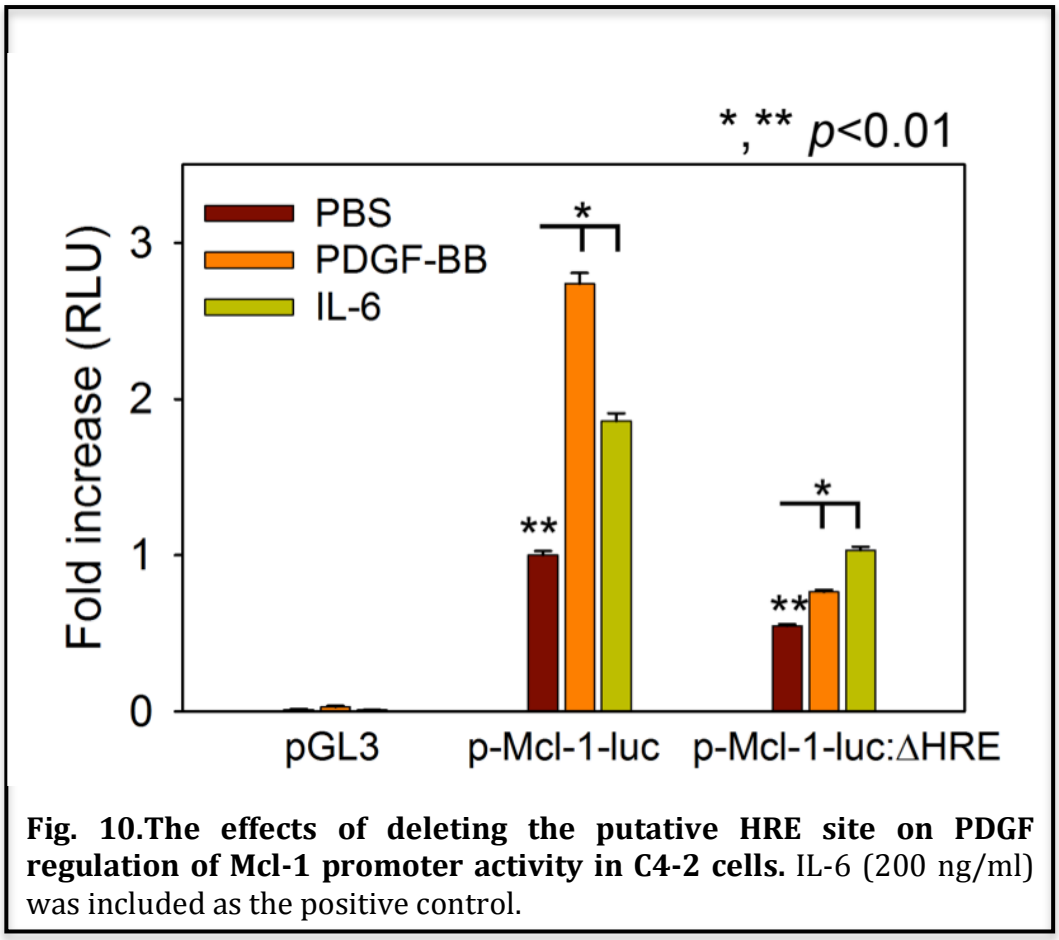

Fig. 10.The effects of deleting the putative HRE site on PDGF regulation of Mcl-1 promoter activity in C4-2 cells. IL-6 (200 ng/ml) was included as the positive control.

was observed (Figure 9D). These results demonstrate that PDGF-BB can facilitate the association of the Mcl-1 promoter with a transcriptional complex consisting of β-catenin and HIF-1α in PCa cells.

Chapter 4

Discussion

This dissertation uncovered the PDGF-Mcl-1 signaling as a crucial survival mechanism in PCa cells (Figure 11). For the first time, this dissertation demonstrated that: 1) PDGF-BB is a novel regulator of Mcl-1 expression; 2) PDGF-BB activation of autocrine PDGFR signaling promotes the interaction between β-catenin and HIF-1α, presumably through a c-Abl-p68-dependent mechanism; 3) a putative HRE motif is required for the basal expression and PDGF-BB activation of Mcl-1 promoter; and 4) inhibition of the PDGFR-Mcl-1 signaling using a small-molecule inhibitor AG-17 could activate apoptotic response in metastatic PCa cells. These results support that targeting PDGF-Mcl-1 pathway may provide a novel strategy for treating PCa metastasis.

Activation of PDGFR signaling may be coupled with multiple downstream pathways in the regulation of cell growth, proliferation, migration and survival (193). In tumor-associated endothelial and fibroblast stromal cells, PDGF has been shown to activate Akt- and MAPK-dependent survival mechanisms (194-197). Yet, it remains elusive on the molecular mechanism by which PDGF exerts its functions in epithelial cancer cells. Recent data have linked autocrine PDGF signaling to the activation of β-catenin pathway. For instance, PDGF-AB was found to induce nuclear β-catenin accumulation via a PI3K-dependent mechanism, thereby protecting hepatocellular carcinoma cells from anoikis during metastatic dissemination (188). In human colon cancer cells, PDGF-BB induces EMT (187) and

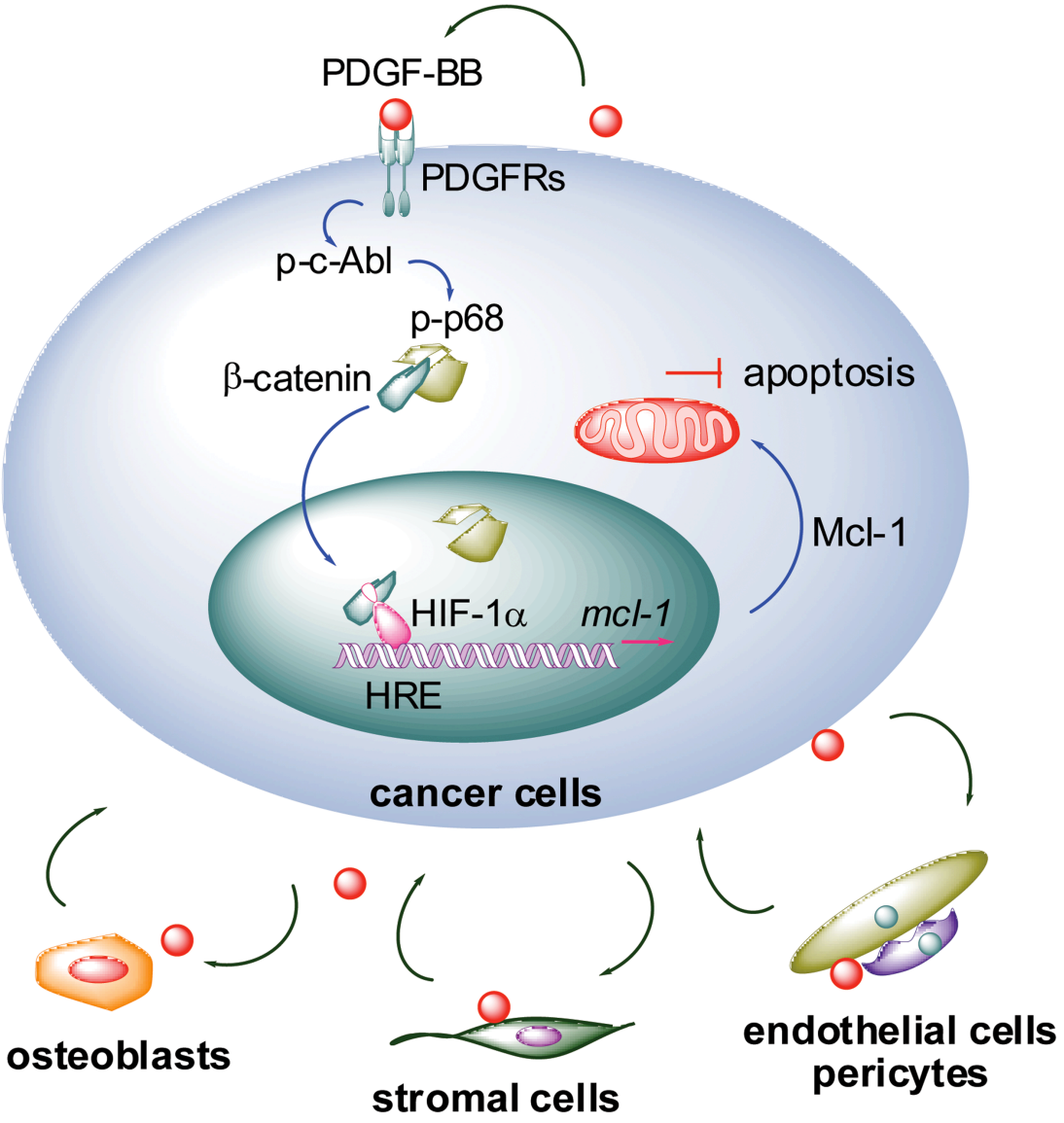

Fig. 11. A proposed model for PDGF-BB regulation of Mcl-1 expression in PCa cells. The engagement of PDGF-BB to PDGFR dimers activates the c-Abl-p68 cascade, which subsequently stablizes β-catenin and promotes its nuclear translocation. In the nucleus, interaction between β-catenin and HIF-1α increases the binding of HIF-1α to the HRE site within Mcl-1 promoter, thereby activating the transcription of Mcl-1 gene. Upregulation of Mcl-1 antagonizes apoptotic signals and confers survival advantages to metastatic PCa cells. Furthermore, tumor-derived and locally expressed PDGF may mediate the interactions between PCa and bone microenvironment. Co-targeting the PDGF signaling in PCa cells (autocrine) and microenvironment (paracrine) could provide a new strategy to disrupt the "vicious cycle" and efficaciously treat metastatic PCa.

upregulates cyclin D1 and c-Myc (198) by activating β-catenin-dependent gene expression. In both cases, PDGF-BB induces the phosphorylation of c-Abl kinase, which subsequently recruits p68, an RNA helicase with ATPase activity, and activates its phosphorylation. Phosphorylated p68 binds β-catenin and promotes its nuclear translocation by displacing Axin from β-catenin and blocking β-catenin degradation, eventually promoting the interacting of β-catenin with TCF/LEF and the assembly of transcription complexes (187). This dissertation provided molecular evidence demonstrating that in PCa cells that express high basal levels of p68 and β-catenin could significantly promote physical interaction and rapid nuclear translocation of p68 and β-catenin. Importantly, p68 depletion in PCa cells led to the inhibition of Mcl-1 expression and induction of apoptosis, as evidenced by the appearance of cleaved PARP (Figure 12). These results, for the first time, underscored a critical role of p68 in the regulation of PCa cell survival. Interestingly, a recent study demonstrated that p68 is actually a novel coactivator of androgen receptor (AR) (199), another transcription factor

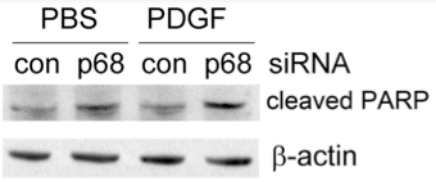

Fig. 12. The effects of p68 siRNA on the expression of cleaved PARP, an indicator of apoptosis in PCa cells. $ARCaP_M$ cells were transfected with p68 or control siRNA (30 nM) for 48 h, serum-starved overnight, and incubated in the presence or absence of PDGF-BB (20 ng/ml) for 72 h.

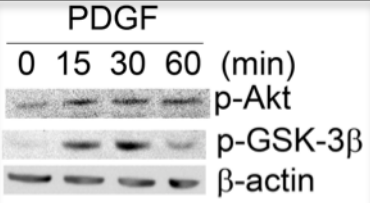

Fig. 13.The effect of PDGF-BB on the Akt-GSK-3β cascade in PCa cells. PDGF-BB treatment (20ng/ml) in $ARCaP_M$ cells increased phosphorylation of Akt and GSK-β at serine residues.

interacting with β-catenin in certain PCa cells (such as LNCaP and C4-2) (200). It would be intriguing to further investigate the dynamic interaction between p68, β-catenin and p68, and its biological consequences in these cells. In addition, other pathways may be involved in the PDGF activation of β-catenin signaling. For example, PDGF-BB treatment was found to induce rapid phosphorylation of both Akt and glycogen synthase kinase 3-β (GSK-3β) (Figure 13), which may also contribute to the elevated intracellular levels and nuclear accumulation of β-catenin (201).

These data confirmed a highly active β-catenin/TCF signaling in ARCaP cells and correlated the TCF reporter activity with the *in vivo* metastatic potential (Figure 6A, 6B), indicating these cells could be used as an excellent model system for investigating β-catenin signaling in PCa progression (202). Though PDGF-BB activated the full-length human Mcl-1 promoter (Figure 6F) in a similar manner to its effect on the luciferase expression driven by an artificial TCF-binding motif (pTOPFlash), it appeared that human Mcl-1 promoter does not contain any consensus sequences of TCF/ lymphoid enhancer-binding factor (LEF). These results suggested that certain transcription factor(s), other than TCF, could be responsible for β-catenin activation of Mcl-1 transcription. One of such candidates was cAMP-response element-binding protein (CREB), which has been implicated in the regulation of Mcl-1 expression through the PI-3K/Akt signaling pathway (169) and highly expressed in ARCaP cell lineage (184). Western blotting analyses, however, could not detect a significant increase in nuclear CREB expression upon PDGF treatment (data not shown), suggesting that CREB may not be involved in the

β-catenin-dependent activation of Mcl-1 transcription. Intriguingly, the transcription factor HIF-1α was found to be rapidly increased in the nucleus and physically interact with β-catenin following PDGF-BB stimulation, which may mediate Mcl-1 transcription by binding to HRE site(s) within the promoter. These data are consistent with a previous study showing that β-catenin can switch its binding partner from TCF4 to HIF-1α and enhance HIF-1α-mediated transcription, and this dynamic reassembly of β-catenin with HIF-1α may allow colorectal cancer cells to rapidly adapt to hypoxic stress and survive (203). It is important to note that unlike the cited work, these studies were performed in normoxic PCa cell cultures. Since ARCaP cells substantially express HIF-1α even under normoxia (184), PDGF may significantly affect the expression of hypoxia-responsive or HRE-containing genes by promoting the interaction between β-catenin and HIF-1α in a Wnt-independent mechanism. Upregulation of Mcl-1, as a consequence, could provide pivotal protection against apoptotic signals during dissemination and colonization when the majority of cancer cells remain under normoxia.

Earlier studies reported high expression of PDGFRs in both localized and metastatic PCa, which could be detected in 88% of primary tumors and 80% of the metastases (204, 205). However, it remains controversial as to which PDGFR isoforms are expressed in PCa cells and primarily responsible for autocrine PDGF signaling (30, 125, 206). These conflicting results may partially arise from the potential non-specificity of antibodies used in the cited studies, but more importantly, may reflect the intrinsic heterogeneity of human cancers, especially when at their late-stages. This dissertation was able to detect the expression of both

PDGFR isoforms in several established PCa cell lines by RT-PCR and Western blot analyses. Given the fact that both PDGFR-α and -β have been implicated in the progression of bone metastatic PCa (125, 189, 204, 205, 207), this study focused on the function of PDGF-BB since it is the only PDGF isoform that binds all the three receptor dimeric combinations (PDGFR-αα, -ββ and -αβ) with high affinity (208, 209). To determine which PDGFR isoform is required for PDGF regulation of Mcl-1, PCa cells were transfected with specific siRNAs against PDGFR-α or -β. Interestingly, the single depletion of neither PDGFR-α nor PDGFR-β inhibited Mcl-1 expression in $ARCaP_M$ cells, suggesting that the PDGF-BB signal could be transduced via the two independent but complementary receptors to activate Mcl-1 expression in PCa cells expressing both isoforms. Supporting this notion, dual depletion of both receptors simultaneously using a mixture of siRNAs against PDGFR-α and -β effectively inhibited Mcl-1 expression. Alternatively, treatment with AG-17 or imatinib, two pan-PDGFR inhibitors that could inhibit the tyrosine kinase activity of both PDGFR-α and -β, also reduced Mcl-1 levels in $ARCaP_M$ cells (Figure 14). Furthermore, in PCa cells that predominantly express one PDGFR isoform (for example, PDGFR-α is the major isoform in C4-2 cells; Figure 5A, right panel), it is plausible to expect that inhibition of the isoform alone could affect Mcl-1 expression. Indeed, transfection of PDGFR-α siRNA in C4-2 cells significantly inhibited Mcl-1 (Figure 15). These findings support

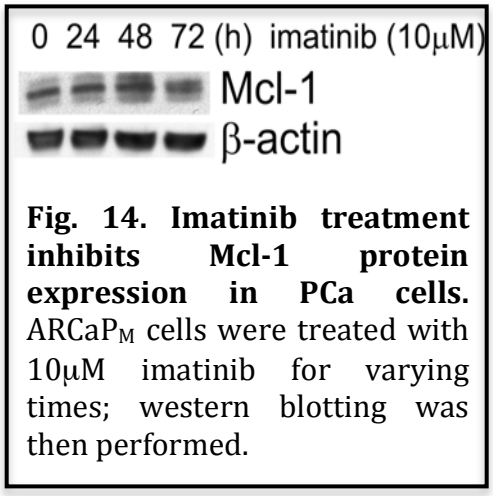

Fig. 14. Imatinib treatment inhibits Mcl-1 protein expression in PCa cells. $ARCaP_M$ cells were treated with 10μM imatinib for varying times; western blotting was then performed.

a model that PDGF-BB could activate both PDGFR isoforms in the regulation of Mcl-1 in PCa cells in a context-dependent manner, which may have important implication in the evaluation of PDGFR expression at tissue levels in clinical PCa specimens.

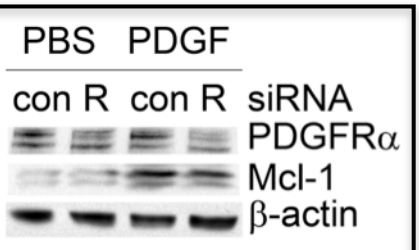

Fig. 15. Depletion of PDGFR-α abrogates PDGF-BB induction of Mcl-1 in C4-2 cells. The cells were transfected with PDGFR-α or control siRNA (30nM) for 48h, serum-starved overnight, and incubated in the presence or absence of PDGF-BB (20 ng/ml) for 72h.

Interaction between PCa and bone microenvironment is crucial to the bone tropism of PCa metastasis, which is identified at autopsy in up to 90% of patients dying from the disease (210). Tumor-initiated bone resorption promotes the release and activation of multiple growth factors immobilized in bone matrix, including PDGF. These locally expressed and tumor-derived PDGF could activate PDGFR signaling in surrounding stroma (including stromal cells, endothelial cells and pericytes) and promote angiogenesis. As a potent mitogen for osteoblasts, PDGF also significantly contribute to the osteoblastic phenotype of PCa bone metastasis (211). These effects, taken together, may provide a favorable microenvironment for the survival and outgrowth of bone metastatic PCa. These facts provided rationale for evaluating the potential of treating PCa bone metastasis with small-molecule PDGFR inhibitors. In earlier studies, imatinib sensitized bone marrow stromal and endothelial cells to paclitaxel treatment and significantly suppressed PCa bone metastasis in experimental models (212, 213). Disappointingly, however, recent clinical trials with imatinib only achieved limited success due to unexpected severe

side effects in patients (207). These observations highlighted the importance of a better understanding of PDGF signaling in bone metastasis PCa. This dissertation delineated a novel signaling axis that may allow PCa cells to escape apoptosis during dissemination and colonization by activating PDGF-Mcl-1 pathway in metastatic cancer cells. It is plausible to hypothesize that PDGF-BB may be crucial in mediating the "vicious cycle" between tumor and bone microenvironment, not only promoting angiogenesis in surrounding stroma but also sustaining survival in PCa cells (Figure 11). Supporting this model, PDGF-BB was found to be elevated in PC3-MM2 cells implanted in the mouse bone cortex, and interestingly, activated PDGFR-β was only detected in tumor lesions growing adjacent to bone and the tumor-associated endothelium (194, 212). Given the clinical significance of both PDGF and Mcl-1 in PCa bone metastasis (161, 211), specific targeting of PDGF-Mcl-1 survival pathway in PCa cells (autocrine signaling) and co-targeting of microenvironment (paracrine signaling) could provide a new strategy to disrupt the vicious cycle and efficaciously treat metastatic PCa.

PDGF/R role in gliomagenesis

Brain tumors or intracranial neoplasms are a vast collection of tumors that can arise from brain tissue or the surrounding environment (e.g. lymph nodes or meninges). A number of intracranial tumors have been identified and classified based on histology (214). The most common brain tumors in adults arise from malignant glial tissue (215) and have a survival rate of approximately 1 year after aggressive therapy of surgical resection, radiation and chemotherapy (216). In

general, malignant gliomas are genetically diverse, progress rapidly and are resistant to therapy (217). PDGFR signaling has been implied in glioblastoma biology through studies based on analyses of human tumor tissue, of cultured glioblastoma cells, and of mouse glioblastoma models (41, 218-221).

During glial development, PDGF signaling functions to maintain glial precursors in an undifferentiated and proliferative state (222). Forced overexpression of PDGF in mature glial cells, that also express the PDGF receptor, induces an autocrine signaling loop to dedifferentiated glial cells into glial progenitor-like cells by altering proliferation rate, cell morphology and gene expression (219). Similarly, *in vivo* data demonstrate that dedifferentiation from PDGF autocrine stimulation results in low-grade gliomas (219, 223-225), suggesting that PDGF signaling alone may be sufficient to induce glioma formation. Furthermore, viruses that express higher levels of PDGF drive cerebral gliomas to form faster, more consistently, and with more malignant features, suggesting that PDGF-driven gliomagenesis is a dose dependent phenomena (226).

Glioma Clinical Pathology and Therapies

In gliomagenesis PDGF was first discovered as a growth-promoting factor in glioma cell lines (227, 228). Subsequent studies revealed that PDGF ligands and receptors are expressed in numerous glioma cell lines (229-231). Further analyses on human glioma tissue revealed the expression of PDGF-A, PDGF-B and PDGFR-α in tumor cells (232, 233). PDGF ligand expression, however, varies among gliomas of different grades, with PDGF-A being expressed in all grades and PDGF-B only in

high-grade gliomas (234), indicating that PDGF-B may be involved in the conversion of low- to high-grade gliomas. Surrounding endothelial cells have also been shown to express PDGF-B and PDGFR-β, suggesting the presence of autocrine and paracrine loops in gliomagenesis (235, 236).

Due to gliomas frequently overexpressing PDGF and PDGFRs in an autocrine/paracrine manner (230, 235, 237, 238), PDGF/Rs have been targeted for therapy. *In vitro* studies have demonstrated that suramin, neutralizing antibodies to PDGF, and dominant-negative mutations of either PDGF ligand or PDGF receptor inhibit glioma progression (239-242). However, in therapy, outer-cell-surface receptor inhibitors such as suramin (243) and neutralizing antibodies have not proven to be practical due to significant levels of functional PDGF/R complex can be formed within the cell cytoplasm (244). STI571 or Imatinib, which targets the PDGF receptor family and the activated Abl oncoprotein appeared to be a lucrative drug therapy to block PDGF induced glioma progression because it is able to block the ATP binding site of the tyrosine kinase proteins to inhibit the transduction of downstream signals (245). Unfortunately, clinical trials have demonstrated that the drug, by itself and in combined therapy, is not effective in reducing cancer progression (246, 247). Current studies in gliomagenesis continue to focus on amplification or over-expression of critical growth factor activated signal transduction pathways, such as EGFR and PDGFR, in combination with activation of PI3K (248) and mutations in genes implicated in cell cycle regulation, such as loss of the tumor suppressor genes *p53*, *p16, RB,* or *PTEN* (249-252).

The potential implication of this thesis on glioma biology is the linkage between PDGFR signaling and activation of HIF-1. The HIF family has been demonstrated to play an imperative role in glioma growth and survival (253). Targeted disruption between PDGF/R activated signaling and the HIF family activation may provide new therapeutic benefits.

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AUTOBIOGRAPHICAL STATEMENT

Shareen Iqbal

EDUCATION

- Ph.D. in Neuroscience, Emory University, Atlanta, Georgia: 2012
- B.A. in Psychology, Indiana University, Bloomington, Indiana; 2003

PUBLICATIONS

- 2011S Zhang, X Wang, **S Iqbal**, Y Wang, A Osunkoya, Z Chen, Z Chen, D Shin, H Yuan, F Marshall, C Ritenour, H Zhau, L Chung, O Kucuk, D Wu. EGF promotes EPLIN protein degradation during epithelial-to-mesenchymal transition. *PNAS. Submitted*.

- 2011 **S Iqbal**, S Zhang, A Driss, ZR Liu, HE Zhau, LWK Chung, D Wu. Platelet-Derived Growth Factor Regulation of Myeloid Cell Leukemia-1 Expression in Bone Metastatic Human Prostate Cancer Cells. *PLoS One, accepted with revision.*

- 2011 S Zhang, X Wang, AO Osunkoya, **S Iqbal**, Y Wang, Z Chen, S Muller, Z Chen, S Josson, IM Coleman, PS Nelson, YA Wang, R Wang, DM Shin, FF Marshall, O Kucuk, LWK Chung, HE Zhau, D Wu. EPLIN downregulation promotes epithelial-mesenchymal transition in prostate cancer cells and correlates with clinical lymph node metastasis. *Oncogene*. May 30.

- 2010 S Zhang, HE Zhau, R Wang, **S Iqbal**, FF Marshall, LWK Chung and D Wu. Vascular endothelial growth factor regulates myeloid cell leukemia-1 expression through neuropilin-1-dependent activation of c-met signaling in metastatic human prostate cancer cells. *Mol Cancer*. Jan 19;9:9.

- 2008 S Seo, L Gera, HE Zhau, WP Qian, **S Iqbal**, N Johnson, S Zhang, M Zayzafoon, J Stewart, LWK Chung and D Wu. BKM1740, an acyl-tyrosine bisphosphate amide derivative, inhibits the bone metastatic growth of human prostate cancer cells through the inhibition of surviving expression. *Clinical Cancer Research* Oct 114 (19):6198-206.

- 2007 D Wu, HE Zhau, W-C Huang, **S Iqbal**, FK Habib, O Sartor, L Cvitanovic, FF Marshall, Z Xu, and LWK Chung. cAMP-responsive element-binding protein regulates vascular endothelial growth factor expression: implication in human prostate cancer bone metastasis. *Oncogene* 26: 5070-5077.

- 2001 SPS Monga, Y Tang, F Candotti, A Rashid, O Wildner, B Mishra, **S Iqbal** and L Mishra. Expansion of hepatic and hematopoietic stem cells utilizing

mouse embryonic liver explants. *Cell Transplantation*, Jan-Feb;10(1):81-9.

ORAL PRESENTATIONS

- 2011 **Centers for Disease Control**: Chagas Disease, Atlanta GA. "Medical Practioner Knowledge and Attitudes on Chagas Disease in Atlanta: A Pilot Study."

- 2006 **Digestive Disease Week**, Los Angeles, CA. **ShareenIqbal**, Andrew Gewirtz, Dean Jones and Shanthi Srinivasan. "Altered Gastrointestinal Motility and Enteric Neuronal Apoptosis in Diabetes Is Due to Increased Oxidative Stress."

POSTER PRESENTATIONS

- 2011 **Global Health Institute Scholar Symposium**. Atlanta, GA. Shareen Iqbal, Rebecca Minneman, Chester Pennock, and Juan Leon. "Attitudes and Knowledge of Chagas' Disease among Latin American Immigrants and Medical Care Providers in Georgia."

- 2010 **Society for the Basic Urological Research (SBUR)**. Atlanta, GA. **Shareen Iqbal**, Shumin Zhang, Adel Driss, Zhi-Ren Liu, Haiyen E. Zhau, Leland W.K. Chung, Daqing Wu. "Platelet-Derived Growth Factor Regulation of Myeloid Cell Leukemia-1 Expression in Bone Metastatic Human Prostate Cancer Cells."

- 2008 **American Association for Cancer Research.** (AACR), San Diego, CA. **ShareenIqbal**, Daqing Wu, Nicole Johnson and Leland Chung. "PDGF Induced Mcl-1: Signaling is a Survival Pathway in Bone-Metastatic Human Prostate Cancer Cells."

- 2007 **WCI 5th Annual Research Symposium.** Atlanta, GA., **Shareen Iqbal**, Seongil Seo, Weiping Qian, Lajos Gera, John Stewart, Haiyen Zhau, Leland Chung and Daqing Wu. "Inhibiting Human Prostate Cancer Cell Growth in Bone with Acyl-Tyrosine Biphosphonate Amide Derivatives."

- 2006 **WCI 4th Annual Research Symposium, Atlanta, GA. Shareen Iqbal,** Seong Il Seo, Haiyen Zhau, Leland Chung and Daqing Wu. "VEGF Autocrine Signaling is a Survival Pathway in Bone-Metastatic Human Prostate Cancer Cells."

- 2005 **GTCBIO: Therapeutic Strategies Against Neurodegenerative Conditioning.** Burlington, MA. **Shareen Iqbal**, Dean Jones and Shanthi Srinivasan. "Glial Derived Neurotrophic Factor Prevents Oxidative Stress in Diabetic Enteric Neuropathy.

HONORS/AWARDS

- 2011 Global Health Multidisciplinary Field Team Scholar. "Attitudes and Knowledge of Chagas' Disease among Latin American Immingrants and Medical Care Providers in Georgia."

- 2010 Travel Award. Society for Basic Urological Research, Atlanta, GA.

- 2006 Travel Award. Digestive Disease Week, Los Angeles, CA.