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Investigating the Functions of EphB4 in Prostate Cancer

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"Investigating the Functions of EphB4 in Prostate Cancer"

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ABSTRACT

Prostate Cancer is the most commonly diagnosed cancer and the second most common cause of cancer death in Canadian men after lung cancer. Factors that promote metastasis in prostate cancer include the ability of prostate cancer cells to proliferate uncontrollably, and migrate and invade into tissues away from a localized prostate adenocarcinoma. Eph receptors are the largest family of receptor tyrosine kinases and their role in cancer progression has recently taken interest due to their identification, expression and activity in several cancer types. The work presented herein examines the potential impact of EphB4 tyrosine kinase receptor on proliferation, migration and invasion of prostate carcinoma cell lines, PC3, Du-145, and LNCaP. Comparison of EphB4 protein levels between prostate tumor cell lines and normal prostate epithelial cells indicated that EphB4 was generally overexpressed in prostate cancer. To understand the role of EphB4 in prostate cancer, we employed siRNA to deplete EphB4 expression, or alternatively, by activating EphB4 receptor by exogenous addition of EphrinB2-Fc. Proliferation of prostate carcinoma cell lines was not affected when EphB4 was reduced using siRNA, nor when EphB4 was activated with EphrinB2. Additionally, activating EphB4 was not found to promote downstream activation of the PI3/AKT pathway hence did not affect cell survival. Cellular migration of PC3, Du-145 and LNCaP cells, as determined by Wound-healing migration assays, was significantly decreased when EphB4 protein was reduced. However, stimulating the EphB4 receptor using EphrinB2-Fc did not show significant changes in migration. A closer examination of Rho-GTPase proteins that regulate cytoskeleton reorganization and motility led to our finding that activation of EphB4 by EphrinB2-Fc increases activity of Rac and Cdc42. Furthermore, EphB4 plays an important part in invasion of PC3 cells as it was found that cells with reduced EphB4 expression had decreased invasion, while increased invasion was observed after EphB4 was activated with EphrinB2-Fc. These findings suggest that EphB4 could play important roles in prostate cancer metastasis and, with further investigations, it may be possible to develop cancer therapies based on targeting the EphB4 receptor.

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LIST OF ABBREVIATIONS
(As in the order of Appearance in Text)

(PCa) – Prostate Cancer
(AIPC) – Androgen Independent Prostate Cancer
(PSA) – Prostate Specific Antigen
(DRE) – Digital Rectal Exam
(BPH) – Benign Prostatic Hyperplasia
(EGFR) – Epidermal Growth Factor Receptor
(RTK) – Receptor Tyrosine Kinase
(Eph) – Erythropoietin Producing Hepatoma
(VEGFR) – Vascular Epidermal Growth Factor Receptor
(TGF) – Transforming Growth Factor
(TKI) – Tyrosine Kinase Inhibitor
(SNP) – Single Nucleotide Polymorphisms
(PTEN) – Phosphatase and Tensin Homolog
(BRCA) – Breast Cancer 1 gene
(MAPK) – Mitogen Activated Protein Kinases
(ATP) – Adenosine Triphosphate
(SAM) – Sterile Alpha Motif
(GPI) – Glycosylphosphatidylinositol
(FAK) – Focal Adhesion Kinase
(PCR) – Polymerase Chain Reaction
(NSCLC) – Non-Small Cell Lung Cancer
(IGF-IR) – Insulin-like Growth Factor Receptor
(GTP) – Guanosine Triphosphate
(PDGF) – Platelet Derived Growth Factor
(MMP) – Matrix Metalloproteinases
(ECM) – Extracellular Matrix
(ROCK) – Rho Kinase
(MLC2) – Myosin II Light Chain
(siRNA) – Short Interference RNA
(RdRP) – RNA dependent RNA Polymerases
(DNA) – Deoxyribonucleic Acid
(RISC) – RNA Induced Silencing Complex
(DMEM) – Dulbecco's Modified Eagles Medium
(PBS) – Phosphate Buffered Saline
(EDTA) – Ethylenediaminetetraacetic acid
(IPTG) – Isopropyl Thiogalactoside
(PMSF) – Phenylmethylsufonyl Fluoride
(DTT) – Dithiothreitol
(TBST) – Tris-Buffered Saline Tween-20
(PrEC) – Prostate Epithelial Cells
(HUVEC) – Human Umbilical Vein Endothelial Cells
(EFN) – Ephrin

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1. INTRODUCTION

1.1 Prostate Cancer statistics

In 2007 it was recorded that 218,890 men were diagnosed with prostate cancer (PCa) in the United States and 9% (27,050) of all cancer deaths in men were related to complications from this disease (1). One of 6 men is currently diagnosed with prostate cancer in the United States and an estimated 15%-30% of men over 50 could harbor microscopic, undiagnosed prostate cancer (1). In Canada, an estimated 22,300 men are diagnosed with prostate cancer and each year ~4,300 will die from it (Table 1) (2). It is the most common type of cancer in Canadian men and 1 in 8 have a chance of developing prostate cancer in their lifetime (2). Even though cancer awareness and education programs have reduced mortality by 25% over the last decade, many challenges still remain in providing an appropriate diagnosis of prostate cancer, executing safer and comprehensive treatments, reducing prostate cancer morbidity, and understanding metastasis and irregularities in cellular pathways that lead to androgen-independent prostate cancer (AIPC).

Most common methods for diagnosing prostate cancer are elevated levels of prostate specific antigen (PSA) and an abnormal digital rectal exam (DRE) followed by a biopsy of the prostate tissue. PSA is normally produced in serum by prostate epithelial cells in small concentrations. An excessive production of prostate specific antigen, above the cutoff of 4ng/ml, could be an indication of tumor cancer cells (1,4). Unfortunately, a PSA screening showing elevated prostate-specific antigen levels cannot distinguish between malignant cancer tumor and benign prostate disorders, such as inflammation or benign prostatic hyperplasia (BPH). Neither can a PSA screening determine the severity and stage (Grade) level of cancer, such as microscopic benign cancer on the gland, a clinically relevant cancer

Table 1. Prostate Cancer Statistics in USA and Canada. Adapted from The Canadian Cancer Society data 2007 (2).

Prostate Cancer (PCa) Statistics - 2007

Men diagnosed with Prostate Cancer –	218,890
Deaths due to Prostate Cancer –	27,050

Table 1.

that can cause morbidity in the future, or a metastatic cancer that has spread to other organs (1). Moreover, an estimated 30%-50% of the time, patients are overdiagnosed using PSA screening with prostate cancer and have to suffer morbidities associated with the therapy (1).

1.2 Limitations of common therapies for Prostate Cancer and search for novel treatments.

The most common treatment of prostate cancer includes hormone ablation, external beam radiation, brachytherapy, chemotherapy, surgery, or a combination of these therapies. Androgen ablation is most often the initial form of treatment for prostate cancer, but its response is usually temporary. Many tumors can develop androgen blockage and may produce a hormone-unresponsive phenotype after 12-18 months of endocrine treatment (3). Surgical removal of localized prostate cancer is a common primary therapy, although, positive surgical margin rates have decreased in the past decade with biochemical recurrence rate reported at 4%-11%, three years after radical prostatectomy (11). Adverse effects include decreased quality of life for patients, including damage to urinary sphincter or erectile nerves. Even though complications from surgery are minimal, radical prostatectomy most often requires a combination of this treatment with radiation and largely benefits patients with localized prostate cancer as opposed to a metastasized stage (4). Radiation therapy, including brachytherapy, is another cancer treatment procedure which requires careful planning to minimize risk of irradiating normal tissues while calculating a dose response for radiation to target prostate tumor (12). Ionizing radiation damages genetic makeup of quickly dividing cells to prevent their growth and proliferation. Complications from external beam radiotherapy may include inflammation, swelling, and incidence of radiation proctitis ranging

from 2% to 39% of patients (4). With radiation therapy, there are also increased occurrences for gastrointestinal toxicities (5). To minimize radiation's effect on the surrounding tissues, accurate knowledge of the prostate's location is essential (4).

As a result of the limitations of the current therapeutic strategies, particularly for aggressive or metastatic PCa, a deeper understanding about the progression and metastatic properties of prostate cancer is necessary to help develop a novel treatment, or a combination of treatments, which would target cellular receptors often involved in cancer cell migration, invasion and proliferation occurring in tissues away from the solid tumor mass. For example, a recent completion of a phase II clinical trial showed that Docetaxel, a first-line chemotherapy agent, can be safely used with Erlotinib, an EGFR tyrosine kinase inhibitor, in androgen-independent prostate cancer (AIPC) (8,13). This type of study could open new doors for using chemotherapy in combination with molecular targeted treatments to increase survival rates and decrease metastasis in several types of lethal cancer tumors where cancer cells have metastasized.

1.3 Properties of cancer cells and instigators of metastasis

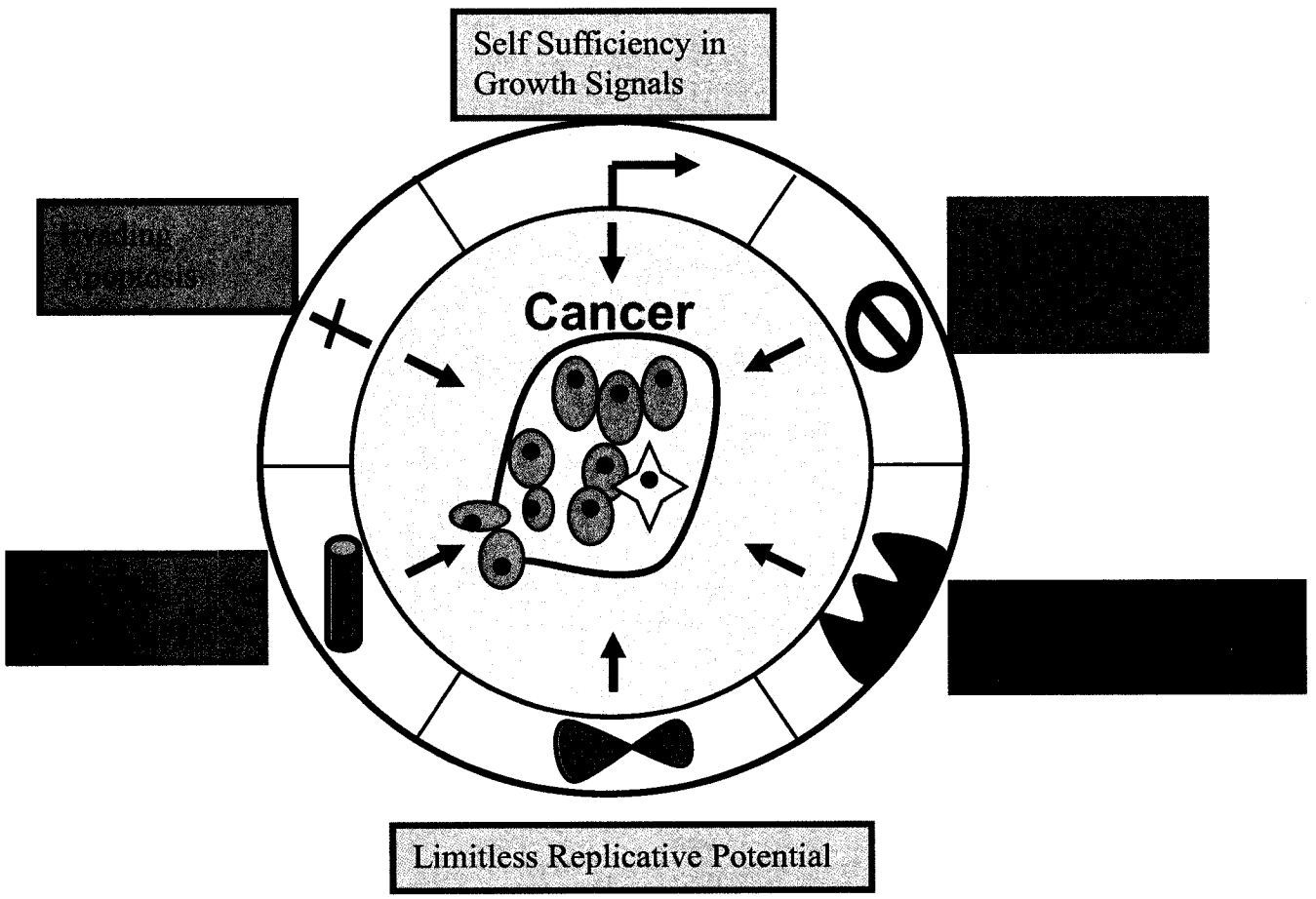
Genetic mutations, carcinogens, heredity, modifications in cellular pathways, and irregular expression of plasma membrane receptors, could be a few of the reasons why normal epithelial cells would change their physiology to evade apoptosis, proliferate uncontrollably, and migrate and invade into tissues away from a localized prostate adenocarcinoma (6) (Figure 1). In advanced stages of prostate cancer, a primary tumor mass produces pioneer cells that migrate to adjacent tissues by way of entering the bloodstream. These cancer cells then utilize their invasive properties to metastasize into distant sites where

they start to form new colonies of their own with the help of some, or all, of the other five properties of cancer cells. Furthermore, it is estimated that 90% of human cancer deaths are due to the ability of cancer cells to escape the primary tumor mass and migrate, invade and metastasize into distant tissues forming new settlements of tumor cells called metastases (6). In prostate cancer, the most common tissues where metastasized cancer cells have been found to locate are bone, lymph nodes, and brain tissue. It has been estimated that bone is the primary site of metastasis (80%-85%) in prostate cancer (7). The 5 year patient survival rate is only 31% in cases where prostate cancer has metastasized to distant sites, as opposed to a survival rate of nearly 100% in cases without metastasis (7).

Recent work has determined that several classes of proteins are overexpressed in cancer cells that can contribute to the properties of cancer metastasis. Some of these include cell membrane proteins like the receptor tyrosine kinase (RTK) class of receptors, which includes the Erythropoietin-Producing Hepatoma (EPH) receptor family, the ErbB family of receptors like Epidermal Growth Factor Receptors (EGFR), and Vascular Endothelial Growth Factor Receptors (VEGFR), and additionally the production of growth factors such as Transforming Growth Factor α (TGFα), EGF and VEGF (7,8,9). RTKs are activated upon a ligand binding to the extracellular domain of the receptor and subsequent phosphorylation of the intracellular kinase domain leading to downstream signal transduction events; hence production of both the growth factors and their relevant receptors creates an autocrine growth loop in tumor cells. In addition, several adhesion proteins, such as E-cadherin, are also elevated in cancer and play a role in cancer metastasis by activating phosphorylation of Eph receptors, as has been shown for example with the EphA2 receptor (10). Although many events can lead to increased levels or activity of RTKs in tumor cells, the most common

ways that cancer cells modulate the expression or activity level of membrane RTKs are a result of mutations in exon regions of kinase domain that lead to increased receptor activity, mutations in genes that produce tyrosine kinase inhibitors (TKI), or genetic polymorphisms of RTK genes that can regulate protein expression. For example, a variety of point mutations, in-frame deletions, and EGFR polymorphisms (such as CA-SSR1, CA Simple Sequence Repeat 1, a highly polymorphic locus, and two SNPs, Single Nucleotide Polymorphisms, in the promoter region) have been linked to decreased activity of RTK inhibitors and increased EGFR mRNA and protein expression in adenocarcinomas of lung, breast, and head and neck cancers (14, 15). Irregular DNA transcription and overexpression of membrane proteins would normally commit the cell towards apoptosis, or programmed cell death. However, malfunctions of the "caretaker" mechanisms in sensing, repairing DNA, and assuring correct chromosomal segregation are present and thus allow the cancer cells to survive and proliferate. In fact, many cancer cells of the breast, lung and prostate carcinoma, that contain irregular expression of receptor tyrosine kinases, also have been reported to have mutations in either the tumor suppressor protein p53 and BRCA genes that are associated with damaged DNA repair signaling, or mutations in PTEN, an enzyme that dephosphorylates AKT to inhibit cell survival and promote apoptosis (6, 16, 17). Therefore, the loss of "caretaker" mechanisms produces genomic instability and generation of mutant cells with selective advantages, such as expression of membrane receptors that promote proliferation, invasion, and metastasis.

Figure 1. Properties of Cancer Cells and Factors that Promote Metastasis. Adapted from Hanahan D. et al.



Characteristics of Cancer Cells	Examples
Self Sufficiency in Growth Signals	Production of PDGF and TGF α
[redacted]	[redacted]
[redacted]	[redacted]
Limitless Replicative Potential	Mutation in p53 and Telomere Maintenance

Figure 1.

1.4 Receptor Tyrosine Kinases and their role in cancer

A Receptor Tyrosine Kinase is generally composed of an extracellular ligand binding structure, a trans-membrane region, and an intracellular kinase domain responsible for downstream signaling pathways. After successful ligand binding, dimerization with adjacent RTKs is triggered leading to autophosphorylation of tyrosine residues in the kinase domain of each receptor. Downstream signaling pathways activated by RTKs can include Mitogen-Activated Protein Kinases (MAPK) and the PI3K-AKT pathway, which are also known to be involved in cancer progression (18). Recently, molecular therapies that target RTK activity have been developed, with the most well characterized approaches being those that target EGFR. These therapies have included both antibodies and competitive inhibitors to EGFR, such as gefitinib and erlotinib, and have shown promise in increasing survival and reducing metastasis. However, little is known about the largest family of RTKs, the Eph receptors and their association with cancer progression. Given the promise of targeting RTKs as a therapeutic approach in cancer therapy, investigation into the role of Eph receptors in cancer and their potential as a therapeutic target is warranted.

1.5 Erythropoietin Producing Hepatoma (Eph) Receptor

(1.5.1) Background

In 1987, Hirai et al. discovered a new type of tyrosine kinase receptor while working on cancer cell proliferation and oncogenesis using an erythropoietin-producing hepatocellular carcinoma cell line, from which the Eph receptors received their name (19,20). In these studies, the Eph receptor gene was identified; cDNA was extracted, and subsequently characterized by molecular cloning. The Eph proteins were unique compared to other RTKs

because of the presence of a cysteine rich extracellular region. The investigators also linked the expression of Eph receptor to several other human carcinomas suggesting that Eph receptors could play a role in neoplastic tumor formation (19). Since the initial identification of the gene, the Eph family has expanded into two subfamilies of receptors and two subfamilies of ligands in humans. The ligands of Eph receptors are different from other growth factors because they are attached to the cell membrane, unlike ligands of other tyrosine kinase receptors which tend to be secreted proteins. Although Eph receptors have some conserved regions within their genome that provide a 30%-70% structural similarity of the extracellular domain, there are functional differences in ligand binding properties between EphA and EphB subfamilies, which separate them from each other, and other RTKs, in tissue expression, function and their role in cancer progression (22,93).

(1.5.2) Eph Receptors: Classification, structure and expression

Eph receptor tyrosine kinases are the largest family of RTKs with two subfamilies, termed EphA and EphB, based on their interaction with the EphrinA and EphrinB ligands, respectively. There are 16 total Eph receptors identified to date. In humans, there are nine classified EphA receptors ranging from EphA1-8 and EphA10, five EphB receptors ranging from EphB1-4 and EphB6, five EphrinA ligands and three EphrinB members (Figure 2) (23). EphB5 and EphA9 have only been found in organs of chickens and are considered to play a biologically active role primarily in development of chick embryos (24,29). Before the existence of their classification and coherent nomenclature, each receptor and ligand was given a unique name upon its discovery which was sometimes redundant and confusing. Therefore, in 1997, an Eph protein nomenclature committee was established to develop a unified system for naming these receptors based on their homology in gene sequence

conservation, ligand interaction, and their date of publication (21). The work performed herein will concentrate on EphB4 receptor, although an introduction on the family of Eph receptors is also provided.

The structure of the Eph family of receptors is comparable to other RTKs with respect to having a single transmembrane spanning domain, an extracellular ligand-binding region and a cytoplasmic kinase domain that is inherently phosphorylated upon ligand binding and dimerization. The only exception to this being the EphB6 receptor that lacks all catalytic activity of the intracellular tyrosine kinase domain due to several genetic deletions and substitutions (22), that results in six amino acids in the conserved kinase domain being substituted with other amino acid residues that impair enzymatic activity (lysine in the ATP-binding site and aspartic acid in the phosphotransfer site are substituted with glutamine and serine, respectively) (27,28). The main difference between the Eph receptors and other RTKs is that both EphA and EphB receptors have extracellular components comprised of an immunoglobulin ligand-binding domain at the amino terminus, followed by a cysteine rich region and two groups of fibronectin type III repeats near the transmembrane segment (Figure 3) (24-26). The intracellular region of Eph receptors include a juxtamembrane region, followed by a conserved tyrosine kinase domain, a carboxy-terminal Sterile Alpha-Motif (SAM) and a less conserved PDZ binding motif (PSD-95 post-synaptic density protein, Discs large, Zona occludens tight junction proteins) (22,24-26).

A precise regulation of transcription and translation of Eph and Ephrin genes determine the pattern and quantity of receptor and ligand expression in various tissues and organs. The Eph and Ephrin genes are regulated by a variety of transcription factors and homeobox genes. For example, Vax2 is a homeobox gene that has been shown to regulate expression of

EphB2 and EphrinB2 in mouse retina (35). Eph receptor expression may also be modulated by gene amplification mechanisms. It is interesting to note that the EphB4 gene locus is on 7q22 located on chromosome 7q (80), amplification of which is a common occurrence in androgen-independent prostate cancer (81). The mechanisms that regulate transcription of EphB receptors in cancer are not clearly understood, however, tumor suppressor genes such as p53 and PTEN have been shown to lower expression of EphB4 in PC3 prostate carcinoma cells (3).

(1.5.3) Differences between EphA and EphB receptors:

EphA and EphB receptors are mainly differentiated by their selectivity for ephrin ligand binding and conformation of their ligand-binding domains. EphA receptors preferentially bind with EphrinA ligands while EphB receptors preferentially bind to EphrinB ligands. X-ray crystallography of the ligand-receptor complex shows a high affinity of interaction whereby an ephrin protein loop is selectively inserted into an Eph receptor cleft via an induced-fit mechanism (20,31). Normally there is a strict binding of EphA receptors by EphrinA ligands and EphB receptors by EphrinB ligands, however, there are a couple of exceptions in complementary binding that have been shown to occur. EphA4 has been shown to bind both EphrinA and EphrinB2 ligands, and EphrinA5 ligand can interact with EphB2 in addition to EphA receptors (22,30). EphB receptors bind multiple EphrinB ligands with similar affinity, however EphB4 is unique as compared to the other EphB receptors as EphB4 is highly selective for binding with EphrinB2 as opposed to other ephrin ligands (25). Other differences between EphA and EphB receptors are found with respect to their ability to recruit different guanine nucleotide exchange factors after receptor activation and phosphorylation of the tyrosine kinase domain. EphA receptors have been shown to activate

Rho GTPases through downstream interactions with Ephexin, a nucleotide exchange factor that binds to the kinase domain (20,32). On the other hand, EphB receptors interact with nucleotide exchange factors Kalirin and Intersectin at their kinase domain activating other family members of Rho GTPases like Rac and Cdc42 (33,34). Due to different recruitment of exchange factors, activation of EphA and EphB receptors are known to influence various behaviors in cells.

1.6 Ephrin ligands: Structure and signaling

Ephrins consist of two subfamilies of ligands that selectively bind to Eph receptors and not other receptor tyrosine kinases. As described previously, A-type ephrins can interact with EphA receptors and B-type ephrins bind with EphB receptors. In humans, the five EphrinA ligands are classified as EphrinA1-5 and three EphrinB ligands are described as EphrinB1-3. The first ephrin ligand was identified in 1994 as EphrinA1 which was found to bind and activate EphA2 receptor (37,38). The characterization of ephrin ligands came about following isolation of an extracellular domain of EphA2 that was used as an affinity reagent for receptor chromatography by fractionating the supernatant of selected cell lines expressing EphrinA1 (37). Both EphrinA and EphrinB contain a conserved 20kDa extracellular receptor-binding domain at the N-terminus (39). Differences in protein structure and cellular expression also occur between EphrinA and EphrinB ligands. For instance, EphrinA ligands are anchored to the cell membrane by glycosylphosphatidylinositol (GPI) linkage, whereas EphrinB ligands are transmembrane proteins that are attached to the cell surface by a hydrophobic transmembrane region and a short cytoplasmic domain. In addition, B-type ephrins also possess conserved tyrosine phosphorylation sites in their cytoplasmic region

with a PDZ binding motif at the carboxy-terminus (39). Since both ephrin ligands and Eph receptors are attached to cell membrane, their interaction occurs only at sites of cell-cell contact.

Investigators have suggested signs of what is referred to as "reverse signaling", whereby following binding of EphB receptors, the EphrinB ligand undergoes phosphorylation of tyrosine residues in its cytoplasmic region by an as yet unidentified mechanism (40,41). This feature appears to be unique to EphrinB ligands and does not appear to occur in EphrinA ligands. Interestingly, both A and B-type ephrin ligands are also known to be cleaved from the cell membrane and internalized along with the Eph receptor in a phagocytosis like manner (86). The vesicles containing the Eph/ephrin complexes appear to enter the endosomal system and may perpetuate the signaling (86). It is suggested that the complexes are later degraded (86). Evidence for phosphorylation of three conserved tyrosines, perhaps by Src family kinases, and binding of the adaptor protein Grb4 to the intracellular domain of EphrinB1, suggests that EphrinB "reverse signaling" can play a role in modifying cell morphology through rearrangement of the actin cytoskeleton (20). For instance, activation of EphrinB1 by soluble EphB2 receptor-Fc recombinant protein has been shown to increase FAK catalytic activity, resulting in the redistribution of the FAK binding protein paxillin, loosening of focal adhesions, cell rounding, and disassembly of F-actin containing stress fibers (20). Conventionally, 'forward' signaling is the term for a response within the cell expressing EphB receptor while 'reverse' signaling is for the cell expressing EphrinB ligand.

Figure 2. Eph A and Eph B Receptors and EphrinA and EphrinB Ligands.

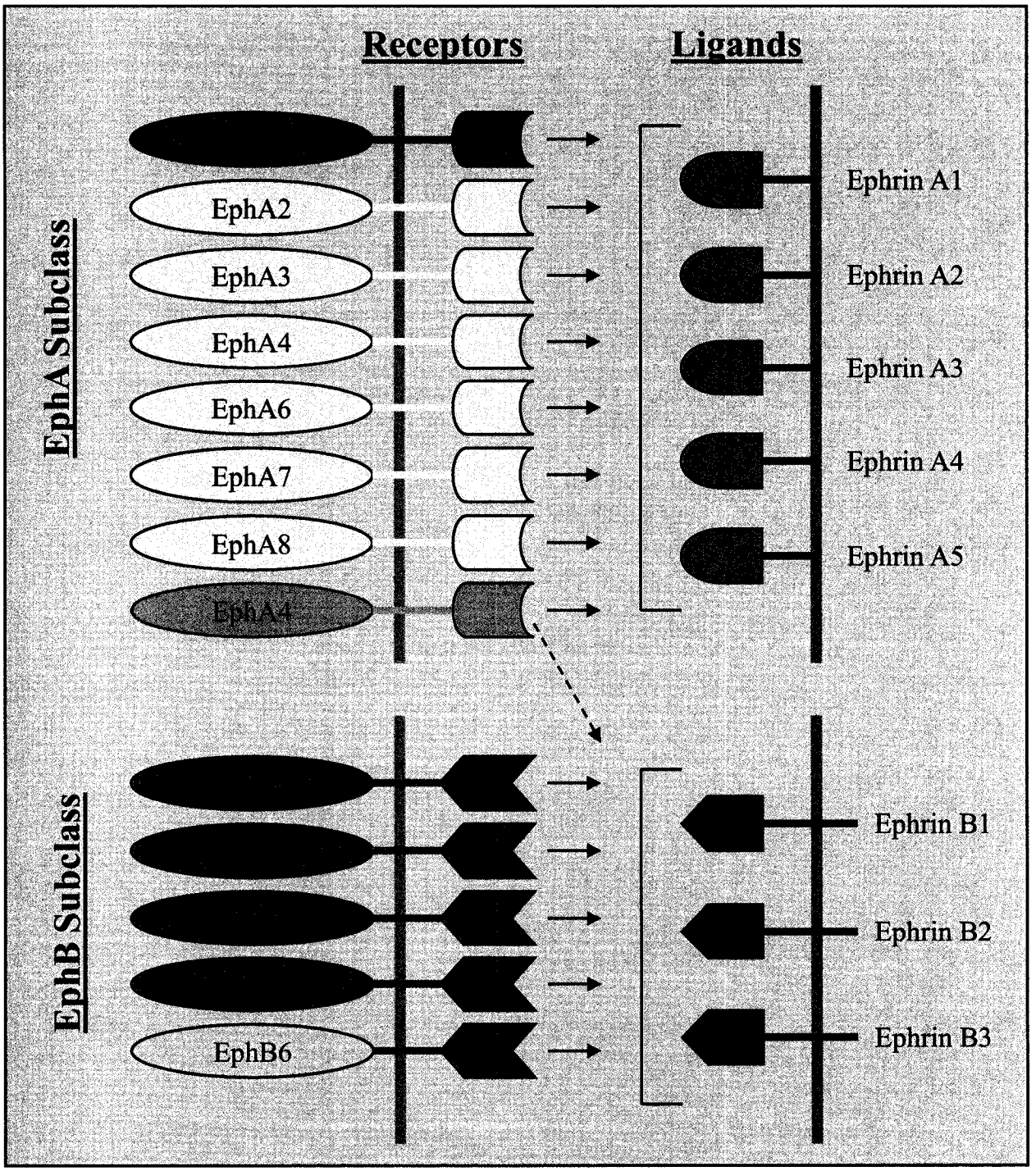

Receptors
Ligands
EphA Subclass
EphA2
EphA3
EphA4
EphA6
EphA7
EphA8
EphA4
Ephrin A1
Ephrin A2
Ephrin A3
Ephrin A4
Ephrin A5
EphB Subclass
EphB6
Ephrin B1
Ephrin B2
Ephrin B3

Figure 2.

Figure 3. Structural Domains of Eph receptors and Ephrin Ligands.

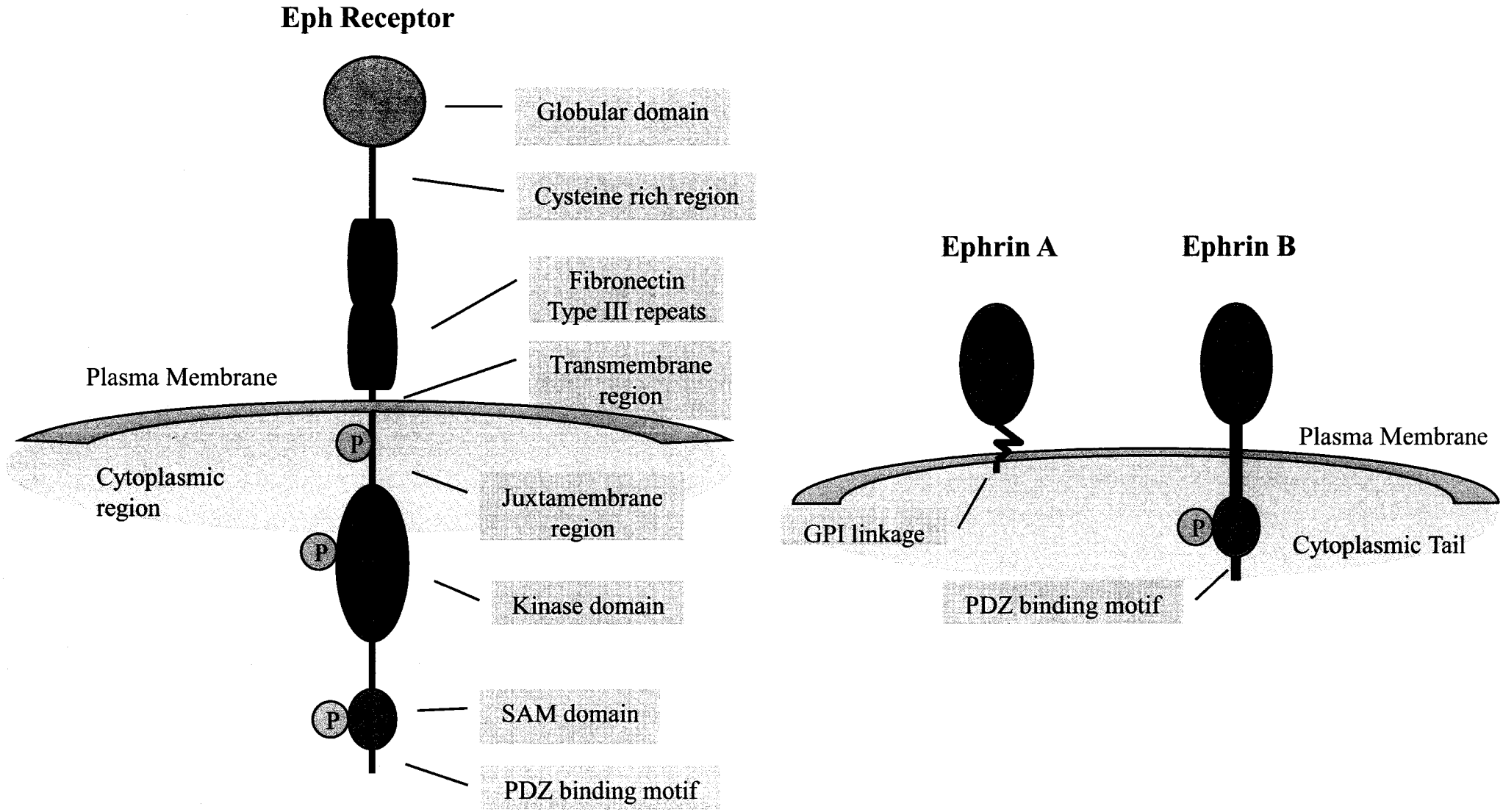

Eph Receptor
Globular domain
Cysteine rich region
Fibronectin
Type III repeats
Transmembrane
region
Plasma Membrane
P
Cytoplasmic
region
Juxtamembrane
region
P
Kinase domain
P
SAM domain
PDZ binding motif
Ephrin A
Ephrin B
Plasma Membrane
GPI linkage
P
Cytoplasmic Tail
PDZ binding motif

Figure 3.

1.7 Eph/Ephrin interaction, phosphorylation, and cellular functions:

Eph receptors and ephrins have important functions in neural development, angiogenesis, axon growth cone, bone homeostasis, organ development and, recently, tumorigenesis. In contrast to other receptor tyrosine kinases, activation and phosphorylation of Eph receptors does not appear to lead to proliferative responses, but rather rearrangements in cell cytoskeleton and subsequent cell movement. A role for Eph receptors and ephrins in cell movement has been previously characterized whereby interactions mediate repulsion in axons while also promoting attraction and adhesion in neurons and other cell types (20,28,30). A knockout of EphrinB1 protein in the mouse embryo results in perinatal lethality occurring from a number of abnormal phenotypes such as defects in neural crest cell-derived tissues and irregular skeletal patterning (42). Spine morphogenesis is also controlled by Eph receptors and ephrins through actin polymerization and reorganization of dendritic structures with help from RhoGTPases, such as Cdc42, which have been shown to be simultaneously activated upon phosphorylation of EphB2 receptor in mouse hippocampal neurons (34). EphB4/ephrinB2 interaction also plays important roles in angiogenic remodeling of blood vessels, and the development of the heart and lymphatic vessels (48). EphrinB2 knockout mice lacked interdigitation of arterial vessels in formation of primary capillary plexus (48,43). Knockout of EphA3 protein in atrioventricular endocardial cushion cells of mice shows reduced migration and decreased formation of stress fibers when compared to wildtype cells, suggesting the requirement of protein expression for cell function in system (44). Addition of supposed mixture of EphA ligands to EphA5 expressing retinal ganglion axons induced retraction and collapse of migrating growth cones (45). These examples

highlight a few ways how Eph receptors and ephrins primarily function in regulating cell motility, changes in cell morphology, and spatial arrangement of cells within body systems.

Initiation of modulation of cellular functions by Eph receptors begins at the site of a cell-cell contact with an adjacent cell expressing the ephrin ligand. Upon receptor recognition of the appropriate ligand, studies show that dimer pairs form 2:2 heterotetramers, leading to oligomers and, eventually, clusters at the cell surface (31). For example, crystallography studies of 2:2 tetramers of EphB2 and EphrinB2 show that this complex forms a ring like structure with each receptor associated with 2 ligands and each ligand with 2 receptors (31). The cytoplasmic intracellular domain of Eph receptors contain multiple phosphorylation sites, located in the juxtamembrane region and kinase domain (20). Crystal structure of an inactive EphB2 shows an unphosphorylated juxtamembrane region that is tightly attached to the N-terminal lobe of the kinase domain forming a loop structure that inhibits kinase activation (49). After the Eph receptor is firmly attached to the ligand, phosphorylation of the juxtamembrane tyrosine residues relaxes constructional constraints on the intracellular region opening the active site on the kinase domain (49). With the juxtamembrane region phosphorylated, this can also establish binding sites for SH2 domain proteins such as cytoplasmic tyrosine kinases Fyn, Src, Nck and Crk (50). Autophosphorylation of three tyrosine residues in the kinase domain provides further stabilization of this intracellular domain and creates more sites for possible binding of SH2 domain proteins in addition to PDZ binding proteins at the C-terminal PDZ motif. As a result of ligand binding, phosphorylation and association with nucleotide exchange factors, such as Kalirin, Intersectin, and Ephexin, Eph receptors can now modulate important cellular functions

initiating a cascade of downstream cell pathways that may directly or indirectly play an important role in rearrangement of actin filaments, cell movement, and migration (20).

Interestingly, cancer cells also possess many characteristics, such as invasion and metastasis, which occur as a result of improper regulation of cell motility, a function that appears to be one of the primary responsibilities the Eph receptors manage. Furthermore, recent evidence has suggested that Eph receptors are implicated in prostate, breast, and lung cancers playing roles in angiogenesis of tumor vasculature, tumorigenesis, and cell attachment, shape and motility. However, conflicting reports of their expression and activity have been the primary hurdles in resolving their mystery. Therefore, it is important to review and study the role of Eph receptors in cancer.

1.8 Eph Receptors in cancer

(1.8.1) Breast Cancer

In a normal human adult, location of EphB receptors on epithelial cells has been suggested to play a role in differentiation or maintenance of secretory epithelium of mammary and bronchial tissues (51). For example, immunohistochemistry of normal breast tissues has shown moderate to high expression of EphB2 and moderate to weak expression of EphB4 in the areas of the cell membrane, cytoplasm and nucleus of secretory epithelial cells (52). However, a large number of breast carcinoma cell lines, including MCF-7, T47-D, and MDA-MB-231, along with breast tumor tissue have been reported to show high expression of EphB2 and EphB4 receptors (51-53). For example, in breast carcinoma tissues, forty-eight of ninety-four tumors (51%) examined by immunohistochemistry showed strong expression of EphB2, while EphB4 had a strong expression in 33 (35%) and moderate expression in 28

(30%) of the 94 tumors tested. (52). Furthermore, high expression of EphB2 was associated with poor overall survival of breast cancer patients and EphB4 expression was positively correlated with increased aggressiveness of breast cancer (52). These studies suggest that EphB2 and EphB4 might play a role in promoting tumorigenesis in breast cancer.

(1.8.2) Lung Cancer

Expression of certain Eph receptors in lung cancers has been suggested as a diagnostic marker and a target for antibodies or RTK inhibitors as a possible method for treatment. One study determined that mRNA expression of EphA3 was upregulated 26-fold in lung tumors compared to normal tissue as determined by quantitative PCR (23). In other studies, EphA2 was found to only be present in lung tumors and not in normal adjacent lung tissue (55). The levels of EphA2 tested in tumor samples from lung cancer patients also correlate with the clinical outcome of lung cancer. Expression of EphA2 increased proportionally with increased stages of Non-Small Cell Lung Cancer (NSCLC) starting from Stage I to Stage IV (56). Higher levels of EphA2 were also able to predict decreased survival rates and increased chances of tumor recurrence (56). A correlation also existed whereby patients with the highest average intensity of EphA2 in clinical specimens also tended to develop brain metastases (56). In addition to NSCLC, Small Cell Lung Cancer (SCLC) cell lines were also found to express EphB4 and EphB2 along with other EphB receptors, but the expression of these receptors varied from high to low depending on the receptor type and cell line. All of the SCLC patient tumor samples that were analyzed showed moderate levels of EphB4 transcript, and the tumor specimen expressed higher levels of EphB4 than their corresponding derivative cell lines (57).

(1.8.3) Prostate Cancer

Western blot analyses of three prostate cancer cell lines PC-3, LNCaP and Du-145 show that the relative EphA2 levels increase proportionally according to the metastatic potential of each cell line (58). EphA2 levels were highly overexpressed in PC-3, intermediately expressed in Du-145, and undetectable in LNCaP (58). EphB4 is expressed in higher levels in prostate carcinoma cell lines compared to benign prostatic hyperplasia, and, in general, has higher mRNA expression than other subfamily of EphB receptors (3,82). Immunofluorescent staining of prostate carcinoma cell lines has shown membrane and cytoplasmic localization of EphB4 suggesting that activation of EphB4 may cause endocytosis of the receptor-ligand complex in cells (59). Immunohistochemical staining of clinical samples for EphB4 has shown overexpression of the receptor in tumors as compared to normal prostate tissue (59). Furthermore, the up-regulation of EphB4 protein expression in PC-3 cells has been linked to several tumor suppressor genes and growth factors. For example, Gill et al. have performed a study showing that re-introducing wildtype p53 or PTEN in PC-3 cells, which contain mutations in p53 and PTEN tumor suppressor genes, resulted in a reduction of the expression of EphB4 by ~75% (3). EphB4 expression has also been linked to growth factor receptor-ligand interactions, whereby upon use of inhibitors of EGFR and insulin-like growth factor-IR (IGF-IR), EphB4 expression was reduced by ~50% in PC3 cells (3).

(1.8.4) Paradox of EphB Receptors in Cancer

Overexpression of EphB4 has been observed in breast cancer and its upregulation has been directly associated with increased metastasis (52,53). New evidence has suggested that Eph receptor activation could possibly suppress breast cancer tumorigenicity (60). Pasquale

et al. have shown that MDA-MB-435 and MCF-7 breast cancer cell lines have higher expression of EphB4 compared to non-transformed MCF-10A human breast epithelial cells. In return, these breast cancer cell lines possess significantly lower levels of EphrinB2 compared to normal breast epithelium (60). Interestingly, upon intratumoral treatment with EphrinB2-Fc in xenograft mice models of MDA-MB-435-derived tumor growth, the volume of the EphrinB2 treated tumors was decreased and contained reduced tumor vasculature. These data show that EphB4 may play a tumor suppressor role in breast cancer by decreasing motility and invasion (60). A separate study conducted on Du-145 prostate cancer cells revealed truncating mutations in EphB2 were responsible for a loss of a functional kinase domain. In-vitro transfections of wild-type EphB2 in Du-145 cells decreased proliferation and colony formation as much as transfecting with p53 gene (61). These are contradictory results compared to the common understanding from earlier studies which widely suggested that Eph receptors are directly linked to tumor promoter activities. Therefore, to further define the role of EphB4 in tumorigenesis, more work is needed to completely understand the role of EphB4 in cancer.

1.9 Understanding Migration and Invasion

Cell migration and invasion play an important role in cancer metastasis and inhibition of cell motility has been a recent target in cancer therapy. Cellular pathways of the proteins which regulate motility are common in both non-neoplastic and cancer cells (62). Movement in cells occurs by way of remodeling and reorganizing of the actin cytoskeleton to produce the force necessary to move cells. Actin reorganization is regulated by members of the family of Rho GTPases, such as Cdc42, Rac and the Rho proteins (Figure 4A, Materials and

Methods chapter). These small GTPases are activated following exchange in binding of Guanosine Diphosphate, GDP, to Guanosine Triphosphate, GTP, downstream of activation of kinase receptors.

Directed cell migration is important in many physiological processes including development, wound-healing, immunity, and angiogenesis. However, in cancer, extensive production of chemotactic factors, such as Platelet-Derived Growth Factor (PDGF) or EGF, can activate cell-surface receptors and induce various intracellular responses that lead to metastasis (62). Directed cell migration on 2-D surfaces involves a coordination of the following four basic steps in cell movement: membrane protrusion at the leading edge, attachment of protrusion to substrate, translocation of cell body, and retraction of trailing edge (62). Rho family of GTPases are mainly involved in reorganization of the actin cytoskeleton. Rho, Cdc42, and Rac are responsible for formation of stress fibers, filopodia, and lamellipodia respectively, with Cdc42 and Rac acting mainly in the front of the cell to control direction and promote protrusion, while Rho stimulates actin-myosin contraction in cell body (63). Rho also mediates contractility at the trailing edge of the cell through regulation of downstream molecules such as ROCK/Rho kinase (Figure 4A) (63).

Cancer cells can migrate both in a collective and an independent fashion. Collective migration can be observed in epithelial tumors where cell-cell junctions are present and cells move together in a sheet-like structure over a surface (62). This is often observed in wound repair by epithelial cells or in angiogenesis by endothelial cells. Independent migration occurs from mainly a reduction in function of cadherins, cell-cell junction proteins, and individual epithelial cancer cells separate from one another allowing single cells to move independently (62). This transition from collective to independent migration is called

epithelial-mesenchymal transition and is considered an important indicator of cancer progression (62). In a 3-D model growth culture, cancer cells migrate by elongating their protrusions and degrading the extracellular matrix (ECM) by producing matrix metalloproteinases (MMPs) at the leading edge of the cell (62). These proteases are responsible for the breakdown of proteins, a process called proteolysis, that make-up the ECM. Cancer cells then switch to a rounded-morphology where they move in an integrin-independent manner, decrease their attachment to the surface, change their shape and then migrate into the gaps created in the ECM (62). While in this rounded-morphology, activated Rho signals to downstream effectors such as Rho-kinase (ROCK) that then phosphorylates myosin II light chain (MLC2) which in turn regulates actin-myosin contraction (64). In elongation, active Cdc42 signals myotonic dystrophy kinase related Cdc42 binding kinase (MRCK) to phosphorylate MLC2 (64).

1.10 Short Interference RNA (siRNA)

Since the discovery of RNA interference in 1998, researchers have been testing its benefits in cancer therapy and many other diseases to reduce irregular expression of cancer related genes. The Tuschl group first published their paper on the ability of double stranded RNA molecules to silence gene expression in mammalian cells (68). The short 25 nucleotide long double-stranded RNA molecules with 2 nucleotide overhangs at the 3'end are inserted into mammalian cells by a variety of transfection methods to deplete the expression of a complementary mRNA from a gene of interest (69). After insertion of the double-stranded Short-Interfering RNA (siRNA) in the cytoplasm of a cell, the sense and antisense strands become separated in the cytoplasm. There are two competing models for reducing gene

expression: Random Degradative PCR model and Endonucleolytic Cleavage model (70). The Random Degradative PCR model shows that siRNA binds with complementary target mRNA and are then extended by nucleotide addition to make dsRNA with help from RNA dependent RNA polymerases (RdRP) (70,71). The dsRNA is later fragmented by the Dicer nuclease thus eliminating the target mRNA and generating new siRNA templates (70,71). This process continues several times in post-transcription within the cytoplasm thus effectively lowering gene specific mRNA levels and subsequent protein expression. The Endonucleolytic Cleavage model determines that siRNA act as a guide in cleaving mRNA in association with formation of a complex called RISC (70). The model shows that single siRNA sequences combine with proteins to form the ribonucleoprotein complex RISC (RNA-induced silencing complex). After formation, the RISC complex recognizes complementary sequences on mRNA and cleaves target mRNA with help from endonucleases (70).

In this study, we will take advantage of the novel siRNA technique to target reduction in EphB4 expression in various prostate carcinoma cell lines, namely PC3, LNCaP and Du-145. Following elucidation of the optimal methods for siRNA interference in these cells and achievement of a significant reduction in EphB4 expression, the role of EphB4 in the proliferation, migration and invasion of prostate carcinoma cell lines will be studied.

HYPOTHESIS

EphB4 activity plays an important function in regulating cell proliferation, migration, and invasion in prostate cancer by activation of downstream signaling proteins.

APPROACH

Recent work on Eph receptor tyrosine kinases, in general, has shown conflicting data on level of expression and activity of such receptors in cancer progression and metastasis of tumor cells. We hope to understand and add supporting evidence that would clarify the role of EphB4, an EphB receptor expressed in all prostate carcinoma cell lines, in activating downstream pathways and regulating proliferation and migration. Prostate carcinoma cell lines will be subjected to siRNA treatment to reduce protein levels of EphB4 and, conversely, stimulated with EphrinB2-Fc to activate the receptor. The objectives of this project were to investigate the potential impact of EphB4 receptor in prostate cancer progression by studying proliferation, migration, and invasion in PC3, LNCaP and Du-145 cells by addressing the following aims;

1) To determine the relative expression of EphB4 receptor and its ligand EphrinB2 amongst prostate carcinoma cell lines as compared to normal prostate epithelium.

2) To examine the kinetics and method of EphB4 activation following stimulation with recombinant EphrinB2-Fc.

3) To determine whether EphB4 protein expression can be specifically and effectively reduced in prostate carcinoma cell lines using siRNA approaches, and to assess the effective concentration of siRNA and duration of effective suppression of EphB4 using this approach.

4) To examine whether proliferation is affected in PC3, LNCaP, and Du-145 cells after reducing EphB4 or alternatively, activating EphB4 with EphrinB2-Fc and whether this modulation affects cell survival through activation of AKT.

5) To determine whether migration of prostate cancer cells is modulated by EphB4 expression or activity and to identify the putative downstream pathways that regulate the cytoskeleton in response to EphB4.

6) To examine whether prostate cancer cell invasion is modulated by EphB4 expression or activity.

2. MATERIALS AND METHODS

2.1 Cell Culture

Human Prostate Carcinoma Cell lines (PC3, LNCaP, Du-145) were obtained from ATCC Bioresource Center (Manassas, VA). Cells were maintained at 37°C and 5% CO_2 in plastic tissue culture dishes (BD Falcon, Mississauga, ON). PC3 and Du145 were propagated in Dulbecco's Modified Eagles Medium (DMEM)/High Glucose (HyClone Cat. SH30207.01, Logan, Utah) supplemented with heat-inactivated Fetal Bovine Serum (FBS, Invitrogen, Burlington, ON) to a final concentration of 10%. LNCaP were propagated in RPMI-1640 medium (HyClone Cat. SH30255.01, Logan, Utah) supplemented with heat-inactivated 10% FBS. Normal human Prostate Epithelial Cells (PrEC) (Lonza, Basel, Switzerland) were maintained in Prostate Epithelial Growth Medium (PrEGM, Lonza Bioscience, CC-3165, Basel, Switzerland), supplemented with nutrients, growth factors, and cytokines (BPE, hydrocortisone, hEGF, epinephrine, insulin, triiodothyronine, transferrin, gentamycin/amphotericin-B, retinoic acid) contained in provided BulletKit (Lonza Bioscience, CC-4177, Basel, Switzerland), and were cultured at 37°C in 5% CO_2. Cells were split in sterile conditions every 2-3 days as required by washing 2x with Phosphate Buffered Saline (PBS, NaCl 9g/L, sodium phosphate diabasic 0.8g/L, potassium phosphate monobasic 0.14g/L, HyClone SH30256.01, Logan, Utah), trypsinizing with 2ml of 37°C Trypsin-EDTA solution (0.25% trypsin, 1mM EDTA, Invitrogen, Burlington, ON) for ~5min to dislodge cells from surface of the plate. Trypsin was then neutralized by addition of medium containing serum, and cells were collected in 50ml conical tubes (BD Falcon, Mississauga, ON), centrifuged 1200xg 4°C for 3min in a table top centrifuge, resuspended in fresh medium containing serum, and subsequently plated into additional plastic culture dishes.

2.2 Protein Extraction

Prostate Carcinoma cell lines and Prostate Epithelial cells were washed 2x with Phosphate Buffered Saline in tissue culture plates. PBS was aspirated and cold protein lysis buffer (25mM Tris, pH 7.4, 150mM NaCl, 1% Triton X-100, 5mM EDTA) containing protease inhibitors (1mM sodium orthovanadate, 10mM sodium fluoride, 2 ug/ml aprotinin, and 10mM leupeptin) (Sigma, Oakville, ON) was spread on sub-confluent petri dishes. Cells were scraped with a rubber policeman and protein lysates collected using a Pipetman-200 pipette (Gilson, Middleton, WI) into sterile 1.5ml microcentrifuge tubes (Fisher Scientific, Ottawa, ON). Protein lysates were sonicated (Misonix 3000, Mandel Scientific, Guelph, ON) for ~20sec and centrifuged on maximum speed ~18,000xg at 4°C for 30 mins. Supernatant was separated using a pipette into separate microcentrifuge and stored at -20°C.

2.3 Western Blotting

Protein concentration was measured using the Bradford Assay. In a 1.5ml microcentrifuge tube, 5ul of total protein lysate was diluted in 795ul of sterile ddH_2O and mixed with 200ul of Coomassie Brilliant Blue G-250 dye (Bio-Rad Cat 500-0006, Hercules, CA). Tubes were shaken and contents poured in disposable cuvettes (Bio-Rad Cat. 223-9955). Absorbance was measured using a spectrophotometer (Biomate 3, Thermo Electron/Fisher Scientific, Ottawa, ON) at 595nm compared with blank controls containing only the lysis buffer and dye. Subsequently, 20-40ug of total protein was resolved on 8% SDS-polyacrylamide gel (2.5ml 1.5M Tris-HCl, pH 8.8, 100ul 10%SDS, 2.5ml 30% stock acrylamide mix, 100ul 10% ammonium persulfate, 5ul TEMED) (Bio-Rad, Hercules, CA) and 4% stacking gel (2.5ml 0.5M Tris-HCl, pH 6.8, 100ul 10%SDS, 1.33ml 30% stock

acrylamide mix, 100ul 10% ammonium persulfate, 10ul TEMED, 6.1ml ddH_2O) (Bio-Rad, Hercules, CA) at 150 Volts for ~75mins with 1xRunning buffer (stock 10x, 30g Tris base, 144.0g glycine, 10.0g SDS, 1 L ddH_2O) (Fisher Scientific, Ottawa, ON). Gel was electroblotted at 100 Volts for 60 mins onto Hybond C nitrocellulose membrane (Amersham Pharmacia Biotech, Quebec, Canada) using 1xTransfer buffer (stock 10x, 30.29g Tris base, 144.15g glycine, 1 L ddH_2O) (Fisher Scientific, Ottawa, ON) supplemented with 20% Methanol (VWR, Brampton, ON) using Bio-Rad Mini-Protean II Electrophoresis Cell kit system (Bio-Rad, Hercules, CA). Nitrocellulose membranes were washed with washing buffer TBST (10mM Tris-HCl, pH 8.0, 150mM NaCl, 0.05% Tween-20) (Fisher Scientific, Ottawa, ON) and non-specific binding was reduced by incubating membranes with 5% skim milk in TBST for 1 hr at room temperature on a vertical shaker. The respective primary antibodies were prepared as indicated in antibody protocols and applied to the membrane in a plastic boat overnight at 4°C on a vertical shaker. The membranes were then washed 3x, for 5 mins each time, with TBST prior to incubation with horseradish peroxidase (HRP) conjugated goat anti-mouse secondary antibody (Calbiochem, Cat # DC02L, La Jolla, CA) diluted 1:2000 in 5%Milk/TBST for 1hr at room temperature on a vertical shaker. Nitrocellulose membrane was washed 3x for 5 mins each time, and the proteins were visualized by the HRP-chemiluminescent reaction using the Immobilin Western ECL kit (Millipore, Billerica, Mass). Blots were exposed to X-ray film (Kodak, Rochester, NY) or, alternatively, using the GeneGnome Bio Imaging system for chemiluminescence (Frederick, MD). Equal loading and transfer of proteins in each lane was determined by blotting of membranes for B-actin protein. Nitrocellulose membranes were washed with TBST and stripped using Restore western blot stripping buffer (Thermo Scientific, Rockford, IL) for 15

mins while shaking at room temperature. Membranes were washed again with TBST, blocked with 5%Milk/TBST for 1hr at room temperature, and incubated with primary monoclonal B-actin antibody (Sigma, Cat. A5316, St. Louis, Missouri) diluted 1:5000 in 5%Milk/TBST at room temperature for 1 hr. Membranes were washed 3x with TBST, incubated with goat anti-mouse secondary antibody, and visualized using the HRP detection method.

2.5 Antibodies and Reagents

Monoclonal anti-human EphB4 primary antibody (clone #265) was obtained from Vasgene Therapeutics (Los Angeles, CA), thanks to Dr. V.Krasnoperov, and used at a concentration of 0.3ug/ml. For western blotting, antibody conditions included blocking membrane with 5%Milk/TBST, incubation with EphB4 Ab diluted in 5%Milk/TBS 0.05%Tween-20 overnight at 4°C, and using goat anti-mouse secondary antibody. Anti-mouse Ephrin-B2 antibody (R&D Systems, Minneapolis, MN) was used at concentration of 0.2ug/ml diluted in 5%Milk/TBST overnight at 4°C and detected with secondary sheep anti-goat antibody (Sigma, A 9452, St. Louis, Missouri). For immunoprecipitation, anti-human EphB4 antibody (Vasgene Therapeutics, Los Angeles, CA) clone #131 was used at the concentration indicated below. Mouse anti-phosphotyrosine Ab (P-Tyr-100) was obtained from Cell Signaling (Cat. # 9411, Boston, MA). Monoclonal anti-human Rac antibody was obtained from BD Biosciences (Cat #610650, Franklin Lakes, NJ). Rabbit polyclonal anti-human Cdc-42 antibody was obtained from Santa Cruz Biotechnologies (Cat #sc-87, Santa Cruz, CA). Purified human IgG was obtained from Sigma (Prod # I4506, St. Louis, Missouri). Recombinant mouse EphrinB2-Fc and mouse EphB4-Fc homodimeric proteins

were obtained from R&D Systems (Cat #496-EB and #446-B4 respectively, Minneapolis, MN). On-Target® Specificity Enhanced 2'-hydroxyl, annealed and desalted siRNA duplex were obtained from Dharmacon (Lafayette, CO) with the following sequences: EphB4 siRNA #1 sense strand 5'-P GUACUAAGGUCUACAUCGA UU (42% GC), antisense strand 5'-P UCGAUG UAGACCUUAGUAC UU – EphB4 siRNA #2 sense strand 5'-P UGGGAAGAUA CGAAGAAAG UU (42% GC), antisense strand 5'-P CUUUCUUCGUAUCUUCCCA UU – siCONTROL Non-Targeting siRNA #1 5'-UAGCGACUAAACACAUCAA-3' – siCONTROL Nontargeting siRNA #2 5'-UAAGGCUAUGAAGAGAUAC-3' – GFP targeting control sense strand AGAACGGAAUCAAGGUUAAUU, antisense strand 5'-P UUAACCUUGAUUCCGUUC UUU.

2.4 Tyrosine Kinase Assay and Immunoprecipitation

To determine phosphorylation of kinase domain of EphB4 receptor, PC3 cell lines were compared by stimulating with recombinant EphrinB2-Fc (R&D Systems, Minneapolis, MN) or IgG control (Sigma, St Louis, Missouri). A sub-confluent 10cm dish of prostate cancer cell lines were serum starved with DMEM/0.5%FBS for 4 hrs in 37°C incubator. Cells were washed 2x with phosphate buffered saline solution. 3ug/ml EphrinB2-Fc and 3ug/ml IgG control were mixed in separate 5ml DMEM/10%FBS media and applied on top of monolayer. Cells were replaced in incubator at 37°C and 5% CO_2. At designated time intervals from 0hrs to 24hrs, cells were lysed using cold lysis buffer (25mM Tris, pH 7.4, 150mM NaCl, 1% Triton X-100, 5mM EDTA) containing protease inhibitors (1mM sodium orthovanadate, 10mM sodium fluoride, 2 ug/ml aprotinin, and 10mM leupeptin) (Sigma,

Oakville, ON) at 4°C and protein lysates were collected using a rubber policeman. Protein was extracted using the protocol outlined earlier in section 2.2. For immunoprecipitation, 400ug of total protein as measured using Bradford Assay, as described in section 2.3, and transferred to a separate centrifuge tube. Protein lysate was kept under ice as much as possible throughout the whole procedure to prevent denaturation. Protein lysate was diluted in 500ul of lysis buffer, incubated with 2ug/ml EphB4 antibody (Vasgene, Clone #131, Los Angeles, CA) and left to rotate overnight at 4°C. Gammabind G-Sepharose beads (GE HealthCare, Baie d'Urfe, Quebec) were washed 3x with lysis buffer including fresh protease inhibitors in a new centrifuge tube. Beads were spun in centrifuge for 30 sec at 4°C ~1500xg. Supernatant was extracted above bed of sepharose. Beads were then diluted in lysis buffer with fresh protease inhibitors to reach a final concentration 50% beads and slurry mixture. Beads were mixed thoroughly by pipetting. Subsequently, 50ul of Protein G Sepharose slurry (=25ul of bed volume) was added to each centrifuge tube containing protein lysate and the EphB4 antibody mixture. Tubes were then rotated at 4°C for 2 hrs. Protein G Sepharose beads were centrifuged for 5 min at 4°C ~4000xg in table-top centrifuge. Supernatant was removed and kept for analysis of antigen-antibody pull-down efficiency using western blotting. Beads were washed 2x with 200ul of cold lysis buffer, centrifuging and extracting the supernatant each time. Following washes, 25ul of 5x Sample buffer (50mM Tris-HCl pH 6.8, 10% glycerol, 2%SDS, 2% β-mercaptoethanol, 1% Bromophenol blue) diluted 4:1 with lysis buffer was added to beads which were then boiled for 5min to release complexes from Protein G-Sepharose. Microcentrifuge tubes were centrifuged for 30 sec at room temperature to collect the beads. A 20ul aliquot of supernatant was then loaded from each tube for SDS-PAGE and western blotting analysis. Following transfer, membranes were blocked using 5%

Milk/TBST at room temperature for 1 hr, washed 3x for 5min using TBST 1% Tween, and then probed with anti-phosphotyrosine antibody (P-Tyr-100, Cell Signaling, Boston MA) diluted 1:2000 in 5% BSA overnight at 4°C. Next day membranes were washed 5x for 5min using TBS 1% Tween and probed with anti-mouse secondary antibody diluted in 5% Milk/TBST at room temperature for 1 hr. Blots were washed 3x for 5 mins with TBS 1%Tween and visualized by chemiluminescence using the ECL kit (Millipore, Billerica, Mass). To determine equal protein loading in lanes, blots were stripped using stripping buffer, blocked with 5% Milk/TBST for 1 hr at room temperature, and reprobed with EphB4 primary antibody (Vasgene, Los Angeles, CA) at 4°C in 5%Milk/TBST overnight. Next day, blots were washed and reprobed with anti-mouse secondary antibody for 1hr at room temperature and visualized using ECL. A "no antibody" negative control and an EphB4-Fc positive control were used to ensure the specificity of the immunoprecipitation technique. In a "no antibody" negative control, the EphB4 antibody was not mixed with the cell lysate. For the positive control, the cell lysate was replaced with EphB4-Fc and allowed to react with EphB4 antibody.

2.5 SiRNA- Short Interfering RNA methods

PC3, LNCaP, and Du-145 cell lines were treated with EphB4 siRNA from Dharmacon along with the lipid-based Oligofectamine reagent from Invitrogen (Cat 12252-011, Burlington, ON) to reduce EphB4 mRNA and, thereby, lowering EphB4 protein levels. Protocols for siRNA experiments were followed according to directions from Invitrogen for use of Oligofectamine reagent. Briefly, $1x10^5$ PC3 and Du-145 cells ($3x10^5$ LNCaP cells) were counted using a hemacytometer and plated in 6-well plates with DMEM/10%FBS

medium and incubated at 37°C. After 6 hrs and at 50-60% confluency, a mastermix of oligofectamine and siRNA oligonucleotide complexes was prepared for cell transfection. For each well in a 6- well plate, 3ul of Oligofectamine was mixed with 12ul of Opti-MEM reduced serum medium (Cat #11058-021, Invitrogen, Burlington, ON) in a microcentrifuge tube to get a final volume of 15ul. The solution was then allowed to sit at room temperature for 10 mins. In the meantime, 2.5ul of 20uM stock of EphB4 siRNA oligonucleotide was diluted in 182.5ul of Opti-MEM reduced serum media in a separate microcentrifuge tube. Subsequently, 15ul of diluted oligofectamine reagent was pipetted and gently mixed with 185ul of diluted siRNA oligonucleotide and incubated at room temperature for 20 mins to form siRNA and oligofectamine complexes. Media was aspirated from wells and cells were washed once with 1ml of Opti-MEM reduced serum media. For each well in a 6-well plate, 800ul of Opti-MEM reduced serum media was added. EphB4 siRNA and oligofectamine complexes were mixed gently and 20ul of complexes were evenly distributed onto cells in each well. The 6-well plate was gently rocked back to forth and side to side to evenly distribute the siRNA and oligofectamine complexes and cells were incubated at 37°C / 5%CO_2. After 4 hrs of incubation, 500ul of DMEM growth medium containing 30% FBS was added to cells without removing the transfection mixture. Subsequent to transfection with siRNA, cells were tested for reduction in EphB4 protein expression using western blotting and assayed for migration and invasion activity.

2.6 Rac and Cdc42 activity assays: Generation of GST-p21 activated kinase (PAK)

To measure activity of Rac and cdc42, a GST-fusion protein pulldown assay was used as published before by EE. Sander et al (72). A 50ul inoculum of glycerol stock of GST-protein producing bacteria (a gift from Luc Sabourin Lab) was added to 50ml of Luria Bertani (LB) broth (5g yeast extract, 10g tryptone, 10g NaCl, 1L ddH_2O) (Fisher Scientific, Ottawa, ON) and grown overnight in 75ug/ml Ampicillin at 37°C with shaking. The next day, this volume of bacterial stock was diluted 1:10 in a separate LB broth with 75ug/ml Ampicillin and shaken at 37°C for 1 hr to prepare for induction of GST-protein production. Following this, Isopropyl Thiogalactoside (IPTG) (Fisher Scientfic, Ottawa, ON) to a 0.1mM final concentration was added to bacterial stock, which was then further incubated for 3 hrs at 30°C to induce GST-protein production. IPTG strongly binds and inhibits the Lac repressor thereby artificially activating the Lac operon. Bacterial stock was poured into plastic ultracentrifuge bottles and centrifuged at ~2000xg for 10mins at 4°C to collect bacterial cells. Supernatant was poured out and bacterial cell pellet was then resuspended in 10ml of bacterial lysis buffer (20%Sucrose, 10%glycerol, 50mM Tris pH 8.0, 2mM dithiothreitol (DTT), 0.2mM sodium sulfite ($Na_2S_2O_5$), 2mM $MgCl_2$) supplemented with fresh protease inhibitors (1mM phenylmethylsulfonyl fluoride (PMSF), 1ug/ml aprotinin, 1ug/ml leupeptin, 1mM Benzamide.) Bacterial lysate was incubated on ice for 30 min, sonicated for 3 mins and centrifuged at 4°C for 20 mins at ~10,000xg. Supernatant was then transferred to 10 ml plastic centrifuge tubes (BD biosciences, Mississauga, ON). GST-Pak fusion protein was precipitated by adding 1ml of 50% Glutathion-Sepharose 4B beads slurry (Amersham Biosciences, Uppsala, Sweden) previously washed 5x in bacterial lysis buffer and subsequently rotated for 2 hrs at 4°C. Beads were centrifuged at ~2000xg at 4°C and washed 3x with washing buffer (50mM Tris-HCl ph 8.0, 150 mM NaCl, 5mM $MgCl_2$, 1mM DTT,

1mM PMSF, 1ug/ml aprotinin). Supernatant was then removed and beads were resuspended in washing buffer including 10% glycerol and stored at -80°C until use in Rac and cdc42 activity assays.

2.7 Rac and cdc42 activation and affinity precipitation assay.

A total of $1x10^6$ PC3 cells were plated in 6-well tissue culture plates (BD Falcon, Mississauga, ON). Next day 2 hrs before stimulation, medium was aspirated, PC3 cells were washed with PBS, and medium was replaced with DMEM/0.5% FBS. PC3 cells were then stimulated using 3ug/ml EphrinB2-Fc (R&D Systems, Minneapolis, MN) or a negative control, 3ug/ml human IgG, (Sigma, St. Louis, MO) supplemented in medium DMEM/0.5% FBS and incubated at 37°C for periods of 0min, 15min and 45 min. Cells were washed with cold PBS and lysed with 500ul of cold GST-fish buffer (10%Glycerol, 50mM Tris pH 7.4, 100mM NaCl, 1% Nonident P-40 (NP-40), 2mM $MgCl_2$, 1ug/ml leupeptin, 1ug/ml aprotinin). Cells were scraped and lysates collected in 1.5ml microcentrifuge tubes and then sonicated. Lysates were incubated on ice for 5 min and centrifuged at 4°C for 10 min at ~10,000xg in a table top centrifuge. Supernatant was transferred to a new tube and protein concentration was quantified using Bradford assay as described in section 2.3 (Bio-Rad, Hercules, CA). Cell lysates can be stored at -80°C or immediately used in affinity precipitation assay with GST-PAK. A total of 800ug of protein was diluted in GST-Fish buffer and subsequently incubated with 50ul of GST-PAK bound bead slurry for 2 hrs at 4°C while rotating. For a positive control, cell lysate was incubated with GTPδS in excess. In a 1.5ml centrifuge tube, 100ul of cell lysate containing GTPase was mixed with 12ul of 0.2M EDTA and 12ul of 1mM GTPδS and incubated at 30°C for 10 min. The exchange reaction

was stopped by adding 8ul of 1M $MgCl_2$(Fisher Scientific, Ottawa, ON), followed by incubation with 50ul of GST-PAK slurry, and rotation at 4°C for 2 hrs. Cell lysates containing beads were centrifuged for 2 min at 4°C at ~2000xg and supernatant was extracted. Beads were washed 3 times with GST-Fish buffer with repeated centrifugation and extraction of supernatant. The bead pellet was resuspended in 4x Laemmli sample buffer (50mM Tris-HCl pH 6.8, 10% glycerol, 2%SDS, 2% β-mercaptoethanol, 1% Bromophenol blue), boiled for 5 min, centrifuged for 15 sec and separated on 15% SDS-PAGE gel. Proteins were then transferred onto nitrocellulose membrane, blocked in 5% Milk/TBST for 1 hr at room temperature, and membrane was treated with 10ml of 5%Milk/TBST with anti-Rac antibody (diluted 1:4000) overnight at 4°C. Membranes were washed 3x 5min and probed with secondary anti-mouse antibody. To check for cdc42 activity, cell lysates were incubated with GST-PAK and procedure was carried out as described earlier in this section. Nitrocellulose membrane was blocked using 5%MILK/TBST for 1 hr at room temperature, and membrane was probed with polyclonal anti-cdc 42 antibody (SantaCruz Biotech, Santa Cruz, CA) diluted (1:400) in 5%Milk/TBST overnight at 4°C. Membranes were washed 3x 5min with TBS 1%Tween and probed with secondary anti-rabbit antibody (diluted 1:10,000) (Sigma, St. Louis, MO) in 5%Milk/TBST.

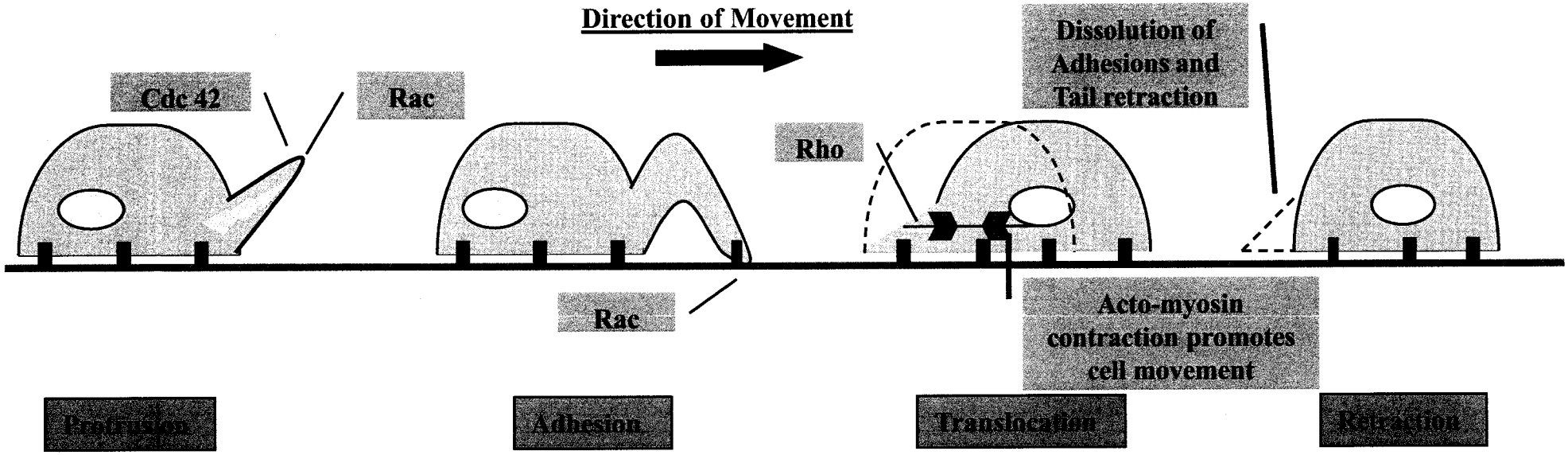
2.8 Scratch Wound-Healing Migration Assay

A common economical and efficient method to determine the in-vitro cell migration potential of cancer cells is using a Scratch Wound-Healing Assay (Figure 4B). A gap (or wound) is created within a monolayer of cancer cells using a pipette tip or cell scraper of set diameter, which thus stimulates the cells on the leading edge of the newly created gap to

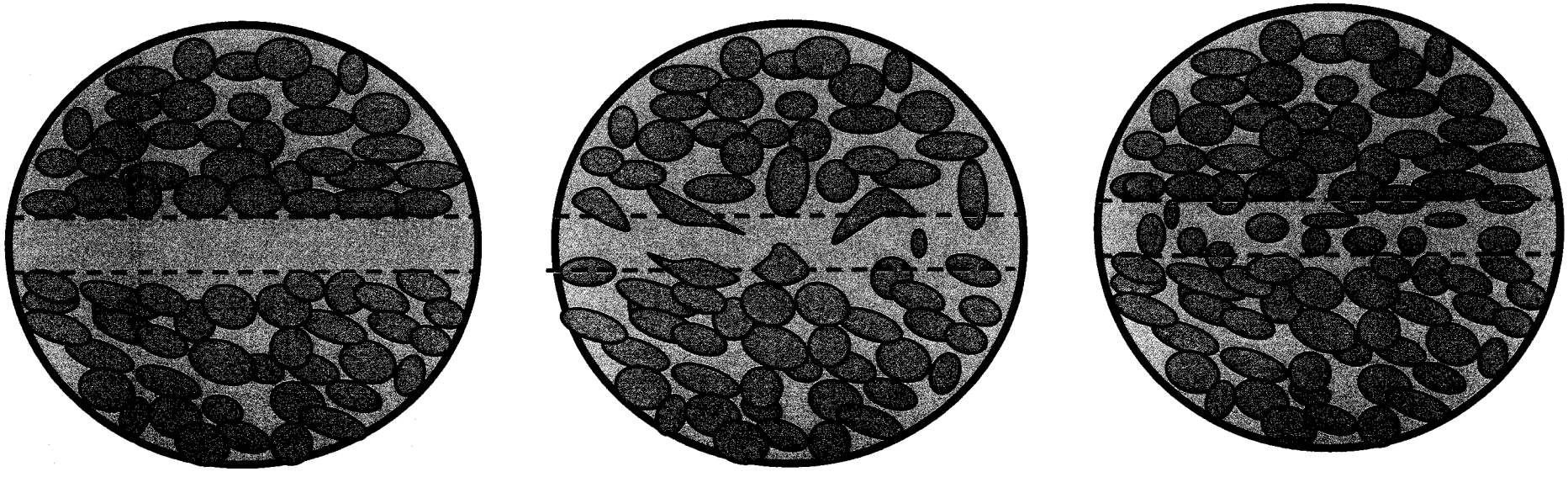
move into the scratched area until new cell-cell contacts are established (Figure 4B) (65). A comparison of images recorded at regular intervals determines the rate of cell migration. A major advantage of this assay is that it mimics in-vivo migration (65). For instance, after removing a part of blood vessel endothelium, endothelial cells are induced to migrate into the wound to close the damaged area. Another benefit of this assay is that it is an in vitro model to study both in-vivo patterns of loosely connected, independent migration and the collective, sheet-like cell migration observed in epithelial and endothelial cells (65). Furthermore, a wound-healing assay is also suitable to study factors which regulate cell migration such as cell-cell contact and interaction of cells with the ECM (65).

For scratch wound assays, $1x10^5$ prostate cancer cells were plated in 6-well plates and treated with EphB4 siRNAs and control siRNAs at 50-60% confluency as described above in Section 2.5. After 48 hrs post-transfection and 100% confluency, the monolayer of cells was ready for scratch-wound healing assay. Using a p200 pipette tip (Fisher Scientific, Ottawa, ON), the cell monolayer was scraped in a straight line to form a "scratch". It is important to make scratches in a straight line and of approximately similar size in assessed and control cells to minimize any possible variation caused by difference in width of scratches. A new pipette tip was used for making each scratch. Media was aspirated to remove debris and cells were washed once with PBS. Fresh growth medium (8ml of DMEM/10%FBS) was replaced into the wells of 6-well plate. Photos of the wound were taken at 100x magnification using a phase-contrast microscope (Nikon Eclipse TE-2000 U) attached to a digital camera (Coolpix P80 Nikon) with a MDC Lens (Coolpix, Nikon) at time intervals starting from 0 hrs (time of first scratch). To obtain the same field during photo

Figure 4. Process of Cell Migration. Panel A- Functions of Rac, Rho, and Cdc42 in directional cell migration. Panel B- Cell migration as seen in a typical Wound-healing assay over time. Yellow line shows the original scratch.



Panel A



Panel B

Figure 4.

acquisition at later time intervals, markings were created to be used as reference points underneath the plates and close to the scratch using the fine tip of a permanent marker. Four markings were placed on each scratch at approximate equal distances from each other. After the markings were placed, the first set of photos was taken so that the reference points were just outside the field of image but within the eye-piece field of view. The plates were returned to the incubator at 37°C / 5%CO_2. Wounds were then photographed periodically matching the reference point and aligned to the photographed region from the first images. The images acquired for each sample were analyzed quantitatively by measuring the width of the wound or the distance between one side of the scratch and the other using a millimeter rule at a minimum of four different points for each photograph of the wound. Percent wound closure was calculated, as described in results section, by entering all widths in Microsoft Excel file and using the following calculation; [(Width at time 0hrs – width at time Xhrs) / Width at time 0hrs] x 100%.

2.9 Invasion Assays

A Matrigel ChemoInvasion assay is a commonly used model to characterize in-vivo cell invasion into basement membrane, which is another important indicator of tumor cell migration and metastatic potential (66). The basement membrane is composed of thin sheets of extracellular matrix that provide a barrier for tissues and organs from invading molecules and cells. Matrigel is a soluble basement membrane preparation taken from Engelbreth-Holm-Swarm sarcoma which contains ECM proteins, collagenases, and growth factors (67). It is in a soluble, liquid state at temperatures below -20°C, but solidifies upon incubation at room temperature or higher. The reconstituted basement membrane forms a thin layer when

placed on top of filters, which contain microscopic pores, inside the transwell chambers of tissue culture plates (67). When invasive cancer cells are placed above the matrigel, they produce matrix degrading proteases that breakdown the thin layer of matrigel and allow cells to migrate to the underside of the filter towards a chemoattractant (usually serum). A count of the amount of cells that have invaded through the matrigel and the filter determines the rate of invasion. The invasion assay is thus an efficient method that mimics the ability of cancer cells to invade tissues and organs (66, 67).

Twenty-four well plates with transwell inserts containing growth-factor reduced matrigel was purchased from Becton-Dickson (BD) Biosciences (Cat. 354483, Missaussauga, ON). Transwell inserts had filters containing 8um pores. Plates were stored at -20°C until time of use. Cells were treated with siRNAs to reduce EphB4 protein levels as described previously in section 2.5. After 48 hrs of transfection, cells were ready for plating in transwell inserts. Twenty-four well transwell plates were removed from freezer and 100ul of warm serum free DMEM was placed in transwell inserts to facilitate thawing and gelling of matrigel. Cells were trypsinized, spun down, and media was replaced with serum free DMEM. Cells were counted using a hemacytometer and diluted to $1x10^6$cells/ml. A total volume of 600ul of DMEM/10% FBS was placed in the lower chamber of the transwell plates. Media was aspirated from upper transwell inserts and 100ul cell suspension ($1x10^5$ cells) was pipetted slowly on top of a thin layer of matrigel. Plates were incubated at 37°C/5%CO_2 for 8 hrs. Transwell plates were removed and prepared for staining with Diff-Quick solution (Dade Behring, Newark, DE). Media was carefully aspirated from the upper chamber so not to disturb the matrigel. Transwell inserts were dipped in Diff-Quick fixative solution (methanol) for 3 mins and removed. The inside of the transwell inserts and the area

top of filter was gently scraped in a consistent manner with a cotton swab (BD Biosciences, Mississauga, ON) to remove the matrigel and non-invading cells. Transwell inserts were dipped in Diff-Quick solution I (1g/L Xanthene dye, 0.01% sodium azide) for 2 mins and then dipped once in distilled water before staining with Diff-Quick solution II (0.625g/L Azure A, 0.625g/L methylene blue) for 2 mins. Inserts were dipped once again in distilled water before leaving to dry. Inserts were viewed with 200x magnification under phase-contrast microscope (Nikon Eclipse TE-2000 U) attached to a digital camera (Coolpix P80 Nikon) with a MDC Lens (Coolpix, Nikon). The number of cells invading through the filter was counted. Each condition in an experiment of the invasion assay was conducted in triplicate using three transwell inserts and the average numbers of cells invading through the matrigel and the filter were plotted to compare invasion over assessed and control cells. For experiments testing invasion of PC3 cells by activating EphB4, PC3 cells were diluted to $1x10^6$ in serum free DMEM and 100ul of cell suspension was plated in the upper transwell with 600ul of DMEM/10%FBS in the lower compartment. Activation of EphB4 was tested with 2 separate concentrations of EphrinB2-Fc, either 1ug/ml EphrinB2-Fc or 3ug/ml EphrinB2-Fc that was added to the upper transwells and compared to controls consisting of addition of 1ug/ml IgG or 3ug/ml IgG, respectively.

3. RESULTS

Upon review of recent literature, it was noted that EphB4 was reported to provide a survival advantage in breast and head and neck carcinoma, but very little evidence was available regarding its role in prostate cancer. In order to determine whether EphB4 expression in prostate tumor cells could influence their growth or tumor-associated angiogenesis, we embarked upon a series of studies designed to characterize its expression in prostate tumor cell lines and whether or not EphB4 expression could influence the phenotype of tumor cells directly. To this end, we investigated the following: characterization of the levels of expression of EphB4 and its ligand EphrinB2 in various prostate carcinoma cell lines as compared to normal prostate epithelial cells; effects of addition of exogenous EphrinB2 on the phosphorylation and activity of EphB4, and the resulting effect on cell proliferation and migration; and use of individual siRNA complexes to reduce expression of EphB4 directly, and the subsequent effects of reduced EphB4 on prostate tumor cell viability, cell migration and invasion .

3.1 Comparison of EphB4 receptor expression in prostate tumor versus normal prostate epithelial cells.

Prior studies comparing expression of the Eph receptor family of tyrosine kinases in cancer cells compared to the normal epithelium have shown evidence of either over-expression or under-expression of such receptors in relation to cancer progression. Such changes in expression have been mostly observed using immunohistochemistry data obtained from tumor sections of patients suffering from lung and breast cancer. We thus hypothesized that the aggressive metastatic properties of prostate carcinoma cell lines could be due, in part,

to overexpression of EphB4 protein. Although during the course of our studies, reports describing EphB4 expression in prostate cancer cell lines, as detected following immunocytochemistry, demonstrated that prostate tumor cells expressed EphB4 protein on the cell surface (59), a comparison of the levels of EphB4 in prostate tumor cells as compared to normal prostate epithelial cells was not performed. We thus decided to compare relative levels of EphB4 protein expressed in three different prostate carcinoma cell lines, specifically PC3, Du145 and LNCaP, with normal prostate epithelial cells (PrEC), and to other previously examined tumor cell lines using immunoblotting. New prostate epithelial cells were thawed and lysed at passage 3 to prevent any changes in morphology due to extensive culturing of primary cells, which is a common occurrence in epithelial cells. The monoclonal anti-human EphB4 antibody, obtained as a gift from Vasgene Therapeutics, is specific for binding to the extracellular domain of EphB4 receptor and does not bind with any other EphB and EphA receptors (3). A single band at ~120kda was clearly observed in PrEC, PC3, LNCaP and Du145 and corresponded in size with reactive bands observed in the positive control cell lines used, namely, three different breast cancer cell lines (MCF-7, BT549, T47D) and human umbilical vein endothelial cells (HUVEC) (Appendix Figure 1). As predicted , the breast cancer cell lines tested had a significantly high expression of EphB4. The three breast cancer cell lines and HUVEC were included in the analysis as these cell lines were previously shown to express EphB4 and thus served as positive controls. Membranes were striped and re-probed for β-actin and equal loading of protein in each lane was confirmed. We observed that the EphB4 expression in PC3 and LNCaP cells was significantly elevated in comparison with PrEC and the Du145 prostate cancer cell line. To confirm these results, the experiment was repeated two additional times using cell lysates

obtained at different cell passages for PC3, LNCaP, and Du-145 cells, and independently obtained lysates for PrEC obtained at passage 3, all of which were subsequently analyzed by western blot under similar conditions (Figure 5). The results confirmed that PC3 and LNCaP have a nearly two-fold overexpression of EphB4 when compared with normal prostate epithelial cells, and that these levels were not affected by serial passaging of the tumor cell lines in culture. The second set of prostate carcinoma cell lines, PC3, LNCaP, and Du-145, were lysed four passages after the first set of cell lines and the results obtained were similar.

3.2 Expression of EphrinB2 in prostate cancer and normal prostate epithelial cells.

Endogenous expression of EphrinB2 has been determined in many types of tumor cells using immunohistochemistry and its role has been mainly studied in angiogenesis and tumor growth (73). However, very little is known about the endogenous levels of EphrinB2 expressed in prostate cancer and prostate epithelial cells. At the time of cell-cell contact, EphB4 has been found to most commonly associate with the ligand EphrinB2 (25). Since EphrinB2 is the main ligand for EphB4 receptor and its concentration has previously been shown to be varied amongst different breast cancer cell lines (60), we examined the level of expression of EphrinB2 in our prostate cancer and normal prostate epithelial cell lines. Subconfluent PC3, LNCaP, and Du-145 cells were lysed in tissue culture plates, total protein lysates were generated and then analyzed using immunoblotting with a polyclonal anti-mouse EphrinB2 antibody. Human and mouse EphrinB2 extracellular domains share approximately 98% homology (74), thus the antibody can cross react with human EphrinB2. Results shown indicate that although all prostate cancer cell lines tested expressed EphrinB2 to some level, as indicated by the strong immunoreactive band

Figure 5. Relative Expression of EphB4 protein in Prostate Carcinoma cell lines compared to PrEC. Cells were lysed in subconfluent conditions and 40ug of total cell lysates were resolved using SDS-PAGE gel analysis and immunoblotting. Two individual sets of PC3, LNCaP, Du-145 and PrEC were tested. PC3 and LNCaP cells have a significant increase in EphB4 expression compared to PrEC, while Du-145 cells showed a marginal increase in EphB4 receptor. After the membranes were stripped, β-actin was used as a control for equal loading. Densitometry values are a representation of raw absorption volumes from the bands normalized to PrEC* and then to B-actin using Genetools by Syngene.

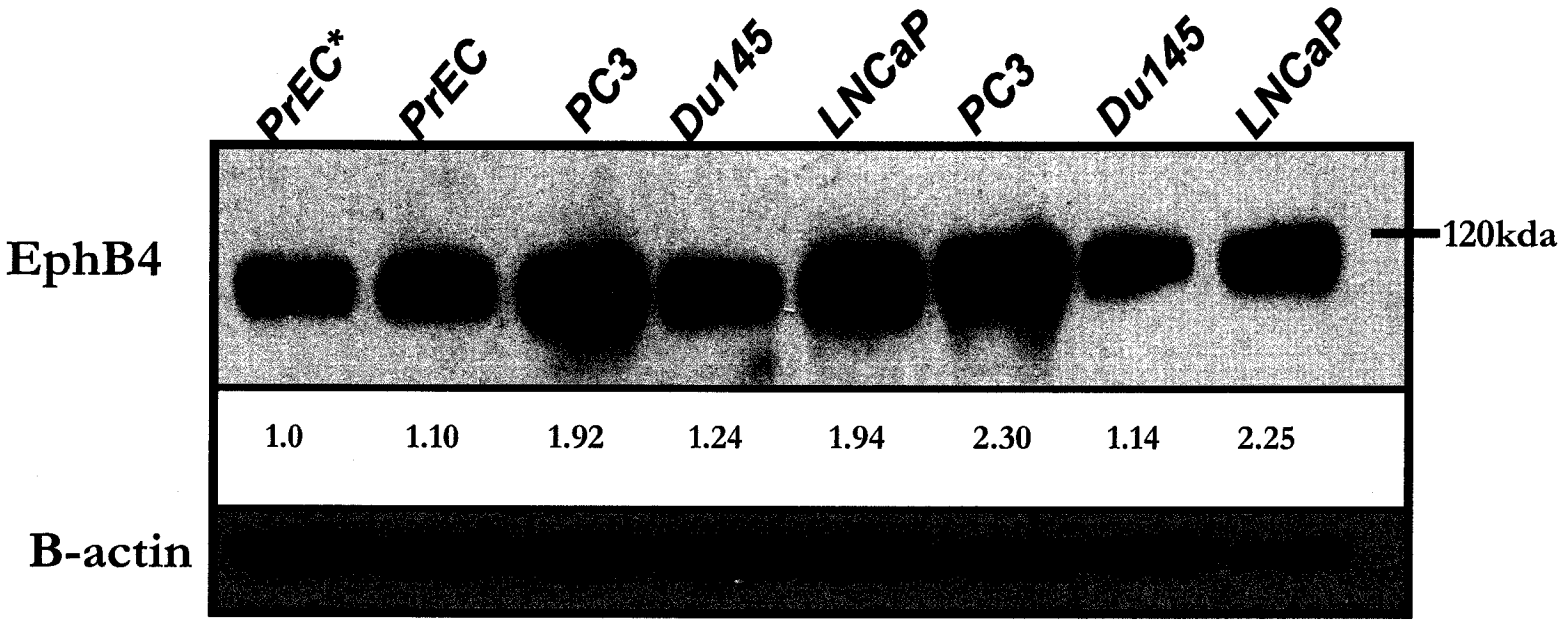

PrEC*
PrEC
PC3
Du145
LNCaP
PC3
Du145
LNCaP
EphB4
120kda
1.0
1.10
1.92
1.24
1.94
2.30
1.14
2.25
B-actin

Figure 5

clearly observed at ~37kda, PC3 cells had the lowest concentration of EphrinB2 protein when directly compared with the other tumor lines Du-145 and LNCaP (Appendix Figure 2). It is worth mentioning that the EphrinB2 expression in Du-145 cells was significantly higher than PC3 and LNCaP (Appendix Figure 2). Equal loading of total protein was again confirmed using β-actin protein as a control. To confirm the results, the experiment was repeated twice using independently isolated protein lysates collected from different PC3, LNCaP, and Du-145 cells (Figure 6). Cell lysates from normal prostate epithelial cells were also included as a comparison to test the relative EphrinB2 protein expression with prostate cancer cell lines. The results showed that prostate epithelial cells, PrEC, express noticeable amounts of EphrinB2, and in general these levels are lower than that found in the various tumor cell lines (Figure 6). Interestingly, the size of EphrinB2 protein detected in PrEC was slightly larger, ~42kDa, as compared to that observed in prostate tumor cells where the immunoreactive band migrated at ~37kDa. The typical size of EphrinB2 compared to other cells and as previously reported in literature is 37kDa (87). This variation in size could be due to differential glycosylation of EphrinB2 or different isoforms such as the one detected previously for EphrinB2 in newborn mouse brain cells (76,94). A more detailed explanation is presented in the Discussion chapter of this thesis.

3.3 Immunoprecipitation and phosphorylation of EphB4.

As mentioned previously, binding of EphrinB2 to EphB4 results in phosphorylation of the cytoplasmic domain and activation of its kinase activity, thus eliciting downstream signaling events. Following activation, the EphB4 receptor and the extracellular domain of EphrinB2 are internalized and slowly degraded (86). Although the kinetics of EphB4

Figure 6. Expression of EphrinB2 ligand in Prostate Carcinoma cells compared to PrEC. A total of 40ug of total protein, independently isolated from different PrEC, PC3, Du-145, and LNCaP, was resolved by immunoblotting and anti-EphrinB2 antibody was used to detect expression levels of the ligand. The amount of EphrinB2 expressed in PrEC was generally lower than most of prostate cancer cells. The size of EphrinB2 detected in PrEC was larger (~42kDa) than in prostate cancer cells (37kDa). Densitometry results were normalized to PrEC* and then to β-actin.

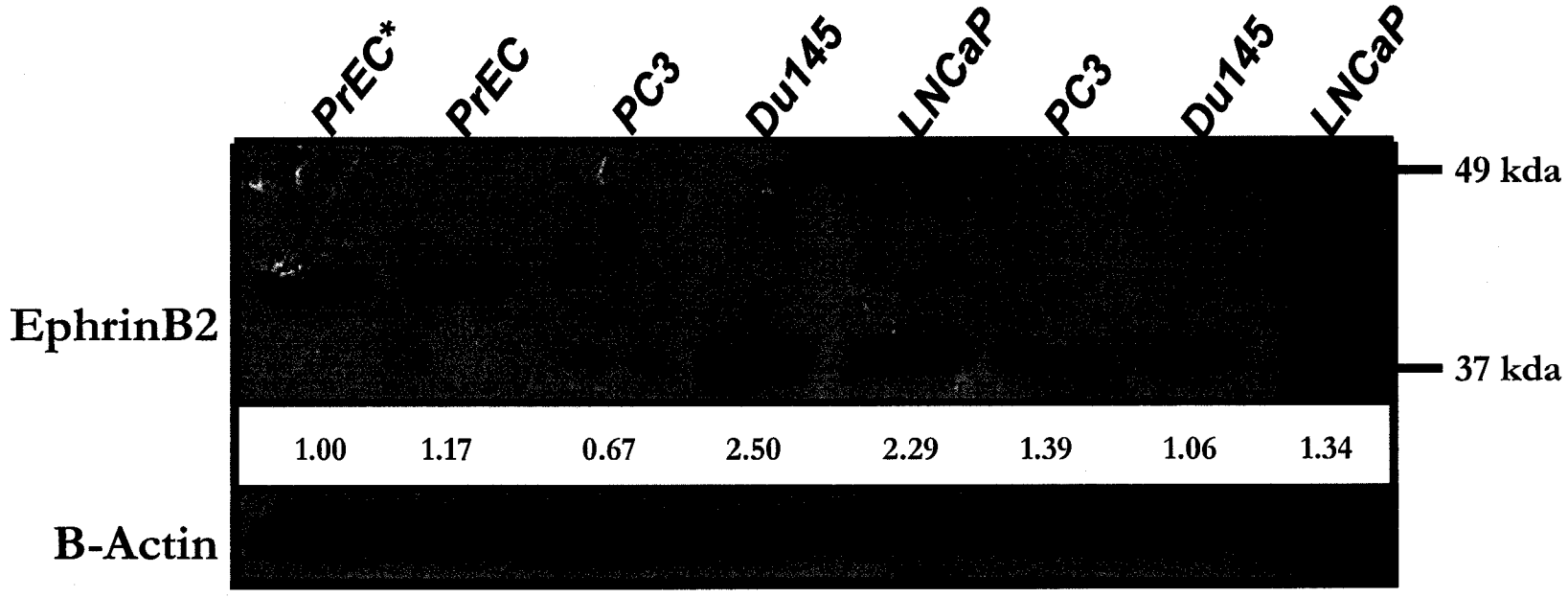

PrEC*
PrEC
PC3
Du145
LNCaP
PC3
Du145
LNCaP
49 kda
37 kda
EphrinB2
1.00
1.17
0.67
2.50
2.29
1.39
1.06
1.34
B-Actin

Figure 6

activation and phosphorylation in prostate cancer cell lines has yet to be elucidated, in other systems Eph receptors are phosphorylated within the first 30 to 45 min following ligand binding. Here, we used soluble recombinant EphrinB2-Fc to activate EphB4 and to determine the length of time that EphB4 remained phosphorylated in our system. To monitor EphB4 phosphorylation, anti-EphB4 antibody was first used to immunoprecipitate the protein (Figure 7), and followed subsequent SDS-PAGE electrophoresis and immunoblotting with anti-phosphotyrosine (Figure 8), the level of EphB4 phosphorylation was determined. A strong immunoreactive band at ~120 kDa was clearly observed in PC3 cell lysates regardless of which concentration of antibody was used in the immunoprecipitation, and the specificity of the antibody for EphB4 in the immunoprecipitation was confirmed by pulling down EphB4-Fc as a positive control (Figure 7). After trouble-shooting the conditions for our immunoprecipitation, PC3 cells were stimulated with 3ug/ml of EphrinB2-Fc and subsequently lysed at time intervals of 0min, 30min and 2hrs post stimulation, and total protein extracts were obtained. Equal amounts of total protein were subjected to immunoprecipitation for EphB4 as described in materials and methods, and the immunoprecipitated proteins were probed for phosphotyrosine using western blot analysis (Figure 8). The results from Figure 8 show that EphB4 is phosphorylated 30 min after stimulation with EphrinB2-Fc and remains in an un-phosphorylated state following addition of the IgG negative control. Furthermore, EphB4 remains phosphorylated, although at somewhat decreased levels at 2 hrs post-stimulation with EphrinB2-Fc as compared with IgG control (Figure 8 and Appendix Figure 3). These results suggest that EphB4 is activated by addition of exogenous EphrinB2, thus demonstrating the utility of this approach for use in examining the role of activation of EphB4 in migration and invasion assays.

Figure 7. Immunoprecipitation of EphB4 from PC3 cells. A total of 400ug of total protein was incubated with two different concentrations of anti-EphB4 antibody to determine the amount of antibody required for immunoprecipitation. Negative Controls: Lane 1 and 2 are without the cell lysate showing that EphB4 antibody bound to G-Sepharose beads does not produce immunoreactive bands at the same size as the 120kDa EphB4 protein. Lane 3 is without the antibody showing G-Sepharose beads do not pull down non-specific proteins. Lane 4 and 5 clearly show that the antibody is specific for EphB4 protein. Control: Lane 6 EphB4-Fc was incubated with anti-EphB4 antibody and extracted with G-Sepharose beads to determine the specificity of the antibody in pulling down EphB4 protein.

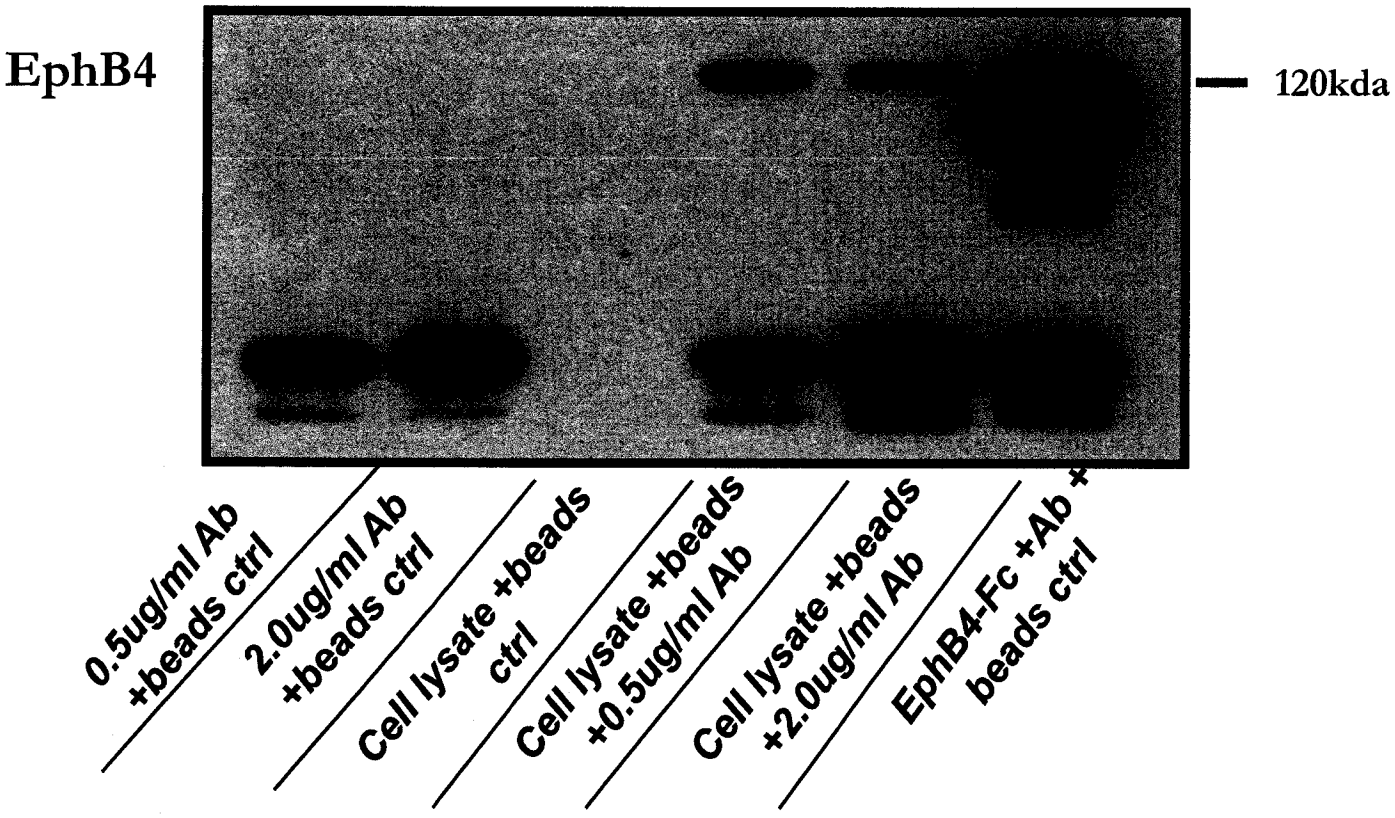

EphB4
120kda
0.5ug/ml Ab +beads ctrl
2.0ug/ml Ab +beads ctrl
Cell lysate +beads ctrl
Cell lysate +beads +0.5ug/ml Ab
Cell lysate +beads +2.0ug/ml Ab
EphB4-Fc +Ab + beads ctrl

Figure 7

Figure 8. Phosphorylation of EphB4 receptor using EphrinB2-Fc. PC3 cells were serum starved and then stimulated with 3ug/ml EphrinB2-Fc or with 3ug/ml IgG-Fc in DMEM/5%FBS. Total protein lysates were then generated at various times post-stimulation. Subsequent immunoprecipitation and western blotting with anti-phosphotyrosine antibody (P-Tyr-100) detected a strong band at 120kDa 30min after stimulating with EphrinB2-Fc. No phosphorylation was observed in PC3 cells stimulated with IgG control. Membrane was stripped and re-probed with anti-EphB4 antibody to confirm equal levels of EphB4 from immunoprecipitation.

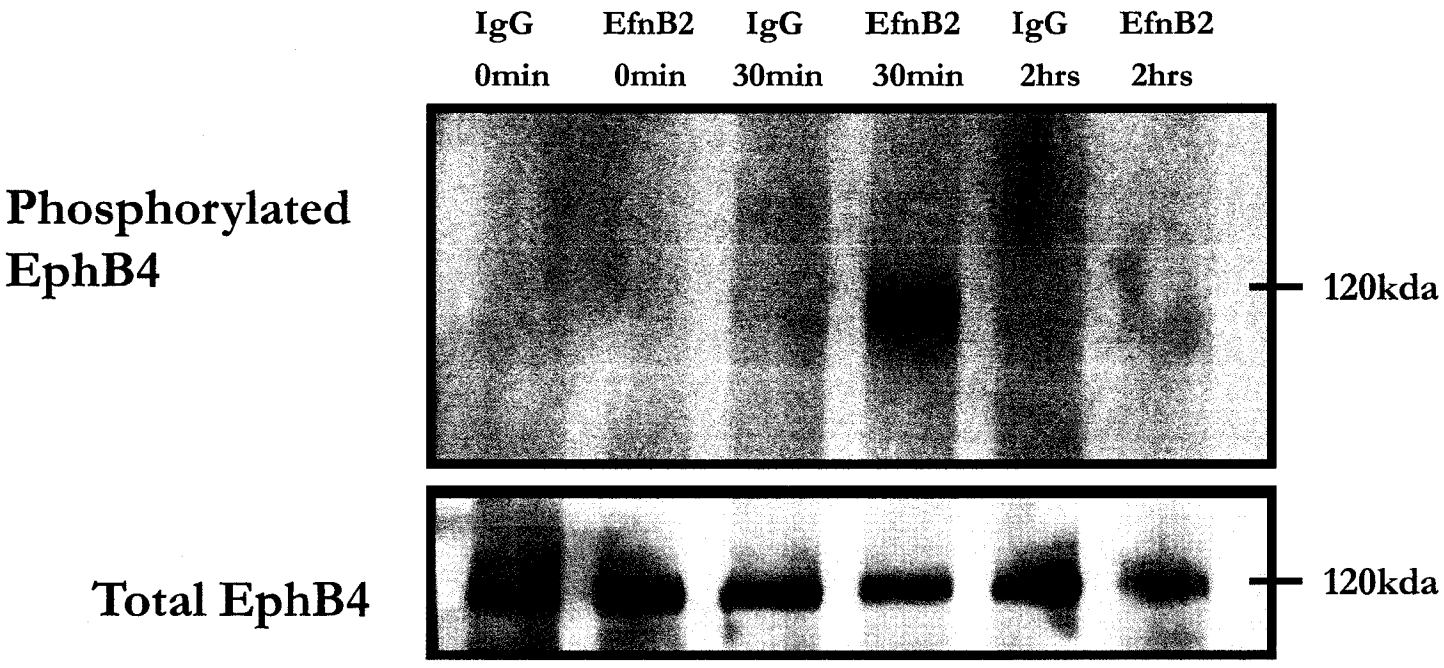

IgG 0min
EfnB2 0min
IgG 30min
EfnB2 30min
IgG 2hrs
EfnB2 2hrs
Phosphorylated EphB4
120kda
Total EphB4
120kda

Figure 8

3.5 Determining Efficiency of EphB4 depletion in PC3, LNCaP and Du-145 cells.

RNA Interference is an effective but temporary technique requiring optimization of conditions such as siRNA concentration and length of time for reducing protein levels in cells. Properties, such as serum and lipid-based transfection reagents, and conditions, such as degradation of oligonucleotides and the cells inherent ability to recycle a protein, may have an effect on the length of time for which reduction of EphB4 protein is sustained. Therefore experiments to determine the optimal siRNA concentration and length of time for reduction of EphB4 expression levels were performed in PC3, LNCaP, and Du-145 cells. Briefly, PC3, LNCaP and Du145 cells were transfected with final concentrations of 10nM, 20nM, 50nM, or 100nM siRNA using either SMARTpool EphB4 siRNA, individual siRNA#1, or siRNA#2 to reduce EphB4 protein levels. Negative controls used were mock transfection whereby PBS was substituted for oligonucleotide, and siRNA to green fluorescent protein (GFP), a non-existing sequence of mRNA in prostate cancer cells. Immunoblot analysis indicates that 50nM or more of SMARTpool siRNA, siRNA#1 or siRNA#2 produced a significant reduction in EphB4 protein levels in all prostate cell lines tested when compared to mock and GFP-targeted siRNA controls (Figures 9A, 10A, and 11A). We further tested the duration of reduction in EphB4 protein levels using the 50nM EphB4 siRNA concentration. Decreased EphB4 was observed within 24 hrs post-transfection with a maximum decrease by 48 hrs in PC3, LNCaP and Du-145 cell lines (Figures 9B, 10B, and 11B respectively). In some cases, reduction in EphB4 protein levels was sustained for up to 96 hrs after transfection. In LNCaP cells, we achieved significant reduction in EphB4 protein levels at 48hrs after transfection so that the protein was essentially undetectable by western blot with the 10nM, 20nM, 50nM,

and 100nM concentrations of SMARTpool EphB4 siRNA, siRNA#1 or siRNA#2 individual complexes when compared to mock and GFP siRNA negative controls (Figure 10, Panel A).

Using these data sets, we were able to determine that 50nM siRNA would be an appropriate concentration to use in future assays involving RNA Interference because all prostate cancer cell lines showed a measurable amount of reduced EphB4 protein levels using this siRNA concentration, while control siRNAs did not appear to influence the level of EphB4 under similar conditions. Additionally, as it appeared that for all cell lines, EphB4 reduction was optimal at 48 hrs post-transfection, but still persisted to some degree at 96 hrs post-transfection, we determined that these conditions would result in sufficient reduction of EphB4 protein to examine its role in proliferation, migration and invasion using this methodology. Furthermore, it allowed us to conclude that the best time to conduct migration and invasion assays is 48hrs after transfection with siRNA in prostate cancer cell lines.

3.6 Measure of Prostate Cancer Cell Viability and Proliferation.

Prostate cancer proliferation and survival has previously been linked to changes in expression and increased activation of tyrosine kinase receptors. We wanted to confirm whether reduced EphB4 expression in prostate cancer would result in decreased proliferation in our system. The results from this experiment would help us confirm that any changes that we would see in our migration and invasion assays are not due to changes in proliferation or cell survival. PC3, Du-145 and LNCaP cells were seeded in triplicate in 12-well plates and transfected with EphB4 siRNAs, control siRNAs or mock transfected using PBS to test if reduced levels of EphB4 would have a significant effect on proliferation.

Figure 9. Reduction of EphB4 Protein Levels in PC3 Cells Using siRNA Interference. Panel A- PC3 cells were tested for transfection efficiency using Oligofectamine reagent with 10nM, 20nM, 50nM and 100nM EphB4 siRNA 1 and EphB4 siRNA 2. Results were compared to mock and same concentrations of the Smartpool EphB4 siRNA, as a positive control, and GFP siRNA 1, as a negative control. Panel B- EphB4 depletion was tested over time. Transfection with 50nM EphB4 siRNA 1 or EphB4 siRNA 2 was compared with negative controls; mock, 50nM GFP siRNA, and two separate 50nM Nontargeting control siRNA sequences (siControl 1 and siControl 2 (Dharmacon)). Optimal protein reduction was detected using 50nM concentration of siRNA beginning at 24 hrs and lasting generally until 96 hrs post-transfection of PC3 cells with EphB4 siRNA 1 and EphB4 siRNA 2.

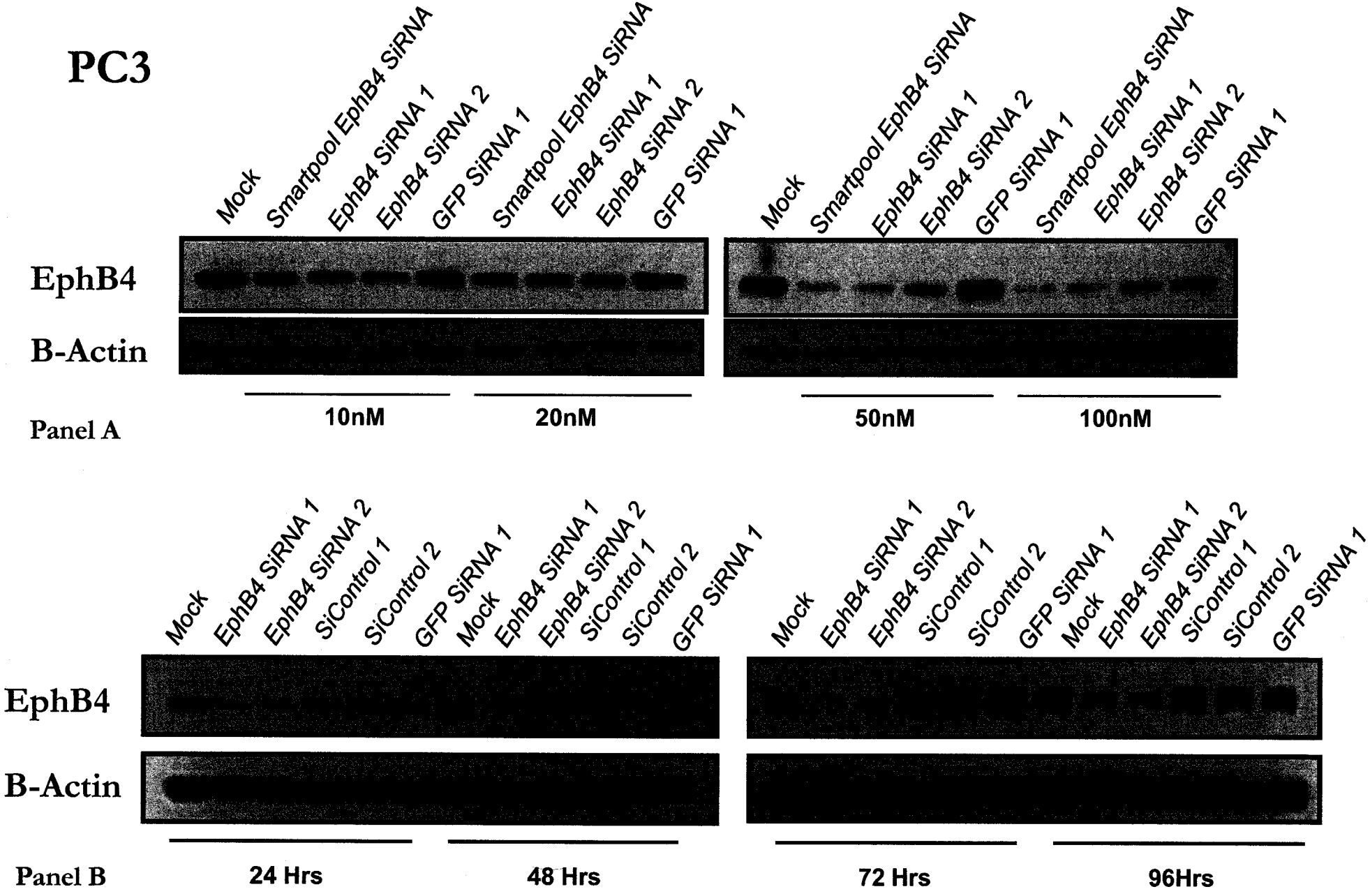

PC3
Mock
Smartpool EphB4 SiRNA
EphB4 SiRNA 1
EphB4 SiRNA 2
GFP SiRNA 1
Smartpool EphB4 SiRNA
EphB4 SiRNA 1
EphB4 SiRNA 2
GFP SiRNA 1
Mock
Smartpool EphB4 SiRNA
EphB4 SiRNA 1
EphB4 SiRNA 2
GFP SiRNA 1
Smartpool EphB4 SiRNA
EphB4 SiRNA 1
EphB4 SiRNA 2
GFP SiRNA 1
EphB4
B-Actin
10nM
20nM
50nM
100nM
Panel A
Mock
EphB4 SiRNA 1
EphB4 SiRNA 2
SiControl 1
SiControl 2
GFP SiRNA 1
Mock
EphB4 SiRNA 1
EphB4 SiRNA 2
SiControl 1
SiControl 2
GFP SiRNA 1
Mock
EphB4 SiRNA 1
EphB4 SiRNA 2
SiControl 1
SiControl 2
GFP SiRNA 1
Mock
EphB4 SiRNA 1
EphB4 SiRNA 2
SiControl 1
SiControl 2
GFP SiRNA 1
EphB4
B-Actin
24 Hrs
48 Hrs
72 Hrs
96Hrs
Panel B

Figure 9

Figure 10. Reduction of EphB4 Protein Levels in LNCaP Cells Using siRNA Interference. Panel A- LNCaP cells were tested for transfection efficiency using Oligofectamine with 10nM, 20nM, 50nM or 100nM EphB4 siRNA 1 or EphB4 siRNA 2. Results were compared to mock and same concentrations of the Smartpool EphB4 siRNA, as a positive control, and GFP siRNA 1, as a negative control. Panel B- Reduction of EphB4 protein levels was tested over time. Transfection with 50nM EphB4 siRNA 1 or EphB4 siRNA 2 was compared with negative controls; mock, 50nM GFP siRNA, and two separate 50nM Nontargeting control siRNA (siControl 1 or siControl 2 (Dharmacon)). Optimal protein reduction was detected using 50nM siRNA concentration at 48 hrs and 72 hrs. EphB4 siRNA 2 still had significant protein reduction at 96 hrs but EphB4 siRNA 1 displayed resurgence of EphB4 receptor at 96 hrs.

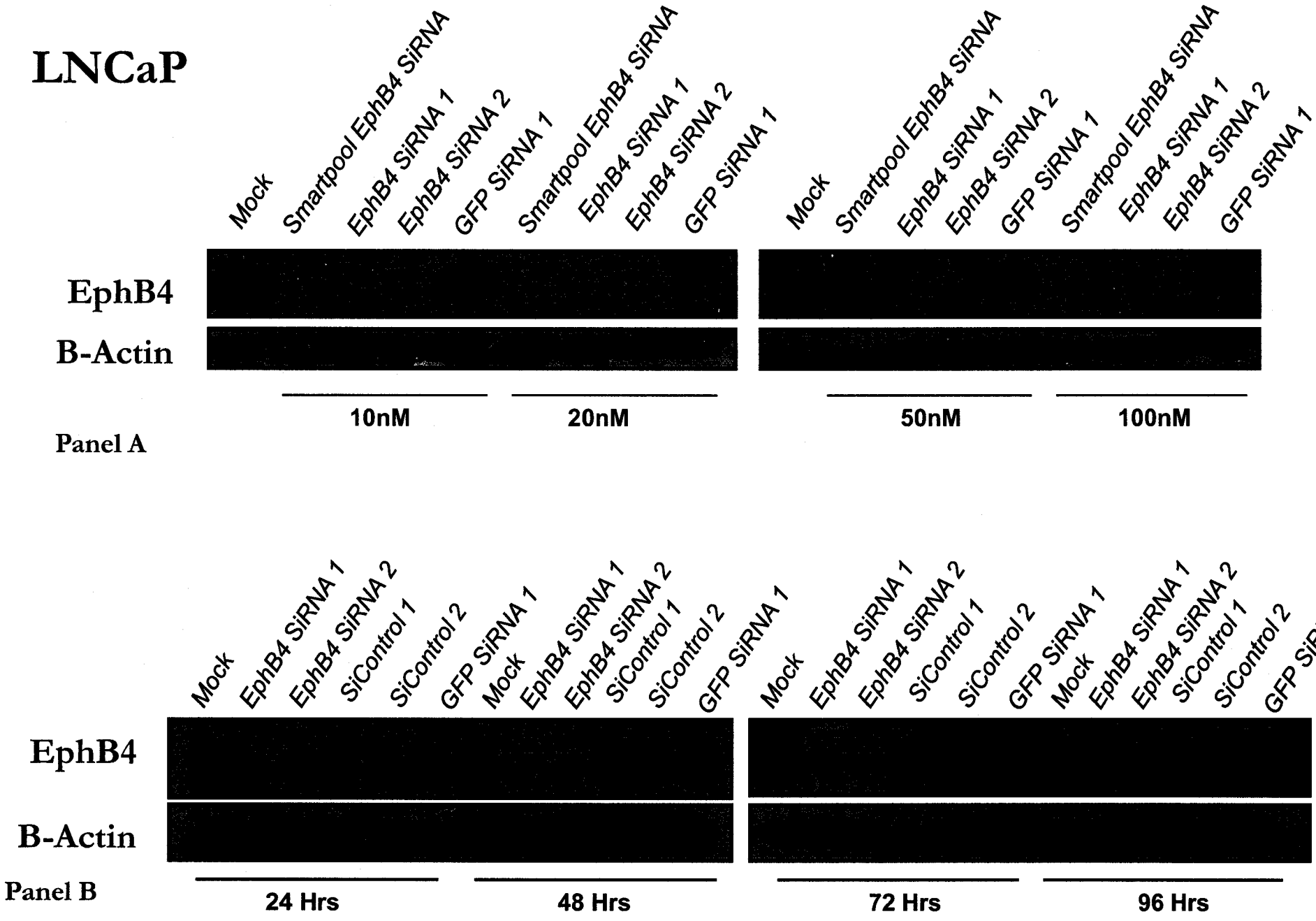

LNCaP
Mock
Smartpool EphB4 SiRNA
EphB4 SiRNA 1
EphB4 SiRNA 2
GFP SiRNA 1
Smartpool EphB4 SiRNA
EphB4 SiRNA 1
EphB4 SiRNA 2
GFP SiRNA 1
Mock
Smartpool EphB4 SiRNA
EphB4 SiRNA 1
EphB4 SiRNA 2
GFP SiRNA 1
Smartpool EphB4 SiRNA
EphB4 SiRNA 1
EphB4 SiRNA 2
GFP SiRNA 1
EphB4
B-Actin
10nM
20nM
50nM
100nM
Panel A
Mock
EphB4 SiRNA 1
EphB4 SiRNA 2
SiControl 1
SiControl 2
GFP SiRNA 1
Mock
EphB4 SiRNA 1
EphB4 SiRNA 2
SiControl 1
SiControl 2
GFP SiRNA 1
Mock
EphB4 SiRNA 1
EphB4 SiRNA 2
SiControl 1
SiControl 2
GFP SiRNA 1
Mock
EphB4 SiRNA 1
EphB4 SiRNA 2
SiControl 1
SiControl 2
GFP SiRNA 1
EphB4
B-Actin
24 Hrs
48 Hrs
72 Hrs
96 Hrs
Panel B

Figure 10

Figure 11. Reduction of EphB4 Protein Levels in Du-145 Cells Using siRNA Interference. Panel A- Du-145 were tested for transfection efficiency using Oligofectamine with 10nM, 20nM, 50nM or 100nM EphB4 siRNA 1 or EphB4 siRNA 2. Results were compared to mock and same concentrations of the Smartpool EphB4 siRNA, as a positive control, and GFP siRNA 1, as a negative control. Panel B- EphB4 protein expression was tested over time. Transfection with 50nM EphB4 siRNA 1 or EphB4 siRNA 2 was compared with negative controls; mock, 50nM GFP siRNA, and two separate 50nM Nontargeting control siRNA (siControl 1 or siControl 2(Dharmacon)). Optimal reduction in EphB4 expression was detected using 50nM siRNA concentration at 24 hrs and 48 hrs. EphB4 siRNA 2 still had marginally reduced protein levels at 72 hrs.

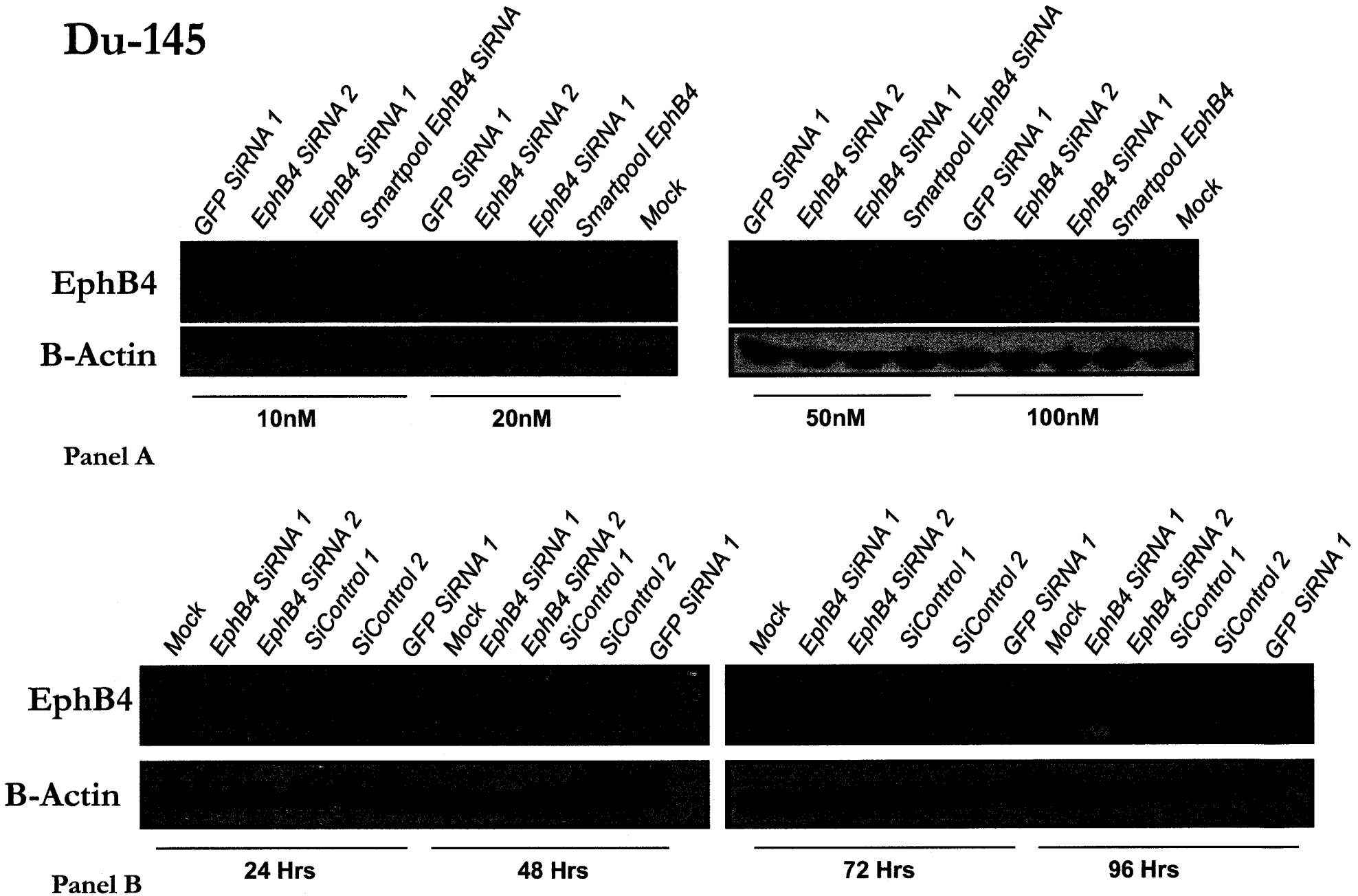

Du-145
GFP SiRNA 1
EphB4 SiRNA 2
EphB4 SiRNA 1
Smartpool EphB4 SiRNA
GFP SiRNA 1
EphB4 SiRNA 2
EphB4 SiRNA 1
Smartpool EphB4
Mock
GFP SiRNA 1
EphB4 SiRNA 2
EphB4 SiRNA 1
Smartpool EphB4 SiRNA
GFP SiRNA 1
EphB4 SiRNA 2
EphB4 SiRNA 1
Smartpool EphB4
Mock
EphB4
B-Actin
10nM
20nM
50nM
100nM
Panel A
Mock
EphB4 SiRNA 1
EphB4 SiRNA 2
SiControl 1
SiControl 2
GFP SiRNA 1
Mock
EphB4 SiRNA 1
EphB4 SiRNA 2
SiControl 1
SiControl 2
GFP SiRNA 1
Mock
EphB4 SiRNA 1
EphB4 SiRNA 2
SiControl 1
SiControl 2
GFP SiRNA 1
Mock
EphB4 SiRNA 1
EphB4 SiRNA 2
SiControl 1
SiControl 2
GFP SiRNA 1
EphB4
B-Actin
24 Hrs
48 Hrs
72 Hrs
96 Hrs
Panel B

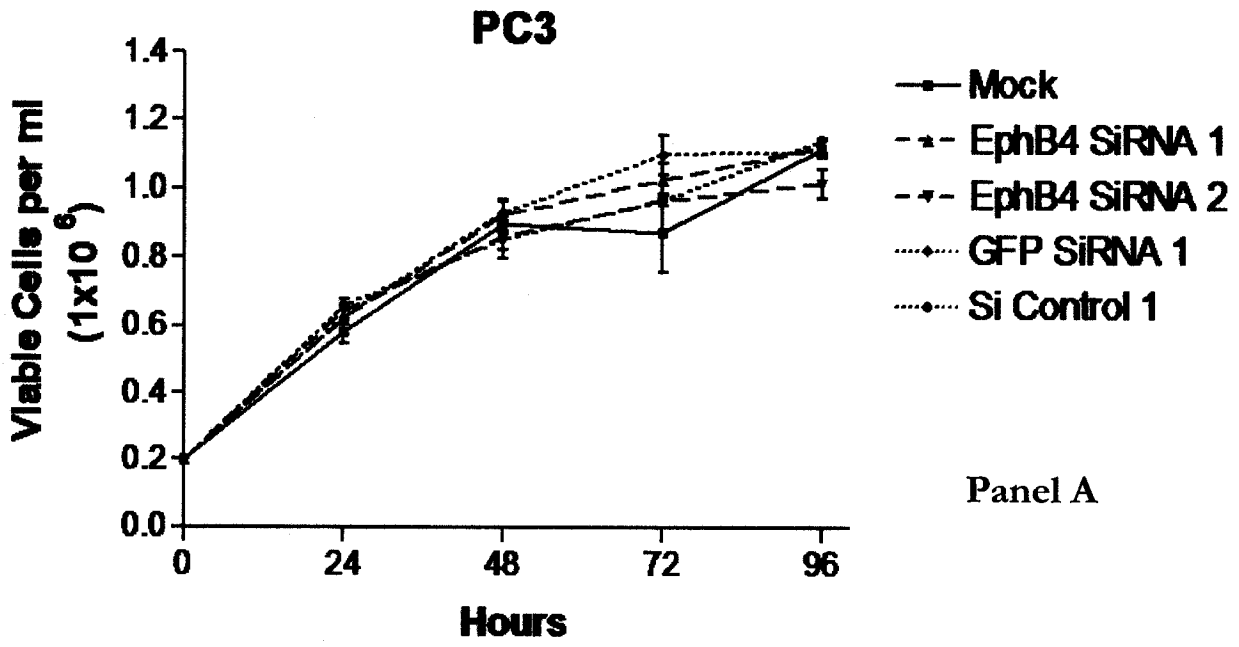
Figure 11

To test proliferation of prostate cancer, the total number cells attached to the plate were counted at 24, 48, 72 and 96 hrs post-transfection using the ViCell XR Coulter counter. Transfection of PC3, Du-145 and LNCaP cells with either EphB4 siRNA 1 or 2 did not significantly affect cell viability as compared to mock, GFP siRNA or Nontargeting siRNA controls (Figure 12).

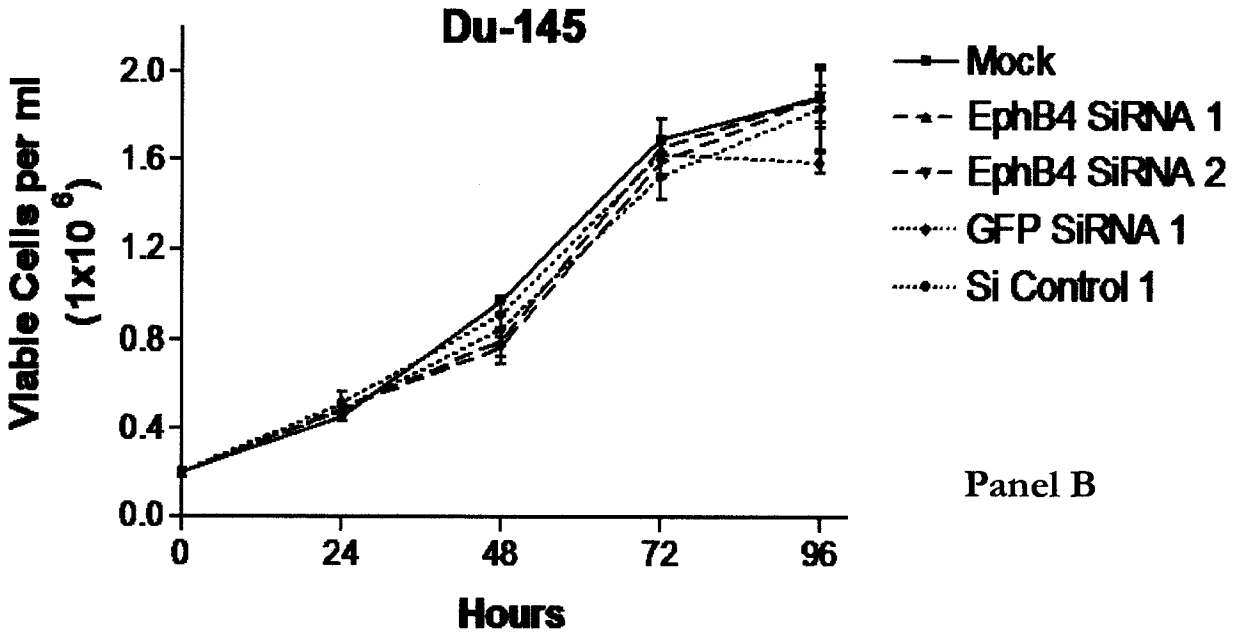
To determine effects on proliferation of prostate carcinoma cell lines following activation of EphB4, exogenous EphrinB2-Fc was added to PC3 and Du-145 cells grown in triplicate in 12-well plates. Treated cells were compared to unstimulated or IgG-stimulated controls over 24, 48, and 72 hrs. Three concentrations of EphrinB2-Fc and IgG were used to determine if increasing the concentration would show a trend in proliferation. Total number of PC3 and Du-145 cells was then determined using ViCell XR Coulter counter. Addition of EphrinB2-Fc did not significantly affect proliferation of prostate carcinoma cell lines when compared to IgG control (Figure 13). Furthermore, no particular trend in proliferation, be it increased or decreased, was observed in PC3 and Du-145 cells when the concentration of EphrinB2-Fc increased from 0.1, 1.0, to 3.0ug/ml.

As a confirmation of the lack of effect on cell viability we observed in our previous studies, we also examined whether stimulation of EphB4 with EphrinB2-Fc resulted in any changes in the phosphorylation of AKT. As recently mentioned in a breast cancer study, stimulating EphB4 with EphrinB2-Fc in MCF-7 cells increased phosphorylation of AKT and hence may provide advantages in increased cancer cell survival (53). We decided to test this in our prostate cancer study. Activation of AKT in our system was measured using western blot analysis of phospho-serine 473 AKT as compared to total AKT levels in PC3 and Du-145 cells stimulated with 3.0ug/ml EphrinB2-Fc as compared to same concentration of IgG

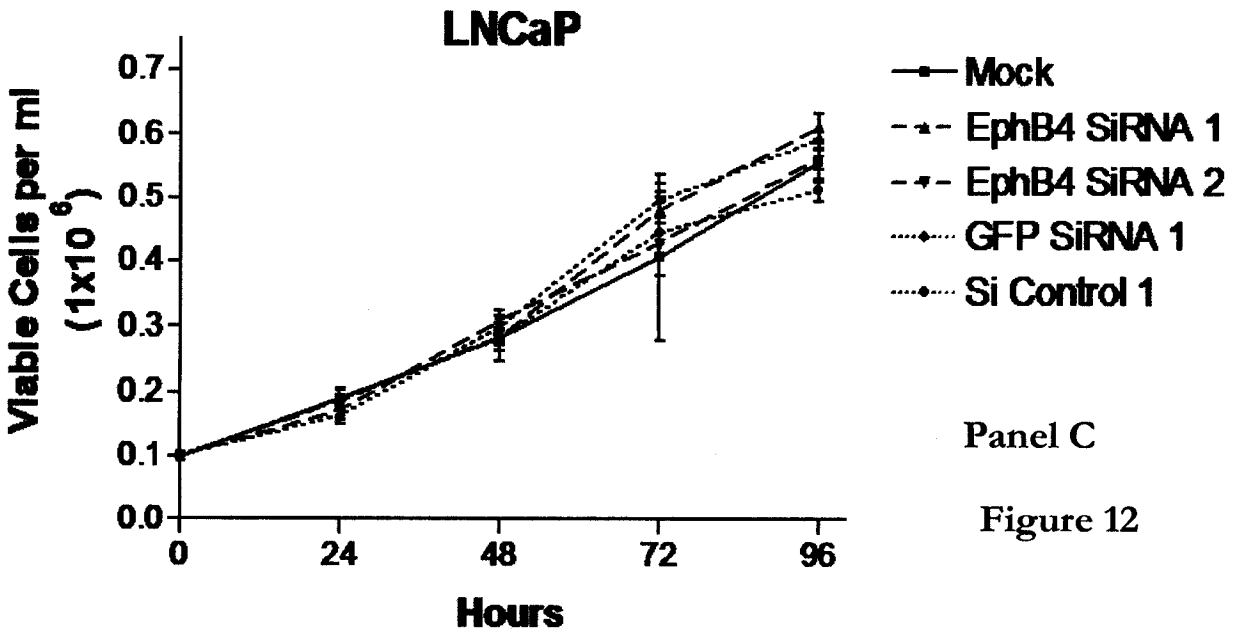
Figure 12. Proliferation of Prostate Cancer Cell Lines after Depletion of EphB4 Using RNA Interference. PC3 (A), Du-145 (B), and LNCaP (C) cells were plated in triplicate and treated with 50nM of various EphB4 specific siRNAs. Negative controls used were mock (PBS) transfection, GFP siRNA 1 and Nontargeting Control siRNA (siControl 1). Dead and floating cells were aspirated, and attached cells were collected at 24, 48, 72 and 96 hrs with trypsin-EDTA, and counted using a Coulter counter. Statistical analysis (unpaired students t-test) did not reveal significant changes in cell proliferation following siRNA reduction of EphB4 protein when compared to negative controls. Graph is representative of the mean of triplicate wells and error bars are 95% Confidence Intervals. The experiment was repeated two independent times for each prostate carcinoma cell lines and similar results were obtained.



Panel A



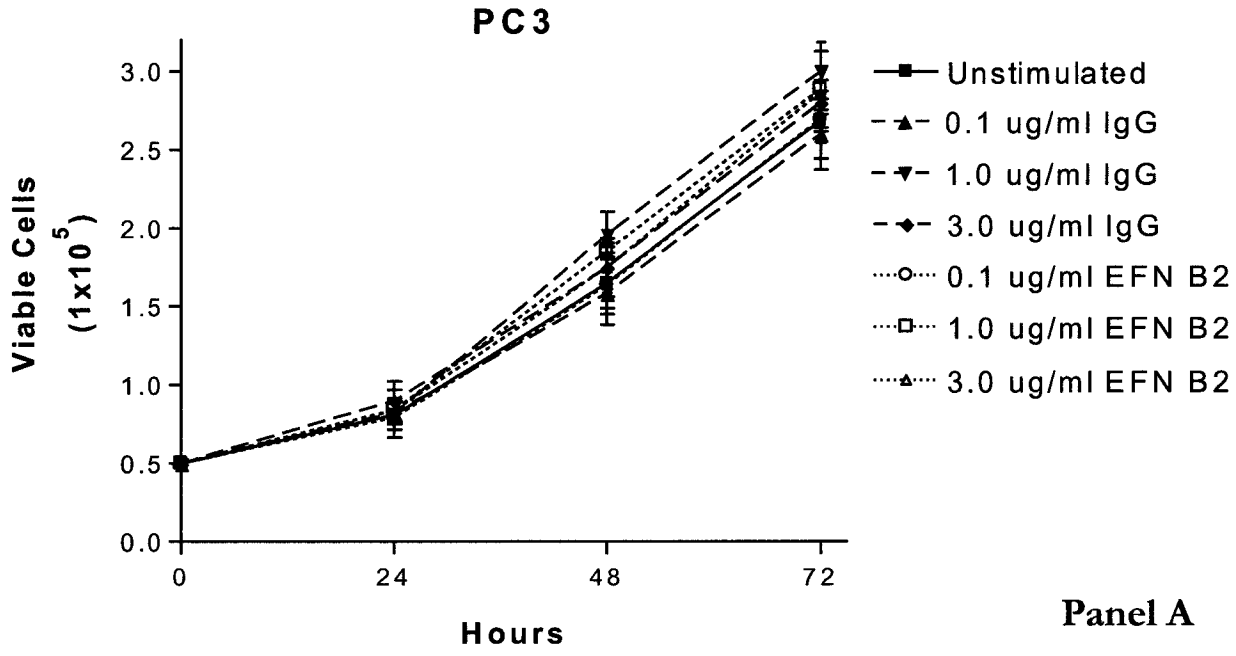
Panel B



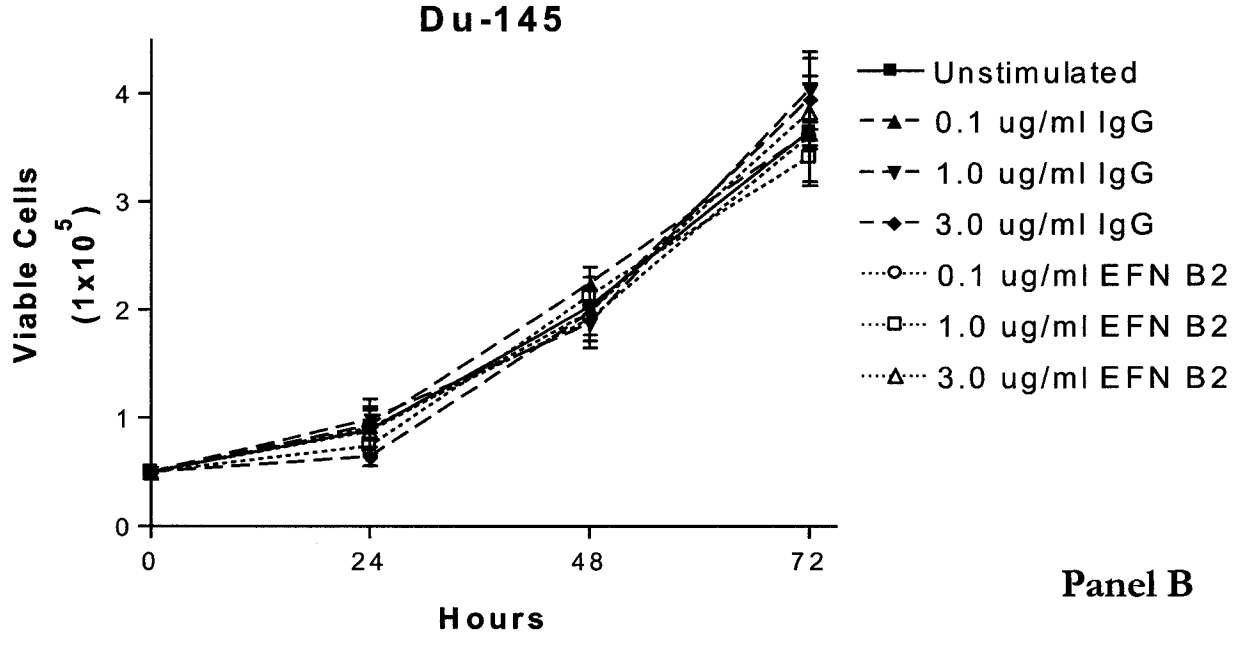
Panel C

Figure 12

Figure 13. Effect of EphrinB2-Fc stimulation of EphB4 on Cell Proliferation. PC3(A) and Du-145(B) cells were plated in triplicate and treated with varying concentrations of EphrinB2-Fc or IgG as a control. At 24, 48, and 72 hrs post-stimulation, media was aspirated to remove dead and floating cells, and attached cells were collected with trypsin-EDTA and counted using a Coulter counter. Statistical analysis did not reveal any significant differences in proliferation of PC3 and Du-145 cells between EphrinB2-Fc and IgG treated cells. Graphs are a representative of the mean of three wells and error bars signify 95% Confidence Intervals. The experiment was repeated independently twice with PC3 and Du-145 cells with similar results.



Panel A



Panel B

Figure 13

as a negative control. The anti-phosphoserine AKT antibody detected activated AKT just below the 60kDa mark, however no significant differences in serine 473 phosphorylation was observed in PC3 cells treated with EphrinB2-Fc or IgG (Figure 14). We next tested AKT phosphorylation in the Du-145 cell line which have wild type PTEN expression. Again, no significant differences in AKT phosphorylation were observed between EphrinB2-Fc and IgG treated Du-145 cells (Figure 15). Although, a noticeable increase in AKT phosphorylation was observed at 45mins post-stimulation, the increase was equivalent in both EphrinB2-Fc and IgG treated cells and thus is not a result of EphB4 activation by EphrinB2-Fc (Figure 15, Lanes 5 and 6). This is likely a result of the addition of media containing 1%FBS at the start of the experiment. A positive control using 5%FBS was included in the experiment to intentionally phosphorylate AKT to show that AKT in Du-145 cells may be phosphorylated. Indeed, our results confirmed a darker band at 6hrs in lysates stimulated with media containing 5%FBS compared to 6hrs with 1%FBS. Taken together, our results demonstrate that manipulating EphB4 levels does not significantly affect cell viability or AKT phosphorylation. Therefore, we believe that EphB4 does not play a direct role in prostate cancer cell proliferation or survival.

3.7 Measure of Tumor Cell Migration

One of the important indicators of aggressive metastatic prostate cancer is the inherent ability of prostate carcinoma cells to migrate and invade. We have previously observed significant overexpression of EphB4 in prostate cancer cell lines, particularly in PC3 and LNCaP cell lines, and marginal overexpression in Du-145 cells when compared to the level of EphB4 expression in normal prostate epithelial cells (Figure 5). As EphB RTKs play an

Figure 14. Effect on AKT Phosphorylation Following Stimulation of EphB4 Receptor With EphrinB2-Fc in PC3 Cells. PC3 cells were serum starved overnight and then stimulated with DMEM/1%FBS supplemented with either 3ug/ml EphrinB2-Fc or IgG as a control. Cells were lysed at various timepoints post-stimulation and 40ug of total protein was immunoblotted and probed with anti-phosphoAKT antibody to detect phosphorylation at serine 473. No significant differences in phosphorylation of AKT were observed between 3ug/ml EphrinB2-Fc and 3ug/ml IgG treated PC3 cells. Membranes were stripped, checked with ECL, and re-probed with anti-AKT antibody to determine equal loading. (Lanes 13, 14) PC3 cells were also stimulated DMEM/5%FBS with EphrinB2-Fc or IgG (respectively) as additional controls to intentionally phosphorylate AKT with serum. The experiment was repeated twice with similar results.

PC3

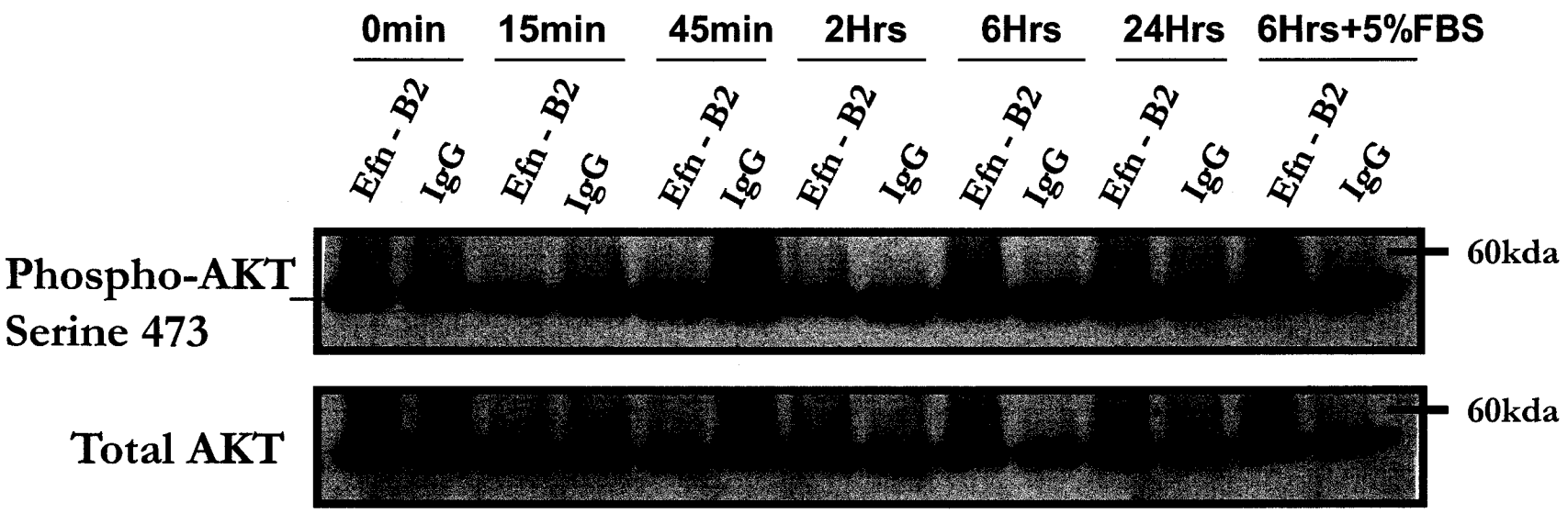


Figure 14

Figure 15. Effect on Phosphorylation of AKT after EphB4 Receptor Stimulation with EphrinB2-Fc in Du145 Cells. Du-145 cells were tested for AKT activation after treating with 3ug/ml EphrinB2-Fc or 3ug/ml IgG as a control. Cells were serum starved and then stimulated with DMEM/1%FBS serum containing either EphrinB2-Fc or IgG. Cells were then lysed at various timepoints post-stimulation. A total of 40ug of total protein was immunoblotted and probed with anti-phosphoAKT antibody to detect phosphorylation at serine 473. No significant changes in phosphorylation of AKT were observed between EphrinB2-Fc or IgG treated Du-145 cells. Membranes were stripped, checked with ECL, and re-probed with anti-AKT antibody to determine equal loading. Controls; Lanes 13 and 14 show significant phosphorylation of AKT when Du145 cells were given DMEM/5%FBS as compared with DMEM/1%FBS although this overexpression was equivalent regardless of whether cells were stimulated with EphrinB2-Fc or IgG.

Du-145

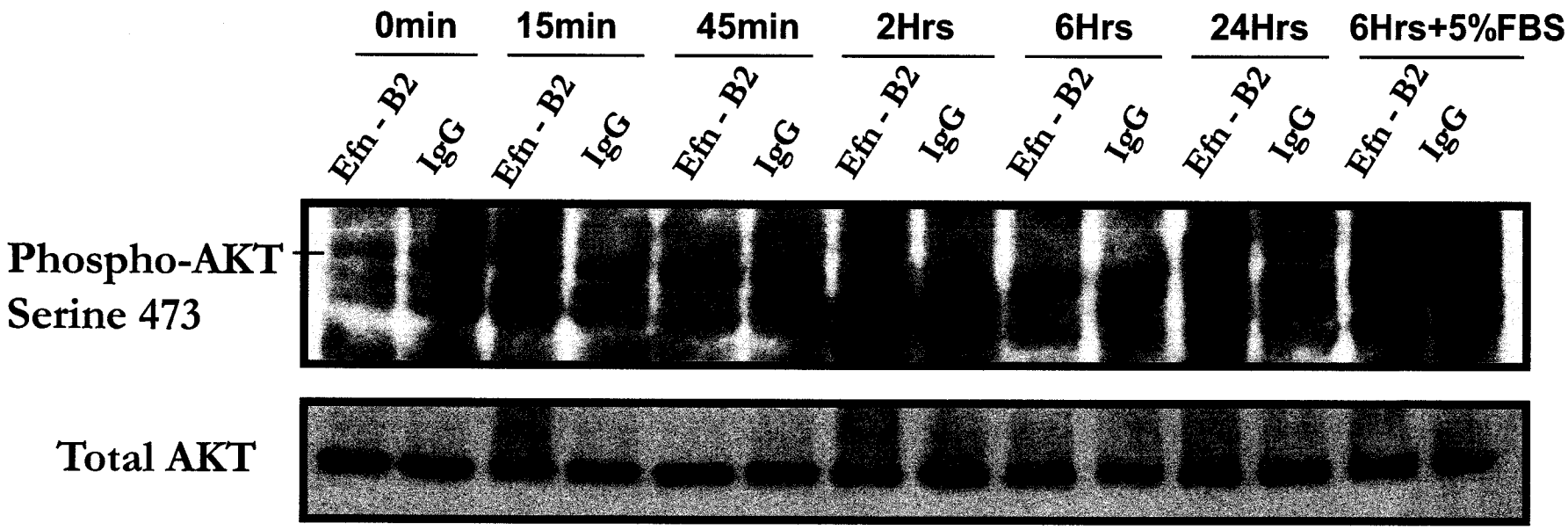


Figure 15

important role in directing axonal growth cone and cell migration during early development and maturation of the nervous system, and overexpression of EphB4 specifically, has been previously implicated in increased migration of vascular endothelial cells (22), we hypothesized that EphB4 receptor signaling plays an important role in cell migration and hence tumor cell metastasis. In support of this, EphB4 overexpression has also been correlated with more malignant and metastatic tumors (89). As this correlation may be a result of an inherent increased ability to migrate and invade, we were interested to examine whether EphB4 played a role in these important functions in PC3, LNCaP and Du-145 prostate carcinoma cells.

In order to examine the role of EphB4 in the migration of prostate cancer cell lines, we used a scratch wound-healing migration assay. Initially, we reduced EphB4 expression using siRNA treatment and, alternatively, we activated EphB4 using EphrinB2-Fc stimulation. In order to assure that EphB4 expression and its activity were not affected by scratching cell monolayers and the generation of the wound itself, two initial control experiments were conducted. Monolayers of PC3 cells were scraped multiple times making straight wounds, and were subsequently lysed at 0, 2, 12 and 24 hrs to generate total protein extracts which were then resolved on SDS-PAGE gel. Western blot analysis of EphB4 showed that monolayer wounding by scratching did not directly result in changes in EphB4 receptor expression over time (Appendix Figure 4A). In addition, EphB4 was also immunoprecipitated from total protein lysates, and western blot analysis for phosphotyrosine determined that no significant differences in phosphorylation of EphB4 occurred as a result of wounding the cell monolayer (Appendix Figure 4B).

(3.7.1) Effect of EphB4 reduction on cell migration

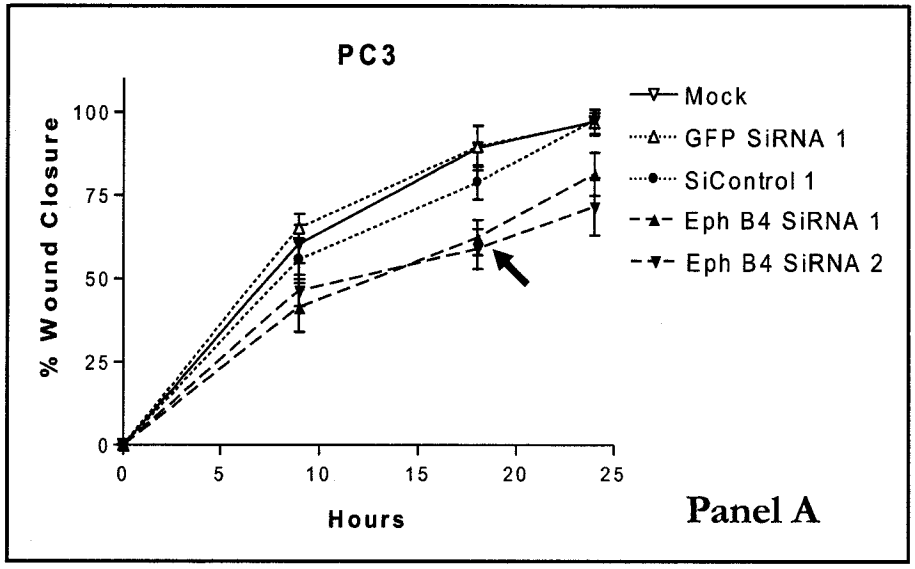
The scratch wound-healing assay was used to assess the effect of EphB4 in cell migration. Targeting EphB4 in PC3 cells decreased wound closure (Figure 16A and 17). The significant decrease in PC3 cell migration was confirmed in the experiment conducted using both sequences of the EphB4 siRNAs (EphB4 siRNA 1 and EphB4 siRNA 2) and had a p value of <0.05 at 18 and 23 hrs when compared to GFP siRNA 1 and siControl 1. The decrease in migration could be noted as early as 10 hrs after wounding, which was equivalent to 58 hrs after siRNA transfection, and stayed below the mean cell migration in control conditions throughout the duration of the assay (Figure 16A). To avoid bias and show consistency in measuring wound closure, the wound closure was independently measured by a second person in a blinded fashion. The results from the independently conducted blind measurements supported our initial findings that knocking down EphB4 protein in PC3 cells reduced cell migration (Figure 16B). Similarly, statistically significant differences in migration were observed in 3 independent experiments following pooling of the results (Figure 16C). To provide an indication for the kinetics of EphB4 depletion and ensure reduced levels were maintained throughout the duration of our migration assay, western blot analysis confirmed that efficient reduction of EphB4 protein as compared to mock and control siRNA occurred at time of wounding and persisted at reasonable levels during the assay (Figure 16D). Furthermore, photographs taken from the wound-healing migration assay at 23 hrs show that the wound did not completely close in PC3 cells treated with EphB4 specific siRNA when compared to mock and control siRNAs (Figure 17).

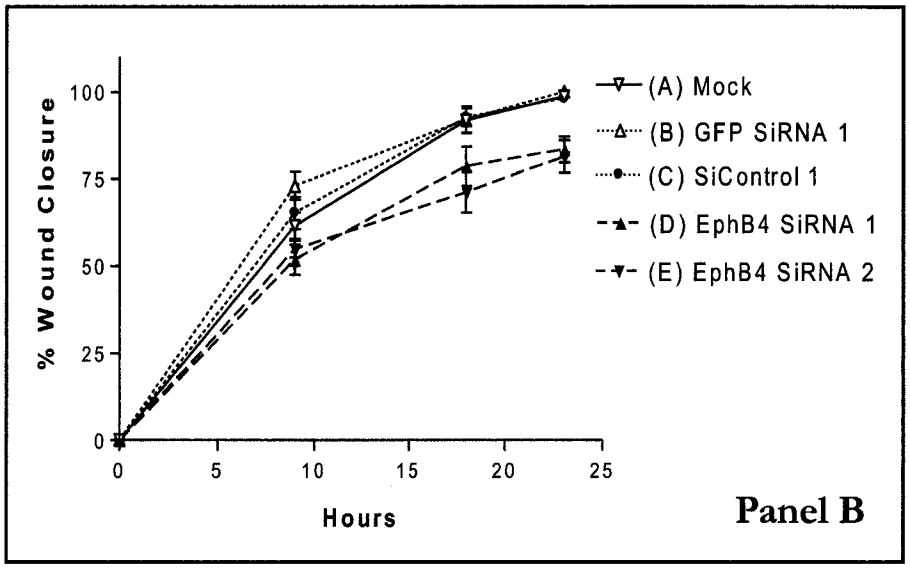
Other prostate cancer cell lines were also tested in a similar fashion to determine whether the role of EphB4 protein in cell migration was specific to PC3 cells or could be

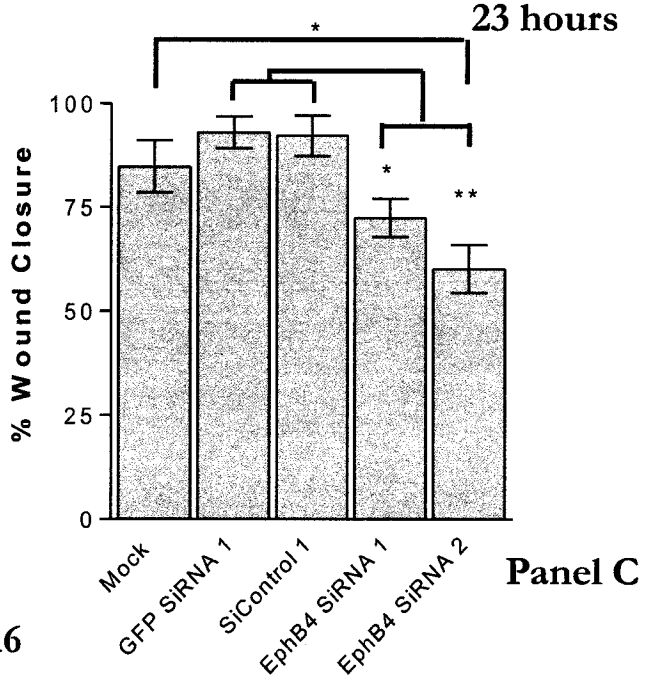
generalized to a variety of prostate cancer cells. To this end, we performed similar scratch wound assays in other prostate cancer cell lines, specifically Du-145 and LNCaP. Similar to what we observed in PC3 cells, we noted significant decreases in cell migration with both Du-145 (Figure 18, 19) and LNCaP (Figure 20, 21) cells following treatment with EphB4 specific siRNAs. Du-145 cells treated with EphB4 siRNA 1 or EphB4 siRNA 2 showed only ~75% wound closure compared to completely closed wounds in control siRNA and mock transfected cells (Figure 18A). Independent scoring, in a blinded fashion, of wound healing migration assay confirmed our initial results (Figure 18B). Western blot analysis of protein lysates taken from Du-145 cells confirmed that EphB4 protein was still reduced at the end of the migration assay, 31 hrs post wounding (55 hrs post transfection) (Figure 18D). Figure 19 is a representative collection of photographs showing Du-145 cells with impaired migration after being transfected with EphB4 siRNA 1 or EphB4 siRNA 2 compared to control siRNA and mock treated cells. LNCaP cells with reduced EphB4 protein levels particularly showed a large decrease in cell migration when compared to mock and Nontargeting control siRNA treated cells (Figure 20A). A blinded and independently scored wound-healing migration assay confirmed that LNCaP cells with reduced EphB4 indeed had impaired migration compared to controls (Figure 20B). The percent wound closure measured in EphB4 siRNA 1 and EphB4 siRNA 2 treated cells was approximately 75% and 50%, respectively, at 48 hrs post scratching of the wound (96 hrs post transfection), which is significantly lower than complete wound closures observed in mock and Nontargeting control siRNA conditions (Figure 20C, 21). Western blot analysis of protein lysates taken from LNCaP cells confirmed that EphB4 protein was still reduced at the end of the migration assay (Figure 20D).

Figure 16. Effect of Reduced EphB4 Levels Using siRNA Treatment on Cell Migration in PC3 Cells. PC3 cells were treated with 50nM concentrations of EphB4 siRNA 1, EphB4 siRNA 2, Nontargeting Control siRNA (siControl 1), GFP siRNA 1, or were mock treated by addition of transfection reagent alone. Cell monolayers were wounded 48 hrs after transfection and the percentage wound closure was measured and calculated at various times post wounding. (A) Line graph representing the mean wound closure with associated standard error over time for the various conditions. The data points at each time interval of every variable are averages of 16 measurements taken from 4 photographs (4 measurements per picture) across a single wound. Results show that cell migration is decreased starting at 10 hrs and remains decreased up to 23 hrs in PC3 cells that are treated with EphB4 siRNA when compared to control siRNAs, and mock. (B) Results from an independently blindly measured wound assay showing similar decreases in cell migration following EphB4 reduction. (C) Bar graph representing the mean percent wound closure with associated standard error from average results of three independent experiments measured at 23 hrs. A significant decrease in cell migration is again observed with the pooled data in PC3 cells treated with siRNA to reduce EphB4 levels. (D) A representative western blot of EphB4 demonstrating reduced protein levels at the end of the wounding assay. At 48 hrs cells were lysed from the migration assay and probed for EphB4 using western blotting. Error bars in line graphs and bar graphs represent standard error of measurements (SEM). * represents $p<0.05$ ** represents $p<0.01$.

PC3







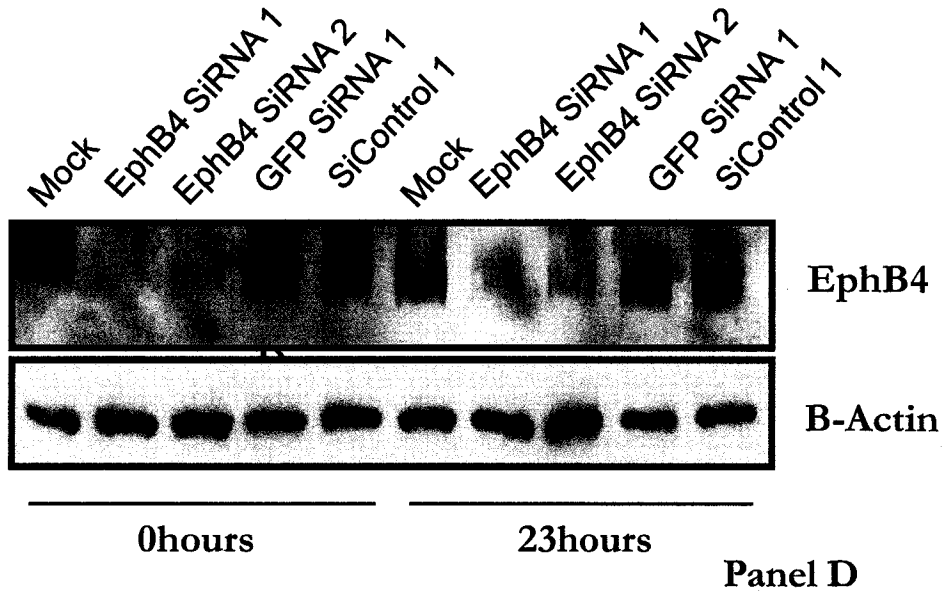


Figure 16

Figure 17. Representative Photographs of Migrating PC3 Cells in Scratch Wound Assay. Representative photographs of PC3 cells taken at 100x magnification with a digital camera attached to the phase contrast microscope showing a typical result from the wound-healing migration assay. Results show that PC3 cell migration is reduced when EphB4 levels are decreased as noted by the incompletely closed wound at 23 hrs in PC3 with reduced EphB4 protein as compared to controls.

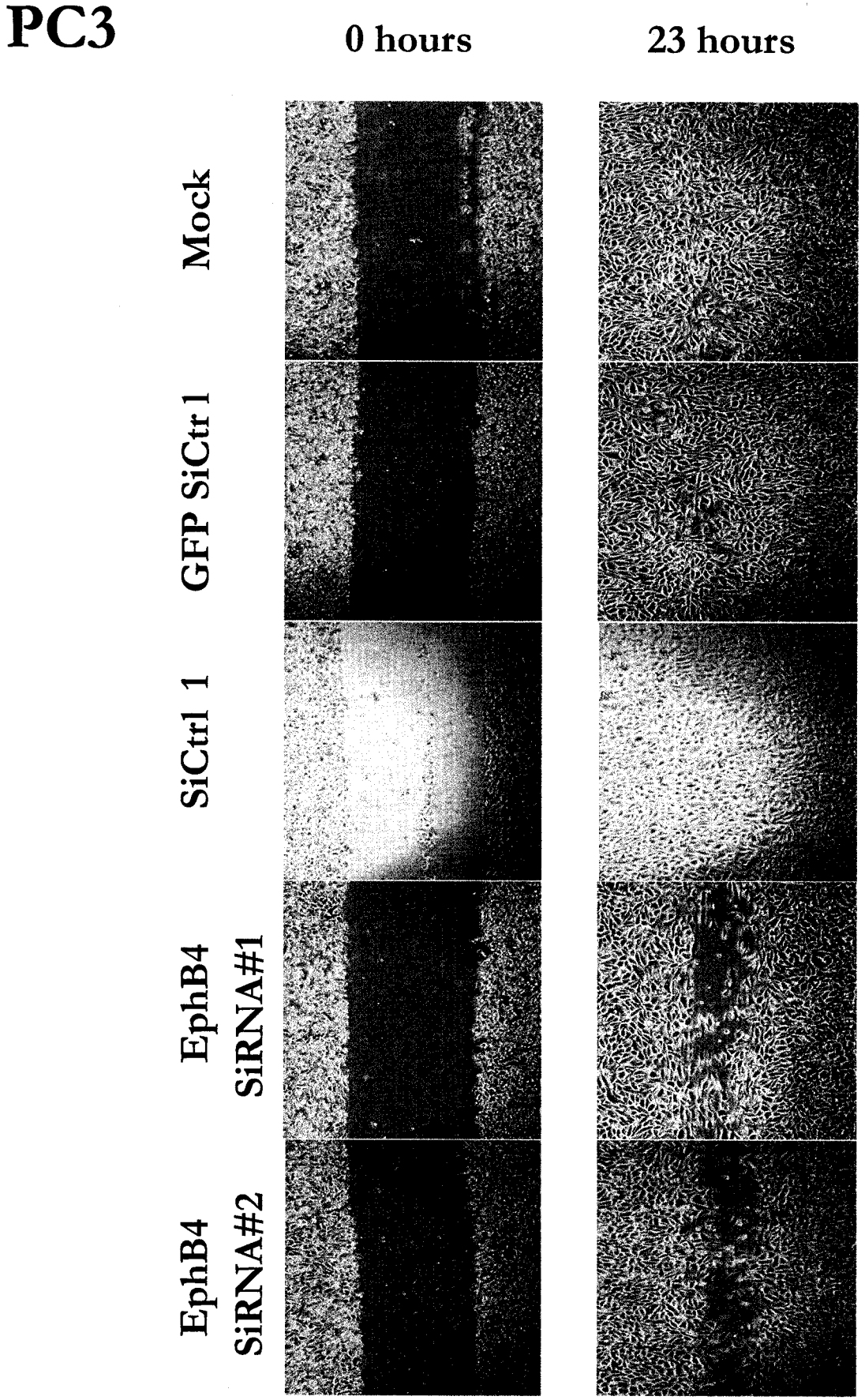
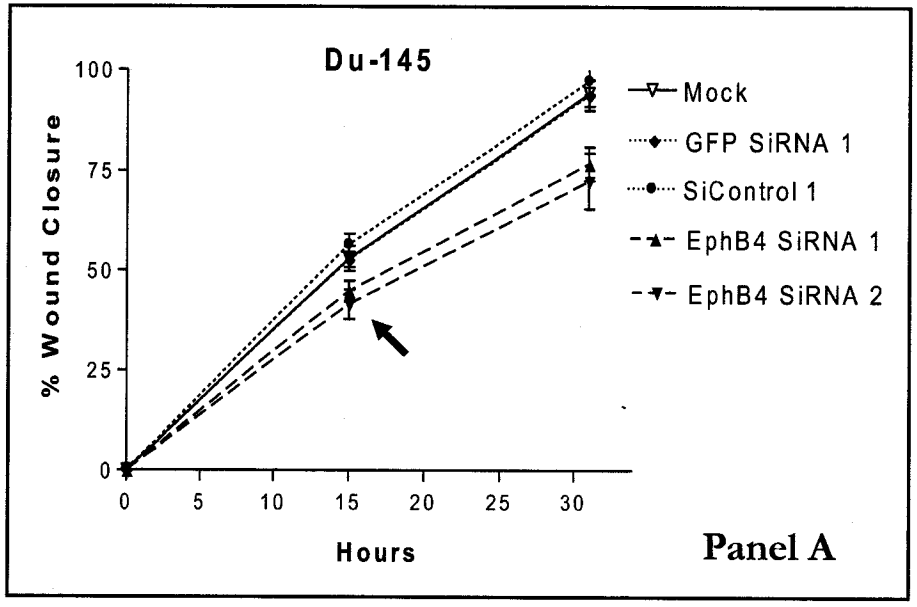
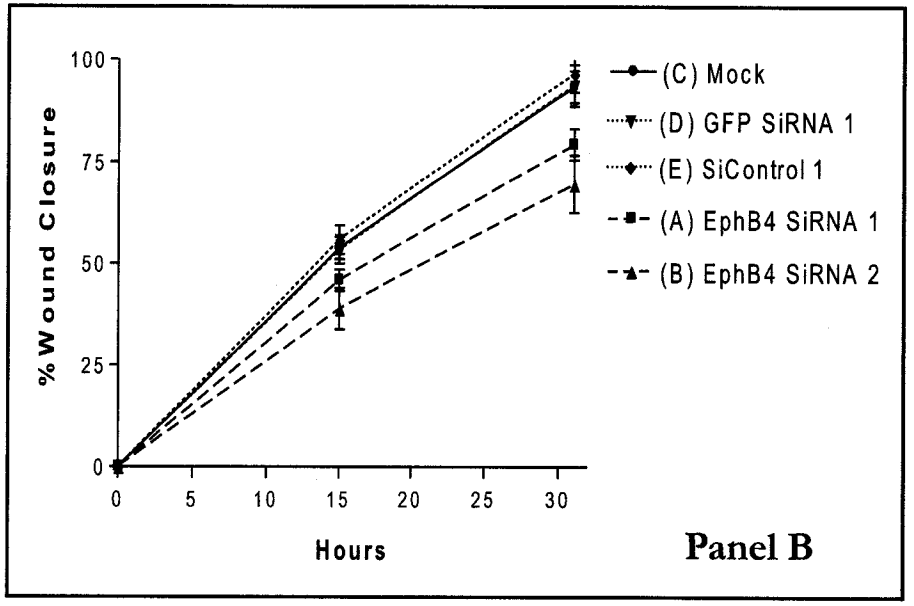

PC3
0 hours
23 hours
Mock
GFP SiCtr 1
SiCtrl 1
EphB4 SiRNA#1
EphB4 SiRNA#2

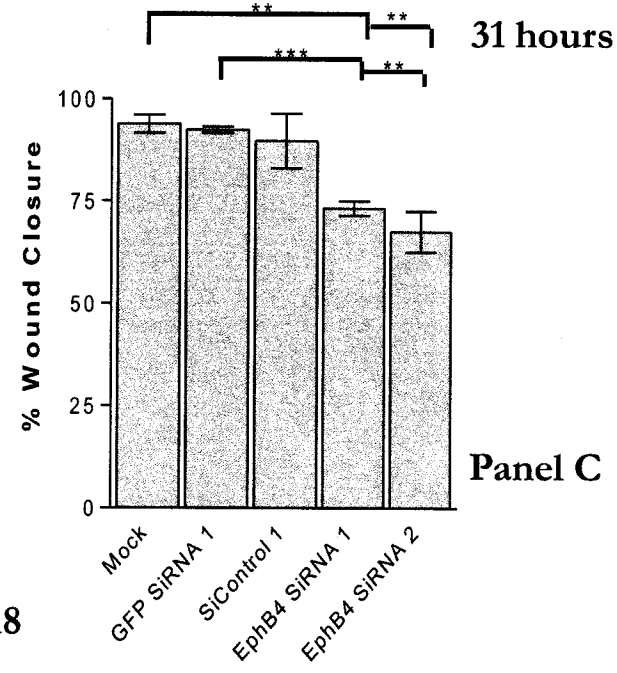
Figure 17

Figure 18. Effect of Reduction of EphB4 Protein Levels Using siRNA on the Migration of Du-145 Cells. Du-145 cells were treated with 50nM concentrations of EphB4 siRNA 1, EphB4 siRNA 2, Nontargeting Control siRNA (siControl 1), GFP siRNA 1, or were mock transfected. Cells were wounded 24 hrs after transfection. (A) Line graph representing the mean wound closure with associated standard error over time for the various conditions. The data points at each time interval of every variable are averages of 16 measurements taken from 4 photographs (4 measurements per picture) across a single wound. Results show that cell migration is decreased starting at 15 hrs and remains decreased up to 31 hrs post wounding in Du-145 cells that are treated with EphB4 siRNA when compared to control siRNAs, and mock. (B) Results from an independently blindly measured wound assay showing similar decreases in cell migration following EphB4 reduction. (C) Bar graph representing the mean percent wound closure with associated standard error from average results of three independent experiments measured at 31 hrs. A significant decrease in cell migration was again observed from the pooled data in Du-145 cells treated to reduce EphB4. (D) A representative western blot of EphB4 to demonstrate that reduced levels of EphB4 have persisted to the end of the scratch wound assay. At 48 hrs cells were lysed from the migration assay and probed for EphB4 using western blotting. Error bars in line graphs and bar graphs represent SEM. ** represent $p<0.01$ *** represents $p<0.001$.

Du-145







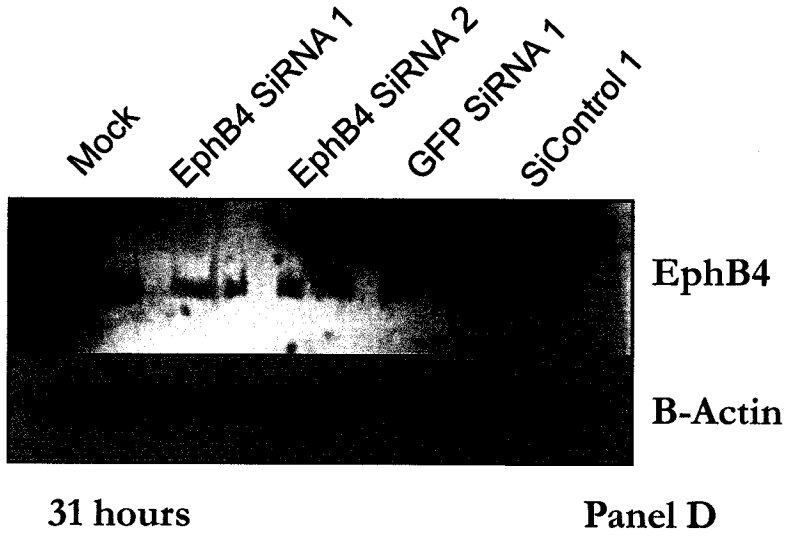


Figure 18

Figure 19. Representative Photographs of Migrating Du145 Cells in Scratch Wound Assay. Representative photographs of Du145 cells taken at 100x magnification with a digital camera attached to the phase contrast microscope showing a typical result from the wound-healing migration assay. Results show that Du145 cell migration is reduced when EphB4 levels are decreased as noted by the incompletely closed wound at 31 hrs in Du145 cells with reduced EphB4 protein as compared to controls.

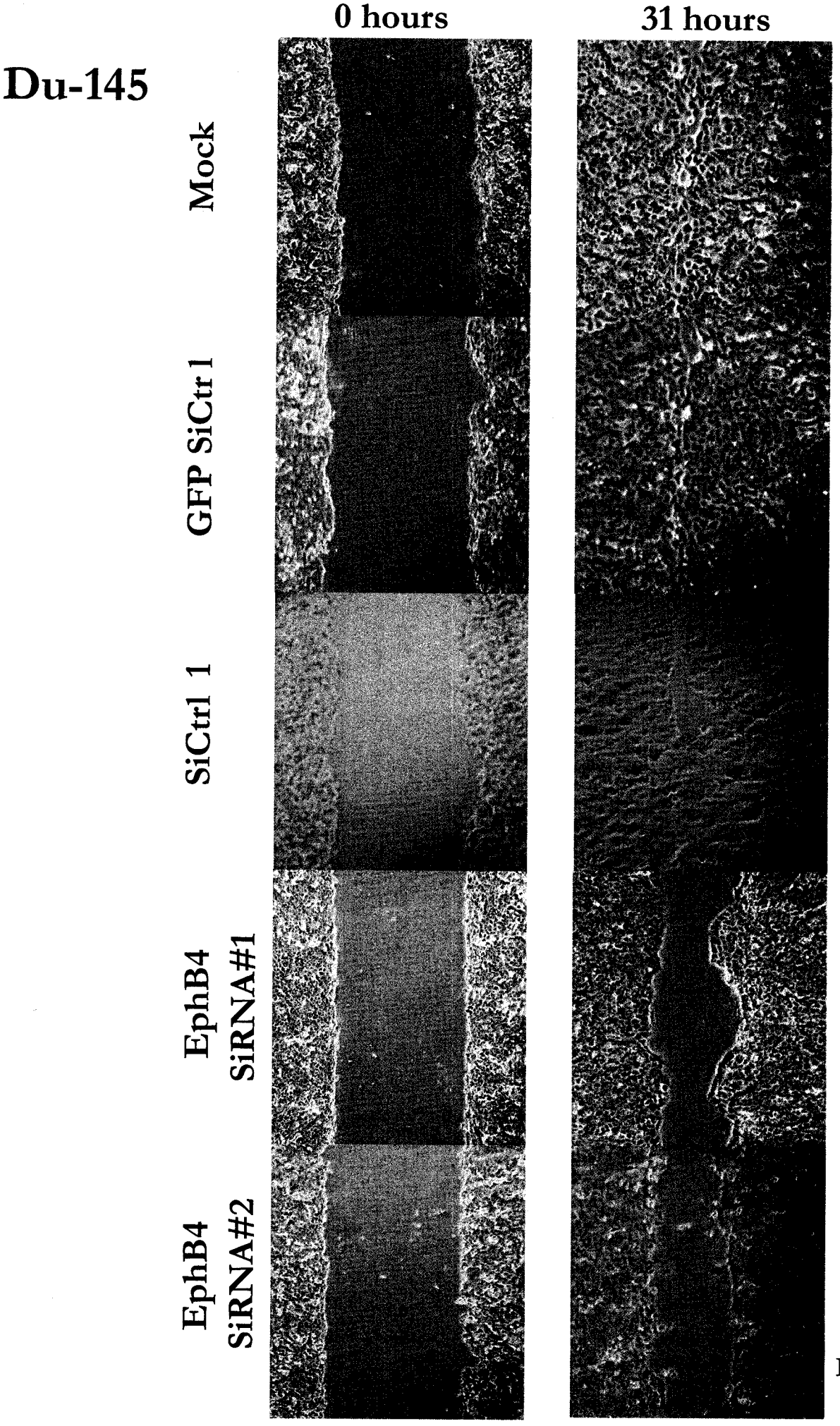
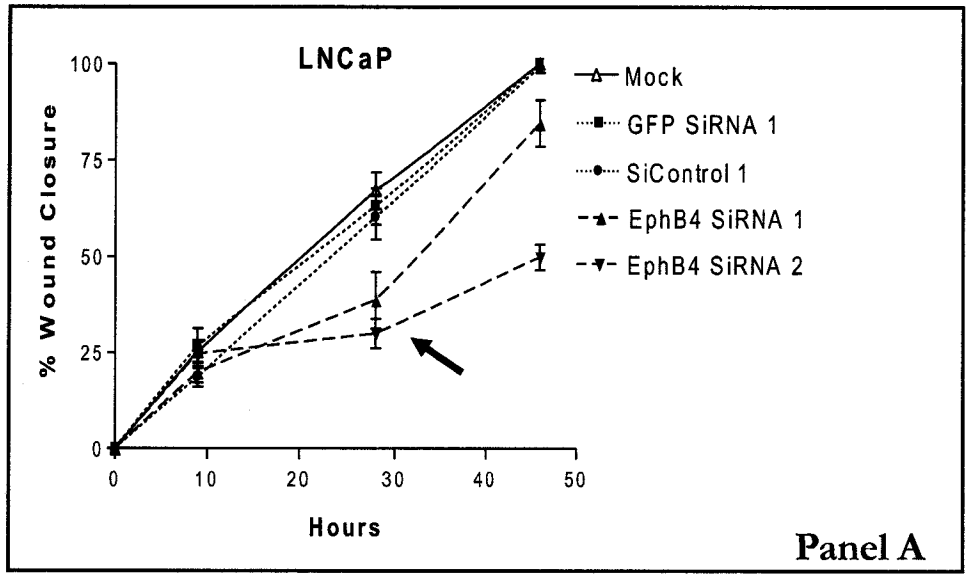
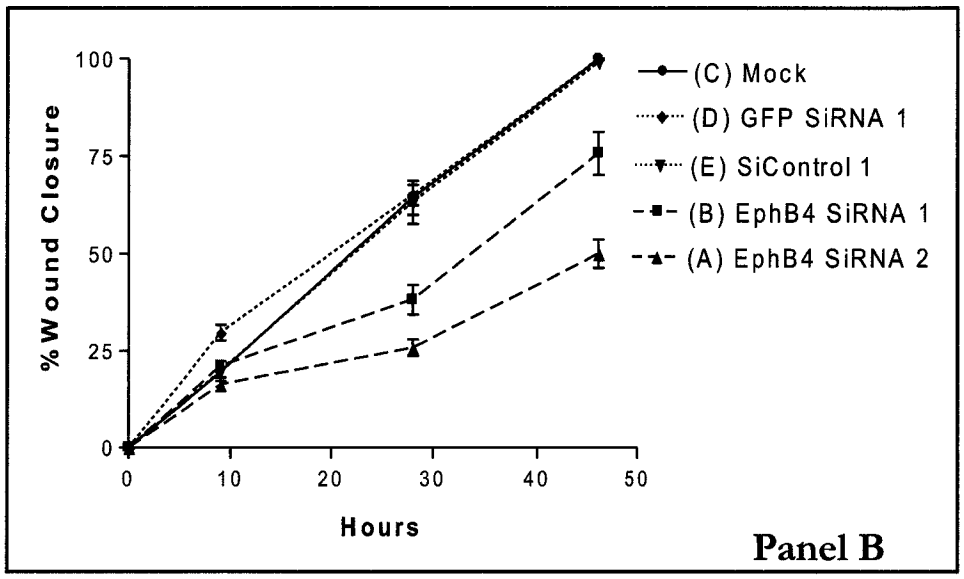

Du-145
0 hours
31 hours
Mock
GFP SiCtr 1
SiCtrl 1
EphB4 SiRNA#1
EphB4 SiRNA#2

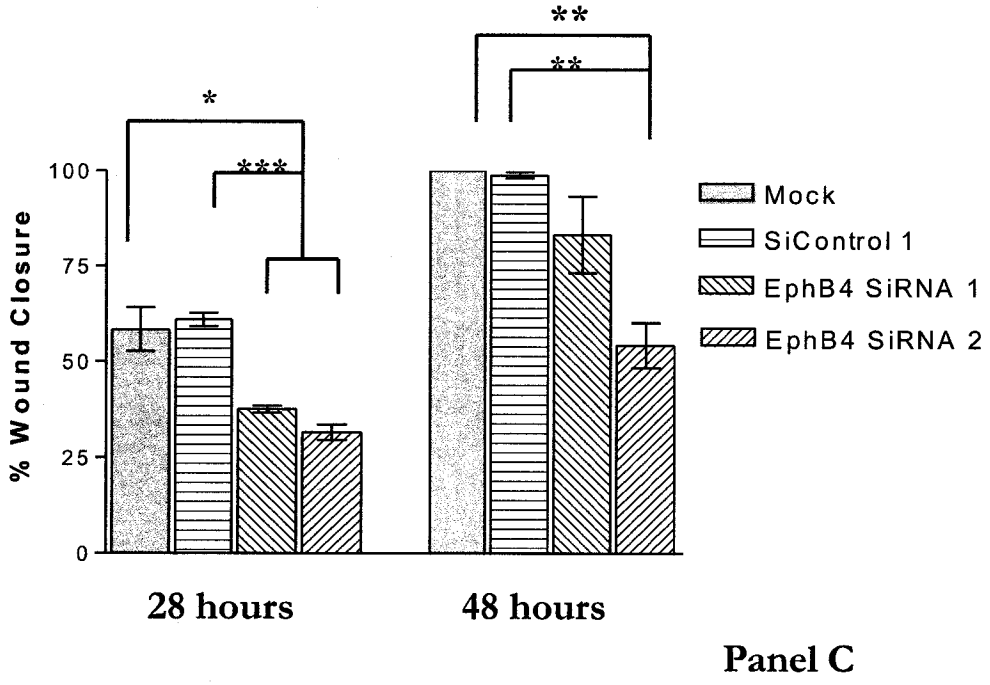
Figure 19

Figure 20. Effect of Reduction of EphB4 Protein Levels Using siRNA on Migration of LNCaP Cells. LNCaP cells were treated with 50nM concentrations of EphB4 siRNA 1, EphB4 siRNA 2, Nontargeting Control siRNA (siControl 1), or were mock transfected. Cells were wounded 48 hrs after transfection. (A) Line graph representing the mean wound closure with associated standard error over time for the various conditions. The data points at each time interval of every variable are averages of 16 measurements taken from 4 photographs (4 measurements per picture) across a single wound. Results show that cell migration is decreased in LNCaP cells that are treated with EphB4 siRNA when compared to control siRNAs, and mock. (B) Results from an independently blindly measured wound assay showing similar decreases in cell migration following EphB4 reduction. (C) Bar graph representing the mean percent wound closure with associated standard error from average results of three independent experiments measured at 28 and 48 hrs. A significant decrease in cell migration is again observed with the pooled data in LNCaP cells treated to reduce EphB4. (D) A representative western blot of EphB4 to demonstrate that reduced levels of EphB4 have persisted to the end of the scratch wound assay. At 48 hrs cells were lysed from the migration assay and probed for EphB4 using western blotting. Error bars in line graphs and bar graphs represent SEM. * represents $p<0.05$, **represents $p<0.01$ and *** represents $p<0.001$.

LNCaP







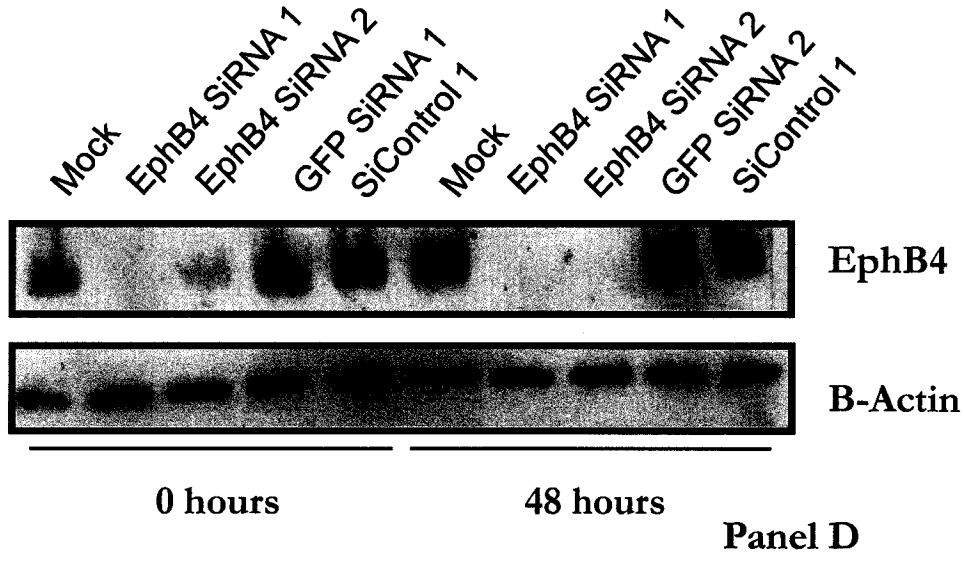


Figure 20

Figure 21. Representative Photographs of Migrating LNCaP Cells in Scratch Wound Assay. Representative photographs of LNCaP cells taken at 100x magnification with a digital camera attached to the phase contrast microscope showing a typical result from the wound-healing migration assay. Results show that LNCaP cell migration is reduced when EphB4 levels are decreased as noted by the incompletely closed wound at 48 hrs in LNCaP cells with reduced EphB4 protein as compared to controls.

LNCaP

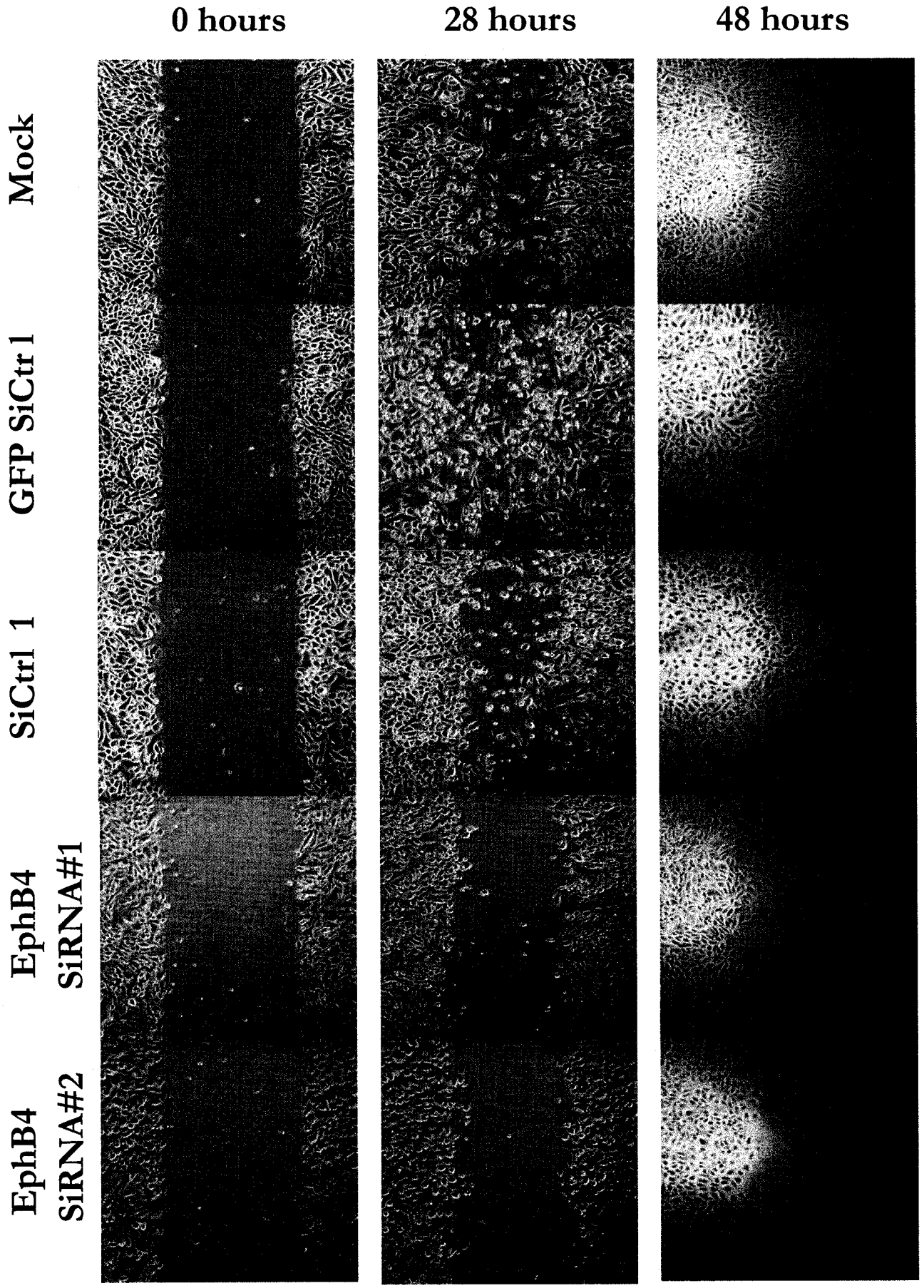


Figure 21

(3.7.2) Effect of EphB4 receptor stimulation on cell migration

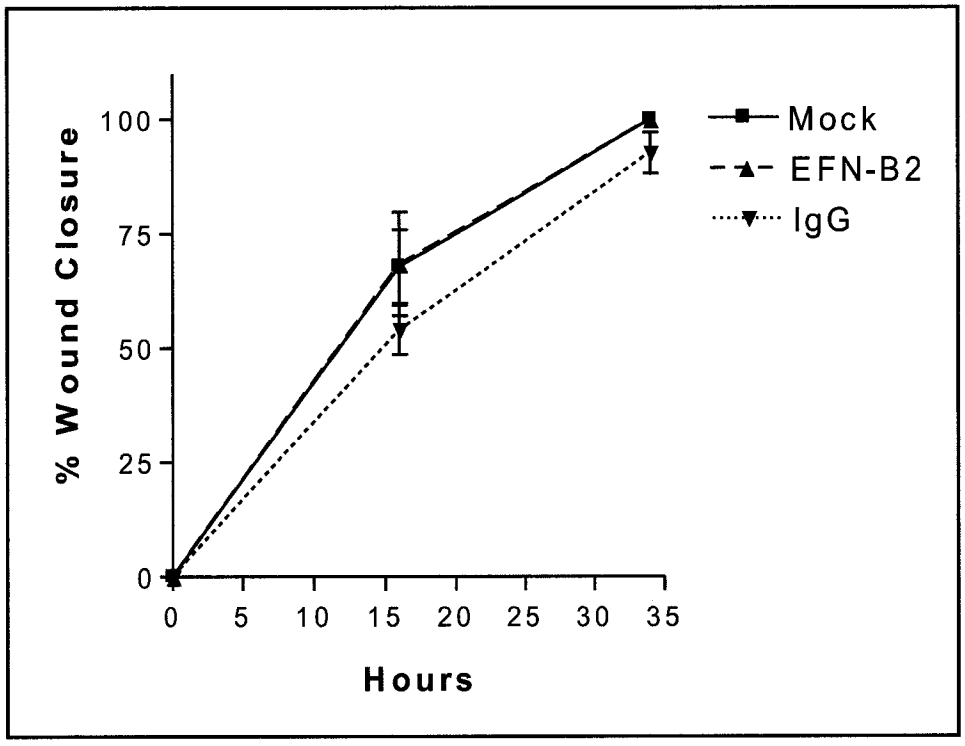
The effect of activation of EphB4 on cell migration in prostate carcinoma cell lines following stimulation with exogenous EphrinB2-Fc was also tested using scratch wound-healing migration assays. PC3 cell lines were grown into a confluent monolayer, and were scraped with a p-200 pipette tip to create the wound. Subsequently, 3ug/ml EphrinB2-Fc, to activate EphB4 receptor signaling, or 3ug/ml IgG, as a negative control, was added to the media. Results from the migration assay determined that cell migration did not increase in EphrinB2-Fc treated PC3 cells when compared to the appropriate IgG controls. The modest differences observed in migration were not statistically significant when compared to mock controls (Figure 22A, 23). Similar experiments were independently conducted using PC3 cells and the average percent wound closure from three experiments confirmed that no significant differences in cell migration were observed following activation of EphB4 with EphrinB2-Fc (Figure 22B). We were interested to confirm this result in another prostate carcinoma cell line, hence performed similar experiments using Du-145. However, Du-145 cells treated with 3ug/ml EphrinB2-Fc did not show an increase in cell migration compared to 3ug/ml IgG (Figure 24A, 25), although the pooled data from three separate wounds displayed a marginal, yet statistically insignificant increase in cell migration (Figure 24B).

(3.7.3) Downstream activation of Rho GTPases with EphrinB2-Fc

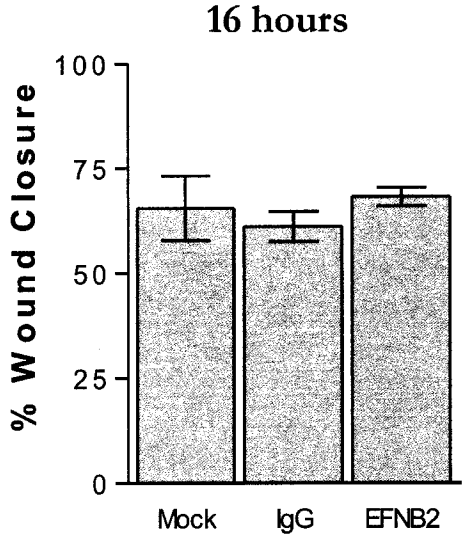
Cell migration is controlled by remodeling of the actin cytoskeleton following activation of downstream pathways such as the members of the Rho GTPases. Activated Rac and Cdc42 have previously been reported to play a major role in prostate cancer cell migration. We were interested to determine if activation of the EphB4 tyrosine kinase

Figure 22. Migration of PC3 Cells Following Stimulation of EphB4 Receptor with Soluble EphrinB2-Fc Ligand. PC3 cell monolayers were wounded by scratching to create a wound of set diameter, and were subsequently stimulated with 3ug/ml EphrinB2-Fc or 3ug/ml IgG or were mock treated by addition of PBS. Photographs were taken at initial time of wounding (0hrs), at 16hrs, and 34hrs post wounding. (A) Line graph representing the mean wound closure with associated standard error over time for the various conditions. The data points at each time interval of every variable are averages of 16 measurements (4 measurements per picture) taken from 4 photographs across a single wound. Migration of PC3 cells was marginally increased in EphrinB2-Fc stimulated cells as compared to IgG, although, not statistically significant. (B) Bar graph representing the mean percentage wound closure and associated standard error from average results of three independent experiments after 16 hrs post stimulation of PC3 cells with EphrinB2-Fc or IgG. The experiment was performed three independent times with similar results. Error bars in line graph and bar graphs represent SEM.

PC3



Panel A



Panel B

Figure 22

Figure 23. Representative Photographs of Migrating PC3 Cells in Scratch Wound Assay. Representative photographs of PC3 cells taken at 100x magnification with a digital camera attached to the phase contrast microscope showing a typical result from the wound-healing migration assay. Results show that PC3 cell migration is slightly increased when EphB4 is stimulated by addition of EphrinB2-Fc as noted by reduced width of the wound at 16hrs as compared to controls, however subsequent quantification of differences did not result in statistical significance.

PC3

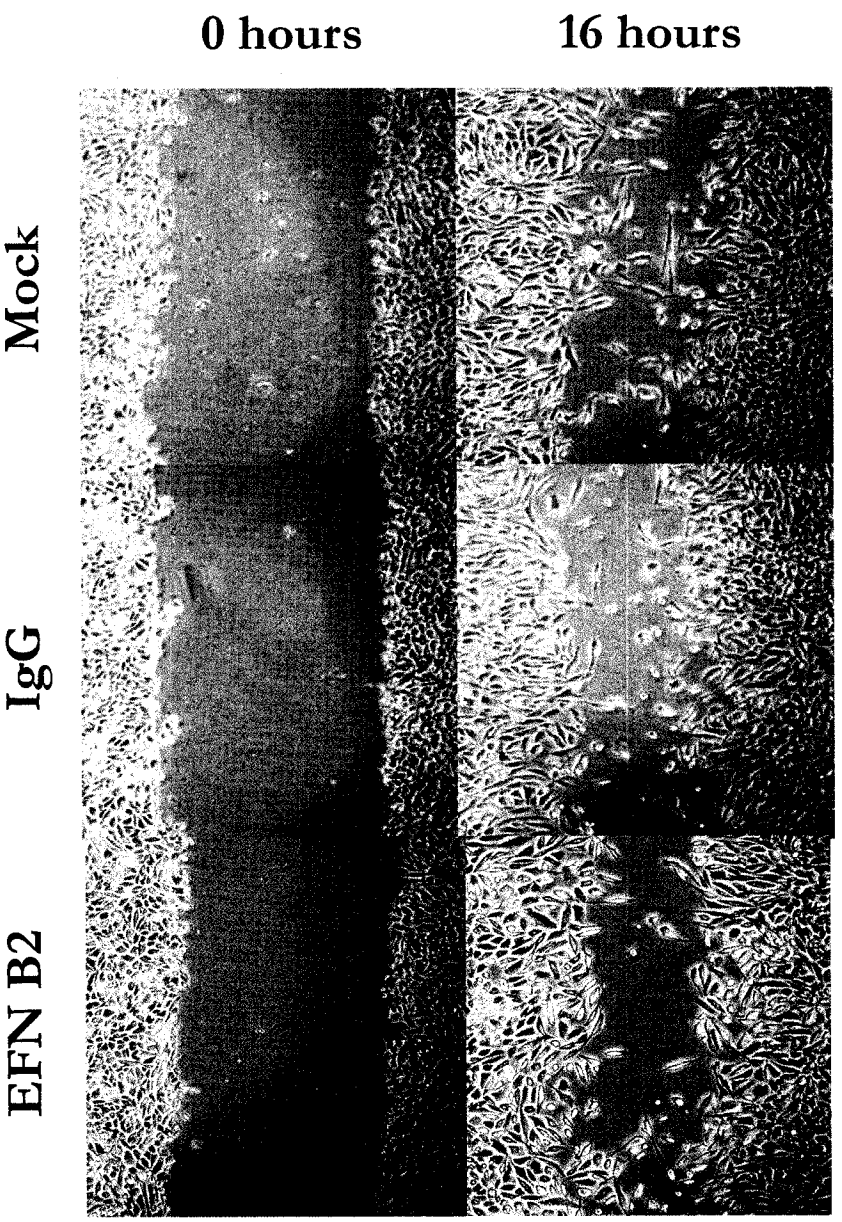
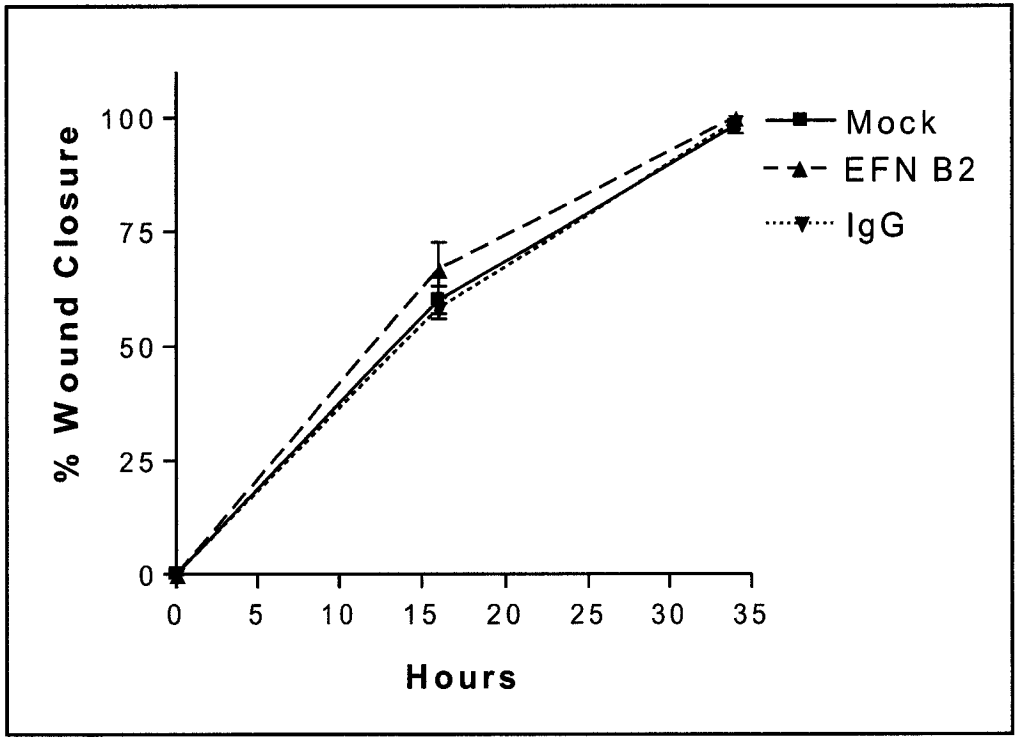


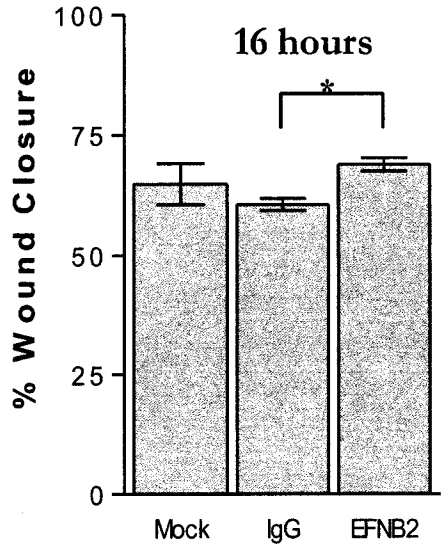
Figure 23

Figure 24. Migration of Du-145 Cells Following Stimulation of EphB4 Receptor with Soluble EphrinB2-Fc Ligand. Monolayers of Du-145 cells were scratched to create a wound of set diameter, and were subsequently stimulated by addition of 3ug/ml EphrinB2-Fc or 3ug/ml IgG, or were mock treated by addition of PBS. Photographs were taken at initial time of wounding (0hrs), at 16hrs, and 34hrs post wounding. (A) Line graph representing the mean percentage wound closure and associated standard error measured from 16 data points obtained from measuring the width of the wound at each time point and comparison to wound width at time zero. Migration of Du145 cells was marginally increased in EphrinB2-Fc stimulated cells as compared to IgG or mock controls. (B) Bar graph representing the mean percentage wound closure and associated standard error of average results from three independent experiments 16 hrs post stimulation and wounding of Du145 cells with EphrinB2-Fc or IgG. The experiment was performed three independent times with similar results. Error bars represent SEM (A, B). * represents $p<0.05$.

Du-145



Panel A



Panel B

Figure 24

Figure 25. Representative Photographs of Migrating Du145 Cells in Scratch Wound Assay. Representative photographs of Du145 cells taken at 100x magnification with a digital camera attached to the phase contrast microscope showing a typical result from the wound-healing migration assay. Results show that Du145 cell migration is increased when EphB4 is stimulated by addition of EphrinB2-Fc as noted by reduced width of the wound at 16hrs as compared to controls. However, following quantification results did not reach statistical significance.

Du-145

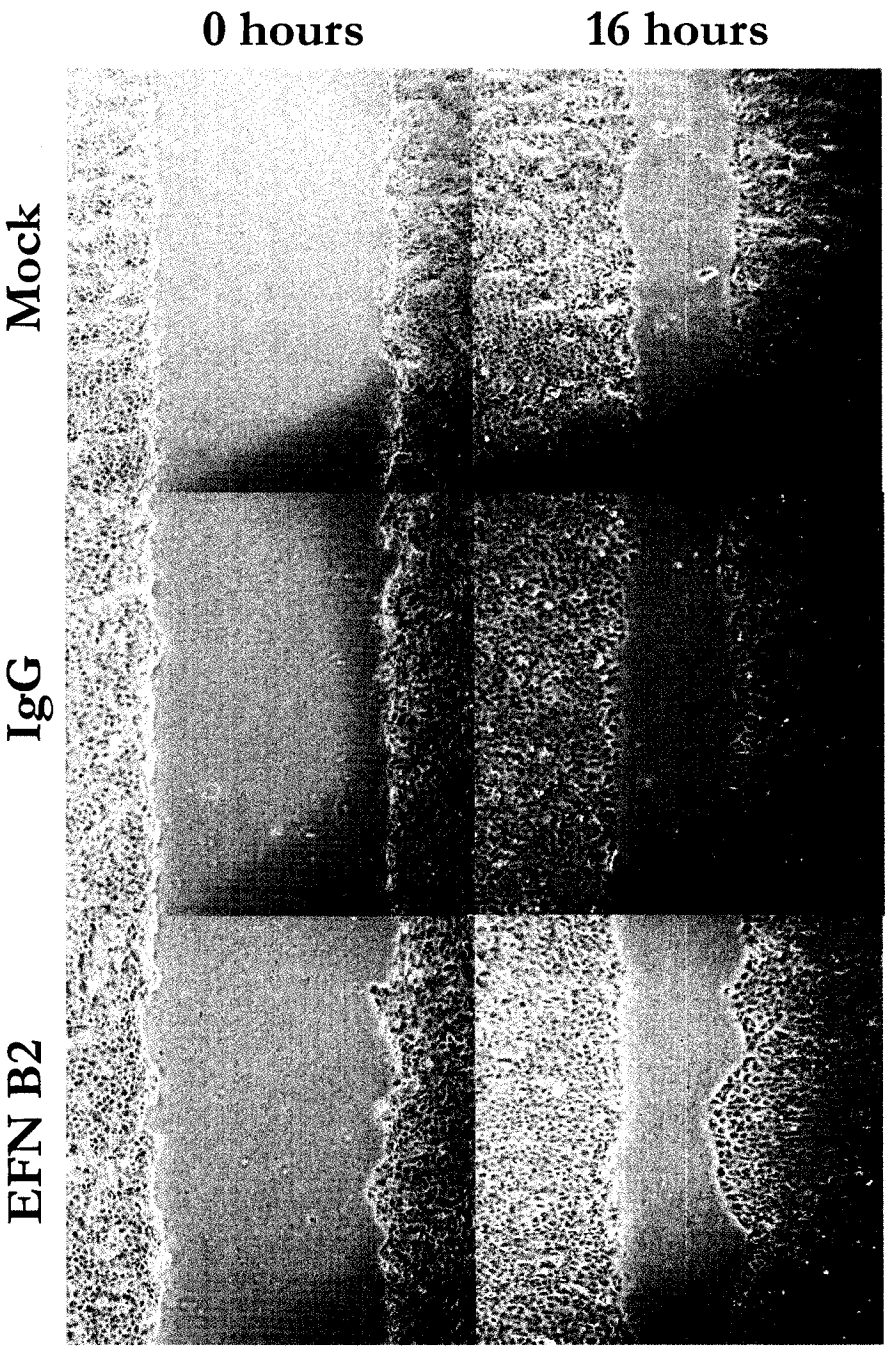
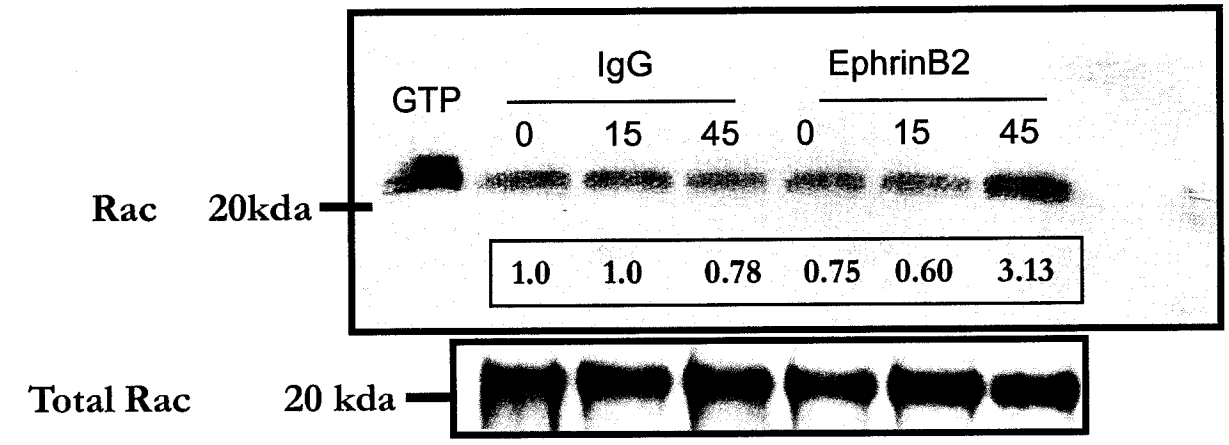


Figure 25

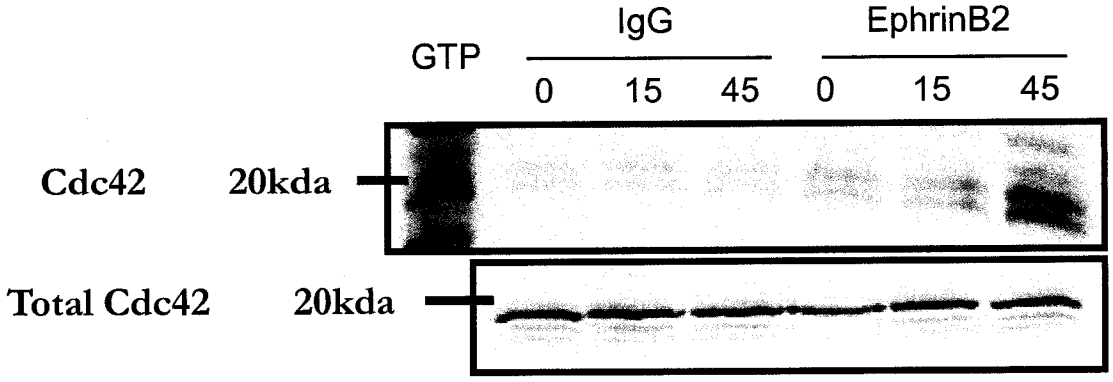
receptor by its ligand EphrinB2 played any role in activating downstream Rac and Cdc42, and thereby promoting cell migration in prostate cancer cell lines. PC3 cells were thus stimulated with 3ug/ml EphrinB2-Fc and the activation of Rac, as compared to that observed following stimulation with 3ug/ml IgG as a negative control, was examined over time. A GST-PAK fusion protein pull-down assay was conducted on total cell lysates and subsequently analyzed by western blot analysis to determine if EphrinB2-Fc activation of EphB4 led to increased activation of Rac or Cdc42. When lysates obtained 45 min post stimulation of PC3 cells were examined, we noticed an increase in the level of activated Rac observed in cells treated with EphrinB2-Fc as compared to IgG control. This was approximately a 3-fold increase when compared to IgG stimulation at the same time point (Figure 26A). PC3 cells treated with 3ug/ml EphrinB2-Fc also showed an increase in the activation of Cdc42 when compared to 3ug/ml IgG treated cells at 45 min (Figure 26B). Therefore, our data supports the contention that activation of EphB4 by its ligand results in the promotion of cell migration through downstream activation of Rac and Cdc42.

In summary, we discovered that reduction of EphB4 protein levels using siRNA interference in prostate carcinoma cell lines, PC3, Du-145, and LNCaP significantly reduced their ability to undergo directional cell migration. In support of this, we also observed that stimulation of EphB4 receptor activity, using addition of soluble EphrinB2-Fc ligand, resulted in a small increase in cell migration in Du-145 cells when compared to IgG controls, although, there wasn't a significant difference in migration seen in PC3 cells. EphB4 protein could be playing a part in cell migration possibly through activation of downstream RhoGTPase family of proteins, namely Rac and Cdc42, which are widely-known proteins involved in actin cytoskeleton reorganization.

Figure 26. Effect of EphB4 Activation on Rac and Cdc42 Activity. PC3 cells were stimulated with 3ug/ml of EphrinB2-Fc or 3ug/ml IgG over 0min, 15min, or 45min, at which time cells were lysed in GST-FISH buffer and 800ug of total protein lysate was incubated with 50ul GST-PAK bound beads to bind activated Rac or Cdc42. Beads were then washed with GST-FISH buffer and the bead pellet was resuspended in Laemmli buffer before subjecting to SDS-PAGE gel electrophoresis and subsequent western blot analysis. (A) Levels of active Rac were determined following western blot analysis using a specific anti-Rac antibody that detected Rac slightly above 20kDa as indicated by the positive control, GTP (Lane1). Rac was significantly activated at 45 min as demonstrated by the increased level of active Rac isolated by the GST-PAK binding assay. Densitometry was used to quantitate the results which were normalized to IgG at 0min using Genetools by Syngene. (B) Levels of active Cdc42 were determined in a similar manner to that described above, except that anti-Cdc42 antibody was used to detect Cdc42, which was visualized by immunoreactive bands at ~20kDa. Similar to Rac, Cdc42 appeared to be noticeably activated at 45 min following addition of EphrinB2-Fc ligand as compared to IgG controls. The positive control represents the total amount of GTP-bound GTPase present in cell lysate. The total amount of Rac and Cdc42 in PC3 cells following treatment with either EphrinB2-Fc or IgG was not affected. The experiment was repeated twice with similar results.



Panel A



Panel B

Figure 26

3.8 Measure of Prostate Cancer cell Invasion

The ability of prostate carcinoma cells to invade through basement membrane of tissues and organs is an important indicator of the metastatic potential of cancer. Since we noticed a significant reduction in cell migration following reduction of EphB4 protein in prostate carcinoma cell lines, we hypothesized that EphB4 may also be linked to cell invasion. We tested this hypothesis using PC3 cells, first by using siRNAs to reduce EphB4 protein levels, and secondly, by activating EphB4 receptor using addition of exogenous EphrinB2-Fc ligand.

(3.8.1) Effect of EphB4 depletion in cell invasion

PC3 cells were treated with siRNA to EphB4 or various control siRNA constructs, and 48hrs post-transfection, cells were harvested and $1x10^5$ cells were plated on the top of the matrigel coated transwell filter. After 8 hrs the invading cells were fixed, stained and counted. The results showed that PC3 cells in which EphB4 levels had been reduced using 50nM concentrations of EphB4 siRNA 1 and EphB4 siRNA 2, showed a significant decrease in cell invasion as compared to cells that had been treated with GFP siRNA 1 or mock transfected controls (Figure 27A,B). The bar graph is a representation of the mean of triplicate transwell filters counted for the number of cells that invaded through the matrigel (Figure 27A). Photographs of PC3 cells were taken using a digital camera attached to a phase-contrast microscope and representative pictures from transwell filters are displayed according to their respective treatments (Figure 27B). It is also apparent from the photographs that PC3 cell invasion was reduced as a result of decreased EphB4 protein expression when compared to controls (Figure 27B).

(3.8.2) Effect of EphB4 receptor stimulation on cell invasion

To determine the effect of EphB4 stimulation and activation on PC3 cell invasion, EphB4 protein was stimulated using 1ug/ml or 3ug/ml of recombinant EphrinB2-Fc or 1ug/ml or 3ug/ml IgG as a control, following addition of $1x10^5$ cells to the upper chamber of the transwell filters. The number of cells that had invaded in response to increased concentrations of FBS was determined following fixing, and subsequent staining and counting of cells on the filters. Interestingly, our results showed that cell invasion was significantly increased when PC3 cells were treated with EphrinB2-Fc as compared to IgG (Figure 28A,B). The bar graph shows that the mean number of PC3 cells invading through the matrigel significantly increased with concentrations of 1ug/ml and 3ug/ml EphrinB2-Fc used in comparison to 1ug/ml and 3ug/ml IgG, respectively (Figure 28A). Representative photographs of a quadrant section from the transwell filters are displayed according to their respective treatments, and also show an increase in cell invasion after EphrinB2-Fc stimulation of EphB4 receptor (Figure 28B).

In summary, our results from the matrigel-invasion assay confirmed that EphB4 plays a role in cell invasion of PC3 prostate carcinoma cell lines. The number of PC3 cells invading through the matrigel decreased when EphB4 protein levels were reduced using siRNA, and this data was supported by additional experiments that showed stimulation of EphB4 receptor with its exogenous ligand EphrinB2-Fc, resulted in increased invasion of PC3 cells.

Figure 27. Effect of Reduced Levels of EphB4 Following siRNA Treatment on Cell Invasion. PC3 cells were treated with 50nM each of EphB4 siRNA 1, EphB4 siRNA 2, GFP siRNA 1 or were mock transfected in 6 well plates. Forty-eight hours after transfection, $1x10^5$ PC3 cells were placed in the top portion of the transwells in DMEM in the absence of serum. The bottom well of the transwell inserts was filled with DMEM containing 10%FBS as a stimulus for invasion. After 8 hrs, the PC3 cells that invaded through the matrigel to the underside of the filter were fixed with methanol solution and stained with Diff-Quick solutions. (A) Bar graph is a representation of the mean of the total number of cells and associated standard error that had invaded from triplicate wells. The experiment was repeated three independent times with similar results. * represents $p < 0.05$. (B) Representative photographs of a quadrant section of the filter viewed under the microscope at 200x magnification showing the cells that have invaded.

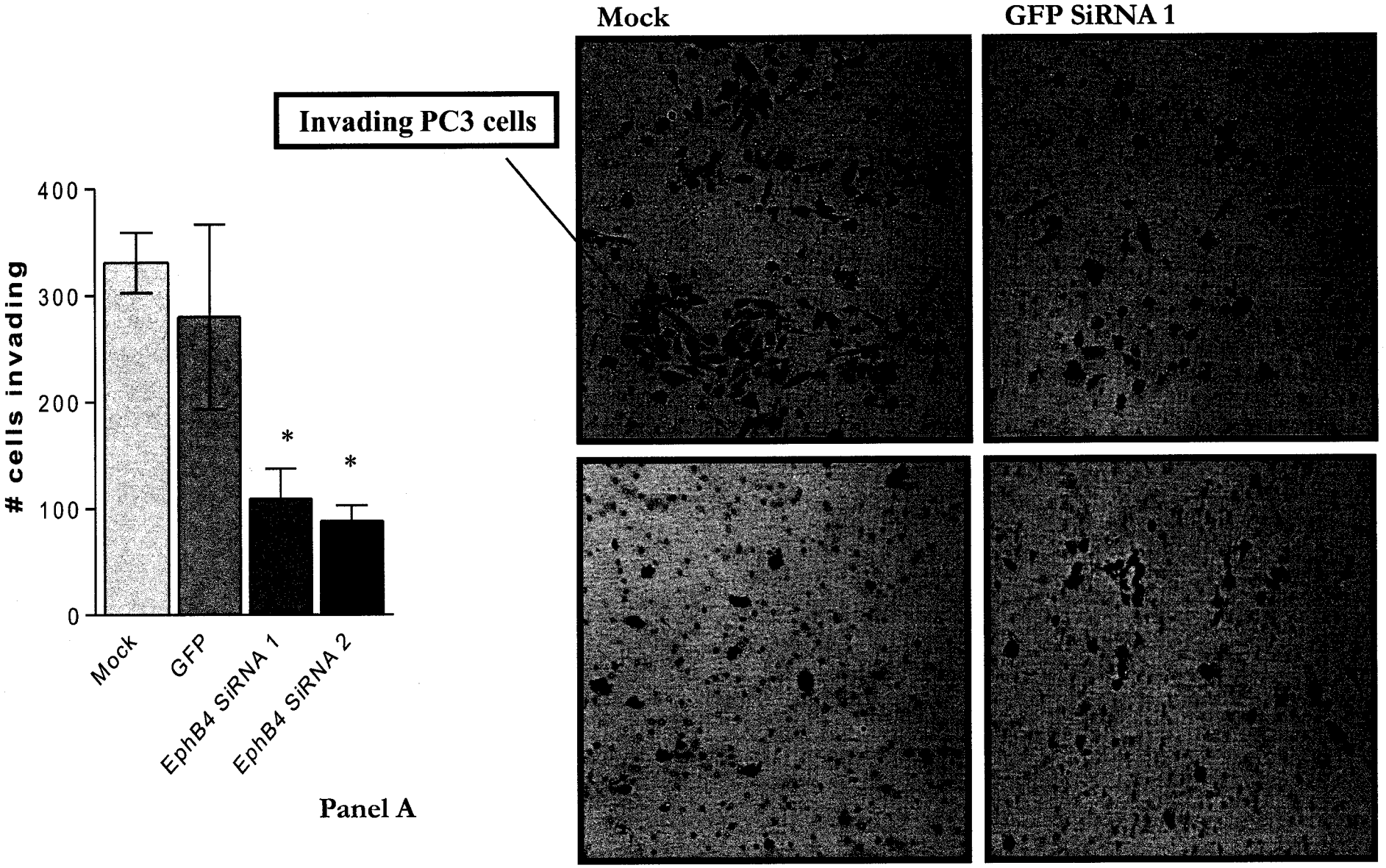
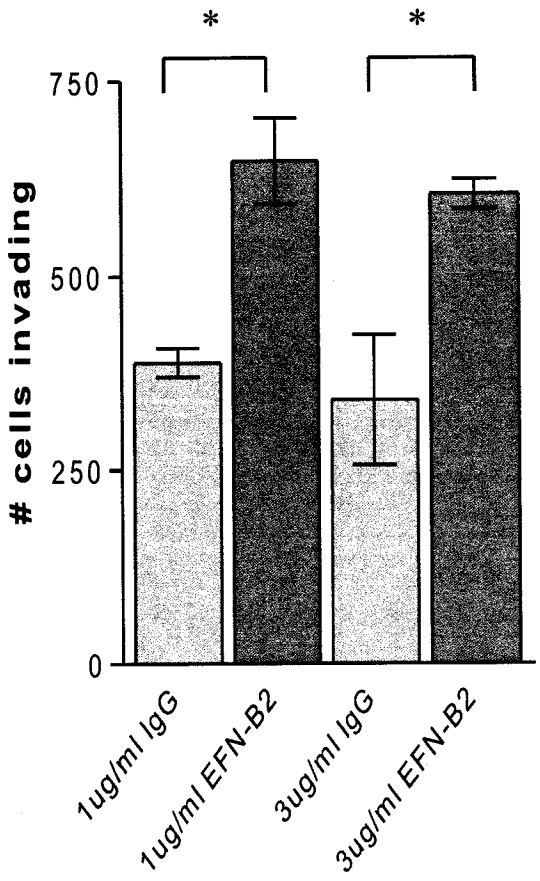
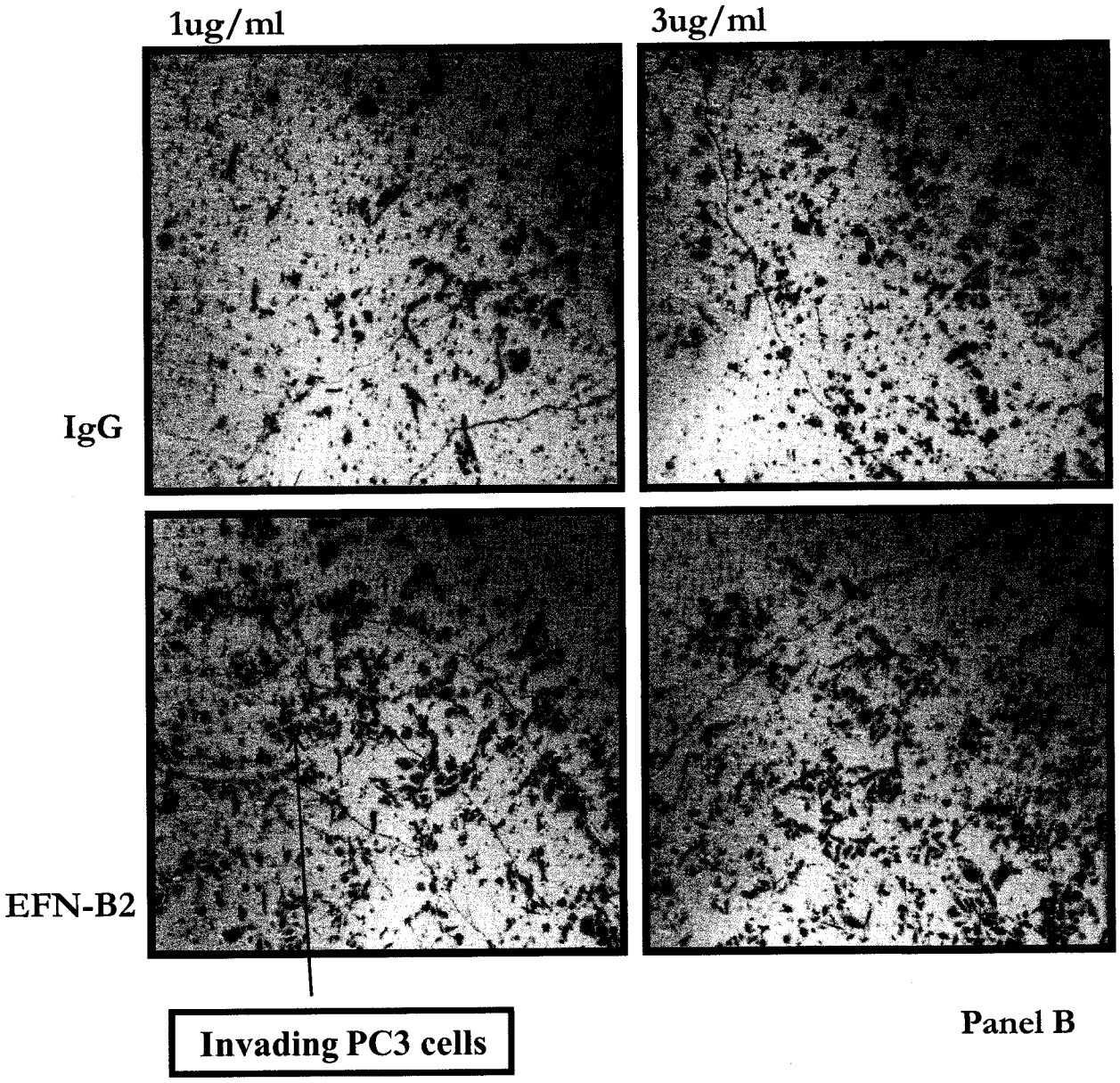

Invading PC3 cells
400
300
200
100
0
cells invading
*
*
Mock
GFP
EphB4 SiRNA 1
EphB4 SiRNA 2
Panel A
Mock
GFP SiRNA 1
EphB4 SiRNA 1
EphB4 SiRNA 2
Panel B

Figure 27

Figure 28. Effect on Cell Invasion After Stimulating EphB4 Receptor with EphrinB2-Fc. A total of $1x10^5$ PC3 cells were placed in the top portion of transwells in DMEM in the absence of serum. The bottom well of the transwell inserts was filled with DMEM containing 10% FBS as a stimulus for invasion. PC3 cells in the top chamber were additionally stimulated with either 1ug/ml or 3ug/ml of EphrinB2-Fc or similar concentrations of IgG as a negative control. After 8 hrs, the PC3 cells that invaded through the matrigel to the underside of the filter were fixed with methanol solution and stained with Diff-Quick solutions. (A) Bar graph is a representation of the mean and associated standard error of total number of invading cells from triplicate wells. The experiment was repeated three independent times with similar results. * represents $p < 0.05$. (B) Representative photographs of a quadrant section of the filter viewed under the microscope at 100x magnification showing the cells that have invaded.



Panel A



Panel B

Figure 28

4. DISCUSSION

This project was designed to investigate the role of EphB4 tyrosine kinase receptor in regulating the phenotype of prostate carcinoma cells. After we initially discovered that EphB4 protein was overexpressed in PC3 and LNCaP cell lines compared to normal prostate epithelium, PrEC, we hypothesized that the elevated levels in cancer cells indicated a positive regulatory influence on tumor progression (Figure 5). To determine what aspects of tumorigenesis EphB4 might be regulating, we chose to look at proliferation, migration and invasion of three prostate carcinoma cell lines, PC3, Du-145, and LNCaP, making use of techniques previously tested in our laboratory, such as cell proliferation and viability assays, scratch wound healing migration assays, matrigel invasion assays, siRNA and stimulation of the receptor with exogenous addition of extracellular ligand fused to the Fc portion of human IgG. In our model systems, our results indicated that EphB4 receptor may not be involved in regulating proliferation or cell survival of prostate carcinoma cell lines (Figures 12-15). Instead, our results support a primary role for EphB4 receptor in regulating cell migration and invasion of prostate carcinoma cell lines based on our observations of decreased migration and invasion when EphB4 protein levels were reduced using siRNA (Figures 16-21, 27). This proposed role is also supported by the results obtained following stimulation of the EphB4 receptor using EphrinB2-Fc which promoted tumor cell migration and invasion. Our results also suggest that EphB4 activity controls these processes through activation of downstream pathways, including such proteins as Rac and Cdc42, which are known to promote cytoskeleton reorganization in migration and invasion (Figures 26, 28).

Eph receptors and ephrins have been previously studied in ovarian, lung, breast, and prostate cancers (22). Higher expression of Eph receptors in carcinoma cells and cancerous

tissues have often been associated with increased aggressiveness and decreased overall survival in patients with breast, lung and prostate cancer (52, 56, 59). Researchers have previously detected high expression of EphB2 and EphB4 receptors in a large number of breast carcinoma cell lines, such as MCF-7, T47-D, and MDA-MB-231 (51, 52). In our results, we confirmed high levels of expression of EphB4 in MCF-7 and T47-D, although we did not have access to the primary breast mammary epithelial cells to compare these levels with (Appendix Figure 1). Although previous studies had detected expression of EphB receptors in prostate cancer, these studies did not compare the levels with that of primary normal prostate epithelium. Comparison of EphB4 expression in our prostate carcinoma cell lines with normal prostate epithelial cells revealed that PC3 and LNCaP had elevated levels of EphB4, a 120 kDa protein, as compared to that found in normal epithelial cells, suggesting that increased EphB4 expression may be associated with increased tumorigenesis. These data are also supported by results obtained by Gill et al (3), where increased expression of EphB4 receptor in PC3 cells and, even more increased EphB4 expression in its metastatic clone, PC3M, were found, as compared to cells isolated from benign prostatic hyperplasia (BPH) tissue. Subsequent studies by Gill et al. using formalin-fixed prostate tumor tissue microarrays found that detectable levels of EphB4 receptor was expressed in ~66% of tumor samples compared to in only ~15% in normal tissues (3). DNA microarray evidence from tissues of prostate cancer surgical specimens also showed that EphB4 was expressed in 64 (89%) of 72 cancer patients and with a higher signal intensity than either EphB1 or EphB2 (3).Changes in the levels of expression of either Ephrin ligands or Eph receptors in several different cancer types have also been shown to correlate with tumor aggressiveness and act as an indicator of cancer progression. For example, in a study conducted by Fujimoto et al. of

68 uterine endometrial cancers, histoscores and mRNA levels of both EphrinB2 and EphB4 were significantly elevated in all cases when compared to normal endometrium tissue samples (75). Increased expression of EphrinB2 and EphB4 was found to correlate with increasing levels of clinical stages and myometrial invasion (75). In that study, the 60 month survival rate for the group of 34 patients classified as having high expression of EphrinB2 was 59%, while the second different group of 34 patients having low EphrinB2 expression had a survival rate of 85%. Similarly, the group of 34 patients with a high EphB4 expression produced a survival rate of 62%, while the second group having low expression of EphB4 had a survival rate of 82% (75). Taken together, these results support a role for EphB4 in tumorigenesis and aggressiveness, and suggest a putative role in prostate cancer progression.

As many other tumor types have been shown to express EphrinB2 ligand, it was important to determine whether the cell lines used in our study also expressed this ligand which could then also be contributing to the activation of EphB4 endogenously. The results from our study showed that all prostate carcinoma cell lines expressed endogenous EphrinB2 and a comparison of EphrinB2 protein levels in PrEC versus prostate carcinoma cells determined that normal prostate epithelial cells generally had lower concentration of EphrinB2 as compared to tumor cell lines (Figure 6). It is also worth mentioning that the molecular weight of EphrinB2 in PrEC was higher than the EphrinB2 in PC3, Du-145, or LNCaP, which was ~37kDa in size even though protein extractions were performed in the same lysing buffer in a similar manner. The predicted molecular size of EphrinB2 as reported in the literature is ~37kDa (53). However, modifications in glycosylation of EphrinB2 have been reported following transfection of OP9 stromal cells with full-length EphrinB2. This resulted in a decrease in electrophoretic mobility from ~46 kDa to ~48 kDa (76), however, it

is unlikely this is the explanation for the differences we observed as the shift in protein size was more dramatic. Our results could also be explained by the presence of different splice variants of EphrinB2 such as the one detected previously for EphrinB2 in newborn mouse brain cells where a conserved region of 31 amino acid residues including a cysteine residue was deleted (94). As splice variants of other Ephrin ligands, such as EphrinA1 in human brain, tonsil cells and adenocarcinoma, and EphrinA3 and A5 in adult rat brain, have also been detected previously (79, 77, 78), this seems like a more plausible explanation for our observations, and raises the interesting question that the splice variant found in tumor cells may affect EphB4 differently than the variant found in normal epithelial cells.

We demonstrated that the EphB4 expressed in PC3 cells is a functional receptor, as we could show a marked increase in phosphorylation of EphB4 from basal levels following stimulation with the ligand EphrinB2-Fc (Figure 8). This result is important as it has been previously shown that Du-145 prostate carcinoma cells express elevated levels of EphB2 receptor, however Huusko et al identified mutations in the EphB2 gene of Du-145 prostate cancer cells that render it inactive due to a nonsense mutation. The mutant receptor mutation prevents phosphorylation of the cytoplasmic domain and downstream signal transduction (61). The authors thus speculate that EphB2 expression and functional receptor activation inhibits prostate tumor progression. Although it is interesting to note that mutations of EphB2 receptor may be involved in cancer progression, to date these findings are restricted to Du-145 cells. In support of this contention, it has also been shown that although prostate tumor cells may express EphB2, the relative expression of EphB4 mRNA is significantly greater as compared to EphB2 in all three prostate cancer cell lines used in our studies (82).

To test the functional significance of EphB4 receptor activity in prostate cancer, we took two approaches; we employed siRNA techniques to specifically decrease EphB4 expression and we stimulated the receptor using EphrinB2-Fc ligand to activate its downstream signaling pathways. To ensure the specificity of our system, measures were taken to ensure that off targets effects resulting from our siRNA approach were minimized. Short siRNA duplexes were designed with help from Whitehead Institute of Biomedical Research at MIT using their online tool http://jura.wi.mit.edu/bioc/siRNAext/home.php). Sequences were kept to 21nt bases and contained between 30% to 60% GC content, which has been reported to be needed for stability of the duplexes. Putative siRNA sequences were BLAST searched against the NCBI database, and only sequences with the least mismatches with other human mRNA were chosen for further analyses. EphB4 siRNA #1 had only a single match of its 13nts out of 21nts to another mRNA (CREG2) with unknown expression and function in prostate cancer. EphB4 siRNA #2 did not have any matches with other human mRNA. Both sequences were also later found to be individual components contained within the EphB4 SMART pool siRNA that is commercially available through Dharmacon and widely used for RNA interference studies. To minimize the possibility that off-target effects were mediating the observed phenotypes, we used 2 different EphB4 siRNAs , together with 2 different control siRNA sequences and a mock transfection with Oligofectamine. EphB4 siRNA 1 targeted the 5'UTR region and EphB4 siRNA 2 targeted the open reading frame region of EphB4 mRNA. Negative controls included a siRNA sequence that targets GFP, which is an mRNA sequence that does not exist in our human tumor cell lines, and Non-Targeting siRNA Control 1 obtained from Dharmacon. To our knowledge, the Non-Targeting siRNA Control 1 and the GFP control we used do not directly

affect the expression or activity of proteins and downstream pathways associated with EphB4 (90). In addition to ascertaining the specificity of our siRNA approaches, we also confirmed that the use of EphrinB2-Fc molecules did indeed lead to the activation of the EphB4 receptor (Figures 8). As a receptor tyrosine kinase, it is known that EphB4 becomes phosphorylated upon dimerization and activation. Our studies indeed showed that EphrinB2 stimulation resulted in a time and concentration dependent phosphorylation of EphB4, and hence theoretically its activation. Although we did not perform these assays, to ensure that EphB4 kinase activity is also induced following EphrinB2-Fc stimulation, one could perform immunoprecipitations of EphB4 followed by in vitro kinase assays of downstream target proteins to confirm its activation.

Our work, which supports a role for EphB4 activity primarily in modulating cell migration and invasion and not influencing tumor cell proliferation, is also supported by the work of Pasquale et al (83). In their work, the authors demonstrated that activation of Eph receptors did not appear to have a major impact on cell proliferation, but Eph receptors, in conjunction with their ligands, had a significant impact on the directional movement of cells by modulating cytoskeleton reorganization (83). In contrast to these and our findings, Gill et al showed that decreasing EphB4 in PC3 cells reduced cell viability 48 hrs post-transfection (3). Although, the siRNA sequences used in their studies were different from our sequences, BLAST searches of the siRNA sequences used in their work revealed lower overall total scores and both of their sequences mismatched with mRNA sequences of 6 other proteins in homosapiens with unknown expression and function in prostate cancer. Their siRNA sequences mismatching with other mRNA sequences could raise the possibility of off-target RNA interference resulting in different phenotypes. In addition to the sequence differences,

there are a few other notable differences in their methods as compared to ours. In their study, Lipofectamine 2000 was used as a transfection reagent while we used Oligofectamine. Oligofectamine has the advantage of being one of the least cytotoxic transfection reagents available for insertion of short oligonucleotides (84). In addition, in the study by Gill et al, the number of viable cells was measured using the MTT assay while we counted our cells using Trypan blue exclusion in the Vi-CellXR Coulter Counter over a time period from 24 to 96 hrs. As the MTT assay is based on the metabolic activity of mitochondrial reductase enzymes, which are active in reducing MTT to formazan, the readout of cell viability is affected by changes in cellular metabolic activity. It is possible that changes in metabolic activity, either as a result of off-target effects of the siRNA used in their studies or as a result of siRNA treatment itself, may have led to large differences in MTT readouts, while if the number of viable cells was directly counted using methods such as ours, the number of viable cells observed may have remained constant (85).

In addition to the siRNA approaches we employed to decrease EphB4 expression, we also used EphrinB2-Fc to activate this receptor, and similar to our findings with EphB4 depletion studies, we found that EphrinB2-Fc stimulation of EphB4 also did not modulate prostate cancer cell proliferation. Although not investigated in prostate cancer, activation of cellular pathways that may increase cell proliferation and promote survival has been noted following EphB4 activation with EphrinB2 in breast cancer and mesothelioma models (53, 87). Since we did not observe changes in proliferation of PC3 or Du-145 cells when exogenous EphrinB2-Fc was given in varying doses over a given time period (Figure 13), but were limited to using a maximum dose of 3ug/ml, we cannot exclude the possibility that tumor cell proliferation may be modulated by EphB4 activity following stimulation with

EphrinB2-Fc in excess of this dose. Alternatively, it remains possible that there is already too much EphrinB2 in the model systems we have chosen; as we have demonstrated that the prostate tumor cell lines used in this system already express fairly high levels of EphrinB2 endogenously, and both endogenous and exogenous EphrinB2 can stimulate EphB4, the system may be saturated, thus addition of exogenous ligand cannot influence cellular proliferation above that which the endogenous ligand is controlling. Thus we speculate that engagement of EphB4 by endogenous EphrinB2 may have activated much of the Eph receptors and initiated internalization and degradation of the Eph/ephrin complex prior to addition of exogenous ligand in our system (86). However, as recent observations in breast cancer and mesothelioma demonstrated that reducing EphB4 in MCF-7 and H28 cells, respectively, resulted in a decrease in phosphorylation (and hence activation) of AKT (53, 87), we decided to explore the possibility that EphB4 may be involved in cell survival by activating downstream phosphorylation of AKT in prostate carcinoma cell lines. Both the androgen independent cell line, PC3, and androgen dependent cell line, Du-145, used in our experiments did not show significant differences in phosphorylation of AKT after using EphrinB2-Fc to stimulate the EphB4 receptor. As it is well known that PC3 cells carry a mutated PTEN gene resulting in constitutively active and phosphorylated PI3/AKT pathway (88), it is not surprising that we did not observe any effects of EphrinB2-Fc stimulation on the levels of phosphorylated AKT in this system. However, the PTEN-positive cell line, Du-145, also did not show significant differences in AKT phosphorylation following EphrinB2-Fc stimulation of the EphB4 receptor, suggesting that in these prostate cancer cells, the EphrinB2/EphB4 signals do not affect AKT activity. Taken together, our results support the

contention that EphB4 activity does not play a role in cell proliferation or survival in prostate cancer.

EphB4, and other EphB receptors, have been reported to play a role in migration and invasion of cancers such as head and neck carcinoma, melanoma, breast cancer and prostate cancer. Using the three prostate carcinoma cell lines, PC3, LNCaP, and Du-145, we were able to show that decreasing EphB4 levels using siRNA significantly reduced their migration (Figures 16-21). Furthermore, invasion of PC3 cells was significantly reduced after reduction of EphB4 levels (Figure 27). Our data correlates with a handful of other studies such as the ones conducted in breast cancer, head and neck carcinoma, and prostate cancer (53, 89, 3, respectively). In vitro studies of MCF-7 or PC3 cells treated with a similar dosage of EphB4 siRNAs (~50nM), as compared to the concentration used in our study, resulted in decreased migration of those cell lines as measured by chemotaxis in a Boyden Chamber Assay or scratch wound healing migration assay (53, 3, respectively). Decreases in invasion of MCF-7 and PC3 has also been previously observed using methods similar to our experiments whereby EphB4 expression was depleted using siRNAs, and subsequently cells were placed on Matrigel coated filters and the number of cells that traveled to the underside of the membrane was subsequently counted (53, 3). Even though studies exist to support our observations, a study with contradictory findings exists in breast cancer where the authors demonstrated that stimulating EphB4 receptor using EphrinB2-Fc in MDA-MB 435 cancer cells resulted in the inhibition of migration and invasion (60). The authors further show that this was thought to occur through Crk phosphorylation and increased activity of Abl kinase that then prevented Crk-CAS coupling and, therefore, inhibited migration (60). We attempted to examine the activity of Abl kinase and Crk phosphorylation in our model system using the

prostate carcinoma cells, however we observed no differences in the states of these proteins following EphrinB2-Fc stimulation (data not shown). Thus although the role of this pathway is not clearly defined in prostate cancer as of yet, we have no evidence that supports a role of EphB4 inhibiting prostate cancer cell migration via activation of this pathway.

As metastatic prostate cancer has a significantly lowered survival rate compared to localized prostate cancers that can be surgically removed, identification of factors that promote metastasis can be useful to identify potential novel therapeutic targets. We have assessed the role of EphB4 in migration and invasion of prostate cancer cell lines, and have determined that EphB4 promotes these processes, both of which are prerequisites for the metastatic phenotype. It was previously reported in Swiss 3T3 fibroblast cells that EphB4 and EphB2 receptors can activate downstream Rho-GTPases such as Rac and Cdc42 to form cytoskeletal extensions, lamellipodia and filopodia respectively (91). In melanoma cells, EphB4 has been found to promote cell migration through RhoA mediated cytoskeleton reorganization (92). In our study, we also observed activation of Rac and Cdc42 in PC3 cells 45 min after stimulating EphB4 receptor with EphrinB2-Fc (Figure 26). It is interesting to note that activation of the Rho-GTPases Rac and Cdc42 (Figure 26) occurred after phosphorylation of EphB4 was first observed at 30 min (Figure 8), suggesting that they were activated downstream of receptor activation. Thus taken together our results suggest that at the site of cell-cell contact upon engagement by EphrinB2 ligand, EphB4 receptor becomes activated and plays a role in increasing motility and invasion of prostate cancer cells via activation of downstream pathways that modulate the cell cytoskeleton, including, but not limited to, activation of Rac and Cdc42.

Based on the currently available information, a model describing the hypothetical interaction between EphB4 and EphrinB2 is shown in Figure 29. In the event that EphB4 receptors bind to EphrinB2 ligands, a 2:2 circular heterotetramer is formed causing phosphorylation of the receptor juxtamembrane region (31, 20). Autophosphorylation of the juxtamembrane tyrosine residue relaxes the constraints on the intracellular region resulting in the opening of the active site of the kinase domain (49). With the juxtamembrane region phosphorylated, binding sites for SH2 domain proteins such as cytoplasmic tyrosine kinases Fyn, Src, Nck and Crk are also established (50). Subsequent autophosphorylation of three additional tyrosine residues in the kinase domain provides further stabilization of this intracellular domain and creates more sites for possible binding of SH2 domain proteins in addition to PDZ binding proteins at the C-terminal PDZ motif of the receptor. As a result of ligand binding, phosphorylation promotes the association of the receptor with nucleotide exchange factors, such as Kalirin and Intersectin. Eph receptors are thus linked to the cytoskeleton via these interactions and can modulate important cellular functions initiating a cascade of downstream cell pathways that may directly and indirectly play an important role in rearrangement of actin filaments, cell movement, and migration (20). In this study, we have made important observations, using PC3, LNCaP and Du-145 cell lines, regarding the biological functions of EphB4 in prostate cancer. The role of EphB4 needs to be further explored in mouse xenograft tumor models to determine whether EphB4 modulates similar effects in vivo and impacts prostate cancer progression and metastasis. Additionally, in vivo models of prostate cancer tumorigenesis could help ascertain whether or not EphB4 could someday serve as a therapeutic target either by inactivating it using antibody inhibitors or specific receptor tyrosine kinase inhibitors as therapeutic approaches.

Figure 29. A hypothetical model showing several proteins and interactions of EphB4 receptor and EphrinB2 ligand before (A) and after (B) binding with each other.

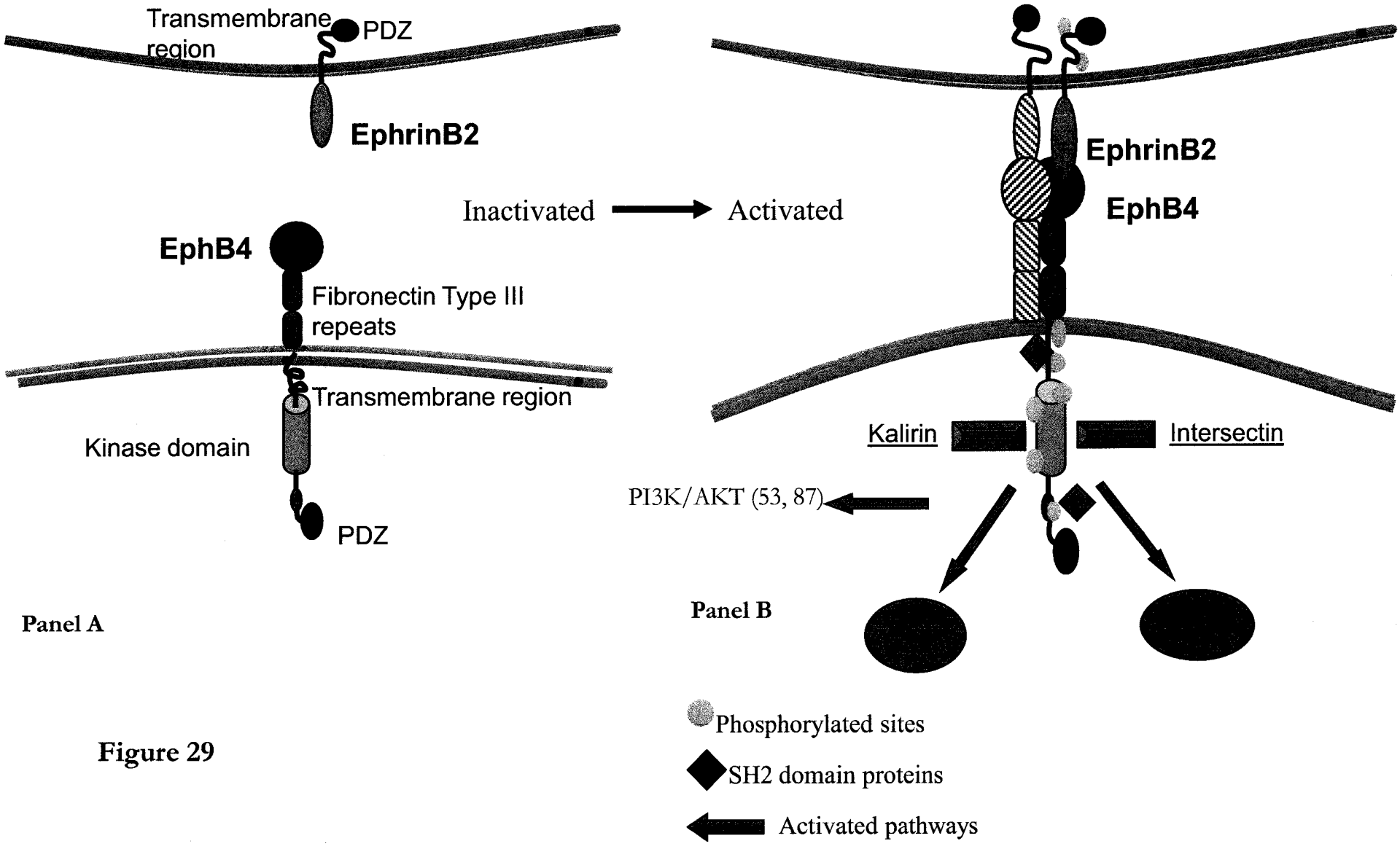
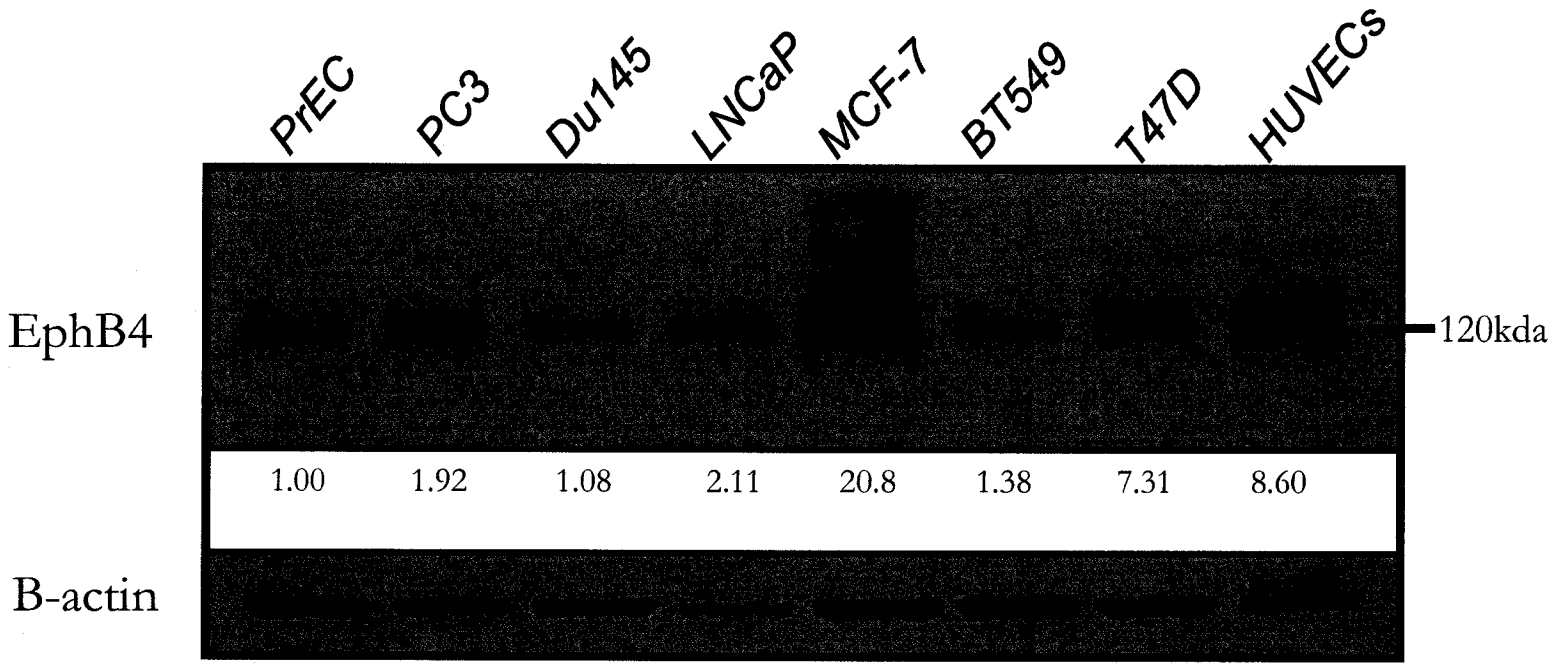

Transmembrane region
PDZ
EphrinB2
EphB4
Fibronectin Type III repeats
Transmembrane region
Kinase domain
PDZ
Inactivated
Activated
EphrinB2
EphB4
Kalirin
Intersectin
PI3K/AKT (53, 87)
Panel A
Panel B
Phosphorylated sites
SH2 domain proteins
Activated pathways

Figure 29

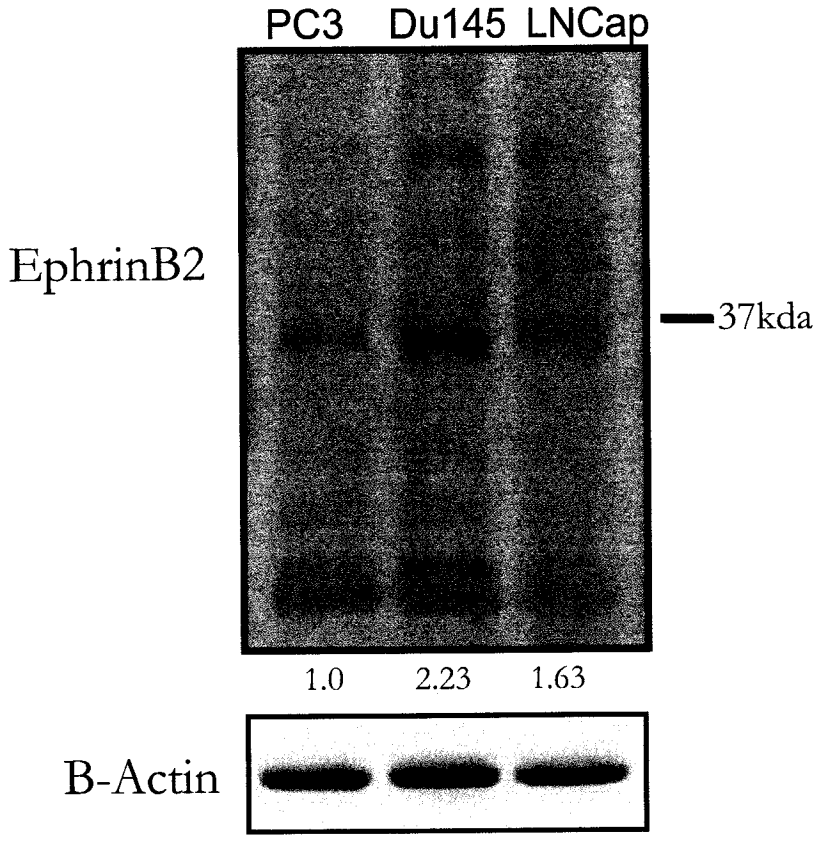
5. APPENDICES

Appendix Figure 1. Relative Expression of EphB4 Protein in Prostate Carcinoma Cell Lines. Cells were lysed in subconfluent conditions and 40ug of total cell protein were analyzed by immunoblotting. Monoclonal anti-EphB4 antibody detected increased expression (~2 fold) of EphB4 in PC3 and LNCaP compared to PrEC. Other tumor cell lines, such as MCF-7 and T47D, also showed high expression of EphB4 receptor. β-actin was used as a control for equal loading of total protein. Densitometry values are a representation of raw absorption volumes from the bands normalized to PrEC and then to β-actin using Genetools by Syngene.



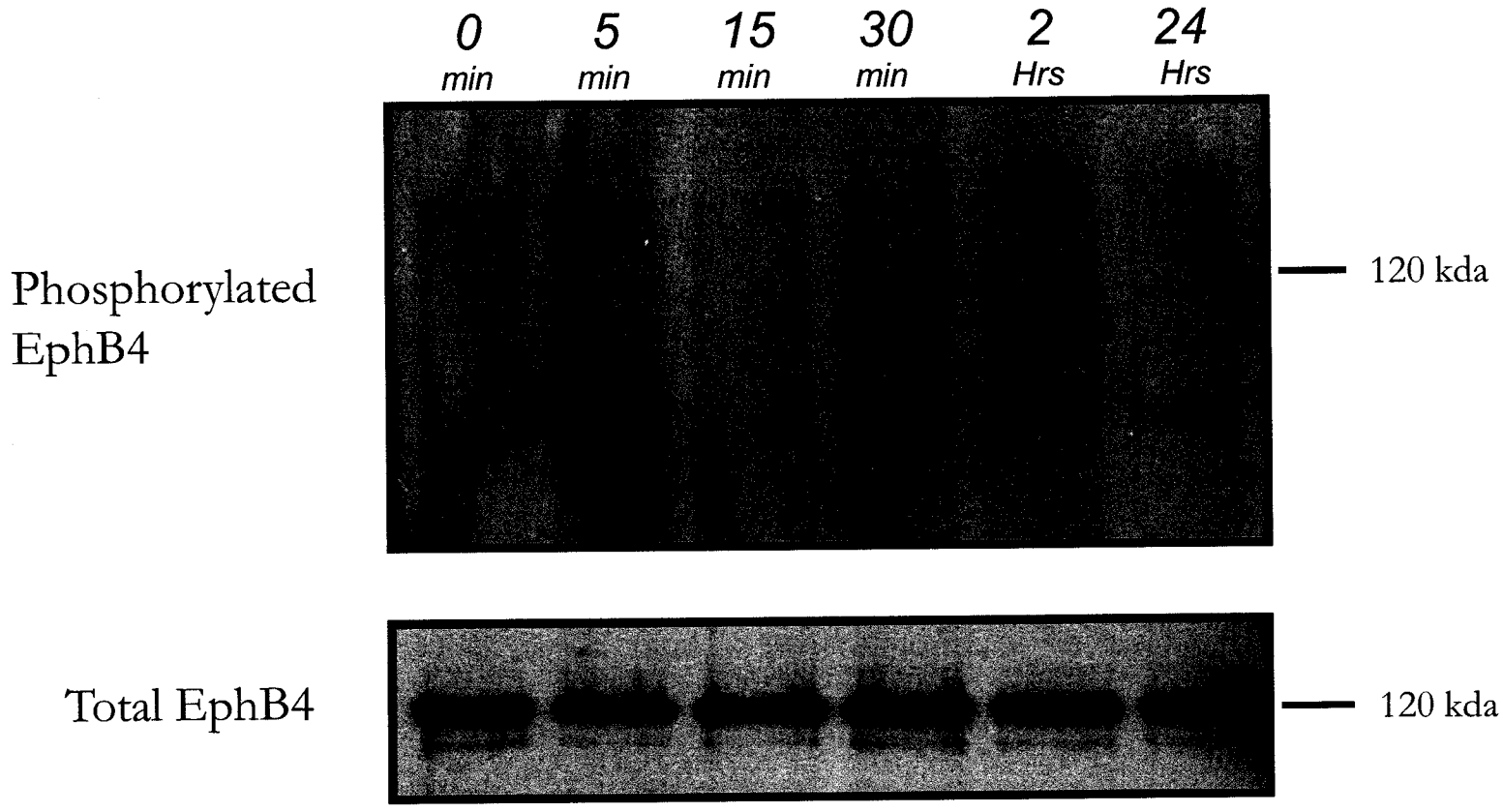
Appendix Figure 1

Appendix Figure 2. Expression of EphrinB2 in Prostate Carcinoma Cell Lines. PC3, Du-145, and LNCaP cells were resolved following SDS-PAGE gel electrophoresis and EphrinB2 was detected using immunoblotting. Anti-EphrinB2 antibody detected endogenous expression of the ligand in all cell lines tested. Du-145 cells showed higher levels of EphrinB2 expression compared to other prostate carcinoma cell lines. Densitometry results were normalized to PC3 and then to β-actin.



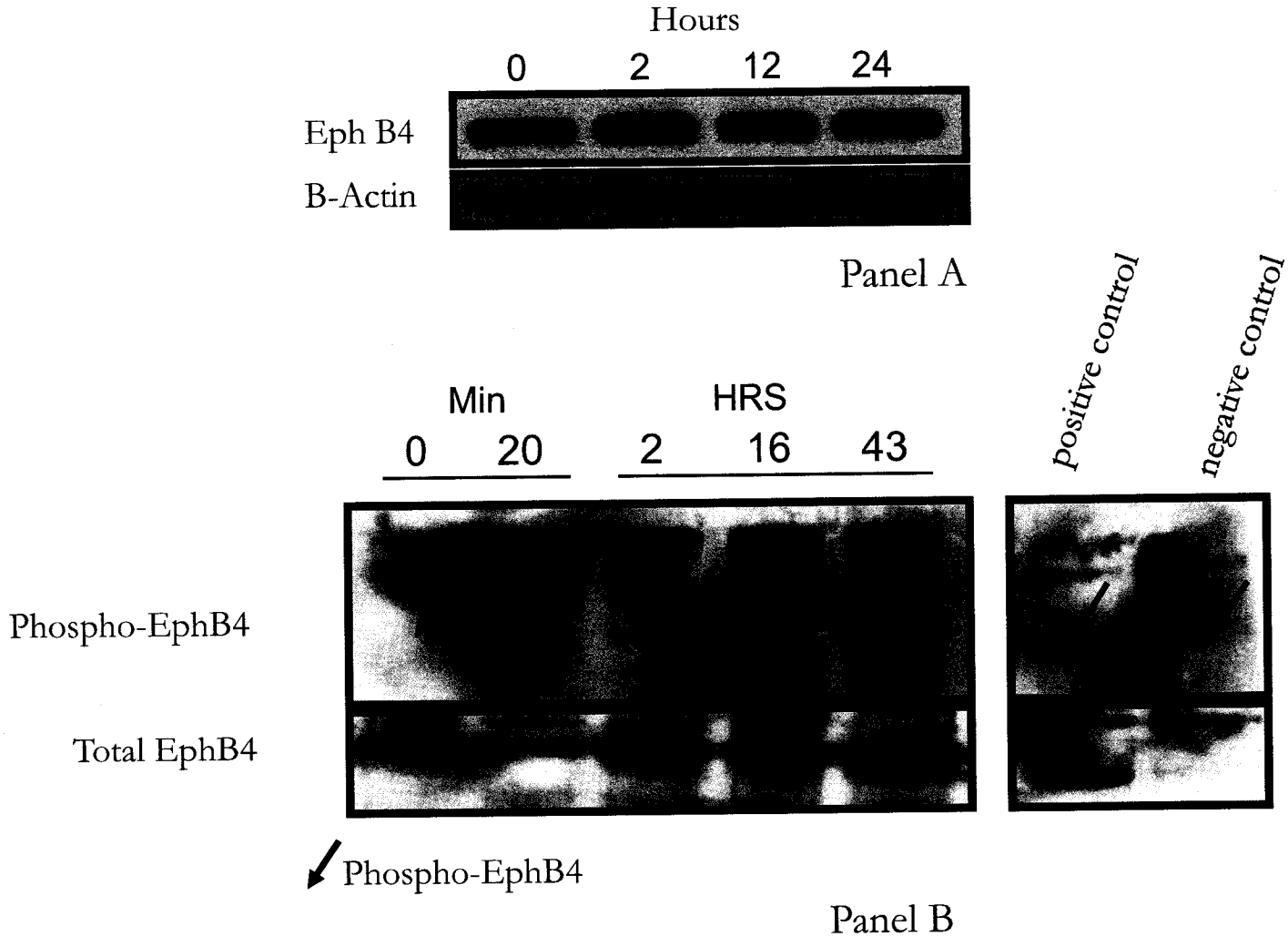
Appendix Figure 2

Appendix Figure 3. Phosphorylation of EphB4 Following Activation with EphrinB2-Fc. PC3 cells were stimulated with 3ug/ml EphrinB2-Fc and cells were lysed at 0min, 5min, 15min, 30min, 2hrs and 24hrs post stimulation. A total of 400ug of total protein was immunoprecipitated to extract EphB4 and following SDS-PAGE gel electrophoresis and electrophoretic transfer of proteins, membranes were probed with anti-phosphotyrosine antibody. Compared to 0min, phosphorylation of EphB4 was first detected at 30 min and continued increasing to 2 hrs post stimulation with EphrinB2-Fc. Equal total protein loading was confirmed by stripping the membrane and re-probing for total EphB4 protein.



Appendix Figure 3

Appendix Figure 4. Characterization of EphB4 Expression and Activity Following Monolayer Wounding. (Panel A) Total cellular EphB4 expression after wounding and migration of cells. PC3 cells were grown to a monolayer in 10cm tissue culture plates and multiple wounds were generated by scraping. Cells were then lysed over time (0, 2, 12, and 24 hrs post wounding) and 40ug of total protein was resolved with SDS-PAGE gel electrophoresis. Subsequent western blot analysis using anti-EphB4 antibody determined that no changes in the level of expression of total EphB4 protein occurred as a result of the wounding and induced cell migration itself. (Panel B) Effect on phosphorylation of EphB4 receptor due to wounding and migration of cells. PC3 cells were serum starved and then monolayers wounded as described above. Cells were kept in reduced serum conditions (DMEM/1%FBS) and lysed over time (0, 20 min, 2 hrs, 16 hrs, and 43 hrs post wounding). Immunoprecipitation with anti-EphB4 and western blotting with anti-phosphotyrosine antibody showed that EphB4 phosphorylation did not change as a result of the wounding procedure. A positive control, tested separately, using cell lysate from PC3 cells that had been stimulated with 3ug/ml of EphrinB2-Fc was used to determine phosphorylation of EphB4. A no-antibody control was added to show that no non-specific proteins were precipitated by the antibody in this process.



Appendix Figure 4

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