Role Of Thromboxane Receptor-Alpha In Prostate Cancer Progression

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ROLE OF THROMBOXANE RECEPTOR-ALPHA IN PROSTATE CANCER PROGRESSION

by

PRASANNA EKAMBARAM

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2012

MAJOR: CANCER BIOLOGY

Approved by:

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Advisor Date

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DEDICATION

I would like to dedicate this dissertation to my wife, Arulselvi and my kids Tejas and Abhi for standing strong by my side at good times and difficult times in life and helping me work hard toward achieving my goal of being a research scientist; To my parents for their blessings, and prayers and for helping to come to Unites States to complete my graduate studies.
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ABBREVIATIONS

PCa....................................................Prostate cancer

PSA.................................................Prostate-Specific Antigen

AA...................................................Arachidonic Acid

COX................................................Cyclooxygenase

LOX................................................Lipoxygenase

LT..................................................Leukotrienes

HETEs..............................................Hydroxyeicosatetraenoic acids

TXA2..............................................Thromboxane A2

TXA2S..............................................Thromboxane synthase

TP..................................................TXA2 receptor, or T-Prostanoid, receptor

GPCR..............................................G-Protein Coupled Receptors

NSAIDS........................................Nonsteroidal Anti-Inflammatory drugs

EGFR..............................................Epidermal Growth Factor Receptor

ErbB...............................................Erythroblastic Leukemia Viral Oncogene Homolog

HER.................................................Heregulins

EGF...............................................Epidermal Growth Factor

HB-EGF..........................................Heparin binding-Epidermal Growth Factor
TGF-alpha...........................................Transforming Growth Factor-Alpha

EREG...........................................Epiregulin

AREG...........................................Amphiregulin

NRG...........................................Neuregulins

ERK...........................................Extracellular signal Regulated Kinase

MAPK..........................................Mitogen-Activated Protein Kinase

Src...........................................Sarcoma

CD...........................................Cluster of differentiation

RHO-A........................................Ras homolog gene family, member A

HEK...........................................Human Embryonic Kidney

PKA..........................................cAMP dependent Protein kinase

AMPK.........................................AMP-activated protein kinase

FFPE........................................Formalin-Fixed Paraffin-embedded
CHAPTER 1: INTRODUCTION

Prostate Cancer:

Prostate cancer is one of the most common cancers among American men and is the second leading cause of death among them in the United States [1]. Prostate cancer is slow growing and the majority of deaths are from metastatic disease. Metastatic prostate cancer generally localizes in the bones and lymph nodes [2]. The causes leading to prostate cancer metastasis and the mechanisms by which cells escape from the primary site and intravasate into the circulation to spread to distant organs, e.g. bone, is poorly understood [2].

Diagnosis of Prostate Cancer:

Recent advances in diagnostic methods have enabled detection of prostate cancer at an early stage thereby reducing the number of deaths from metastasis. These methods include the PSA test, Digital Rectal Examination (DRE), trans-rectal ultrasound and trans-rectal biopsy [3]. Two of the most commonly used methods for diagnosis are the PSA test and DRE [2, 4].

Blood Test for Prostate-Specific Antigen (PSA): PSA, also known, as Kallikrein is a serine protease enzyme that was discovered by Dr. Richard J. Ablin and has been approved by FDA for annual screening of prostate cancer in men of age 50 and older. Serum PSA levels are higher in patients who have prostate cancer compared to men with healthy prostates. PSA is measured from a blood sample by Enzyme Linked Immuno-Absorbance Assay (ELISA), where the normal level of PSA in human blood is 4 ng/ml. Free PSA in blood beyond 10 ng/ml indicates additional tests are necessary to
check for cancer. As there were many false positives in some patients, PSA test is more of a predictive test that needs to be verified by additional tests. Success of the PSA test was one factor responsible for the decline in the number of deaths that occurs due to prostate cancer [5, 6].

**Digital Rectal Examination:** This is a physical examination of the prostate where the prostate is directly palpated for irregularities from inside the rectum. However, with this method only 10% of the gland can be examined. Furthermore performance in detecting abnormalities varies greatly, and agreement between examiners is low. Therefore additional markers are necessary to augment existing PCa tests. [2, 3, 7].

**Prostate Cancer Pathology and Grading**

Once diagnosed, prostate cancer is classified into different grades and stages that are used to predict responsiveness to a specific cancer therapy, such as radiotherapy or surgery. The Gleason grading system developed by Dr. Donald F. Gleason is the most commonly used method to grade prostate cancer tissues based on the histologic pattern of arrangement of cancer cells in Hematoxylin and Eosin (H&E) stained prostate tissue sections [8]. Gleason grades include five basic grade patterns (Figure 1.1). To generate a histologic score, ranging from 2 to 10, the primary and the secondary grade patterns are added. “Gleason grade has been linked to a number of clinical end points, including clinical stage, progression to metastatic disease, and survival” [6, 8, 9].
**Figure 1.1: Gleason grading system.** This was developed by Dr. Donald F. Gleason and is the most commonly used method to grade prostate cancer tissues based on the so-called “architecture” of H&E-stained prostatic tissue sections [8].

**Treatment Options:**

There are several methods available for treating men with prostate cancer. These include surgery, chemotherapy, cryotherapy, hormonal therapy, radiation, High Intensity Focused Ultrasound (HIFU), and "watchful waiting" [10][2]. Treatment options vary between patients depending on the patient’s age, stage and grade of the disease.
The treatment that is best for one man may not be best for another, and yet another may have a combination of treatments [2].

**Risks Factors for Prostate Cancer:**

Common risk factors that are associated with prostate cancer include: age, race and diet [2, 11].

**Age over 65:** Age is the main risk factor for prostate cancer. In the United States, most men with prostate cancer are over 65. This disease is rare in men under 45. There is a racial component and African-American men should be screened earlier for prostate cancer at age 40 [2, 11].

**Race:** Prostate cancer is more common among African-American men than Caucasians or Hispanic men. It is less common among Asian and Native American men. Also the rate of men dying from prostate cancer has varied, depending on their race and ethnicity where recent data shows that, African-American men were more likely to die of prostate cancer than any other ethnic group. Caucasian men had the second highest rate of deaths from prostate cancer, followed by Hispanic, Native American, Alaskan Native, and Asian/Pacific Islander [12, 13].

**Diet and Prostate Cancer:** Diet is also considered an important risk factor for prostate cancer. Epidemiologic studies have suggested that high consumption of fat and red meat, and lower consumption of fruits and vegetables contributes to morbidity [11, 14-17]. Animal fat is especially rich in arachidonic acid, whose metabolites have been shown to affect prostate cancer progression [16, 17].
Arachidonic Acid Metabolism:

Arachidonic acid (AA) is an essential component of mammalian cell membranes and plays a critical role in the synthesis of eicosanoids [18]. Arachidonic acid and its precursor, linoleic acid, are major components in animal fats and many vegetable oils [17]. Arachidonic acid is mobilized by phospholipase A2 (PLA$_2$) from cellular membrane glycerolipid pools in response to many stimuli such as cytokines and growth factors (15). Arachidonic acid is present in membrane phospholipids and is released by phospholipase A$_2$ and can be oxidized by three distinct enzymes Cyclooxygenase (COX), Lipoxygenase (LOX), or P450 epoxygenase into three distinct pathways to form a variety of eicosanoids as shown in Figure 1.2 [18]. “Eicosanoids are 20-carbon lipid molecules derived from the enzymatic breakdown of membrane lipid precursors, chiefly arachidonic acid” [18]. These bioactive metabolites of arachidonic acid are potent in several physiological and pathological processes such as inflammation, asthma, and cancer [19, 20]. Lipid mediators generated by these enzymes are involved in a wide variety of cellular and molecular pathways, including but not limited to apoptosis, cell survival, proliferation, chemotaxis and senescence [19, 20].

Lipoxygenase Pathway:

In the lipoxygenase pathway, AA is oxidized by several enzymes resulting in the formation of hydroxyeicosatetraenoic acids (HETEs), leukotrienes, lipoxins, and hepoxilins. “The chief enzymes of the LOX pathway are the 5-LOX, 12-LOX, and 15-LOX enzymes whose names are derived from the position in which molecular oxygen is inserted into the arachidonic acid backbone” [21].
bioactive eicosanoids. Eicosanoids are generated via the oxidation of the 20-carbon chain present on arachidonic acid or other related fatty acids. During processes, such as inflammation, arachidonic acid is released from the cell membrane through the activation of phospholipase A2. Arachidonic acid is metabolized by the CYPω-hydroxylases to 7-, 10-, 12-, 13-, 15-, 16-, 17-, 18-, 19-, and 20-HETEs, the principal metabolite being the pro-inflammatory 20-HETE.

**Figure 1.2:** Schematic overview of major bioactive lipids derived from arachidonic acid metabolism by three classes of enzymes (Cyclooxygenase, Lipoxygenase and Cytochrome P450) into three pathways [22].

The predominant bioactive products of the 5-LOX pathway are the leukotrienes (LT). Leukotrienes include LTA4, LTB4, LTC4 and LTD4 [21]. LTA4 is the primary leukotriene formed by action of 5-LOX on AA, which is then hydrolyzed to LTB4 by LTA4 hydrolase [21]. Action of LTC4 synthase on LTA4 can also give rise to the cysteinyl leukotrienes, namely LTC4 and LTD4 [21, 23].

Another key enzyme of the LOX family is 12-LOX. The three important isoforms are platelet-type, epidermal, and leukocyte-type 12-LOX that act on AA to result in the synthesis of 12(S)-HETE [21]. It is known that 12-LOX and its product 12(S)-HETE is involved in the disease process of a variety of cancers including colon carcinoma,
melanoma, human glioma, prostate and breast cancer [18, 20]. Studies have also shown that 12(S)-HETE has a wide range of biological functions and has been implicated in prostate cancer metastasis through its action on endothelial cell retraction and integrin regulation [18, 24, 25]. Another key member of LOX family is 15-LOX, which exists as two isoforms; 15-LOX-1 and 15-LOX-2 [18, 20]. Action of 15-LOX on arachidonic acid gives rise to 15-HETE [21].

**P450 Epooxygenase Pathway:**

Cytochrome P450-dependent metabolism of AA includes two distinct pathways: the ω-hydroxylase and epoxygenase pathways [22]. The ω- hydroxylases of the 4A and 4F gene families of cytochrome P450 (CYP4A and CYP4F) convert arachidonic acid to autacoids such as hydroxyeicosatetraenoic acids (HETEs) [22, 26]. 20-HETE is the principal isoform of this pathway and can be vasoconstrictive [27, 28]. The epoxygenase pathway occurs in several tissues including liver, kidney, and the cardiovascular system [26]. This latter pathway includes two enzymes, CYP2C and CYP2J, which generate epoxyeicosatrienoic acids (EETs) that are known to have vasodilatory activity [22, 26]. Guo et al. have shown that 20-HETE can stimulate endothelial cell proliferation and VEGF expression in vitro suggesting that CYP ω-hydroxylase may play an important role in tumor growth and angiogenesis [29, 30]. In Non Small Cell Lung Cancer (NSCLC) cells, CYP ω-hydroxylase enhanced cellular invasion in vitro as well as angiogenesis and metastasis in vivo and significantly increased the expression of VEGF and MMP-9 in vitro and in vivo [29, 30].
Cyclooxygenase Pathway:

Most prostate cancer biology has focused on the role of COX and LOX enzymes [18, 20, 31, 32]. Cyclooxygenase has two isoforms that differ mainly in regulation of their expression and tissue distribution. COX-1 is widely expressed in most tissues, whereas COX-2 expression is normally absent, but can be induced by numerous stimuli such as growth factors, TNFα and inflammation [33, 34]. COX-2 is over expressed in many human non-endocrine and endocrine tumors including those of the colon, breast, prostate, brain, thyroid, and pituitary [33, 35]. Cyclooxygenase catalyzes the conversion of AA into a prostaglandin intermediate, PGH2, which is in turn oxidized by several other enzymes that can form two classes of eicosanoid products, namely prostaglandins (PGI₂, PGE₂, PGD₂, PGF₂α) or thromboxanes (TXA₂) (Figure-1.3) [36-38].

Prostaglandin E₂ (PGE₂) is formed by the action of prostaglandin E synthase (PGES) on Prostaglandin H₂ (PGH2). Among the COX metabolites, PGE₂ has been widely implicated in various steps of cancer development and progression such as angiogenesis, cell survival, proliferation, and chronic inflammation [21, 31, 35].

PGH2 can also be acted on by thromboxane synthase (TXA₂S) that catalyzes its conversion to thromboxane A₂ (TXA₂), a potent vasoconstrictor and an inducer of platelet aggregation [39, 40] [36, 37, 41]. Additionally the enzyme prostacyclin synthase, antagonizes the effect of TXA₂S by catalyzing the conversion of PGH2 to prostacyclin I₂ (PGI₂) that opposes the action of TXA₂, namely causing vasodilation and inhibiting platelet aggregation [42, 43].
Figure 1.3: Generation of the prostanoids through metabolism of arachidonic acid by the COX pathway. In the first step of the generation of TXA₂, COX-1 and COX-2 isoforms are responsible for the conversion of AA to PGH₂. Thromboxane synthase (TXA₂S) then catalyzes the conversion of the COX product, PGH₂ to TXA₂ [44].

Thromboxane Synthase and TXA₂:

Thromboxane synthase (TXA₂S) is an ER membrane protein that belongs to the P450 epoxygenase family and catalyzes the conversion of PGH₂ to TXA₂ [45]. The enzyme plays a role in several pathophysiological processes including hemostasis, cardiovascular disease, and stroke [46]. TXA₂S is a 60 KDa protein that was first identified as a microsomal enzyme in platelets [46, 47]. TXA₂S expression is detectable in lung, platelets, kidney, stomach, duodenum, colon and spleen [46, 48]. Its expression...
is reported to be closely associated with cardiovascular, renal, and inflammatory diseases [49].

TXA\textsubscript{2} is very unstable in aqueous solution, and becomes hydrolyzed within 30s to the biologically inactive TXB\textsubscript{2} [36, 50]. Due to its very short half-life, TXA\textsubscript{2} functions as an autocrine or paracrine mediator in the nearby tissues surrounding its site of production [48]. TXA\textsubscript{2} is responsible for multiple biological processes through the cell surface TXA\textsubscript{2} receptor, or T-Prostanoid, receptor (TP) [46]. TXA\textsubscript{2}S, and TP are the two important components necessary for the functioning of this potent bioactive lipid signaling pathway [46].

**Thromboxane A\textsubscript{2} Receptor:**

Ushikubi et al. first purified TXA2 receptor protein from human blood platelets in 1989 [51]. In 1991, a cDNA for the human TP receptor was originally cloned from placenta and the platelet-like MEG-01 cell line by Hirata et al. [52, 53]. Raychowdhury et al. later isolated a cDNA encoding a second isoform of the TP receptor from a human umbilical vein endothelial cell (HUVEC) cDNA library [53, 54]. Thus, to date there are two receptors for TXA\textsubscript{2} in humans, termed TP\textalpha{} from human placenta and TP\textbeta{} from endothelial cells [53, 55, 56]. In humans, these cDNAs represent alternative splice products of the same TXA\textsubscript{2} receptor gene. In all other species, it is only expressed as a single isoform, TP\textalpha{} [52, 54, 57]. The physiological significance of the existence of two receptors for TXA\textsubscript{2} in humans, but not in other species is still not well understood.

Human TP\textalpha{} and TP\textbeta{} are typical G-protein coupled receptors (GPCR) that share the first 328 amino acids but differ in their C-terminal tail, where TP\textalpha{} has a shorter C-terminal cytoplasmic tail than the TP\textbeta{} isoform (15 versus 79 residues) [52-54] (Figure-
Figure 1.4: Structures of TPα and TPβ Receptor Proteins. TPα and TPβ are encoded by the single TP receptor gene, on chromosome 19p13n3 [31], and arise by a novel differential splicing mechanism within exon 3, whereby nucleotides 984–1642 of the TPα mRNA behave as an intron (intron 2b) within the TPβ mRNA [52-54].

TXA₂ is responsible for multiple biological processes through its cell surface
receptor TP. Previous studies have shown that activation of TP by TXA$_2$ or its stable synthetic analog, U46619, will result in platelet aggregation [50], contraction of vascular smooth muscle cells [60] or release of prostacyclin from endothelial cells [61]. Ligation of TP by TXA$_2$ can evoke the activation of multiple downstream pathways including phospholipase C activation and a subsequent rise in the intracellular Ca$^{2+}$ concentration, leading to vasoconstriction and platelet aggregation [48, 57]. TXA$_2$ has been implicated in the pathology of a variety of cardiovascular diseases, including atherosclerosis [62], stenosis after vascular injury [63], and hypertension [64] [49].

**Distribution of Thromboxane A$_2$ Receptor (tissues and cells)**

TP can be found in a large number of tissues, and cell types include platelets, lung, vascular smooth muscle, endothelial cells, kidney, brain, spleen, thymus, monocytes, uterus, and placenta [46, 57, 65].
Table 1: Cells in which thromboxane A<sub>2</sub> receptors have been identified based on ligand binding studies [57, 65].

<table>
<thead>
<tr>
<th>Cell/tissue</th>
<th>Ligand used to identify receptor</th>
<th>Effect of TXA&lt;sub&gt;2&lt;/sub&gt; or its mimic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>125I-PTA-OH, 1H-SQ9548, 125I-BOP, 3H-U-46619, 3H-S-145, and 3H-GR32191</td>
<td>aggregation, secretion, shape change, exposure of fibrinogen receptors; activation of phospholipase C-β</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>125I-PTA-OH</td>
<td>shape change</td>
</tr>
<tr>
<td>HEL cells</td>
<td>125I-BOP, 125I-L-PTA-OH, and 3H-S-145</td>
<td>increase in [Ca&lt;sup&gt;2+&lt;/sup&gt;], contraction</td>
</tr>
<tr>
<td>Rat</td>
<td>125I-BOP</td>
<td>increase in [Ca&lt;sup&gt;2+&lt;/sup&gt;], mitogenesis and MAP kinase activation</td>
</tr>
<tr>
<td>Rabbit</td>
<td>125I-BOP</td>
<td>stimulation of the synthesis of PGI&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>Smooth muscle cells</td>
<td>125I-BOP, 3H-S-145</td>
<td>contraction and bronchoconstriction</td>
</tr>
<tr>
<td>Rat aortic</td>
<td>125I-BOP</td>
<td>increase in inositol phosphates</td>
</tr>
<tr>
<td>Guinea pig coronary</td>
<td>125I-BOP</td>
<td>contraction and stimulation of matrix protein synthesis; phosphorylation of MARCKS protein</td>
</tr>
<tr>
<td>Guinea pig lung parenchyma</td>
<td>125I-BOP</td>
<td>vasoconstriction</td>
</tr>
<tr>
<td>Guinea pig heart</td>
<td>125I-BOP</td>
<td>effects on chemotaxis</td>
</tr>
<tr>
<td>Rat glomeruli</td>
<td>1H-SQ29548, 125I-BOP</td>
<td>increases in cAMP and [Ca&lt;sup&gt;2+&lt;/sup&gt;], respectively</td>
</tr>
<tr>
<td>Rat mesangial cells</td>
<td>1H-SQ29548, 125I-BOP</td>
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<tr>
<td>Human placenta</td>
<td>1H-SQ29548, 3H-S-145</td>
<td>increase in water flow</td>
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<tr>
<td>Monocytes</td>
<td>125I-BOP</td>
<td></td>
</tr>
<tr>
<td>Equine and human</td>
<td>1H-BOP</td>
<td></td>
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<td>Mouse thymocytes</td>
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<td>Human uterus</td>
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<td>Toad urinary bladder</td>
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**Thromboxane A<sub>2</sub> Receptor–Ligand Interaction:**

It was previously shown that the transmembrane (TM) region of TP contributes to its ligand binding since point mutations of amino acids in the TM region resulted in diminished ligand-binding activity [66]. The second extracellular loop (eL2) of TP is important for ligand binging, especially amino acids Cys183 to Asp193 [67]. The NMR study demonstrated that Val176, Leu185, Thr186 and Leu187 in eL2 of TP are putative ligand contact points [68]. Point mutations within eL2 of TP indicated that Asp193 is a key amino acid for ligand binding (both for binding to agonist and antagonist) [69]. This study also reported that Phe184, Thr186 and Ser191 are key residues for antagonist...
binding [66]. Pharmaceutical companies have successfully designed TXA\(_2\) competitive antagonists for TP that target this region thereby affecting its functional role.

**Agonists and Antagonists:**

TXA\(_2\) is very unstable in aqueous solution, where it is hydrolyzed with a half-life of 30s to form the biologically inactive thromboxane B\(_2\) (TXB\(_2\)) [36]. More stable functional analogs have been synthesized because of the short half-life of TXA\(_2\) and characterized. Notable agonists include U-46619 [70] [71] and I-BOP [72] [73]. The antagonists include PTA2 [74], SQ-29,548 [75], Ramatoroban (Bay U3405) [76, 77], and seratrodast [78, 79]. The failure of existing TXA\(_2\)S inhibitors and TXA\(_2\) antagonists to show robust clinical benefit in treatment of cardiovascular disorders has largely been due to co-incident use of Nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, as part of the standard of care. Conversely, current therapeutic regimens for cancer incorporate few agents from this class making TXA\(_2\)S/TP antagonism an attractive target with potentially significant therapeutic benefit. Moreover, in contrast to NSAIDs, TXA\(_2\)S/TP inhibitors do not prevent other COX-derived anti-tumor products with beneficial effects, such as PGI\(_2\), from being synthesized.

**Thromboxane A\(_2\) Receptor and its Signal Transduction**

Stimulation of TP receptors in platelets leads to an increase in intracellular free calcium, activation of Phospholipase C, generation of IP3 and diacylglycerol, activation of protein kinase C, stimulation of myosin light chain kinase and exposure of integrin IIb/IIIa binding sites [53]. In platelets, stimulation of the TP\(_\alpha\) receptor isoform results in an increase in cAMP formation. The same observation was made with CHO and COS-7 cells transfected with TP\(_\alpha\) [53, 58]. Ushikubi et al. showed that the presence of TP\(_\beta\)
coupled to Gαi2 in reconstituted vesicles resulted in decreased cAMP formation [80].

**Contribution of G proteins in TP Signaling:**

TXA$_2$ signaling through TPα and TPβ occurs when they couple with G proteins, such as Gq, G11, G12, G13, G15, G16, Gi, Gs and Gh, which in turn regulate effectors such as phospholipase C, guanine nucleotide exchange factor of the small G protein Rho (RhoGEF) and adenyl cyclase (Figure 1.5) [57, 58, 66]. Activation of TP by TXA$_2$ can evoke the activation of phospholipase C leading to phosphatidylinositol turnover and a subsequent rise in the intracellular calcium ion concentration, which in turn leads to vasoconstriction and platelet aggregation (Figure 1.5) [57, 66]. However signaling through Gq and G12/13 appears most relevant to TP function [66]. Gq couples to platelet TP receptors, as evidenced by increases in agonist stimulated GTPase activity [66]. TP-mediated morphological changes in platelets are mainly dependent on G12/13. Platelet aggregation by TXA$_2$ is dependent on Gq (Figure 1.5) [81] [57-59].
Figure 1.5: G protein coupling of thromboxane receptor and signal transduction [66].

TP isoforms have both common and distinct signaling pathways depending on the G protein subunits bound to their C-terminal tail. Therefore, differences in function between TPα and TPβ must manifest due to the complement of G-proteins associated with it [46]. One of the major differences between the two isoforms is the cAMP pathway. When each TP isoform was expressed in CHO cells, IBOP, a TP agonist, increased intracellular cyclic AMP levels in TPα-expressing cells, but decreased cyclic AMP levels in TPβ expressing cells (Figure 1.5) [55]. These studies suggest that the coupling of TPα with Gs-stimulating adenylyl cyclase and of TPβ with Gi-inhibiting adenyl cyclase both induce changes in cyclic AMP levels, and this can be exploited [53].
Furthermore, Gh functionally interacts with TPα, but not TPβ [82]. It has been also shown that TPβ, but not TPα expression is required for the inhibition of vascular endothelial growth factor (VEGF)-induced migration and angiogenesis [83], suggesting that the downstream signaling pathways differ between TP isoforms [57-59].

**Role of Thromboxanes in Cardiovascular Disorders:**

Both TXA₂ biosynthesis and thromboxane receptor (TP) expression is elevated in numerous cardiovascular and inflammatory diseases [49, 56, 84, 85]. By signaling through its receptors, TXA₂ plays a very important role in the pathogenesis of acute coronary artery syndrome, vessel remodeling, thrombosis, renal, pulmonary and atherosclerotic cardiovascular diseases, primarily through its action as a potent vasoconstrictor and an inducer of platelet aggregation and activation [56, 62, 79, 85-88]. Therefore, inhibition of TXA₂S and TP has become central to the therapy of many diseases including myocardial infarction, hypertension, stroke and renal dysfunction [89-91]. Aspirin is the most common drug used to prevent TXA₂ production. Other thromboxane modulators which have been either approved for therapeutic use or under clinical trials for the above mentioned diseases includes terbogrel [92], Ridogrel [93], SQ-29548 [75], Ramatoroban (Bay U3405) [76, 77], and seratrodast [78, 79]. In recent years, several studies revealed an additional functional role for both TXA₂S and TP, namely in cancer progression [46, 94-103].

**Role of Thromboxanes in Cancer Progression:**

Increased COX-2 expression has been described in a variety of diseases [33, 34] and also documented in various cancers including pancreatic cancer [104], lung cancer [105-107], breast cancer [108], colon cancer [109] and prostate cancer [106, 110-112].
Though several reports have documented the antitumor effects of NSAIDs, it is not well understood how COX promotes tumorigenesis and cancer progression. As TXA$_2$ is a downstream metabolite of the COX pathway, this has elicited interest as to whether COX-2 actions are mediated by TXA$_2$ signaling [113]. The eicosanoid products of the COX pathway have dramatically different, sometimes contrasting or antagonizing, biological activities [114-116]. Therefore, the actual profile of the downstream COX metabolites is more relevant than the level of COX protein since the COX metabolites such as TXA$_2$ and PGI$_2$ have diverse and opposing functions [46].

One of the first study to look into the role of downstream metabolites of COX, such as TXA$_2$ in cancer progression and metastasis was by Honn et al., where they had shown inhibition of either TXA$_2$S or TXA$_2$ receptor reduced cancer metastasis in vivo from tail vein injected B16a lung cancer cells. Inhibition of TXA$_2$S also decreased tumor cell proliferation and inhibits DNA synthesis in B16 melanoma cells [116, 117]. Several years later, a study by Nie et al. in 2003, also indicated a functional role for TXA$_2$S in prostate cancer progression. Since then, there have been several reports indicating a functional role for not only TXA$_2$S but also for TP in several cancers that includes breast, bladder, lung and colon cancer [46, 94-103]. Cancer progression involves several key steps such as angiogenesis, cell survival, cell migration, cell invasion and metastasis, and studies have shown that both TXA$_2$S and TP play important roles in one or more of these key process [46, 95, 96, 100, 118-120].
Role of Thromboxanes in Prostate Cancer:

Nie et al. had shown that human PCa cells express TXA\(_2\)S and that this enzyme may contribute to PCa progression through modulation of cell motility [94]. As the expression of COX-2 and COX-1 in prostate cancer has been reported previously, data suggest that human PCa cells express functionally active TXA\(_2\)S and that both COX-1 and COX-2 are essential for providing the substrate for TXA\(_2\)S mediated TXA\(_2\) biosynthesis in PCa cells [94]. Increased TXA\(_2\)S expression and activity in PCa cells augmented cell migration, but had minimal effect on cell cycle progression or survival [94]. Increased TXA\(_2\)S expression and TXA\(_2\) biosynthesis in PCa cells highlighted further a possible functional role for TP in PCa tissues, as the actions of TXA\(_2\) are mediated by its interactions with TP receptors.

In a study conducted on tissue samples from 46 patients, with well-documented histological and molecular data, increased TP, TXA\(_2\)S and COX-2 expression were observed in malignant tissues (High-Grade Prostatic Intraepithelial Neoplasia (HGPIN)) [46, 97]. Similarly, TXA\(_2\)S mRNA and protein expression were higher in prostate carcinomas compared to matched normal tissues [97]. In contrast, epithelial cells in non-tumoral glands displayed almost no TXA\(_2\)S or TP expression [46]. Moreover, a significant association between the expression levels of COX-2, TXA\(_2\)S, and TP and higher Gleason score/pathologic stage of the tumors was observed [97]. Though this study confirms that TP expression is higher in prostate cancer tissues, it did not identify which isoform, TP\(\alpha\) or TP\(\beta\), was predominantly expressed [46].

Tumor cell migration is an important step in the metastatic cascade, at which
time the tumor cells leave the primary organ to enter the circulation, so as to colonize distant tissues [121]. Prostate tumor progression involves several key steps including cell survival, cell migration, cell invasion and metastasis, and studies have shown that TXA₂S or TP play a key role in either one of these processes [46]. Rho GTPases are critical for the dynamic changes in cell shape and adhesion that drive cell migration. Nie and colleagues demonstrated that the TXA₂-TP signaling axis regulated cell migration and cytoskeleton reorganization by promoting Rho-A activation [101]. Recent studies have indicated that the Gα12 family of heterotrimeric G proteins (Gα12 and Gα13) is upregulated in PCa and that activation of Gα12 signaling, through a Rho-dependent pathway, promotes PCa cell invasion. Also Gα12 signaling via Rho is required for TXA₂-stimulated invasion of PCa cells [122]. Therefore, as TP is known to couple to the Gα12 family of heterotrimeric G-proteins, the up-regulation of both TP and Gα12 in PCa suggests a possible role for TP in driving the cell migration and invasion observed in high grade PCa.

There is also a novel constitutive interaction between TPα and TPβ with the Protein kinase C-related kinases (PRK1) [123]. PRK1 is a RhoA effector that has been widely implicated in androgen-associated PCa and ovarian serous carcinomas [123]. It was established that PRK1 directly interacts with endogenously expressed TPα and TPβ in both PC3 and LNCaP cells, and that disruption of PRK1 by siRNA substantially impairs cell migration in response to TXA₂ agonist U46619 in these cells [123]. These findings are all suggestive of a functional role of TP receptor in cell migration in prostate cancer [46].
Crosstalk between Thromboxane and EGFR Signaling Pathways:

Mitogen-activated protein kinases (MAPK) play a central role in regulating cell growth and differentiation. The activation of MAPK may result from stimulation of either Receptor Tyrosine Kinases (RTK), which possess intrinsic tyrosine kinase activity, or G-Protein-Coupled Receptors (GPCR) [124, 125]. The signaling pathways that link GPCRs to MAPK involve both receptor and non-receptor tyrosine kinases as well as Protein Kinase C (PKC) [126]. The Epidermal Growth Factor Receptor (EGFR) tyrosine kinase as an essential link in the GPCR-mediated MAPK activation in Rat-1 fibroblasts, HaCaT keratinocytes, primary mouse astrocytes, and COS-7 cells [127] [128, 129]. Studies also have shown that TP-mediated activation of MAPK signaling involves EGFR by two possible mechanisms: Ligand-Independent (Src kinase-dependent) [127], and Ligand-dependent (shedding of EGFR ligands) [130].

Transactivation of EGFR by TP receptor in a Ligand Independent Manner:

Gao et al. have shown that EGFR plays an essential role in the TP-mediated MAPK activation in a ligand-independent manner. Activation of the Gq-coupled TP receptor by the TXA\textsubscript{2} mimic IBOP in ECV304 cells (bladder cancer cell-line) was found to induce ERK phosphorylation through tyrosine phosphorylation of EGFR in an Src dependent manner (Figure 1.6) [127]. Tyrosine kinases of the Src family are involved in mediating both the tyrosine phosphorylation of EGFR, and MAPK activation from both Gq and Gi coupled receptors [127] [124, 125]. Furthermore, TP receptor activation also increases Src kinase activity, which is blocked by Src Inhibitor PP1, but not by AG1478, indicating that Src activation occurs before phosphorylation of EGFR [127].
Figure 1.6: Activation of MAPK pathways by TP receptor involves transactivation of EGFR in a Src kinase-dependent manner. A proposed model by Gao et al., suggests that stimulation of TPα and TPβ by TXA₂ leads to phosphorylation of ERK (ERK-P), and involves transactivation of EGFR in a Src-kinase-dependent manner in TPα or TPβ-transfected cells [127].

TP receptor mediated Transactivation of EGFR through the shedding of EGFR Ligands:

The work of Galet et al. has shown the existence of an additional mechanism by which TXA₂ stimulation of TPα and TPβ in human aortic smooth muscle cells (hASMC) cells leads to EGFR transactivation resulting in phosphorylation of ERK1/2 in a ligand dependent manner. Transactivation of EGFR is dependent on the cleavage of pro-Heparin-binding EGF-like growth factor (HB-EGF) by matrix metallo proteinase (MMPs) in response to TP receptor stimulation thereby allowing the binding of HB-EGF to its receptor [126]. A broad spectrum inhibitor of MMPs, i.e. BB2516 decreased ERK and EGFR phosphorylation in TPα or TPβ-transfected cells indicating the important role of MMPs in TP-mediated EGFR transactivation (Figure-1.7) [126]. They also demonstrated
that both TP isoforms activated ERK phosphorylation in an Src-kinase-dependent manner in HEK293 cells. This confirms the existence of both ligand-dependent and ligand-independent mechanisms of EGFR transactivation [126].

**Figure 1.7: Crosstalk between Thromboxane and EGFR signaling by shedding of EGFR ligands by MMPs.** A proposed model by Galet et al., suggests that stimulation of TPa and TPβ by TXA₂ results transactivation of EGFR that is dependent on the cleavage of pro-HB-EGF by extracellular MMPs thereby freeing HB-EGF to bind to its receptor [126].

In another study by Uchiyama et al. TP-mediated EGFR transactivation is partially caused by shedding of EGFR ligands such as HB-EGF, which involves furin and ADAM via novel types of PKCs (PKC-δ and PKC-ε) through Gαq/11 proteins in an extracellular Ca²⁺-dependent manner [130]. Activation of TP activated the EGFR pathway by Src-dependent phosphorylation, and also activates EGFR by causing shedding of EGFR ligands. These studies strengthen further the idea that TP receptor mediates EGFR signaling.
**EGFR Family of Receptor tyrosine Kinases:**

The EGFR family of RTKs consists of four members: EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3 and HER4/ErbB4 [131-138]. All EGFR family members share a similar structure that consists of three regions: an extracellular ligand-binding domain, a single trans membrane segment, and a large intracellular domain with protein kinase activity (Figure-1.8) [139-144]. Upon activation by a number of EGF-like growth factor ligands, ErbB receptors form homo or heterodimeric complexes that, which leads to the autophosphorylation of cytoplasmic tyrosine residues in the kinase domain. This leads to activation of down-stream signaling cascades, such as the Ras activated MAPK pathway, and the phosphoinositide 3-kinase (PI3-K)/Akt pathway (Figure-1.8) [139-145].

ErbB receptors regulate multiple cellular responses, including cell proliferation, survival, migration, and differentiation [146]. EGFR and ErbB4 can homodimerize when bound to ligands, or heterodimerize with any of the other ErbB receptors, with ErbB2 being the preferred heterodimeric partner [147]. ErbB2 and ErbB3 each require heterodimerization in order to transduce signals, because ErbB3 does not have an intrinsic kinase, while ErbB2 is not bound by any known ligand (Figure-1.8) [147]. Hence ErbB2 constitutively exists in the active conformation state. Therefore, it is always available for dimerization with other EGFR family members [147]. ErbB signaling is important for normal biological function, but unregulated signaling by EGFR and ErbB2 has been shown to contribute to cancer progression [139-144]. Members of the EGFR family have frequently been implicated in various forms of human cancers and serve both as prognostic markers and as therapeutic targets [139-144].
Figure 1.8: Members of Epidermal Growth Factor Receptor (EGFR) Family: The ErbB receptor family consists of four closely related type 1 transmembrane tyrosine kinase receptors, EGFR, ErbB2, ErbB3, and ErbB4. EGFR and ErbB4 have active tyrosine kinase domains and known ligands. ErbB3 can utilize several neuregulins as ligands, but lacks tyrosine kinase activity. By contrast, ErbB2 possesses an active tyrosine kinase domain, but no ligand has been identified [148].

Epidermal Growth Factor Receptor:

ErbB1 was the first RTK to be sequenced by Ullrich et al. [22]. EGFR is a 170 kDa transmembrane glycoprotein that is present on all epithelial and stromal cells as well as on select glial and smooth muscle cells, though not on hematopoietic cells [131, 141, 143]. EGFR can be activated by binding to growth factors of the EGF-family that are produced by the same cells that express EGFR (autocrine secretion) or by surrounding cells (paracrine secretion) [139, 143]. Upon binding of an extracellular
ligand, the receptor transforms the inactive monomers into homodimers that activate its intracellular tyrosine kinase domain and autophosphorylate its C-terminal tyrosine residues. This stabilizes the active receptor conformation and creates phosphotyrosine-docking sites for proteins that transduce signals within the cell leading to activation of several downstream signaling pathways (Figure-1.9) [131, 141, 143].

The EGFR homodimer possesses multiple sites of tyrosine phosphorylation and couples to multiple downstream signaling effectors (Figure-1.9) [137, 140]. EGFR is most often upregulated in a wide variety of human tumors such as bladder, breast, head and neck, kidney, non-small cell lung, and prostate cancer, and is associated with advanced disease and poor clinical prognosis [141, 146, 149, 150]. Studies of various tumors have revealed that many express either EGFR or one of its common ligands, such as the epidermal growth factor (EGF), Amphiregulin (AREG) and transforming growth factor-α (TGFα). Some tumor cells express both the receptor and one of the ligands [142, 144, 150]. Activation of EGFR by its ligands either in an autocrine or paracrine manner has been shown to play an important role in prostate cancer progression from localized PCa to metastatic disease [141, 146, 149, 150].
Figure 1.9: EGFR tyrosine phosphorylation sites. A schematic representation of the EGFR homodimer binding to its ligand is shown. Sites of cytoplasmic tyrosine (Y) phosphorylation are indicated, as are cytosolic effector proteins that bind to these phosphorylated tyrosine residues, and some of the effector signaling pathways [137, 140].

EGFR Ligands:

EGFR family members are activated by a large group of EGF-related growth factors. Major EGF family members includes EGF, Amphiregulin (AREG), Epiregulin (EREG), Transforming Growth Factor-α (TGF-α), Heparin-binding EGF-like growth factor (HB-EGF), Betacellulin (BTC), Epigen and Neuregulins (NRG-1, NRG-2, NRG-3, NRG-4) (Table-2) (Figure-1.8) [131, 132]. Common to all these growth factors is the
EGF domain with six conserved cysteine residues characteristically spaced to form three intramolecular disulphide bridges [141, 142, 144, 150]. In general, EGF-like ligands are synthesized as glycosylated transmembrane precursors, which are proteolytically cleaved from the cell surface by MMPs to yield the mature growth factor [141, 142, 144, 150].

With respect to ErbB receptor binding, EGF-related growth factors can be divided into three groups (Table-2) [141]. The first group includes EGF, TGF-α and AREG, which bind specifically to the EGFR [141, 142, 144, 150] (Table-2). The second group includes BTC, HB-EGF, and EREG, which show dual specificity by binding both EGFR and ErbB4 [141, 142, 144, 150] (Table-2) (Figure-1.8). The third group is composed of the Neuregulins (NRGs) and can be divided into two subgroups based upon their capacity to bind ErbB3 and ErbB-4 (NRG-1 and NRG-2), or only ErbB4 (NRG-3 and NRG-4). None of the EGF family of peptides binds ErbB-2 [138, 141, 151-153] (Table-2) (Figure-1.8).

<table>
<thead>
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<th>The ErbB receptors and their cognate ligands</th>
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<tr>
<td>ErbB Receptors</td>
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*Table.2: EGFR family of receptors and their ligands* [154].
Amphiregulin- an EGFR Ligand:

Amphiregulin (AREG) is a glycoprotein that is originally synthesized as a trans-membrane precursor that undergoes ectodomain cleavage and is released as a secreted protein [155, 156]. EGFR is believed to be the sole cell surface receptor for AREG in epithelial cells, as AREG has not been shown to interact directly with other members of the EGFR family such as ErbB2, ErbB3 and ErbB4 [157]. AREG binds EGFR with a lower affinity than EGF, and induces receptor autophosphorylation and downstream activation of the ERK signaling cascade [156, 158]. AREG mRNA is expressed in many normal tissues including the placenta and ovary, where it is abundant, as well as in the skin, testis, pancreas, spleen, kidney, lung, breast, and gastrointestinal tract [159].

Several studies identified an active cAMP-Responsive Element (CRE) in the AREG promoter [160, 161]. Other conserved response elements have been identified in the human AREG promoter such as the Serum Responsive Element (SRE), the highly conserved Specificity Protein-1 element (SP1), the WT-1 responsive element (WRE) and the TATA box that all may have roles in regulation of AREG expression [156, 159, 162-166]. AREG can also be regulated by the EGFR itself, where activation of EGFR leads to AREG expression in several cell types [155] [163, 165, 167]. For instance, stimulation of EGFR in prostate stromal cells with mitogenic levels of EGF and HB-EGF increased AREG mRNA expression more than 15-fold [166]. However, the specific pathway leading from EGFR activation to AREG transcription has not been elucidated prior to our study. Similar to EGFR, AREG also has been associated with several types of human cancer [155, 158, 168, 169].
Role of EGFR and their ligands in Prostate Cancer:

EGF family members and their membrane receptor, the EGFR are involved in the pathogenesis of different tumors, including prostate cancer [170, 171]. Overexpression of EGF family members and their receptors results in the progression of normal prostate epithelial cells to a hyper proliferative cancer cell which is a key step in the multi-step process of development of prostate cancer [171, 172]. Changes in gene expression of growth factors and the corresponding changes in their receptor signaling pathways in epithelial and stromal cells, contributes to enhancing tumor cell growth, survival, migration and invasiveness [173, 174].

Studies have confirmed the important role played by the EGFR-driven autocrine pathway in prostate cell growth, as it has been shown that prostate cancer cells express the EGFR in vitro and in vivo, and that TGF or EGF are potent mitogens for prostate cancer cells [154, 171]. Activated EGFR signaling may induce the stimulation of distinct mitotic cascades, including those of the MAPK, PI3K/Akt, and Phospholipase-C (PC) signaling pathways These in turn participate in the stimulation of proliferation, survival, motility and invasion of PCa [174]. There are a number of mechanisms by which growth factors of the EGF family, such as EGF, AREG, and TGF-α could contribute through autocrine and paracrine mechanisms to prostate cancer cell growth and proliferation [154, 158]. The enhanced expression of EGFR and its ligands, EGF, TGF-α, HB-EGF and AREG, has been reported to correlate with high grades of PCa malignancies [158, 174].
Role of Amphiregulin in Prostate Cancer:

AREG has been studied extensively for its role in breast cancer progression but there have been few studies about its possible involvement in prostate cancer until now [155, 168, 169]. Previous studies showed increased expression of AREG in both androgen-dependent and independent PCa cell lines [157, 175].

One of the first hints of a possible role for AREG in PCa was in a study by Sehgal et al. where it was shown that expression of AREG message and peptide in both androgen-sensitive and androgen-independent prostate cancer cell-lines. Androgen-dependent PCa cells, such as LNCaP cells, do express amphiregulin mRNA and peptide and this expression is elevated by androgenic stimulation [157]. Furthermore, TGF-α stimulation of EGFR induces AREG expression in LNCaP cells suggesting the mechanism of androgen-induced AREG expression is dependent on EGFR signaling. Therefore androgenic effect of AREG synthesis may occur through androgen upregulation of the EGFR expression [157].

Another study by Torring et al. has shown a selective upregulation of some ligands belonging to the EGF family in androgen-independent prostate cancer cell lines. Expression of mRNA for the ligands TGF-α, AREG, HB-EGF and EREG were increased 10 to 100 fold in androgen-independent cells and as compared to LNCaP and PNT1A cells (Immortalized normal prostate epithelium) [175]. Similar results were obtained when examining the protein levels as confirmed by ELISA [175].

Bostwick et al. analyzed prostate tissue sections from 93 prostate cancer patients with pathologic stage T2N0M0 intermediate to high-grade (Gleason 6 and higher, by design) and found that AREG expression in prostate increases progressively
from benign epithelium to PIN and to prostate cancer. They were able to conclude that increased expression of AREG might contribute to the development of prostatic adenocarcinoma [158]. There have not been any studies looking further into the mechanisms that result in increased AREG expression in both androgen-dependent and independent cell lines. We have revealed herein a link between TP receptor signaling and AREG in prostate cancer.
Statement, Hypothesis and Specific aims:

Statement and Rationale:

Most of the work performed on TP receptor in prostate cancer thus far has not focused on which of the two TP isoforms is indeed responsible for TP’s role in prostate cancer. Previous studies have indicated different functional roles for both TP isoforms [46, 83]. In lung cancer, TPα is thought to promote angiogenesis [176], whereas TPβ expression promotes increased proliferation, migration, and invasion in bladder cancer cells. It is over-expressed in bladder cancer patients and associated with poorer prognosis in those patients [99]. Expression status of TP isoforms is largely uncharacterized in prostate cancer cells and tissues. One of the objectives of this study is to profile TP isoform expression in prostate cancer cells and tissues, and determine their functional contribution in prostate cancer.

As previous studies had shown that the TPα receptor isoform could promote angiogenesis by up regulating VEGF in the lung cancer cell line A549 overexpressing TPα receptor [176], we initially focused on whether TP signaling could promote VEGF expression and subsequent angiogenesis in PCa cells.

Activation of endogenous TP in DU145 cells by TXA₂ agonist led to an increase in VEGF expression as expected, and revealed for the first time, a novel link to the expression of amphiregulin (AREG) (1.5 Fold increase) in response to TP receptor stimulation. AREG is a specific ligand of EGFR that activates its downstream signaling pathways such as the MAP kinase pathways. We also looked at effect of TPα
stimulation on the predominant ligand for EGFR namely EGF, and found no effects (Figure-1.10).

**Figure 1.10: TP receptor activation in DU145 cells increased expression of AREG, a ligand for EGFR.** DU145 cells were serum starved and treated with IBOP 50nM (A) or ETOH (B) for 16 hrs. Before the conditioned medium was collected to measure the impact of TP receptor signaling on angiogenesis modulators. IBOP activation of TP receptor increased VEGF expression (2 fold) as well as expression of AREG (1.5 fold).

TP receptor activation can lead to transactivation of EGFR through phosphorylation in a both ligand dependent (HB-EGF) or ligand independent (Src kinase) manner thereby implicating TP receptor as a regulatory point in the signaling pathways that are stimulated by EGFR [127] [126]. Both TP and EGFR are highly expressed in PCa and this has been shown to play a role in its progression [46, 101, 174]. Previous studies also have found increased expression of AREG in prostate cells and tissues but the specific pathways leading to AREG expression have not yet been elucidated [157, 158, 175]. Increased expression of AREG in response to TP stimulation can lead to an alternate mechanism of EGFR activation in PCa cells. To the best of our knowledge, this is the first study to explore the direct link between TP signaling and
AREG expression in prostate cancer or any other cancer. As both TP receptor and TXA₂S enzyme are expressed in a wide range of cells and tissues including PCa tissues and endothelial cells [46, 94, 101], increased expression of AREG in response to TP receptor stimulation could lead to constitutive activation of EGFR signaling that could increase tumor growth and progression. These findings led us to hypothesize that the functional role of TP in PCa progression is mediated through activation of EGFR signaling brought about by overexpression of its ligand, AREG.

**Hypothesis**

TP Receptor signaling plays a role in prostate cancer by promoting AREG activation of EGFR signaling.

**Specific Aims:**

- Characterization of thromboxane receptor isoforms in PCa cell lines (*in vitro*) and human tissues (*in vivo*).
- Determine whether stimulation of TP lead to AREG and EGFR overexpression and activation of downstream signaling pathways.
- Determine the functional implications of TP activation in PCa invasion, tumor growth and angiogenesis *in vivo*
CHAPTER-2: MATERIALS AND METHODS

Materials and Reagents:

TXA₂ agonist (I-BOP) and TXA₂ antagonist (SQ29548, BM567) were purchased from Cayman Chemicals (Ann Arbor, MI). PKA inhibitor H89, MEKK inhibitor U0126, EGFR inhibitor AG1478, Src Inhibitor PP2 were obtained from Calbiochem (San Diego, CA). EGFR inhibitor Gefitinib was kindly provided by the laboratory of Dr. Mustapha Kandouz (Department of Pathology, Wayne State University). AMPK inhibitor Compound-C was purchased from Millipore (Billerica, MA). Other chemicals were obtained from Sigma–Aldrich (St. Louis, MO). RPMI-1640 culture medium and heat-inactivated fetal bovine serum (FBS) were purchased from Hyclone (Thermo Fisher, Rockford, IL). Human PCa cells PC3, DU145 and RWPE1 cells were supplied and validated by the American Type Culture Collection (ATCC, Manassas, VA) and PC3M Cells were obtained from Dr. Isaiah J. Fidler (University of Texas, MD Anderson Cancer Center, Houston, TX). ECL Western blotting detection system was purchased from Thermo Scientific (Rockford, IL) as were the horseradish peroxidase (HRP)-linked goat anti-mouse and -rabbit IgGs.

Cell Culture:

PC3, DU145, PC3M and LNCaP cells were grown in RPMI (Hyclone Laboratories, Inc., Logan, UT) containing 10% FBS at 37°C in 5% CO₂ incubators. RWPE-1 cells were grown in Keratinocyte Serum-Free Medium (K-SFM) that contained two growth factors - 0.05 mg/ml BPE and 5 ng/ml EGF. Cell lines were frequently monitored for mycoplasma contamination by mycoplasma PCR detection kit (Sigma–Aldrich, St. Louis, MO).
Generation of Stable Transfectants:

PC3 cells were transfected with plasmids encoding TPα (PC3-TPα), TPβ (PC3-TPβ) or pcDNA 3.1 empty vectors (PC3-Neo) using the TransIT transfection reagent for prostate (Mirus Bio LLC, Madison, WI) as per the manufacturer's instructions. After 48h, cells were grown for selection with G418 (400µg/ml) over two weeks. Clones expressing high levels of TPα were pooled for total protein isolation as were empty vector control cells (Neo). PC3-TPα and PC3-Neo cells were maintained in RPMI-1640 with 10% FBS in the presence of 0.2 mg/ml of G418 (Geneticin, Life Technologies, Grand Island, NY). Real time PCR was done with TP receptor isoform specific Taqman probes (Life Technologies, Grand Island, NY) to validate stable transfectants.

Antibodies:

Rabbit polyclonal antibodies to human TPα and TPβ were kindly provided by Dr. Anthony Ashton (University of Sydney, Sydney, Australia). Rabbit polyclonal antibodies to Pan TP were purchased from Cayman Chemicals or GeneTex, Inc. (Irvine, CA). Rabbit monoclonal antibody specific to EGFR, ErbB2, ErbB3, ErbB4, p-ERK, ERK, p-SRC, SRC, p-AMPK, AMPK and CD31 were from Cell Signaling Technology, Inc. (Danvers, MA). Antibody specific for beta-Actin was purchased from Millipore (Billerica, MA). Horseradish peroxidase (HRP)-linked goat anti-mouse and rabbit IgG were supplied by Thermo Fisher (Rockford, IL).

Primer Design and RT-PCR:

The gene specific primers for human TPα and TPβ and GAPDH were ordered from Invitrogen. Thermo cycling PCR conditions: 95 °C for 2 min (Hot Start), (95°C for
30s, 60°C for 30s, 72°C for 45s) for 40 cycles. Corresponding PCR products were run on a 1.6% Agarose gel with Ethidium bromide and pictures of the gel were taken under UV illumination using a Flour Chem Alpha-Imager Imaging system (Santa Clara, CA).

### Table 3: List of Human specific primers used for RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>For-Forward</th>
<th>Rev-Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP-For [54]</td>
<td>5’- TGGTTTCCGGCCACAAAAATTA -3’</td>
<td></td>
</tr>
<tr>
<td>TP-Rev [52]</td>
<td>5’- GATACCCAGGTGCTCTGTCA -3’</td>
<td></td>
</tr>
<tr>
<td>TP-For</td>
<td>5’- CTTCTGCTGAACACGCTCA -3’</td>
<td></td>
</tr>
<tr>
<td>TP-Rev</td>
<td>5’- GATATACCCAGGGGTCCAG -3’</td>
<td></td>
</tr>
<tr>
<td>TPα-For [177]</td>
<td>5’- TTCCTGCTGAACACGGTC -3’</td>
<td></td>
</tr>
<tr>
<td>TPα-Rev [177]</td>
<td>5’-CGGAGCGCTGCTGAGGTCT-3’</td>
<td></td>
</tr>
<tr>
<td>TP-For [99]</td>
<td>5’-ACCGAGAAGGAGCTGCTATCT-3’</td>
<td></td>
</tr>
<tr>
<td>TPα-Rev [99]</td>
<td>5’-CCAGCCCTGAATCCTCA-3’</td>
<td></td>
</tr>
<tr>
<td>TPβ-Rev [99]</td>
<td>5’-CAAAAGGAAGCAACTGTACCCC-3’</td>
<td></td>
</tr>
<tr>
<td>GAPDH-For</td>
<td>5’-GAGTCAACGGATTTGGTCGT-3’</td>
<td></td>
</tr>
<tr>
<td>GAPDH-Rev</td>
<td>5’-TTGATTTTGGAGGGATCTCG-3’</td>
<td></td>
</tr>
<tr>
<td>B-Actin-For</td>
<td>5’-GGACTTTCAAGCAAGGATGG-3’</td>
<td></td>
</tr>
<tr>
<td>B-Actin-Rev</td>
<td>5’-AGCATGTGTTGGCGGTACAG-3’</td>
<td></td>
</tr>
</tbody>
</table>

**Real-time PCR Taqman Gene Expression Assay:**

Total RNA was isolated from sub confluent cells (60-80% confluent) using Nucleospin-2 kit (Macherey-Nagel, Inc., Bethlehem, PA) following the manufacturer’s protocol and reverse-transcribed into cDNA using the ABI High Capacity RT Kit (Life
Technologies, Grand Island, NY). For real-time detection of target gene expression, TaqMan® Gene Expression Assays (Life Technologies, Grand Island, NY) were used. Taqman Gene expression assays for TPα and TPβ were performed with custom designed probes made with the help of ABI Primer Express software (Life Technologies, Grand Island, NY). The GAPDH housekeeping gene internal control was a commercial assay (Life Technologies, Grand Island, NY). PCR was performed using Applied Biosystems 7500 Fast Real-Time PCR System in a total reaction mixture of 20 μl containing 25 ng of cDNA, 1 x ABI TaqMan PCR Master Mix, and 1.25 μl of probe/primers mixture (TaqMan® Gene Expression Assays). After denaturation at 95 °C for 10 min, 40 cycles were performed at [95 °C for 10 s, 60 °C for 1 min] (universal cycling conditions as per the manufacturer). The values for ΔΔCT were determined and final relative expression analyses were performed using $2^{-\Delta\Delta CT}$ ($\Delta\Delta Ct = \Delta Ctsample - \Delta Ctreference$) as described previously [178].

SYBR Green Assay (AREG, EGF, EGFR, CD31, IL6, ErbB2, ErbB3, ErbB4):

Total RNA was isolated from sub confluent cells (70-80% confluent) (Nucleospin-2, Macherey-Nagel, Inc., Bethlehem, PA) and reverse-transcribed into cDNA using the ABI High Capacity RT Kit (Life Technologies, Grand Island, NY). Primers were designed then synthesized by Invitrogen (Life Technologies, Grand Island, NY) or purchased from Real Time Primers (Elkins Park, PA). Real- time PCR was performed in 10 ul reactions using 96-well plates, 10-25 ng cDNA, and the 2X SYBR Green PCR Master Mix (Life Technologies, Grand Island, NY). Reactions were performed in triplicate using the Applied Biosystems ABI 7500 real-time PCR machine (Life Technologies, Grand Island, NY).
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AREG-For [167]</td>
<td>5’-GTGGTGCTGCTGCTCTTTGATA-3’</td>
</tr>
<tr>
<td>AREG-Rev [167]</td>
<td>5’-ACTCACAGGGAAATCTCACT-3’</td>
</tr>
<tr>
<td>EGFR-For</td>
<td>5’-CTCAGCCACCATTATGTACC-3’</td>
</tr>
<tr>
<td>EGFR-Rev</td>
<td>5’-CGTCCATGTCTTTCTTTCATCC-3’</td>
</tr>
<tr>
<td>GAPDH-For</td>
<td>5’-GAGTCAACGGATTGGGTCGT-3’</td>
</tr>
<tr>
<td>GAPDH-Rev</td>
<td>5’-TTGATTTTGGAGGGATCTCG-3’</td>
</tr>
<tr>
<td>B-Actin-For</td>
<td>5’-GGACTTTCGAGCAAGAGATGG-3’</td>
</tr>
<tr>
<td>B-Actin-Rev</td>
<td>5’-AGCATGTGTTGGCGTACAG-3’</td>
</tr>
<tr>
<td>VEGFA-For</td>
<td>5’-AGACACACCCACCCACCATAC-3’</td>
</tr>
<tr>
<td>VEGFA-Rev</td>
<td>5’-TGCCAGAGCCTCTCATCTCC-3’</td>
</tr>
<tr>
<td>EGF-For [167]</td>
<td>5’-CGCAGGAATGGGAATTCTA-3’</td>
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<tr>
<td>EGF-Rev [167]</td>
<td>5’-TCCACCACCAATTGCTCATA-3’</td>
</tr>
<tr>
<td>IL6 For</td>
<td>5’-AGCGCCTTCCGTCCTGGTG-3’</td>
</tr>
<tr>
<td>IL6 Rev</td>
<td>5’-GTGGCTGTCTGTGTGGGGCG-3’</td>
</tr>
<tr>
<td>IL8 For</td>
<td>5’-TAGCAAATATGAGGCGCAAGG-3’</td>
</tr>
<tr>
<td>IL8 Rev</td>
<td>5’-AGCAGACTAGGGTGCCAGA-3’</td>
</tr>
<tr>
<td>CD31 For</td>
<td>5’-ATTGCAGTGTTATCATCAGGAGTG-3’</td>
</tr>
<tr>
<td>CD31 Rev</td>
<td>5’-CTCGTTGTTGGAGTTCAAGATG-3’</td>
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<tr>
<td>EREG-For [167]</td>
<td>5’-CTGCCTGTGGTTCCATCTTCT-3’</td>
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<tr>
<td>EREG-Rev [167]</td>
<td>5’-GCCATTCAGTGCAGAAGCTACCT-3’</td>
</tr>
<tr>
<td>ErbB2-For</td>
<td>5’-CAGGCACCACGCTCATCTCA-3’</td>
</tr>
<tr>
<td>ErbB2-Rev</td>
<td>5’-TCCAGGTCACCATAATACATC-3’</td>
</tr>
</tbody>
</table>
**SDS-PAGE and Western Blotting**

Cells were extracted in cell lysis buffer, and proteins were loaded on 10% SDS-PAGE gels and transferred onto nitro-cellulose membranes. Blots were then probed with indicated antibodies and developed by enhanced chemi-luminescence (Pierce, Thermo Fisher, Rockford, IL) according to the manufacturer’s instructions.

**Tissue cDNA Arrays:**

Commercial cDNA arrays containing representative sets of normal and cancerous human tissue cDNAs (TissueScan™) were purchased from Origene Technologies, Inc. (Rockville, MD) and used as per manufacturer’s instructions.

**Cell Treatments:**

For treatments, cells were plated in complete growth medium in 100 mm cell culture dishes until they are 50-70% confluent. After 24-48 hrs, spent medium was aspirated and replaced in serum-free medium for serum starvation overnight. Inhibitors were dissolved either in DMSO or ethanol, diluted in serum free medium, and added to the cells at appropriate concentrations for a given amount of time. Once the cells were pre-treated at appropriate concentrations with the inhibitors (EGFR inhibitors- AG1478,
gefitinib, AMPK inhibitor- Compound-C, PKA inhibitor-H89), TXA₂ agonist I-BOP (50 nM) was dissolved in ethanol and added to the cells in serum-free medium for the indicated times.

**Measurement of cAMP:**

Following treatment with I-BOP or antagonist, media samples from cultured cells were collected and stored at -80°C for 1-2 weeks before its assayed. The cAMP enzyme immunoassay (EIA) kit was purchased from Cayman Chemical (Ann Arbor, MI) and used according to the manufacturer’s instructions.

**Antibody Array:**

The relative levels of expression of 55 angiogenesis-related proteins were simultaneously detected using the commercial angiogenesis array (Catalog No: ARY007) (R&D Systems, Minneapolis, MN). DU145 cells and PC3 transfectants were cultured in 100 mm dishes, serum-starved overnight, and subsequently treated with either IBOP (50nm) or ETOH (Vehicle) for 18hr as described. Conditioned culture medium was then screened by antibody array for angiogenesis related proteins as per manufacturer’s instructions.

**Modified Boyden Chamber Cell Invasion Assay**

Cell invasion assays were done in modified Boyden chambers fitted with 8-µm transwell filters as described [179]. PC3 parental or transfected cells were seeded in the top of transwells of the 24 well plates at a density of (2x10⁵ cells) / (0.5 ml of 1% BSA in RPMI medium with ETOH, IBOP or SQ29548). Fetal bovine serum at 1% was used as a
chemo attractant in the bottom chambers. After 18 h, residual, non-migrating cells on the upper side of the membranes were removed using Kim-wipes, and the membranes were fixed and stained according to manufacturer’s instructions for the Diff Quick staining kit (IMEB, Inc., San Marcos, CA). Five fields of 100 cells, from random areas were counted.

**Animal Model and *in vivo* Studies:**

All animal procedures were performed in accordance with the NIH and institutional guidelines established for the Department of Laboratory Animal Research (DLAR) at Wayne State University. Male athymic NCr nude mice were purchased from Taconic Farms, Inc. (Germantown, NY). A total of $2 \times 10^6$ PC3-TP\(\alpha\) cells or PC3-Neo control cells in 200 µl of HBSS were injected sub cutaneous (s.c.) into the right flank of 4-6-week-old male NCr nude mice (Six mice per group). Tumor measurements were made using a vernier caliper, and tumor volume was calculated using the formula: $\text{Volume} = (\text{width})^2 \times \text{length}/2$ [180].

Mice were sacrificed six to seven weeks after injection, and tumors were resected for further analysis [181]. Resected tumors were weighed and split into 10% formalin for immunohistochemistry and in Ambion’s RNAlater reagent (Life Technologies, Grand Island, NY) (frozen at -80°C) for subsequent RNA isolation.

**Immunohistochemistry**

Serial sections from formalin-fixed, paraffin-embedded (FFPE) tissues were processed for immunohistochemistry analysis of CD31 and ki67 through core services provided by University Pathology Research Services (Wayne State University and
Barbara Ann Karmanos Cancer Institute, Detroit, MI)

**Matrigel Implantation Assay for Tumor Cell-induced Angiogenesis.**

The Matrigel implantation assay was performed as described by Ito et al. [182] with the following modifications. Male SCID mice were purchased from Taconic Farms (New York, NY). A total of $2 \times 10^6$ PC3-TPα cells or PC3-Neo control cells in 100 µl of HBSS were mixed with 300 µl of Matrigel BME (Trevigen, Gaithersburg, MD) and injected s.c. into the right flank of 4-6-week-old male SCID mice (six mice per group). Mice were sacrificed 12 days after injection and dissected to expose the implants for recording [183].

**Measurement of AREG in Culture Media:**

Cells were cultured in 6-well plates for measuring secreted AREG and other angiogenic cytokines. Cells were plated at a density of $2 \times 10^5$ cells per well of a 6-well plate. After 24 h, cells were serum starved overnight and then treated with TP agonists, antagonists and chemical inhibitors. Following treatment, spent medium was collected and the levels of AREG were determined using ELISA immunoassay kits from Ray Biotech (Norcross, GA), based on manufacturer’s instructions.

**Statistical Analysis:**

Student's $t$ test (two tailed) was used to analyze the difference between two groups. Differential P values <0.05 were considered significant.
CHAPTER-3: TPα-MEDIATED OVER EXPRESSION OF AMPHIREGULIN IS MEDIATED BY AMP-ACTIVATED PROTEIN KINASE (AMPK)

Prostate cancer cells express TPα receptor:

Though the existence of two TP isoforms was known as early as 1994, most of the earlier studies on TP did not distinguish the functional roles between the two isoforms in cardiovascular disorders and cancer progression [46, 53, 58, 66]. However recent findings suggest a novel role for TPβ in cancer progression especially in bladder cancer [99]. TPα might play a role in tumor growth by promoting angiogenesis in lung cancer [176], whereas TPβ expression promotes increased rate of proliferation, migration, and invasion in bladder cancer cells and over-expressed in bladder cancer patients and associated with poorer prognosis in those patients [99, 184]. In our earlier studies, where we have shown TP receptor plays a role in cell motility by activating Rho-A in prostate cancer was documented using antibodies/probes that bind the N-terminal domain that is common to both TP receptor isoforms. Expression status of TP receptor isoforms and downstream signaling in response to TXA₂ binding is largely uncharacterized in prostate cancer cells and tissues. In this study, we first want to determine which TP isoform is expressed in prostate cancer cell lines and tissues.

To evaluate the potential role of TP receptors in prostate cancer, prostate epithelial cells and PCa cells were screened so as to identify which receptor isoforms are present and to determine their expression levels. RT-PCR (Figure 3.1) and immunoblots (Figure 3.2) on whole cell extracts of the prostate cancer cell lines PC3, DU145, PC3M and LNCaP, revealed that these PCa cells only express the TPα
receptor isoform and not the TPβ receptor isoform. Real-time PCR data also indicated that DU145 had the highest level of TPα expression (10-11 fold) followed by PC3 (3-4 fold) and PC3M (3 fold) compared to RWPE1 or PREC cells (Figure 3.3). TPα expression in LNCaP cells was similar to RWPE1 cells. These data reveal that TPα expression is higher in PCa cells compared to prostate epithelial cells, and that of the cell lines tested, DU145 had the highest expression levels of this isoform.

Expression profile of TPα (mRNA) receptor in PCa cell-lines with TP isoform specific Primer sets (RT-PCR)

<table>
<thead>
<tr>
<th></th>
<th>PC3</th>
<th>DU145</th>
<th>PC3M</th>
<th>LNCaP</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC(+) RT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-RT</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**A: TP isoform specific Primer set-1**

**B: TP isoform specific Primer set-2**

*Figure 3.1: Screen for expression of TPα receptor in prostate cancer cell lines by RT-PCR.* Expression of TPR was verified in several PCa cell lines such as PC3, DU145, PC3M and LNCaP cells and prostate epithelial cells (RWPE1) using RT-PCR. cDNA from PCa cell lines was analyzed with two sets of isoform specific primers designed to detect: TPα, or TPβ (A, B). PCR products were validated as TPα by sequencing. Non-RT samples (samples in which no reverse transcriptase was added) were used as Negative controls. Recombinant TPα and TPβ plasmids were used as positive controls (PC) for TP expression as indicated. Shown here is a representative result from three independent experiments.
A: Expression profile of TPα (protein) receptor in PCa cell-lines (Immunoblot)

B: Expression profile of TPβ (protein) receptor in PCa cell-lines (Immunoblot)

Figure 3.2: Screen for expression of TPα in prostate cancer cell lines by immunoblot. Expression of TPR was verified in several PCa cell lines: PC3, DU145, PC3M and LNCaP cells using Western blot. Total cell lysates prepared from these cell lines were probed with isoform specific TP antibodies that can detect TPα (A) and TPβ (B). Recombinant TPα protein expressed in SF-9 cells using baculovirus system was used as the positive control for TPα, [185] and HUVEC cell lysates served as the positive control for TPβ. β-Actin served as a loading control. The asterisks in A and B indicate non-specific bands. Shown here is a representative result from three independent experiments.
**Figure 3.3: TPα expression was high in PCa cells compared to prostate epithelial cells.**

Real-time PCR was done to compare the expression of TPα mRNA levels between PCa cell lines (PC3, DU145, PC3M and LNCaP) and normal prostate epithelial cells (RWPE1, PREC). Expression of TPα was higher among the PCa cells when compared with prostate epithelial cells (RWPE1, PREC). Among the PCa cell lines, DU145 cells expressed the highest levels of endogenous TPα mRNA expression. Values expressed represent the mean fold change ± SEM, compared to PREC cells and is derived from three experiments normalized in each case to GAPDH gene expression. Similar results were observed in a further two independent experiments.

**Functional status of TPα receptor expressed in PCa cells:**

The multiplicity of effects by TXA₂ stems from its receptor isoforms TPα and TPβ coupling to several and distinct G-protein subunits leading to differences in function between the two isoforms. TPα receptor isoform binds to Gs subunit thereby stimulating adenylate cyclase, resulting in increased levels of the second messenger cAMP, whereas TPβ receptor isoform binds to Gi subunit and inhibits adenylate cyclase, thereby reducing cAMP levels [66]. Therefore, the differential response by two TP
isoforms in terms of second messenger cAMP accumulation can be used as a tool to confirm the expression and functional status of TPα expression in PCa cells. Our data demonstrates that cAMP levels are higher in DU145 cells compared to PC3, PC3M and LNCaP cells which aligns with our earlier PCR and Immunoblotting results (Figure 3.4). Although PC3 and PC3M cells exhibit expression of TPα protein and mRNA, the level of cAMP accumulation in response to IBOP was lower in these cells compared to DU145 cells.

![Functional assay for TPα - cAMP levels](image)

**Figure 3.4: Endogenous TPα expression in PCa cells can elicit a functional response in terms of second messenger.** To verify whether TPα expression was enough to elicit a functional response, PC3, DU145, PC3M and LNCaP cells were treated with vehicle (ETOH) and agonist (IBOP) alone or pretreated with antagonist (SQ29548) before addition of agonist for 20min, and the effect on cAMP synthesis was measured by cAMP specific Enzyme ImmunoAssay (EIA). Results were DU145 had higher cAMP levels in response to IBOP when compared with other PCa cells. The results shown are mean ± standard deviation of triplicate wells, *p <0.05, **p <0.01; Student’s t test. This experiment was repeated at least three times with similar results.
Data-mining analysis confirms increased expression of TPR in Human PCa tissues

TPα plays a critical role in tumor growth, angiogenesis, invasion and inflammation [46, 176, 186, 187]. Moreover, expression of TXA₂S, a rate-limiting enzyme for TXA₂ biosynthesis, is significantly up regulated in prostate cancer tissues and correlates positively with aggressiveness of the disease based on Gleason score [94]. In a study by Dassasse et al., significant association between the expression levels of COX-2, TXA₂S, and TP and higher Gleason score/pathologic stage of the tumors was observed [97]. Increased TP expression in PCa tissues documented in this study was done using antibodies that bind to the common domain of TP, hence did not provide sufficient information about which of the two TP isoforms is predominantly expressed [46].

To further examine the expression of TP isoform in prostate tumor tissues, we have performed data mining analysis for TP expression in the cBio Cancer Genomics Portal from Memorial Sloan-Kettering Cancer Center (MSKCC) that has published the most comprehensive genomic prostate cancer database available to date [188, 189]. Several important observations resulted from these analyses, namely that TP receptor expression appeared to be upregulated in 19% of prostate cancer patients studied (n=85) (Figure 3.5), and was higher in PCa tissues compared to normal patient samples (Figure 3.5). If the Z-score threshold is lowered to 1.5, then TP expression appeared to be up regulated in 31% (n=26) of prostate cancer patients studied. However, the MSKCC studies also do not discriminate between the TPα and TPβ isoforms as the probes designed were for the common domain of TP [188].
Figure 3.5: Database search using Memorial Sloan-Kettering Cancer Center genomics database indicates that TPR expression is amplified in prostate carcinoma. TP expression is upregulated in 19% (14 samples) of total PCa samples (n=85) tested by microarray analysis with a Z-Score threshold of 2 that is statistically significant. Microarray analysis for TP receptor was done with probes that were designed for the common domain of TP and hence do not discriminate between the TPα and TPβ isoforms (n=85) [188]

TPα expression correlates positively with aggressiveness of prostate tumors.

All the work done so far showing increased expression of TP in PCa does not discriminate between the two TP isoforms. We have shown that the PCa cell lines do only express the TPα isoform and not the TPβ isoform. Since we know that TP is overexpressed in PCa tissues based on our database analysis, we hypothesized that it might be the TPα that is up regulated. Human PCa tissue was profiled for TPα utilizing commercial cDNA expression arrays that included representative samples of both
normal and prostate cancer tissues. Tissue cDNAs of each array were synthesized from high quality total RNAs from pathologist-verified tissues, normalized and validated with β-actin in two sequential qPCR analyses. The manufacturer provided these with clinical information and QC data. As there were no isoform-specific Taqman probes available for TPα and TPβ through ABI, the arrays were screened with custom probes that were designed using ABI Sequence detection software. Utilizing the accompanying clinical information TPα expression levels were associated with grades of different tissue samples based on Gleason scores [8] (Figure 3.6).

The TPα isoform was elevated in higher grade, more aggressive prostate cancer seen in patients with a Gleason score greater than 7 (n = 9) versus Gleason 7 (n = 21) or Gleason 6 and below (n = 11) and matched normal tissues (n=8) (Figure 3.6). Based on the difference in primary and secondary patterns, the sum Gleason score 7 can be depicted as GS (4+3) or GS (3+4), where the primary grade must be >50% of the total cancer observed, and the secondary grade must be <50% but at least 5% of the observed cancer [9]. Therefore, even within the same Gleason score differences in gene expression are known to exist [9]. A significant finding from these data was that TPα expression was elevated in GS7 (4+3) (n=7) compared to GS7 (3+4) (n=17), which suggests that differences in TPα expression between primary and secondary patterns (Figure 3.6) can potentially be exploited as a biomarker for discriminating GS7 subtypes when making prognostic determinations. It also implicates TPα as a therapeutic target. Collectively, the data-mining results and the data from qPCR analysis of human tissue arrays make a strong case for the association of TPα expression with the aggressiveness and progression of prostate tumors.
Figure 3.6: TPα expression levels correlate with grades of different cancer tissues that are classified based on its Gleason scores. Real-time PCR for TPα was done on Human Prostate Cancer tissue cDNA arrays using custom designed TPα specific taqman assay probes. The TPα isoform was elevated significantly in higher grade, more aggressive prostate cancer seen in patients with a Gleason score greater than 7 (n=9). TPα expression is elevated significantly in GS7 (4+3) (n=7) compared to GS7 (3+4) (n=17), suggesting stratification of TPα expression between primary and secondary patterns. Values expressed represent the mean fold change ± SEM, compared to Normal prostate tissues and is derived from two experiments normalized in each case to β-Actin gene expression.

Overexpression of TPα receptor in PC3 cells:

To further understand whether TPα signaling mediates increased expression of AREG, that might be responsible for its functional role in PCa, we used two different Cell line models: 1. Wild-type DU145 cells (High endogenous TPα expression, Low
TXA$_2$S expression) 2. PC3 cells overexpressing TP$\alpha$. PC3 cells do express thromboxane synthase (TXA$_2$S) and are a widely used model for in vivo studies. While PC3 cells appeared to express endogenous TP$\alpha$, the level of expression was insufficient to elicit functional responses of TP$\alpha$ signaling such as cAMP activation and phosphorylation of ERK compared to DU145 cells. In order to increase the expression of TP$\alpha$ in PC3 cells to elicit better functional responses, we stably transfected TP$\alpha$ plasmid in PC3 cells to examine the effect of TP$\alpha$ overexpression.

Stable trasfectants were generated and confirmed by Real-time PCR with TP$\alpha$-specific taqman assay probes (Figure 3.7A), and by Western blotting (Figure 3.7B). TP$\alpha$ transfected PC3 cells became responsive to 50 nM I-BOP induction of cAMP synthesis (Figure 3.7C). Whereas, minimal activation of cAMP was measured in control PC3-Neo cells (Figure 3.7C). These data demonstrated that the IBOP-responsive phenotype of DU145 cells could be recapitulated in PC3 cells expressing ectopic TP$\alpha$. 

![Graph showing over-expression of TP$\alpha$ receptor (mRNA) in PC3 cells (Real-time PCR)](image-url)
3.7 B: Over-expression of TPα receptor (protein) in PC3 cells (Immunoblot)

PC3 cells were transfected with TPα or TPβ expression plasmids, or empty vector (pcDNA 3.1) transfectants referred to as PC3-TPα, PC3-TPβ and PC3-Neo were tested for over expression using: real-time PCR with Taqman gene expression probes specific for TPα (A); Values expressed represent the mean fold change ± SEM, compared to vector control and is derived from three experiments.

Figure 3.7: Over expression of TPα receptor in PC3 cells. PC3 cells were transfected with TPα or TPβ expression plasmids, or empty vector (pcDNA 3.1) transfectants referred to as PC3-TPα, PC3-TPβ and PC3-Neo were tested for over expression using: real-time PCR with Taqman gene expression probes specific for TPα (A); Values expressed represent the mean fold change ± SEM, compared to vector control and is derived from three experiments.
normalized in each case to GAPDH gene expression. Similar results were observed in a further two independent experiments. Western blotting was done with antibodies specific for TPα and platelets cell lysates were used as positive control for TPα (B). The asterisks in B indicate non-specific bands (B) Immunoblotting for each protein was performed at least three times using independently prepared lysates, and representative data from one such experiment are shown. The functional status of TPα receptor was verified by measuring cAMP accumulation by cAMP Enzyme Immunoassay (EIA)(C) The results shown are mean ± standard deviation of triplicate wells, **p <0.01; Student’s t test. This experiment was repeated at least three times with similar results.

**Activation of TPα signaling promotes overexpression of AREG in PC3 cells over expressing TPα:**

Subsequently, PC3 prostate cancer cells expressing various levels of TPα were employed to examine the role of TPα in prostate cancer progression. We treated PC3-Neo cells, PC3-TPα transfectants with either ETOH (Vehicle) or TXA₂ agonist IBOP for 16hrs and conditioned medium was collected for further analysis. Supernatants were screened for growth factors such as AREG in response to TXA₂ signaling using a commercial antibody array (R&D Systems, Minneapolis, MN). In comparison to PC3-Neo cells, PC3-TPα transfectants responded to IBOP activation of TPα by overexpressing AREG (Figure 3.8 A-D).

To verify the findings of elevated levels of AREG expression in PC3-TPα transfectants from the antibody array, ELISA for AREG was performed on the conditioned medium collected earlier from PC3 transfectants. Our results confirmed a higher secretion of AREG (5 fold) by the PC3-TPα cells in response to IBOP compared to vector control cells (PC3-Neo) (Figure 3.8E). Pretreatment with TXA₂ antagonist
SQ29548 before addition of IBOP inhibited the increase in AREG expression and levels were comparable to ETOH treated cells (Figure 3.8E). Using Real-time PCR, the effect of TPα stimulation (IBOP) or inhibition (SQ29548) on AREG mRNA expression was tested more directly. The results indicate that AREG mRNA levels increased 6 fold in response to IBOP compared to ETOH treatment (Figure 3.8F). Pretreatment with TXA₂ antagonist SQ29548 before addition of IBOP inhibited the increase in AREG mRNA and levels were comparable to ETOH treated cells (Figure 3.8F).

To verify the findings from the TPα transfected cells, wild-type DU145 cells were treated with either ETOH (Vehicle) or TXA₂ agonist IBOP or TXA₂ antagonist SQ29548 for 16hrs. Elevated levels of AREG expression both at the protein (ELISA) and mRNA levels (1.5 fold) in response to IBOP were also confirmed by in DU145 cells (Figure 3.8 G, H). These findings proved that TPα does play a direct role in increased expression of Amphiregulin, a ligand for EGFR in prostate cancer cells.

3.8 A-D: Effect of TPα stimulation by IBOP on AREG (protein) expression in PC3 transfectants (Antibody Array)
E. Effect of TPα stimulation by IBOP on AREG expression (protein) in PC3-TPα transfectants (ELISA)

F: Effect of TPα stimulation by IBOP on AREG (mRNA) expression in PC3-TPα transfectants (Real-time PCR)
Figure 3.8: Activation of TPα in PC3 cells over expressing TPα increases AREG, a ligand for EGFR. PC3-TPα and PC3-Neo cells were serum-starved overnight followed by treatment with ETOH (A, C) or IBOP (B, D). Conditioned medium was collected and screened by an angiogenesis antibody array. Activation of TPα by IBOP in PC3-TPα cells led to an increase in AREG expression levels but had little effect on EGF expression levels. PC3-TPα and PC3-Neo transfectants were serum-starved overnight and were treated either with ETOH or IBOP (50 nM) only, or they were pretreated with SQ29548 (10 µM) for 30 min before addition of IBOP. Conditioned medium was collected and cDNA isolated from the cell lysates. Data from Real-time PCR (F) and ELISA (E) also confirms the increase in expression of AREG mRNA and protein expression in PC3-TPα cells treated with IBOP when compared with ETOH or SQ29548 treated cells. Similar results were obtained with parental DU145 cells when treated with IBOP (G, H). Real-time PCR values expressed represent the mean fold change ± SEM, compared to...
vector control and are derived from three experiments normalized in each case to GAPDH gene expression. Similar results were observed in a further two independent experiments. The ELISA results shown are mean ± standard deviation of triplicate wells, *p <0.05, **p <0.01; Student’s t test. This experiment was repeated at least three times with similar results.

**Activation of TPα signaling had little effect on EGF expression in PC3 cells over expressing TPα:**

We also looked at the effect of TPα receptor on EGF, which is the predominant ligand for EGFR, and did not find any change in EGF levels (Figures 3.8 A, B, C, D). To determine whether TPα receptor stimulation could lead to increased expression of EGF mRNA, we did Real-time PCR on cDNA samples from PC3-TPα transfectants treated with vehicle (ETOH), agonist (IBOP) or antagonist (SQ29548). We did not find any change in EGF mRNA expression in response to IBOP by Real-time PCR in PC3-TPα transfectants (Figure 3.9A). Similar results were obtained in parental DU145 cells that express higher levels of endogenous TPα (Figure 3.9B). These results indicate that activation of TPα by IBOP selectively upregulates AREG expression at the level of protein and mRNA but has little effect on EGF expression.
**Figure 3.9: Activation of TPα in PC3-TPα transfectant cells over expressing had little effect on expression of EGF, the predominant ligand for EGFR:** PC3 transfectants (A) and DU145 cells (B) were serum starved overnight and cells were treated with ETOH or IBOP (50 nM) only or pretreated with SQ29548 (10 µM) for 30m before addition of IBOP. After 16 hrs. of treatment, cells were lysed in RNA lysis buffer for total RNA isolation. Expression of EGF mRNA in PC3-TPα (A) and DU145 cells (B) was minimally affected by treatment with IBOP. Values
expressed represent the mean fold change ± SEM, compared to vector control cells and is derived from three experiments normalized in each case to GAPDH gene expression. Similar results were observed in a further two independent experiments.

**AREG produced through activation of TPα receptor is functional and can phosphorylate EGFR in PC3 cells:**

AREG selectively binds to EGFR, following dimerization of EGFR, which leads to dimerization of the receptor itself as either a homodimer or as a heterodimer with other EGFR family members. Subsequently EGFR undergoes phosphorylation at specific tyrosine residues within the intracellular domain that in turn activates downstream signaling pathways such as MAP kinase [ERK-1/2]. Therefore, we sought to determine whether the AREG that was induced in response to TPα receptor stimulation in PC3 transfectants was functional. We assayed AREG from PC3-TPα conditioned medium for its ability to bind EGFR and mediate phosphorylation at tyrosine residue (pY1068).

To test whether this led to subsequent activation of ERK1/2, we performed following addback experiments. Conditioned medium was collected from PC3-Neo cells or PC3-TPα transfectants treated with IBOP alone (PC3-TPα+IBOP, PC3-Neo+IBOP) for 16hrs. To determine if TPα mediated AREG production made a difference in the phosphorylation of EGFR or ERK1/2, we compared immunoblots of cells treated with CM from PC3-TPα to that of CM from PC3-Neo cells. We also performed the addback experiments in parental PC3 cells that have endogenous EGFR but have lower TPα receptor levels. To avoid any trans activation of EGFR involving endogenous TPα receptor, PC3 cells were pretreated with TXA₂ antagonist SQ29548 or ETOH for 30 minutes before addition of IBOP-treated-conditioned medium collected from PC3-TPα.
transfectants. Whole cell lysates were prepared and subsequently analyzed for phosphorylation of EGFR and downstream targets such as ERK1/2 (Figure 3.10).

Our results support an active role for TPα receptor driven AREG production that leads to AREG activation of EGFR at pY1068 (Figure 3.10). Our results also support that AREG in the conditioned medium collected from PC3-TPα cells not only binds to EGFR leading to its phosphorylation but can activate downstream signaling pathways such as MAP kinase pathway as evident by phosphorylation of ERK1/2 (Figure 3.10). Therefore our studies reveal a novel means of indirect TPα receptor activation of EGFR through the effects of TPα receptor on AREG.

3.10 Functional status of AREG secreted in response to TPα receptor stimulation in the conditioned medium from PC3 transfectants (Immunoblot)

3.10 A: Effect of AREG on EGFR phosphorylation (Immunoblot)
3.10 B: Effect of AREG on EGFR phosphorylation (Immunoblot)

3.10 C: Effect of AREG on MAPK activation (Immunoblot)
3.10 D: Effect of AREG on MAPK activation (Immunoblot)

<table>
<thead>
<tr>
<th>Time</th>
<th>PC3-TPα CM</th>
<th>PC3-Neo CM</th>
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<tr>
<td>5 minutes</td>
<td>SQ29548+IBOP</td>
<td>IBOP</td>
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<tr>
<td>10 minutes</td>
<td>SQ29548+IBOP</td>
<td>IBOP</td>
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D: ERK1/2

3.10 E: Effect of AREG on EGFR Phosphorylation – Loading control (Immunoblot)

<table>
<thead>
<tr>
<th>Time</th>
<th>PC3-TPα CM</th>
<th>PC3-Neo CM</th>
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<tr>
<td>5 minutes</td>
<td>IBOP</td>
<td>SQ29548+IBOP</td>
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<tr>
<td>10 minutes</td>
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E: β-Actin
**Figure 3.10: AREG secreted in response to TPα receptor activation is active and leads to EGFR phosphorylation.** PC3-TPα and PC3-Neo cells were serum-starved overnight and then treated with IBOP only. Conditioned medium was collected and secreted AREG activity post TPα stimulation was assayed. PC3 parental cells were serum-starved overnight and pretreated with TXA₂ antagonist SQ29548 or ETOH for 30 min. These were subsequently treated with the conditioned medium collected from PC3 transfectants for 5min or 10min. Cell lysates were probed by immunoblot with phospho-specific antibodies for EGFR (A) and ERK1/2 (C). Total EGFR (B) and ERK1/2 (D) were used as loading controls for p-EGFR and p-ERK respectively. Actin served as a protein loading control (E). AREG in the conditioned medium from PC3-TPα cells stimulated increased phosphorylation of EGFR at pY1068 and ERK1/2 when compared to the conditioned medium from PC3-Neo cells. These findings indicate that AREG produced in response to activation of TPα is functional through binding of EGFR leading to its phosphorylation and activation of MAPK pathways. Immunoblotting for each protein was performed at least twice using independently prepared lysates, and representative data from one such experiment are shown.

**Activation of TPα signaling promotes overexpression of EGFR in PC3 cells over expressing TPα:**

After observing the relationship between TPα signaling and increased AREG expression at the level of both protein and mRNA, we looked to see whether TPα affected the expression of EGFR itself from PC3-TPα and PC3-Neo cells that were treated with ETOH, IBOP and SQ29548. After 16 hr, cells were trypsinated and cell pellets were divided into two tubes for protein and cDNA analysis respectively. Real-time PCR and immunoblot analyses revealed that EGFR mRNA and protein expression was significantly elevated in PC3-TPα cells in response to IBOP when compared to the vector control cells (PC3-Neo) (Figures 3.11 A, B). Pretreatment with TXA₂ antagonist SQ29548 before addition of IBOP reduced EGFR expression to the level in untreated
cells, thereby confirming that TPα signaling also regulates EGFR expression in addition to AREG (Figures 3.11 A, B).

We opted to corroborate the findings from PC3 transfectants by examining parental cells that have naturally elevated levels of TPα. These were treated with either ETOH (Vehicle) or TXA₂ agonist IBOP or TXA₂ antagonist SQ29548 for 16hr. DU145 cells stimulated with IBOP had a significant increase in EGFR mRNA (Figure 3.11C) and protein levels (Figure 3.11D) compared to ETOH treated cells. Pretreatment with SQ29548 prior to addition of IBOP reduced EGFR expression back to levels achieved with ETOH alone (Fig 3.11 C, D). Collectively these data reveal that TPα activation with IBOP in PC3-TPα transfectants as well as in parental DU145 cells stimulated AREG production, and also its cognate receptor, EGFR. This demonstrates for the first time that signaling events of the two receptors are coupled in prostate cancer.
3.11 B: Effect of TPα stimulation by IBOP on EGFR (protein) expression in PC3 transfectants (Immunoblot)
3.11 D: Effect of TPα stimulation by IBOP on EGFR (protein) expression in DU145 cells (Immunoblot)

**Figure 3.11**: Activation of TPα in PC3 cells over expressing TPα resulted in an increase of EGFR. PC3-TPα, PC3-Neo and DU145 cells were serum starved overnight and were either treated with ETOH, IBOP (50 nM) only or pretreated with SQ29548 (10 μM) for 30m before addition of IBOP for 16 hr. After treatment, cells were trypsinized, centrifuged and cell pellets were divided into two tubes for protein and cDNA analysis respectively. Data from Real-time PCR.
PCR reflects an increase in expression of EGFR mRNA expression in PC3-TPα cells treated with IBOP (A) when compared with ETOH or SQ29548 treated cells. Results from the immunoblotting suggest that activation of TPα by TXA₂ mimetic IBOP in PC3-TPα cells resulted in an increase of EGFR (B) Similar results were obtained with DU145 cells that have higher endogenous TPα expression (C, D). Immunoblotting for each protein was performed at least three times using independently prepared lysates, and representative data from one such experiment is shown. Real-time PCR Values expressed represent the mean fold change ± SEM, compared to ETOH treated vector control cells and is derived from three experiments normalized in each case to GAPDH gene expression. Similar results were observed in a further two independent experiments.

**Activation of TPα signaling had little effect on expression of ErbB2, ErbB3 and ErbB4 in PC3 cells over expressing TPα**

As EGFR was over expressed in response to TPα stimulation, we then sought to determine whether TPα could affect a change in expression of other EGFR family members such as ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4) that can indeed dimerize with EGFR. PC3 transfectants cells were serum-starved overnight and treated with ETOH, IBOP alone or pretreated with SQ29548 for 30 min followed by addition of IBOP for 16 hr. After treatment, cells were trypsinized, centrifuged and cell pellets were divided into two tubes for protein and cDNA analysis respectively. Real-time PCR (Figures 3.12 A, B, C) and Immunoblot analyses (Figure 3.12 D) revealed that there was very little effect on ErbB2, ErbB3 and ErbB4 mRNA and protein expression in PC3-TPα cells in response to IBOP when compared to the vector control cells (PC3-Neo). Pretreatment with TXA₂ antagonist SQ29548 before addition of IBOP, did not affect the expression of EGFR family members (Figures 3.12A, B, C, D). These studies clearly demonstrate that TPα activation with IBOP in PC3-TPα transfectants resulted in increased expression of EGFR but not other EGFR family members.
3.12 A: Effect of TPα stimulation by IBOP on ErbB2 expression in PC3-TPα transfectants (Real-time PCR)

3.12 B: Effect of TPα stimulation by IBOP on ErbB3 expression in PC3-TPα transfectants (Real-time PCR)
3.12 C: Effect of TPα stimulation by IBOP on ErbB4 expression in PC3-TPα transfectants (Real-time PCR)

3.12 D: Immunoblot: Effect of IBOP on ErbB2, ErbB3, ErbB4 expression in PC3 transfectants
Figure 3.12: TPα activation in PC3-TPα transfectants had little effect on expression of other EGFR Family members, ErbB2, ErbB3 and ErbB4. PC3-TPα and PC3-Neo cells were serum-starved overnight and were treated either with ETOH, IBOP (50 nM), only or pretreated with SQ29548 (10 µM) for 30m before addition of IBOP for 16 hr. After treatment, cells were trypsinized, centrifuged and cell pellets were divided into two tubes for both protein and total RNA isolation. Real-time PCR data indicate that activation of TPα by IBOP in PC3-TPα cells had little effect on expression of ErbB2, ErbB3 and ErbB4 mRNA (A, B, C) when compared with ETOH or SQ29548-treated cells. Additionally results from the immunoblotting suggest that activation of TPα by TXA2 mimic IBOP in PC3-TPα cells had little effect on expression of other EGFR family members, ErbB2, ErbB3 and ErbB4 (D). Real-time PCR Values expressed represent the mean fold change ± SEM, compared to vector control cells and is derived from three experiments normalized in each case to GAPDH gene expression. Similar results were observed in a further two independent experiments. Immunoblotting for each protein was performed at least twice using independently prepared lysates, and representative data from one such experiment are shown.
**EGFR upregulated as a result of TPα activation is functional and actively signals in PC3 transfectant cells:**

As our data from real-time PCR and Immunoblot showed that PC3 cells do express EGFR but exhibited differential expression of other EGFR family members in PC3 transfectant cells. Therefore, we wanted to confirm, whether the EGFR expressed in response to TPα stimulation in PC3 transfectants cells was functional. To test the functionality of EGFR produced by PC3-TPα transfectants in response to TPα stimulation, we treated PC3-Neo and PC3-TPα cells with either EGF or vehicle (PBS) for 5 min. Total protein was harvested from these cells and assayed for the levels of p-EGFR, EGFR, p-ERK and ERK by Western blotting (Figure 3.13).

As evident on Figure 3.13, treatment of PC3-Neo and PC3-TPα cells with EGF resulted in increased phosphorylation of EGFR at pY1068, which is an indication of ligand dependent phosphorylation and a functional receptor (Figure 3.13). Furthermore, signaling pathways that are downstream of EGFR such as MAP kinase were activated as evident by phosphorylation of ERK1/2 (Figure 3.13).
3.13 Functional status of EGFR expressed in PC3 transfectants (Immunoblot)

A: pEGFR (pY1068)

B: EGFR

C: pERK1/2

D: ERK1/2
**Figure 3.13: Add-back experiment proving that EGFR expressed in PC3 transfectants is functional.** PC3-TPα and PC3-Neo cells were serum-starved overnight and then treated with either PBS (vehicle) or EGF for 5min and cells were lysed, and immunoblotted with phospho-specific antibodies for EGFR (A) and ERK1/2 (C). Total EGFR (B) and ERK1/2 (D) were used as a loading control for p-EGFR and pERK1/2 respectively. Actin (E) served as a protein loading control. Our data indicate that EGF treated PC3-Neo and PC3-TPα cells showed increased phosphorylation of EGFR and ERK1/2 concluding that EGFR expressed in both PC3-Neo and PC3-TPα cells is functional. Immunoblotting for each protein was performed at least twice using independently prepared lysates, and representative data from one such experiment are shown.

**Upregulation of AREG by TPα is not dependent on the PKA pathway:**

The molecular mechanisms involved in the regulation of AREG expression by TPα receptor are largely unknown. Our studies show that activation of TPα by IBOP in PC3-TPα cells results in increased levels of the second messenger cAMP (Figure 3.4). Recent studies have shown that the cAMP pathway can induce AREG in T cells as well as in cells of nonlymphoid origin such as H295R (adrenal) and MCF-7 cells (breast cancer) [160]. As it is known that one of the downstream targets of cAMP is PKA, we wondered whether PKA might be involved in the expression of AREG.
To look into the role of PKA and EGFR in TPα mediated AREG expression, we pretreated PC3-TPα and PC3-Neo cells with PKA inhibitor H89 (20 μM) for 30 min before incubation with TXA₂ agonist IBOP for 18 hr. Following incubation, total RNA was isolated from these cells and processed for Real-time PCR analysis of AREG mRNA expression. Treatment with the PKA inhibitor H89 had no effect on AREG expression mediated by activation of TPα receptor (Figure 3.14). This suggests that upregulation of AREG by TPα is not dependent on the PKA pathway.

**Figure 3.14: Upregulation of AREG by TPα is not dependent on the PKA pathway.** PC3-TPα and PC3-Neo transfectants were serum-starved overnight and were treated either with ETOH, IBOP (50 nM) only, or pretreated with SQ29548 (10 μM) and H89 before addition of IBOP for 16 hr. Data from Real-time PCR confirms that PKA inhibitor H89 had no effect on AREG expression mediated by activation of TPα receptor in PC3-TPα cells when compared to IBOP treated cells alone. Values expressed represent the mean fold change ± SEM, compared to ETOH treated vector control cells and is derived from three experiments normalized in each case to GAPDH gene expression. Similar results were observed in a further two independent experiments.
Upregulation of AREG by TPα is dependent on the AMPK pathway.

Though studies have shown that AREG can be induced by the cAMP pathway in T cells as well as in cells of nonlymphoid origin such as H295R (adrenal) and MCF-7 cells (breast cancer) [160], our Real-time PCR data nonetheless showed that pretreatment of PC3 transfectant cells with PKA inhibitor (downstream mediator of cAMP) had no effect on expression of AREG, which suggested an alternate pathway involving cAMP [160]. We wondered whether AMP-activated protein kinase (AMPK), an alternate cAMP-signaling pathway is involved in the overexpression of AREG mediated by stimulation of TPα receptor. As we have previously shown that activation of TPα does increase cAMP levels, the relationship between thromboxane signaling, AMPK and AREG was studied in TPα overexpressing PC3 cells. AMPK is a sensor for changes in cellular energy state and is activated by an increase in the intracellular AMP-to-ATP ratios in response to various stresses such as hypoxia, oxidative stress and glucose deprivation [190-194]. Activation of AMPK requires phosphorylation of Thr172 in the activation loop of the α subunit by at least 2 upstream kinases, LKB1 and Ca\(^{2+}\)/calmodulin-dependent kinase kinase (CaMKK)-β [190-194].

TPα activation increases cAMP levels, as well as intracellular Ca\(^{2+}\) concentration. Therefore we investigated the role of AMPK in TPα mediated AREG expression. Previous studies by Zhang et al. had shown that TP activates the AMPK in vascular smooth muscle cells via hydrogen peroxide [190].

Apart from cAMP being a regulator of AREG expression, studies have shown that AREG also can be regulated by the EGFR itself and activation of EGFR leads to AREG expression in several cell types [155, 157, 160, 166]. As it is known that EGFR
can directly regulate AREG through EGFR-AREG autocrine loop, we questioned whether this might be true in TPα mediated AREG upregulation [155].

To investigate whether TPα mediates AREG upregulation via the AMPK pathway and to identify the role-played by EGFR in the expression of AREG, we pretreated the PC3-TPα and PC3-Neo cells with the AMPK inhibitor Compound-C (20 μM) and EGFR inhibitors Gefitinib (20 μM) or AG1478 (5 μM) for 30 min before incubation with TXA₂ agonist IBOP for 18hrs. Gefitinib or AG1478 is an EGFR inhibitor that binds to the tyrosine kinase domain and inhibits downstream EGFR signaling. Following incubation, total RNA was isolated from these cells converted to cDNA and Real-time PCR was done for AREG mRNA expression. The amount of AREG transcript was estimated based on the Relative quantification method. Treatment with the AMPK inhibitor Compound-C in PC3-TPα cells led to an decrease in the level of AREG as shown in Figure 3.15A to the levels comparable to ETOH or SQ29548 treated cells. This suggests that TPα upregulates AREG mRNA levels through cAMP by stimulating the AMPK pathway (Figure 3.15A).
Figure 3.15: Upregulation of AREG by TPα is dependent on AMPK pathway. PC3-TPα and PC3-Neo transfectants were serum starved overnight and were either treated with ETOH, IBOP 50nM only or pretreated with SQ29548 (10 μM), Gefitinib (20 μM), AG1478 (5 μM) and Compound-C (20 μM) for 30 min before incubation with IBOP for 18 hr. AMPK inhibitor
Compound-C strongly affects AREG expression mediated by TPα in PC3-TPα cells (A). EGFR inhibitors, Gefitinib and AG1478 reduced expression of AREG, mediated by TPα in PC3-TPα cells to a lesser extent than compound-C (A). Values expressed represent the mean fold change ± SEM, compared to vehicle treated vector control and is derived from three experiments normalized in each case to GAPDH gene expression. Data from ELISA supports the Real-time PCR data, that inhibition of the AMPK pathway reduces levels of AREG expression mediated by TPα activation (B). Similar results were observed in a further two independent experiments. The ELISA results shown are mean ± standard deviation of triplicate wells, *p <0.05, **p <0.01; Student’s t test. This experiment was repeated at least three times with similar results.

To verify our findings from Real-time PCR at the protein level, conditioned media was collected from PC3-TPα and PC3-Neo cells treated with AMPK inhibitor or EGFR inhibitors and the levels of AREG were assayed by ELISA and normalized to cell number. We observed that, Compound-C decreased AREG secretion from PC3-TPα cells when compared to IBOP treated cells (Fig 3.15B). Also treatment with EGFR inhibitors had a partial inhibition on expression of AREG mediated by TPα when compared with AMPK inhibitor (Fig 3.15B). The reduction of AREG secretion in PC3-TPα overexpressing cells upon treatment with AMPK inhibitor suggests that the AMPK pathway is required for AREG secretion in these cells (Fig 3.15B). From these experiments, it is evident that AMPK pathway plays a key role in IBOP mediated increase in AREG secretion in the TPα overexpressing cells.

**Activation of TPα signaling results in increased PCa cell invasion.**

To determine whether TPα activity in PC3 cells translates to a more invasive phenotype and AREG does play a role in it, a modified Boyden chamber assay was performed where transwell chambers were coated with Matrigel, and PC3-TPα or PC3-Neo cells were treated with either ETOH (Vehicle), IBOP (agonist), or SQ29548 (antagonist). Of all the treatments, only IBOP increased the invasiveness PC3-TPα cells
Similar results were obtained with DU145 cells that have higher endogenous TPα levels. Treatment of DU145 cells with IBOP also increased the invasive capacity of the cells when compared to treatment with either ETOH or pretreatment with the antagonist SQ29548 (Figure 3.16C). Invasiveness of PC3-TPα and PC3-Neo cells were similar in response to 5% FBS stimulation.

Studies have shown that EGFR signaling pathway plays an important role in cell migration and invasion [195, 196], we wondered whether AREG or EGFR might be involved in increasing the invasive capacity of the cells mediated by TPα. Over expression of AREG mediated by TPα in PCa cells can then activate EGFR and then activate signaling pathways that result in increasing the invasive nature of these cells. To study whether AREG or EGFR has a role to play in it, we pretreated PC3-TPα and PC3-Neo cells with EGFR inhibitor gefitinib (25 μM) or AMPK inhibitor-Compound-C (20 μM) for 30min, before incubation with IBOP for 18 hr and cells were fixed and pictures of invasive cells were taken with a Nikon TE200 inverted microscope (Figure 3.16A, C,). Gefitinib is an EGFR inhibitor that binds to its tyrosine kinase domain and inhibits signaling through the EGFR pathway and Compound-C is an AMPK inhibitor. Binding of gefitinib to EGFR also will prevent AREG to activate EGFR and thereby affecting its role in cellular invasion whereas AMPK inhibitor should affect the overexpression of AREG mediated by TPα receptor. Treatment with Gefitinib or AMPK inhibitor did reduce the invasiveness of these cells, induced by IBOP, indicating that EGFR signaling is involved in TPα mediated invasion (Figure 3.16A, B, C, D). Similar results were obtained with DU145 cells that have higher endogenous TPα levels when treated with EGFR inhibitor gefitinib or AMPK inhibitor (Figure 3.16E, F).
3.16 A: Effect of EGFR and AMPK inhibitors on TPα mediated cell invasion in PC3-TPα cells (Modified Boyden chamber assay)

3.16 B: Effect of EGFR and AMPK inhibitors on TPα-mediated cell invasion in PC3 transfectants (Modified Boyden Chamber Assay)
3.16 C: Effect of EGFR and AMPK inhibitors on TPα mediated cell invasion in PC3-Neo cells (Modified Boyden chamber assay)
3.16 E: Effect of EGFR and AMPK inhibitors on TPα mediated cell invasion in parental DU145 cells (Modified Boyden chamber assay)

Figure 3.16: Activation of TPα signaling by IBOP stimulation results in increased invasiveness of PCa. Modified Boyden chamber assay was done where transwell chambers were coated with matrigel and PC3-TPα and PC3-Neo cells that were serum starved overnight and were either treated with ETOH, IBOP (50 nM) only or pretreated with SQ29548 (10 μM) Gefitinib (20 μM) or AMPK inhibitor Compound-C (20 μM), before IBOP was added to the chamber. After 16 hr of treatment, cells that invaded to the bottom of transwell chamber were fixed and photographed. IBOP treatment stimulated the invasive capability of PC3-TPα cells in
the in vitro invasion assay, whereas the vehicle, antagonist, Gefitinib, Compound-C reduced the invasive capability of PC3-TPα cells (A, B). Similar results were obtained with DU145 cells that have higher endogenous TPα expression (C). Representative phase contrast microscope pictures are shown in correlation with the invasion assay from three independent experiments. It was also quantified by counting the number of cells that invaded to the lower side of the filter in three representative fields in each independent experiment.

**TPα signaling results in larger tumors in a mouse model:**

To examine the physiological relevance of increased colony formation and invasion brought on by TP-α expression and signaling in PCa cells, we introduced PC3-TPα or PC3-Neo cells into athymic mice in an established subcutaneous (s.c.) xenograft tumor model to assess tumor growth in vivo. Prostate cancer cell proliferation in vitro was largely comparable between PC3-TPα and PC3-Neo cells. This suggested that PC3-TPα transfectants did not have a growth advantage over PC3-Neo cells in vitro (Data not shown).

However mice that received PC3-TPα cells formed larger tumors compared to mice injected with PC3-Neo cells, indicating that TPα transfected PC3 cells had an in vivo growth advantage compared to Neo controls. (Figures 3.17, 3.18 A, B). Weekly measurements over a 7-8 week period, revealed that tumors originating from PC3-TPα cells injected into nude mice grew faster compared to PC3-Neo mice. (Figure 3.17) After 7-8 weeks, mice were sacrificed as per the animal protocol, and tumors resected from the mice receiving PC3-TPα were larger and found to weigh approximately twice that of tumors originating from PC3-Neo-injected mice. (Figure 3.18 A, B, C, D)
Figure 3.17: Mice bearing PC3-TPα cells showed greater tumor growth compared to the control group. Increases in tumor growth in mice injected with PC3-TPα transfectants were observed compared to PC3-Neo controls. Tumor growth was plotted for 7-8 weeks and a growth curve was obtained.

3.18: Size and weight Comparison of tumors originating from PC3 vector controls and PC3 cells overexpressing TPα

A: Mice injected with PC3-TPα cells @ week-8

B: Mice injected with PC3-Neo cells @ week-8
Angiogenesis plays an important role in tumor growth and is considered a key step in tumor development. We found significant vascularization in tumors derived from TPα transfected PC3 cells, whereas the PC3-Neo control tumors showed little vessel

Figure 3.18: Size and weight Comparison of tumors originating from PC3 vector controls and PC3 cells overexpressing TPα. PC3 cells over expressing TPα yielded larger tumors in vivo compared to PC3 parental cells carrying the expression vector alone. Mice (n=6) receiving PC3-TPα (A) and mice (n=6) receiving PC3-Neo (B) were photographed on Day-56 after they were sacrificed. Tumors excised from mice injected with PC3-TPα cells were larger in size compared to the PC3-Neo controls (C). Tumors excised from mice injected with PC3-TPα cells displayed twice the weight (D) compared to the PC3-Neo controls. Weight of the mice displayed on the graph is average weight of each group of mice (n=6) (D).
penetration. (Figure 3.19A) Paraffin-embedded tumor sections from mouse xenografts of PC3-TPα cells or PC3-Neo cells were immunostained using antibodies specific for CD31, which is an endothelial cell marker (Fig 3.19C). Real-time PCR on the cDNA from tumors also confirmed the increased expression of CD31 in PC3-TPα tumors compared to controls (Fig 3.19B).

To confirm whether increase in proliferation due to EGFR signaling was responsible for increased tumor size seen in PC3-TPα mice, Paraffin-embedded tumor sections from mouse xenografts of PC3-TPα cells or PC3-Neo cells were immunostained using antibodies specific for Ki67, which is an proliferation marker (Fig 3.19C). Immunohistochemistry analysis of the tumors sections also confirmed the stronger staining for Ki67 in PC3-TPα tumors compared to controls (Fig 3.19D).

Our in vitro data revealed that IBOP activation of TPα enhanced cellular AREG and EGFR levels. Therefore, mouse tumors were examined for expression of these downstream targets. Analysis of cDNA generated from the tumor samples revealed that EGFR, as well as AREG, VEGF and IL6 were in fact over expressed in tumors originating from PC3-TPα transfectants (Figure. 3.20B). Furthermore, it was confirmed that PC3-TPα cells maintained elevated expression levels of TPα compared to PC3-Neo controls over the duration of the six week in vivo growth experiment. (Figure. 3.20A)
C: Staining for CD31 (Endothelial marker) on mice tumor sections
Figure 3.19: Increased neo vascularization and proliferation was observed in tumors from mice harboring PC3-TPα cells compared to those given vector controls. Comparison of vessel density of tumors ex vivo from PC3-TPα mice compared to control mice (A). Presence of CD31, a prominent angiogenesis marker, was increased at the mRNA levels in mice injected with PC3-TPα compared to PC3-Neo controls (B). Immunohistochemical analysis for CD31, an endothelial marker, showed that sections from PC3-TPα tumors were strongly positive for CD31 (C). Arrowheads show the staining regions for CD31. Immunohistochemical analysis for Ki67, a proliferation marker, showed that sections from PC3-TPα tumors were strongly positive for Ki67 (D). Arrowheads show the staining regions for Ki67.
Figure 3.20: Increased expression of downstream target genes known to affect tumor growth. In order to determine whether increased tumor growth in PC3-TPα, injected mice is due to increased expression of either pro angiogenic factors or growth factors, RNA isolated from mouse tumors was converted to cDNA and gene expression was quantified using Real-time PCR. PC3-TPα transfectants maintained high TPα receptor levels in mouse tumors for the duration of the study (A). Elevated TPα levels were associated with increased expression of target genes such as AREG, EGFR, VEGF-A and IL6 (B). Values expressed represent the mean fold change ± SEM, compared to vector control mice and is derived from three experiments normalized in each case to GAPDH gene expression. Similar results were observed in a further two independent experiments.
Activation of TPα also promotes increased angiogenesis in a mouse model:

Increased neo vascularization was seen in PC3-TPα tumors as well as increased expression of VEGF-A mRNA. This implied TPα might also lead to tumor growth by playing a role in angiogenesis.

The increased angiogenicity due to TPα mediated VEGF expression in PC3-TPα tumors was confirmed by the Matrigel implantation assay in an in vivo xenograft mouse model. As shown in Fig. 3.21, within 12 days, TPα transfected PC3 cells (PC3-TPα) in matrigel induced increased angiogenesis, indicated by the accumulation of blood in the gel, compared to the neo control (PC3-Neo). The results clearly illustrate that the TPα transfected PC3 cells are more angiogenic than their neo controls.
**Figure 3.21: Induction of angiogenesis in Matrigel by TPα transfectants:** PC3-TPα cells or PC3-Neo control cells (2x10⁶) were mixed with matrigel BME and injected s.c. into the right flank of 4-6-week-old male SCID mice (n=6). In Mice injected with PC3-TPα cells mixed with Cultrex BME, there was increased vessel penetration into the gel and considerable blood accumulation was observed (A). In contrast, mice injected with PC3-Neo cells mixed with Matrigel BME, the vessel penetration into the gel was minimal, with little blood accumulation (B).
Chapter-4: Summary and Discussion:

Development of prostate cancer (PCa) is a multistep process. A key step in this process is the overexpression of growth factors and their receptors that results in the progression of normal prostate epithelial cells to a hyper proliferative cancer cell [171, 172]. Changes in gene expression of growth factors and their corresponding receptors in epithelial and stromal cells during the different developmental stages of prostate cancer contribute to enhanced tumor cell growth, survival, migration and invasiveness [171, 172]. Among these, the ErbB family of receptors and their ligands, the EGF family of peptide growth factors, has a central role in the pathogenesis and progression of different carcinoma types [141, 144, 146, 150]. In particular, epidermal growth factor receptor (EGFR) signaling has been associated with a wide variety of tumors and causes PCa to progress by becoming invasive and metastatic [170, 171] [173, 174].

In this study, we report for the first time that activation of TPα signaling can lead to over expression of AREG, a ligand of EGFR in prostate cancer cells. Our data also indicate that activation of TPα not only upregulates AREG expression, but also increases the expression of its cognate receptor EGFR. We also have demonstrated that of the two TP receptor isoforms, only TPα receptor is expressed in PCa cells and tissues. These findings suggest an alternate mechanism by which TPα can activate EGFR signaling pathways leading to increased tumor growth. The experimental evidence from this study has revealed molecular mechanisms underlying the enhanced expression of AREG in prostate cancer. Our data indicate that the AMPK pathway plays an important role in the expression of AREG mediated by TPα in PCa cells.
Recent studies have shown that GPCRs are able to utilize the EGFR as a downstream signaling partner in the generation of growth signals that might play an important role in various cancers [124, 125, 128]. Studies have identified that EGFR as an essential link in the GPCR-mediated activation of downstream signaling pathways such as MAPK pathways in several cell types treated with the GPCR agonists [128, 197-199]. The TP receptor also uses EGFR as an essential link in the activation of downstream signaling pathways. Two mechanisms i.e. ligand-dependent (shedding of HB-EGF by MMPs) or ligand-independent (Src kinase mediated) have been proposed that involve transactivation of EGFR by phosphorylation that leads to activation of downstream signaling pathways [127] [126]. These models were established in HEK cells over-expressing TP receptor-EGFR crosstalk has been established using HEK cells overexpressing TP receptor and they have not examined in cancer cells. Therefore alternate pathways involved in activation of EGFR by TP receptor are possible.

In this study we have proposed a novel mechanism by which EGFR can be activated in PCa cells through GPCR. Our data show for the first time that increased expression of AREG in response to TP stimulation by its agonist can lead to EGFR activation in PCa cells. Our data also confirm that increased levels of AREG, seen in response to stimulation of TPα, result in functional binding to EGFR, leading to EGFR phosphorylation and activation of downstream MAPK pathways. Elevated AREG could then lead to constitutive EGFR signaling and activation of downstream signaling pathways involved in cellular proliferation, cell migration, cell survival, and angiogenesis. Overexpression of AREG has been reported in both androgen dependent and independent prostate cell lines as well as tissues. However the specific pathways
leading to AREG expression have not yet been elucidated [157, 158, 175]. Our study sheds light on a novel pathway, where we found that activation of TPα led to increased expression of AREG. This presents a mechanistic explanation for those findings, as we also determined that TPα is over-expressed in PCa cell lines.

We discovered that TPα activation also led to over expression of EGFR. It is known that AREG acts exclusively through EGFR, where it binds EGFR with a lower affinity than EGF, and induces receptor autophosphorylation and downstream activation of the ERK signaling cascade [156, 158]. One of the most studied receptor tyrosine kinases i.e. EGFR and its signaling pathway is central to both physiological and pathophysiological process [139, 140, 143, 144, 150]. Over-expression of EGFR or up-regulation of its signaling pathway has been associated with several cancers and has led to uncontrolled cellular proliferation and autocrine stimulation of tumors producing their own growth factors [141, 146, 149, 150, 200]. The enhanced expression of EGFR and its ligands, EGF, TGF-α, HB-EGF and AREG, has been reported to correlate with high grades of PCa malignancies [158, 174].

The mechanisms responsible for AREG upregulation in transformed cells are not fully understood and might vary from one tumor subtype to another. Recent studies have shown that AREG can be induced by cAMP pathway in various cell types [160]. A cAMP-responsive element (CRE) has been described in the AREG promoter and expression of AREG is regulated by cAMP [160, 161]. AREG can also be regulated by EGFR itself, and activation of EGFR leads to AREG expression in several cell types [155, 157, 166, 168]. We investigated and determined that activation of TPα clearly increases the cAMP levels in PCa cell lines. These findings and other studies point to a
possible role for cAMP and EGFR in over expression of AREG that is induced by TPα signaling.

As our data indicate, blocking EGFR activation with EGFR inhibitors (Gefitinib, AG1478) actually reduced AREG levels in TPα over-expressing cells, that TPα upregulates AREG mRNA by direct activation of EGFR via EGFR phosphorylation. Inhibition of PKA (downstream mediator of cAMP) prior to EGFR inhibition did not affect the expression of AREG. The possibility of an alternate pathway involving cAMP [160] led us to consider whether the AMPK pathway was involved. The thromboxane receptor reportedly can activate the AMP-activated protein kinase in vascular smooth muscle cells via hydrogen peroxide [190]. Treatment with the AMPK inhibitor (Compound-C) led to a decrease in AREG both at the level of mRNA and protein (Figure 3.15). Therefore we have identified the AMPK pathway as the key signaling pathway triggered by TPα to increase AREG expression in human prostate cancer cells.

Aberrant EGFR and AREG signaling has been recognized to affect cellular proliferation, cell migration, cell survival, and angiogenesis [141, 144, 146, 150]. Many of these key steps in cancer progression are also regulated by TP receptor. Previous studies from our lab have shown that activation of TPα receptor by IBOP induces cell motility through the Rho-A pathway [101]. As AREG had been shown to upregulate several genes known to be involved in motility and invasion in breast cancer cells [155, 156, 168], we investigated whether TPα-AREG-EGFR signaling plays a role in PCa cell invasion. Our invasion assays showed that TPα does in fact play a role in cell invasion, and is mediated by EGFR signaling. Our data also confirm that increased expression of AREG induced by TPα receptor promoted cell invasion, as blocking AREG expression
with AMPK inhibitors reduced invasion. These data indicate that PCa cells expressing AREG in response to TPα receptor stimulation require autocrine signaling through EGFR for cell invasion to occur.

Activation of TPα receptor in PC3-TPα cells by IBOP promoted increased expression of growth factors such as AREG and its receptor EGFR thereby playing a role in tumor growth. Using a xenograft mouse model, we demonstrated that TPα transfected PC3 cells formed larger tumors than vector controls, and that the increased tumor volume was positively correlated with enhanced tumor angiogenesis. Tai et al., have shown that overexpression of TPα in A549 lung cancer cells also led to enhanced tumor angiogenesis and growth in a xenograft animal model [201]. Taken together, these studies suggest that expression of TPα in cancer cells can enhance their angiogenic potential through expression of growth factors leading to tumor growth. We found significant vascularization in tumors derived from PC3-TPα cells, compared to PC3-Neo tumors. Formalin fixed paraffin-embedded tumor sections from these mice showed very strong CD31 staining. Analysis of tumors from PC3-TPα mice by real-time PCR showed increased expression of pro-angiogenic growth factors and cytokines such VEGF and IL6.

Angiogenesis is a multistep process that requires the concerted action of numerous growth factors. VEGF is an important angiogenic factor that had been correlated with poor prognosis and improved survival of solid tumors. Expression and secretion of VEGF is triggered by a vast multitude of factors and signaling pathways in cancer cells [202, 203]. In solid tumors, the VEGF and EGFR pathways are linked with respect to angiogenesis [204, 205]. EGFR ligands such as EGF and TGF-α can induce
VEGF expression *via* activation of EGFR in cell culture models and have proangiogenic properties [204, 205]. It is likely that the EGFR pathway modulates angiogenesis by up-regulating VEGF or other key mediators in the angiogenic process [206]. The increased angiogenicity due to TPα mediated VEGF expression in PC3-TPα tumors was confirmed by the Matrigel implantation assay in an *in vivo* xenograft mice model indicating that increased EGFR signaling mediated by TPα to be responsible for it.

In this study, we identified which TP receptor isoform is predominantly expressed in PCa cells and tissues. Though the existence of two TP isoforms was described as early as 1994, most of the earlier studies on TP did not distinguish the functional roles between the two isoforms and it was assumed that most of the functional effects of TP to be mediated by TPα. Recent findings though provide a novel role for TPβ in bladder cancer progression [99]. Until now the expression status of TP isoforms was largely uncharacterized in prostate cancer cells and tissues. Identification of the active isoform in prostate cancer will help us to design isoform-specific antagonists or inhibitors that uniquely target this pathway for greater therapeutic impact.

One of the technical challenges in working with TP isoforms is the lack of isoform-specific antibodies. The only difference between TPα and TPβ is that TPα has a shorter C-terminal cytoplasmic tail than the TPβ isoform (15 versus 79 residues) [52-54]. Currently there are no commercially available antibodies that can discriminate these two isoforms. To distinguish them, we designed Taqman gene expression assays specific for each TP isoform and were able to differentiate their expression level in PCa cells and tissues. Within the detection boundaries of this assay, TPα appeared to be solely expressed isoform in PCa cell lines and tissues. We used several approaches
(Immunoblot, RT-PCR, Real-time PCR, cAMP enzyme Immunoassay) to look into the possible expression of both TP isoforms in PCa, but our data strongly pointed that only TPα expression is seen in PCa cells. Data from our tissue array analysis show that TPα expression is higher in prostate cancer tissues compared to normal prostate tissues. Collectively, the data-mining results and the data from Real-time PCR analysis of human tissue arrays make a strong case for the association of TPα expression with the aggressiveness and progression of prostate tumors. Previous studies by Bostwick et al. were able to conclude that increased expression of AREG might contribute to the development of prostatic adenocarcinoma [158].

EGFR protein was found by Marks et al. to be frequently expressed in tissue from prostate cancer patients undergoing hormone therapy [207]. In 57 of 71 patients undergoing hormonal therapy, there was demonstrable EGFR expression by immunostaining. More studies will be needed to focus on the co-expression levels of both TPα and AREG in prostate cancer tissues in relation to EGFR so as to determine their connectivity. In gastric cancer cell lines, Cetuximab responsiveness against EGFR was associated with AREG levels, and Cetuximab treatment attenuated AREG, while EGF elevated AREG. This implies that the EGFR pathway itself appears to regulate AREG[208]. It is known that TPR activation includes the expression of a nuclear receptor called Nurr1 that can stimulate proliferation of lung cancer cells [119]. In turn, Nurr1 has been shown to regulate pro-inflammatory mediators, including AREG, in synovial fibroblasts [209]. As both TPα and AREG are over expressed in PCa tissues, it will be interesting if links such as those implied by the Nurr1 studies play a role in TPα-mediated expression of in human PCa tissues.
Conclusion:

In summary, our study has identified a novel link between TPα signaling and AREG expression in prostate cancer cells. We report for the first time that activation of TPα signaling in PCa cells can lead to increased expression of AREG and its receptor EGFR, and that AMPK plays an important role in regulation of AREG by TPα. EGFR signaling has been recognized to be at the forefront of aberrant signal transduction pathways, involved in cellular proliferation, cell migration, cell survival, and angiogenesis. Over expression of TPα in PC3 cells can result in increased invasiveness of PCa cells, which might be mediated by EGFR signaling pathways.

Over expression of TPα in PC3 cells led to increased tumor growth and angiogenesis in a xenograft mouse model that is likely associated with increased expression of AREG, VEGF and EGFR signaling. We report for the first time that TPα mRNA expression is elevated in the prostate cancer tissues and conclude that TPα expression in PCa tissues correlates with aggressiveness of the disease. These findings suggest that the functional role of TPα in prostate cancer progression might involve increased expression of AREG leading to increased activation of EGFR signaling promoting angiogenesis, cell invasion and tumor growth.
Project Summary

Figure 4: Summary of TPα receptor mediated AREG expression and EGFR signaling in prostate cancer cells

Future Directions:

Recent emphasis in prostate cancer research has been on identification of candidate genes that are good prognostic or predictive markers. As defined by Busser et al., “Prognostic factors provide information on the outcome of the disease, whereas predictive factors are used to prospectively select responsiveness or resistance to a specific treatment” [169]. TPα appears to be an ideal candidate to be considered as a prognostic marker in prostate cancer. TPα was more highly expressed in aggressive...
forms of PCa and correlated with Gleason scores. A significant finding from our studies was that TPα expression was elevated in GS7 (4+3) compared to GS7 (3+4), which suggests that differences in TPα expression exist between primary and secondary patterns. Further studies are needed in statistically relevant sample set to see whether this can potentially be exploited as a biomarker for discriminating GS7 subtypes when making prognostic determinations.

Further in vivo studies will clarify the actual role of EGFR and AREG in TPα-mediated tumor growth. This could be approached by knocking down either AREG or EGFR in PC3 cells over expressing TPα, injecting them into mice, and following tumor growth. Further elucidation of TPα-mediated pathways that elevate expression of AREG and lead to subsequent tumor cell growth will provide insights into the key steps involved in prostate cancer progression. Inhibition of growth factors and their receptor signaling pathways has proven to be an effective approach to block tumor growth. Therefore, inhibition of TPα signaling by antagonists, or prevention of TXA₂ synthesis by inhibiting TXA₂S, may be a novel approach to develop anticancer, anti-angiogenic therapy [210]. It remains to be determined if TPα and AREG expression in prostate cancer tissue can be correlated to each other.

The low cost of aspirin has kept it in the forefront of treatment in cardiovascular disorders, whereas TXA₂S inhibitors and TXA₂ antagonists have not been employed for clinical benefits. Conversely, current therapeutic regimens for cancer incorporate few agents of the NSAID class, which makes TXA₂S/TP antagonism an attractive target with potentially significant therapeutic benefit. Moreover, in contrast to NSAIDs, TXA₂S/TP inhibitors do not interfere with synthesis of other beneficial COX-
derived anti-tumor products, such as PGI₂ [211-216]. Hence, future work should focus on the advantages of directly targeting TXA₂S and/or developing TP antagonists or taking advantage of the anti tumor effects of PGI₂.
APPENDIX

INSTITUTIONAL ANIMAL
CARE AND USE COMMITTEE
101 E. Alexandrine St.
Detroit, MI 48201-2011
Telephone: (313) 577-1629
Fax Number: (313) 577-1641

ANIMAL WELFARE ASSURANCE # A 3310-01

PROTOCOL # A 05-21-08
Year 2 Annual Review Date: June 1, 2009

TO: Dr. Kenneth V. Honn
Pathology
429 Chemistry Building

FROM: Lisa Anne Polin, Ph.D.
Chairperson
Institutional Animal Care and Use Committee

SUBJECT: Approval of Protocol # A 05-21-08
"Role of Thromboxane in Prostate Cancer Progression"

DATE: June 1, 2009

The Annual Review of your animal research protocol and any applicable grant applications has been conducted and approved by the Wayne State University Institutional Animal Care and Use Committee (IACUC). The species and number of animals approved for the duration of this protocol are listed below.

Species Strain Qty. Cat.
MICE .......... Hsd:Athymic Nude-Foxn1nu, nu/nu, male, 4-6 weeks old ........................................... 50 ......C
MICE .......... Hsd:Athymic Nude-Foxn1nu, nu/nu, male, 4-6 weeks old ........................................... 100 ......D

Species Amendments Strain Qty. Cat.
NONE

Be advised that any change in the procedures used, a change in species, or additional numbers of animals requires prior approval by the IACUC. Any animal work on this research protocol beyond the expiration date will require the submission of a new IACUC protocol form and full committee review.

The Guide for the Care and Use of Laboratory Animals is the primary reference used for standards of animal care at Wayne State University. The University has submitted an appropriate assurance statement to the Office of Laboratory Animal Welfare (OLAW) of the National Institutes of Health. The animal care program at Wayne State University is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).
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ABSTRACT

ROLE OF THROMBOXANE RECEPTOR-ALPHA IN PROSTATE CANCER PROGRESSION

by

PRASANNA EKAMBARA

December 2012

Advisor: Dr. Kenneth.V.Honn
Major: Cancer Biology
Degree: Doctor of Philosophy

Thromboxane A$_2$ (TXA$_2$) is a major arachidonic acid metabolite that signals through TXA$_2$ receptors (TP) to induce platelet aggregation and smooth muscle contraction. TXA$_2$ receptors are expressed as two different isoforms in humans, namely TP-alpha (TP$\alpha$) and TP-beta (TP$\beta$), which have common and distinct signaling pathways. Of the two TP receptor isoforms, studies have shown that TP$\alpha$ impacts tumor growth and progression of lung cancer. Previously our studies demonstrated that activation of Thromboxane receptor by TXA$_2$ agonists could regulate prostate cancer (PCa) cell motility and cytoskeletal reorganization through activation of Rho-A. The primary objective of this study is to investigate the functional role of TP$\alpha$ in prostate cancer progression and metastasis.

Our data indicates that of the two TPR isoforms, human PCa cell lines only express TP$\alpha$ and that expression of TP$\alpha$ is higher in PCa cells compared to cultured normal prostate epithelial cells, such as RWPE1. Furthermore, expression of TP$\alpha$ was observed to be higher in clinical PCa specimens when compared with normal tissue,
and expression was higher in tumor tissues with Gleason scores of 7 and above. In response to the TXA$_2$ mimetic, IBOP, DU145 cells that have higher endogenous levels of TPα compared to other cell lines, up regulated expression of the growth factor Amphiregulin (AREG) and its receptor EGFR. Introduction of an expression plasmid encoding TPα into another PCa cell line, PC3 (PC3-TPα), led to a similar phenotype in response to IBOP, thereby confirming a link between TPα and over expression of AREG and EGFR.

Increase in AREG expression mediated by TPα seems to involve EGFR and AMP activated protein kinase (AMPK) signaling pathways. PC3-TPα cells treated with IBOP were also highly invasive compared to PC3-Neo cells; this invasiveness mediated by TPα was affected by pre treatment with an EGFR inhibitor. In a subcutaneous animal model, mice injected with PC3-TPα exhibited greater tumor growth and increased neo vascularization compared to mice with PC3-Neo cells. Also when mice was injected with PC3-TPα cells and Matrigel BME, increased angiogenesis was observed as indicated by the accumulation of blood compared to mice with PC3-Neo cells. Collectively these data suggest that activation of TPα receptor in PCa cells increases the level of growth factors such as AREG and its receptor EGFR that might be mediated through AMPK, thereby implicating TPα in prostate cancer progression.
AUTOBIOGRAPHICAL STATEMENT

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Education

Ph.D. – Cancer Biology, School of Medicine, Wayne State University, Detroit, MI
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Awards and honors

- Third place prize in the annual Graduate Student Research Day (GSRD) XIII poster session II, September 2009, Detroit, MI
- Travel Award to participate in American Association for Cancer Research's (AACR) 102nd Annual Meeting, held in Orlando, Fla., April 2-6, 2011.
- Travel Award to participate in Bioactive Lipids Eicosanoid International research conference, October 2009, Cancun, Mexico.
- Gold medalist in Plant Breeding and Genetics, BSc (Horticulture), Annamalai University, January 2001, Tamilnadu, India.

Publications

