DIFFERENTIAL SIGNALING AND GENE REGULATION AMONG THREE HUMAN EP_3 PROSTANOID RECEPTOR ISOFORMS

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DEDICATION

This dissertation is dedicated to my family both the ones I was born to and the ones I have acquired over the years. They have provided great patience, support, humor and love. And especially to Douglas Hanniford, he has always been supportive, in spite of the distance, through good times and bad.

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LIST OF ABBREVIATIONS

COX-1 = Cyclooxygenase 1

COX-2 = Cyclooxygenase 2

EGR3 = Early growth response factor 3

Fra-2 = Fos-like antigen 2

G6PD = Glucose-6-phosphate dehydrogenase

GPCR = G-protein coupled receptor

NSAID = Non-steroidal anti-inflammatory

PCR = Polymerase chain reaction

PGD_2 = Prostaglandin D_2

PGE_2 = Prostaglandin E_2

$PGF_{2\alpha}$ = Prostaglandin $F_{2\alpha}$

PGI_2 = Prostaglandin I_2

PGH_2 = Prostaglandin H_2

PGG_2 = Prostaglandin G_2

PKC = Protein kinase C

PI3K = Phosphoinositide 3-kinase

PLA_2 = Phospholipase A_2

RT-PCR = Reverse transcription PCR

LIST OF ABBREVIATIONS-*Continued*

TxA_2 = Thromboxane A_2

TM = Transmembrane

WT-1 = Wilm's Tumor 1

ABSTRACT

Prostaglandin E_2 (PGE_2) is a hormone derived from the metabolism of arachidonic acid whose functions include regulation of platelet aggregation, fever and smooth muscle contraction/relaxation. PGE_2 mediates its physiological and pathophysiological effects through its binding to four G-protein coupled receptor subtypes, named EP_1, EP_2, EP_3 and EP_4. The EP_3 prostanoid receptor is unique in that it has multiple isoforms generated by alternative mRNA splicing. These splice variants display differences in tissue expression, constitutive activity and regulation of signaling molecules. To date there are few reports identifying differential activities of EP_3 receptor isoforms and their effects on gene regulation.

We generated HEK 293 EBNA cell lines expressing the $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, or $EP_{3\text{-}III}$ isoforms. After confirming the functional expression of each of these isoforms, we examined their activation of cellular signal transduction pathways.

We found that each of these isoforms utilize distinct mechanisms to regulate ERK 1/2 phosphorylation and that these differences lead to unique

regulation of the downstream effectors ELK-1 and AP-1. We also found MAPK dependent differences in regulation of cell proliferation. The $EP_{3\text{-III}}$ isoform increases cell proliferation in a MAPK dependent manner while the $EP_{3\text{-Ia}}$ dose dependently regulates cell proliferation via $G\alpha_i$ and not ERK 1/2. Activation of the $EP_{3\text{-II}}$ receptor had no effects on cell proliferation.

To study differential gene regulation by these three EP_3 receptor isoforms, we conducted microarray studies. Over 300 genes were differentially regulated by these isoforms. Quantitative real-time PCR analysis was used to validate 15 candidate genes. Five genes were chosen for further analysis of protein expression using immunoblotting, but only one of these, WT-1, was significantly increased following treatment with PGE_2. WT-1, a transcription factor important for kidney and heart development, was strongly upregulated by PGE_2 stimulation of the $EP_{3\text{-II}}$ receptor, but only weakly by the other isoforms.

In conclusion, these studies show that the human EP_3 prostanoid receptor isoforms are capable of distinct regulation of both signal transduction pathways and gene transcription. Elucidating the differential functions of

EP_3 receptor isoforms may allow for greater understanding of the diverse functions attributed to this receptor and their physiological functions.

CHAPTER ONE

INTRODUCTION, PURPOSE AND AIMS

1.1 Introduction

Prostaglandins are autocrine and paracrine hormone-like compounds derived from the sequential activities of cyclooxygenase (COX) and prostaglandin synthases on the unsaturated fatty acid arachidonic acid. They regulate numerous physiological activities including the contraction and relaxation of smooth muscle, platelet aggregation, modulation of immune system, control of blood pressure and many other activities.

Prostaglandin analogues are used clinically to induce labor, as abortafacients in early pregnancy, to reduce ocular hypertension in glaucoma and to maintain patency of the ductus arteriosus in neonates with certain types of congenital heart defects. Decreasing the biosynthesis of prostaglandins through the inhibition of COX-1 and/or COX-2 is a mainstay for the treatment of inflammation, fever and pain.

The actions of prostaglandins were first observed in the 1930's by several researchers including Raphael Kurzrok and his colleague Charles Lieb at Columbia University, Maurice Walter Goldblatt at Sherrington School of Physiology in London and Ulf von Euler at the Karolinska Institute in

Stockholm [1-3]. The initial observations found that human seminal fluid could contract and relax human uterine strips and rabbit intestine as well as reduce blood pressure in experimental animals. Von Euler coined the term prostaglandin due to the belief, at the time that the prostate produced this compound. In the 1960's and 1970's Sune Bergstrom, Bengt Ingemar Samuelsson and John Robert Vane were largely responsible for the purification and the characterization of the biosynthesis, metabolism and function of the prostaglandins. In recognition of their groundbreaking contributions to the prostaglandin research field they shared the Nobel Prize in 1982 for Physiology or Medicine. for their groundbreaking contributions to the prostaglandin research field.

Prostaglandins are members of a large family of signaling lipids known as eicosanoids. Eicosanoids is collective term that describes bioactive metabolites of arachidonic acid, including the prostanoids. The prostanoid subclass is made up of the prostaglandins and thromboxanes. Prostaglandins are 20 carbon fatty acids composed of a cyclopentane ring, with hydroxyl or keto group modifications on the ring and two side chains [4]. The subscript, for example in PGE_2, refers to the number of double bonds on the two side-

chains of the cyclopentane ring [4]. Thromboxanes are also 20 carbon fatty acids, but they contain a cyclohexane ring. The most physiologically relevant prostaglandins are prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin D_2 (PGD_2), prostacyclin (PGI_2) and thromboxane A_2 (TxA_2).

Prostanoids are not stored within the cell and thus must be synthesized *de novo*. Prostanoid biosynthesis can occur when arachidonic acid is released from the plasma membrane by phospholipase A_2 (PLA_2) in response to cytokines and mitogens [5]. Following its release, arachidonic acid is converted into PGG_2 then reduced to form PGH_2 by the sequential actions of cyclooxygenase (COX). PGH_2 is subsequently converted to the prostaglandins or thromboxane by specific synthases. Figure 1.1 shows the pathways of prostaglandin synthesis. Following synthesis, prostaglandins are metabolized by the enzyme 15-hydroxyprostaglandin dehydrogenase.

Cyclooxygenase exists as two forms, COX-1 and COX-2. COX-1 is constitutively expressed in all tissues and is important for basic functions such as inhibition of gastric acid production and stimulation of gastric

mucous secretion. COX-2 is the inducible isoform whose expression is stimulated by growth factors, serum factors and cytokines and plays an important role in inflammation and the immune response. In recent years there has been evidence indicating that COX-2 is constitutively expressed in some tissues under non-pathological conditions. These tissues include the brain, kidney and gastric mucosa.[6-9]. Likewise there is evidence indicating that COX-1 can be induced in some tissues and can contribute to the inflammatory process [10, 11].

The actions of the COX enzymes are the rate-limiting step for prostanoid synthesis and their actions are the target of a commonly used class of drugs, the NSAIDs. A major indication of non-steroidal anti-inflammatory drugs (NSAIDs) is for treatment of pain and inflammation associated with rheumatoid arthritis. The major categories of NSAIDs inhibitors are the non-selective and selective COX-1 and COX-2 inhibitors.

In 2000, a study published in the New England Journal of Medicine reported that the use of the selective COX-2 inhibitor, celcoxib reduced the number

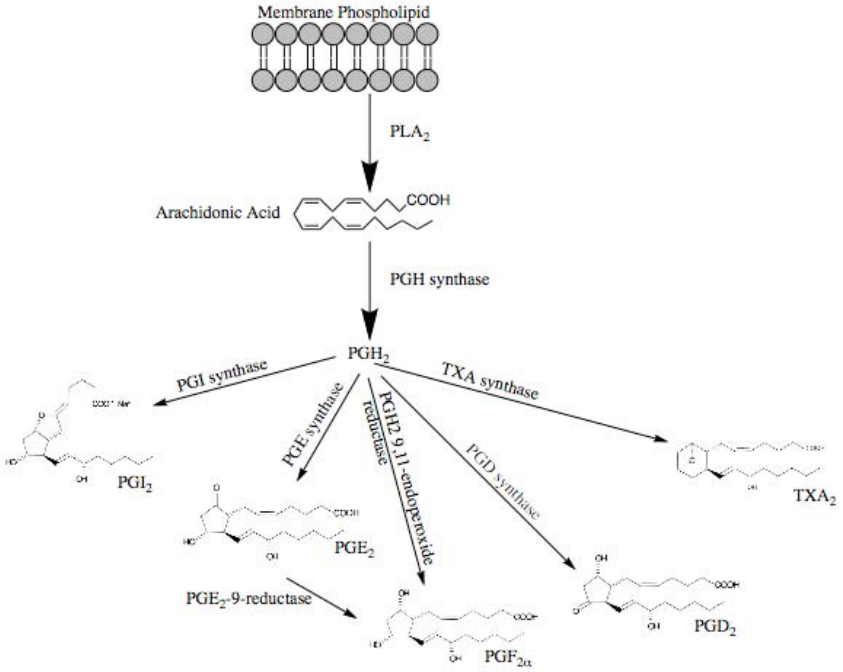


Figure 1.1 Schematic illustration of prostanoid biosynthesis.
Arachidonic acid is released from converted phospholipids in the plasma membrane by phospholipase A_2 (PLA_2). Arachidonic acid is converted into PGH_2 by the actions of PGH synthase/cyclooxygenase (PGH/COX). PGH_2 is subsequently converted to prostaglandins PGE_2, PGI_2, PGD_2, $PGF_{2\alpha}$ or TXA_2 by specific prostaglandin synthases.

of colon polyps in patients with Familial Adenomatous Polyposis (FAP) [12]. Following this report the FDA approved celecoxib as an adjunctive drug for use alongside standard treatment in people with FAP [13]. Subsequent clinical studies investigating the use of high dose COX-2 inhibitors for the prevention of cancer uncovered an increased risk of fatal and non-fatal cardiovascular events. In 2004, the Food and Drug Administration (FDA) removed the COX-2 selective inhibitor rofecoxib from the market after an increased risk of cardiovascular toxicities was identified in people taking the drug for 18 months participating in a clinical trial to prevent colon adenomas [13]. Additionally, NSAID manufacturers were instructed to revise their product labeling to include information detailing potential cardiovascular and gastrointestinal risks. Currently there is much emphasis on circumventing the use of cyclooxygenase as a therapeutic target and instead inhibiting microsomal PGE synthase or selective antagonism of specific prostanoid receptors.

1.2 Prostanoid Receptor Subtypes and Isoforms

Prostanoids mediate their effects by binding to and activating eight distinct heptahelical G-protein coupled receptors (GPCR). GPCRs are a large

superfamily of seven transmembrane domain-spanning proteins that mediate signal transduction by activation of G-proteins. Prostanoid receptors are part of the Class A rhodopsin-like family of GPCRs and share similar structural features including conservation of residues distributed among the seven helical domains. The two most highly conserved regions in prostanoid receptor genes are the seventh transmembrane domain and the second extracellular loop between the fourth and fifth transmembrane domains. It is believed that these regions contribute to ligand binding [14]. The binding of ligand and subsequent activation of these receptors leads to a conformational change of the receptor that allows for the coupling and activation of heterotrimeric guanine nucleotide-binding proteins (G-proteins). G-proteins are composed of three subunits α, β and γ. The β and γ subunits form a stable dimer. The inactive α subunit is bound to GDP and the binding of ligand and subsequent activation of the G-protein allows for the exchange of GDP for GTP. The exchange of GDP for GTP allows the α and $\beta\gamma$ subunits to dissociate and go on to relay a variety of signals to the intracellular environment via the actions of second messenger systems. The diversity of signaling through which GPCRs can induce is partly due to the type of G-protein it couples. There are four major types of Gα subunits as follows,

$G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$. G protein coupled receptors that activate the stimulatory guanine nucleotide binding protein, $G\alpha_s$, increase the activity of adenylyl cyclase, which produces the second messenger adenosine 3' 5-cyclic monophosphate (cyclic AMP). Receptors that couple to the inhibitory guanine nucleotide binding protein, $G\alpha_i$, inhibit the activity of adenylyl cyclase. $G\alpha_q$ coupling activates phospholipase C (PLC) that leads to the production of the second messengers diacylglycerol (DAG) and inositol triphosphate (IP3). $G\alpha_{12/13}$ coupling regulates the activities of the regulator of phospholipase D and RhoGEF.

Prostanoid receptors mediate their actions via activation of their cognate receptors. The use of molecular cloning and recombinant technology has allowed for the identification and characterization of the structural and ligand binding properties of prostanoid receptors. PGE_2 binds EP receptors, $PGF_{2\alpha}$ binds FP receptors, PGD_2 binds DP receptors, PGI_2 binds IP receptors and TxA_2 binds TP receptors. Due to the similarities in chemical structure of the prostanoids there is significant cross reactivity in ligand binding between prostanoids and receptors within the prostanoid receptor family.

Eight distinct genes encode prostanoid receptors and sequence homology between these genes is minimal, usually ranging between 20-30% [15]. Initial phylogenetic analysis of prostanoid receptor cDNA sequences determined that receptors coupling to the same type of heterotrimeric G-protein and subsequently second messenger were more closely related than the receptors sharing the same ligand [16, 17]. Thus IP, EP_2, EP_4 and DP receptors couple to G_s and represent one category. TP, EP_1, and FP couple to G_q and represent a second category and finally EP_3 couples to G_i and represents a third category. Figure 1.2 illustrates the phylogenetic tree of the human prostanoid receptors based on a basic model of prostanoid gene structure. Phylogenetic analyses by other methods including Clustal method have generated variations of the tree presented here [18].

PGE_2 mediates its physiological and pathophysiological effects through its four G-protein coupled receptor subtypes EP_{1-4}. In humans, the EP_1 receptor couples to G_q, EP_2 to G_s, EP_3 to G_i, G_s, G_q and $G_{12/13}$ and EP_4 couples to G_s and G_i [15, 19-21]. The EP_3 receptor is unique among the human EP subtypes in that there are multiple splice variants generated by alternative splicing of the exon encoding the seventh transmembrane domain. In

humans alternative splice variants are also observed in the TP receptor (TPα and TPβ) and the FP (FP and FP_s receptors) [22, 23]. In other species, splice variants for EP_3, TP and FP are known as well as variants of the EP_1 receptor in rats [24].

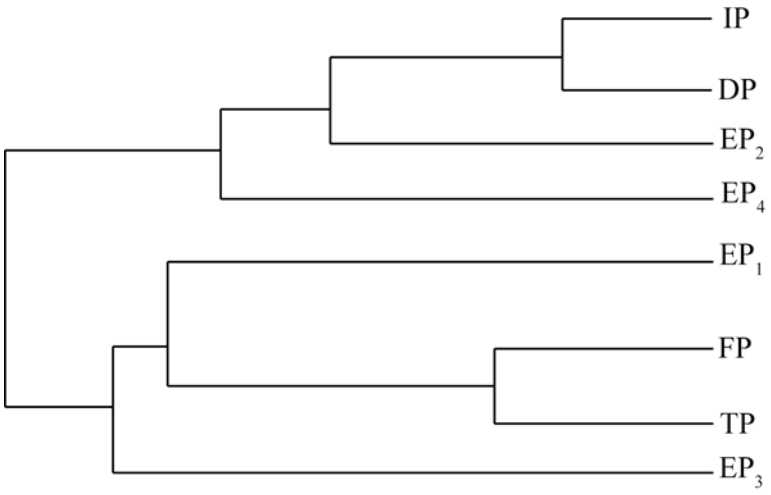


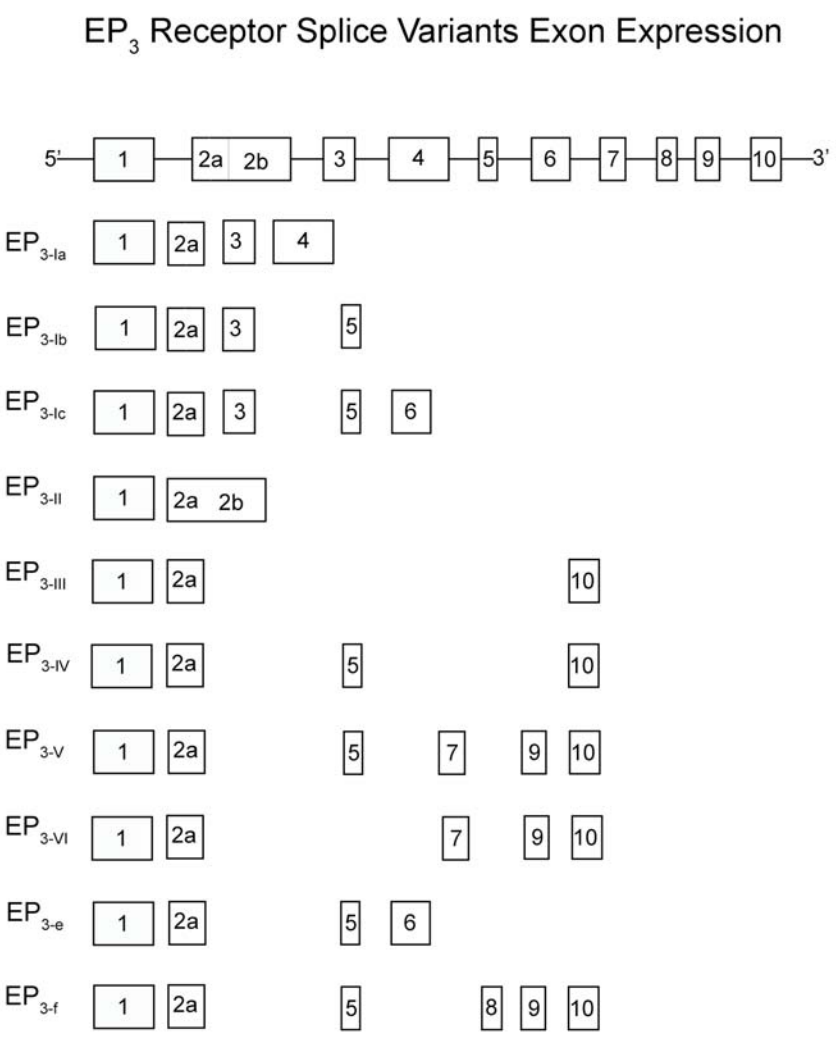
Figure 1.2 Prostanoid receptor gene phylogeny and model of basic gene structure.
Phylogenetic analysis of prostanoid receptor cDNA sequences based on receptors coupling to the same type of heterotrimeric G-protein and subsequently second messenger are more closely related than the receptors sharing the same ligand.

1.3 EP_3 Receptors

The EP_3 gene, PTGER3, is located on chromosome 1p31.2. PTGER3 spans more than 80kb and contains 10 exons separated by 9 introns [25]. As common to all prostanoid receptors, the region of the PTGER3 gene encoding the sixth transmembrane domain is separated by an intron. This suggests that despite there being only 20-30% homology across prostanoid receptors, a common ancestral gene links them all [15, 25].

In a 1994 publication, Kotani et al. provided a detailed analysis of the structural organization of the human EP_3 receptor gene. They found that all isoforms of the EP_3 receptor shared homology at exons 1 and exon 2a, but from here a divergence occurs. Exon 1 encodes the first six transmembrane domains and exon 2a encodes the seventh transmembrane domain. Exon 2a contains the first 180 base pairs of exon 2 followed by a GT internal splice donor site. The remaining 3,461 base pairs of exon 2 are designated exon 2b. The EP_3 receptor isoforms are composed of the shared exon 1 and 2a and several combinations of exon 2b and/or the remaining 8 exons of the gene. Figure 1.3A illustrates the exons in the mRNA transcripts generated by

A.


EP3 Receptor Splice Variants Exon Expression
5'
1
2a
2b
3
4
5
6
7
8
9
10
3'
EP3-Ia
EP3-Ib
EP3-Ic
EP3-II
EP3-III
EP3-IV
EP3-V
EP3-VI
EP3-e
EP3-f

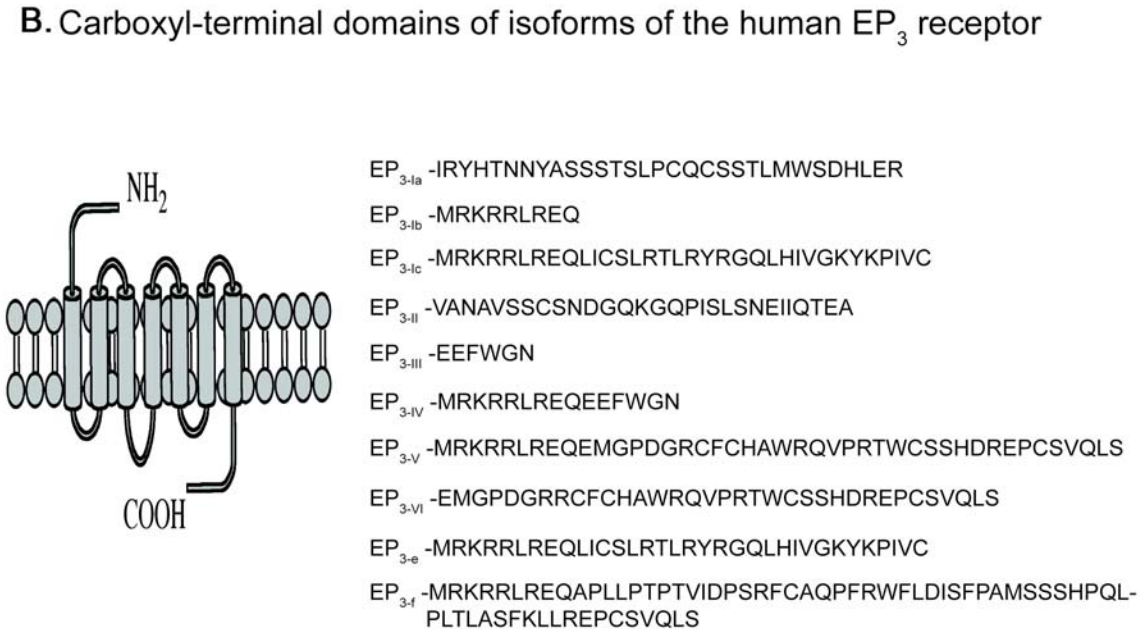


Figure 1.3 Exons expressed in splice variants human EP_3 receptor and corresponding amino acid sequences of carboxyl tails.
(A) Details the exons expressed in the individual mRNA sequences of the ten EP_3 receptor isoforms. Inverted triangles represent the locations of polyadenylation sites. (B) Unique carboxyl tail amino acid sequences of the EP_3 receptor splice variants.

alternative splicing of the human EP_3 gene and Figure 1.3B details the amino acid sequence in the carboxyl tails of the 10 distinct mRNAs. Further structural analysis has revealed that second extracellular loop of the EP_3 receptor confers ligand selectivity and that the seventh transmembrane domain is important G protein coupling [26-29]. Although these studies were carried out using an isoform of the rabbit EP_3 receptor, there is homology between these regions in the human receptor and thus it is expected that these results are applicable to the human EP_3 receptor.

The EP_3 receptor subtype is unique among the EP receptor subtypes in that it has multiple isoforms. In humans, there are 10 distinct mRNA splice variants encoding 8 isoforms generated by alternative mRNA splicing of the carboxyl-terminal tail. A uniform nomenclature for the human EP_3 receptor isoforms and sequence variants has not been agreed upon and established. This document utilizes the nomenclature from Kotani et al. (1997) [25].

Splice variants in other species have been cloned and include the mouse $EP_{3\alpha}$, $EP_{3\beta}$ and $EP_{3\gamma}$ isoforms; the rat EP_{3A}, EP_{3B} and $EP_{3\alpha}$; bovine EP_{3a}, EP_{3b}, EP_{3c} and EP_{3d}; and rabbit 72A, 74A, 77A, 80A and NT (no tail) [30-

35]. Isoforms of the EP_3 receptor do not precisely correspond to each other across species. For example, the human $EP_{3\text{-}V}$ and $EP_{3\text{-}VI}$ isoforms are not found in mouse, rabbit, rat, or bovine [36]. However, there is ~80-90% similarity in the carboxyl tail sequences of the human $EP_{3\text{-}Ia}$ and mouse $EP_{3\text{-}\alpha}$ and the human $EP_{3\text{-}II}$ and mouse $EP_{3\text{-}\gamma}$ isoforms, but this doesn't confer 80-90% identical receptor functions between species [37].

The EP_3 receptor is the most highly expressed EP receptor in humans [14]. Its expression is most abundant in the kidney, uterus, adrenal gland and pancreas, but is also found in the stomach, small intestines, brain, vasculature, prostate, connective tissue and epidermis. Few studies assessing EP_3 receptor expression identify the isoforms expressed in the tissue. Data from published studies indicate that EP_3 receptor isoforms are expressed in groups of two or more rather than singly [30, 32-34, 38, 39]. This makes identification of specific functions of the isoforms difficult. What is clear from these studies is that the $EP_{3\text{-}I}$ and $EP_{3\text{-}II}$ isoforms are the most abundantly expressed EP_3 isoforms in human tissues [30, 34].

Activation of the EP_3 receptor induces a number of diverse actions including inhibition of arginine-vasopressin induced water reabsorption in renal collecting tubules, smooth muscle contraction, inhibition of histamine induced gastric acid secretion, inhibition of lipolysis in mature adipocytes, neurotransmitter release, cell proliferation and neural inflammation [40-49]. EP_3 receptor is important in several diseases including cancer, inflammation, allergy, cardiovascular ischemia, thromboembolism and neurodegeneration [50-54].

Colorectal cancer is one of the most commonly diagnosed forms of cancer and the second leading cause of cancer related death in the United States and the western world [55]. In the early 1980's it was discovered that non-steroidal anti-inflammatory drugs like indomethacin had anti-tumor effects in large bowel tumors rats and reduced the incidence of colorectal cancer. It was later established that COX-2 was upregulated in colon cancer and the anti-cancer activities of NSAIDs were due to the inhibition of COX [56-58]. Colon tissue samples from humans, mice and rats showed a reduction in EP_3 receptor expression in colon cancer samples versus healthy tissue. After establishing a similar trend in human colon cancer cell lines it was

discovered that the down regulation of EP_3 receptor expression was due to hypermethylation of the PTGER3 gene [59, 60]. Further studies concluded that in some forms of cancer the loss of EP_3 receptor expression contributes to increased proliferation and tumor size [59, 61]. Contrastingly, EP_3 receptor activity has also been linked to increasing tumorigenicity due to enhancement of proliferation in skin, lung and endometrial cells [60, 62, 63].

The diversity of activities seen in studies of the EP_3 receptor may in part be due to a variety of circumstances including tissue context. However, it is important to recognize that there are 10 distinct mRNA splice variants of the EP_3 receptor in humans and multiple variants in other species. The initial cloning and characterization of the EP_3 receptor concluded that it coupled to the inhibitory G protein, G_i [64]. Subsequent studies and the cloning of multiple splice variants and their expression in recombinant cell systems unveiled that the receptor was capable of coupling to a variety of G proteins and that some isoforms preferentially coupled to specific G proteins. To date, EP_3 receptor isoforms are known to couple to G_i, G_s, G_q and $G_{12/13}$ [30-33, 36, 64-71].

Many groups have undertaken the task of elucidating the functional differences between EP_3 receptor isoforms. EP_3 receptor isoforms are reported to have differences in cellular localization, constitutive activity, agonist induced internalization patterns, activation of stress fiber formation and regulation of second messengers such as calcium [37, 66, 72]. However, few papers have analyzed if these differences in signal transduction pathway activation among isoforms translates into differences in gene regulation.

Understanding how differences in EP_3 receptor isoform activity affects signal transduction and gene regulation is important for understanding the many divergent actions attributed to the EP_3 receptor.

1.4 Hypothesis and Aims

It is known that EP_3 receptor activation results in the activation of divergent signal transduction pathways, but it is not known how this affects gene regulation. **We hypothesize that PGE_2 stimulation of the $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ receptor isoforms results in activation of distinct signal transduction pathways that result in unique gene regulation programs.** The following aims summarize how these studies were carried out:

Aim 1: Model Design & Characterization

Aim 2: Differential ERK 1/2 Signaling

Aim 3: Comparative Analysis of Gene Expression Patterns among Human EP_3 Prostanoid Receptor Isoforms

To carry out these studies I developed three HEK 293 EBNA cell lines stably expressing the $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ receptor isoforms individually. These isoforms are the most highly expressed isoforms of the human EP_3 receptor. EP_3 receptor isoforms are often found expressed in multiple combinations rather than singly, making the identification of individual signaling capabilities more difficult [30, 36, 39, 72-74]. The use of recombinant systems allows for the identification of distinct differences that may be difficult to identify in native systems. After developing these EP_3 receptor isoform cell lines I performed functional characterization using competitive radioligand binding, cyclic AMP assays and inositol triphosphate assays. Following confirmation of the functional capabilities of these cell lines I undertook the task of using gene reporter assays and microarray analysis to identify genes uniquely regulated by each EP_3 receptor isoform. The aims of this research was to identify differential

signaling pathway activation and gene regulation among three isoforms of the human EP_3 receptor and to determine if these differences result in induction of distinct gene expression programs dependent on the receptor isoform(s) expressed.

CHAPTER TWO

GENERATION AND CHARACTERIZATION OF HEK 293 EBNA CELLS EXPRESSING HUMAN $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$ AND $EP_{3\text{-}III}$ RECEPTOR ISOFORMS

2.1 Introduction

Analysis of unique receptor function can be complicated by the presence of receptors that share a common ligand. In the case of prostanoid receptors, despite the ligand selectivity there is also a significant degree of cross reactivity among the receptors and ligands. Unlike studies that utilize selective agonists and antagonists when comparing prostaglandin receptor subtypes, there are no available ligands that can differentiate between EP_3 receptor isoforms. A further complication resides in the observation that EP_3 receptor isoforms are often expressed in clusters of multiple isoforms rather than singly. Recombinant heterologous expression systems are used to examine proteins in isolation. We cloned cDNAs from individual EP_3 receptor isoforms, into the pCEP-4 expression vector that uses a cytometgalovirus (CMV) promoter to drive transcription of the gene. Utilization of this model system allows for not only analysis of the pharmacological characteristics of the receptor but also the unique signal transduction and gene regulation capabilities of the receptor.

Studies utilizing recombinant heterologous expression of the EP_3 receptor isoforms have been conducted in a number of cell lines including Chinese

hamster ovary (CHO) cells, African green monkey kidney fibroblast cells (Cos-7) and human embryonic kidney (HEK) cells. These previous studies have helped to identify ligand-binding characteristics of the EP_3 receptor isoforms as well as second messenger systems activated by these isoforms. [19, 30, 31, 68-72, 75, 76]

To identify unique functional differences among the three isoforms of the human EP_3 receptor we utilized a human cell system which did not endogenously express any of the prostanoid receptors, thus allowing for examination of the unique signaling and differential gene regulation among the $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ isoforms in isolation. HEK 293 EBNA cells stably expressing the $EP_{3\text{-}I}$, $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ receptor isoforms were generated by excising the cDNA cassette from previously generated pBC12 plasmid and cloning these cDNA's into the pCEP4 plasmid vector. Following transfection of the plasmids into HEK 293 EBNA cells, clones were characterized using radioligand binding, adenosine 3' 5-cyclic monophosphate (cyclic AMP) and inositol triphosphate (IP_3) assays to ensure the clones behaved similarly in regards to PGE_2 binding and G-protein coupling as has previously established both in this laboratory and in

other published studies [30]. Following the establishment of stable clones, I utilized these cells to complete my studies to examine unique signaling and gene regulation capabilities of the $EP_{3\text{-}I}$, $EP_{3\text{-}II}$ AND $EP_{3\text{-}III}$ receptor isoforms.

2.2 Experimental Procedures

Materials.

Dulbecco's Modified Eagle's Medium (DMEM), DMEM without phenol red, G418 and gentamicin sulfate were from Mediatech (Herndon, VA). Trypsin-EDTA (0.25% trypsin; 1 mM EDTA4Na), Fetal Bovine Serum (FBS), OptiMEM, Hygromycin B, pCEP4 vector, and HEK 293-EBNA cells were from Invitrogen (Carlsbad, CA). QIAQUICK GEL PURIFICATION kit was from Qiagen (Venlo, The Netherlands). T4 DNA ligase was from New England Biolabs (Ipswich, Massachusetts). Fugene 6 transfection reagent was from Roche Diagnostics (Indianapolis, IA). [^{3}H] PGE_2, adenosine 3' 5-cyclic monophosphate and [^{3}H] myo-Inositol are from Amersham/GE Healthcare (Buckinghamshire, England). Forkolin and Pertussis Toxin are from Calbiochem (San Diego, CA). PGE_2 and sulprostone are from Cayman Chemical. Safety-Solve is from Research

Products International (Mt. Prospect, Illinois). Protein Kinase A, 3-Isobutyl-1-methylxanthine (IBMX) and cyclic AMP are from Sigma (St. Louis, MO).

Construction of pCEP4 $EP_{3\text{-}I}$, $EP_{3\text{-}II}$, and $EP_{3\text{-}III}$ plasmids.

The EP_3 receptor isoforms were amplified from the pBluescipt (KS+) plasmid constructs KS/EP_{3A}, KS/EP_{3D}, and KS/EP_{3E}, previously generated by polymerase chain reaction (PCR) [30]. EP_{3A}, EP_{3D}, and EP_{3E} are referred herein as $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ respectively. The following primers were used: a common EP_3 sense primer includes nucleotides 1-23 (bold) and contains a HindIII site (italics): 5'-ATTATT*AAGCT*TATGAAGGAGACCCGGGGCTACGG-3'. All EP_3 anti-sense primers contain an Xho-1 site (italics). $EP_{3\text{-}Ia}$ anti-sense primer represented nucleotides 1154-1173 (bold) 5'-CCGCCG*CTCGAG*TTATCTTTCCAAATGGTCGC-3'. $EP_{3\text{-}II}$ anti-sense primer represented nucleotides 1143-1167 (bold) 5'-CCGCCG*CTCGAG*TCATGCTTCTGTCTGTATTATTTC-3'. $EP_{3\text{-}III}$ anti-sense primer represented nucleotides 1077-1098 (bold). 5'-CCGCCG*CTCGAG*TTAATTTCCCCAAAATTCCTCC-3'. PCR products were electrophoresed on 1.5% agarose gel, excised and purified using

QIAQUICK GEL PURIFICATION kit. Following purification, the individual EP_3 receptor PCR products were sequenced at the University of Arizona Genomic Analysis and Technology Core facility. After sequence confirmation, the individual EP_3 receptor cDNA fragments and the pCEP4 expression vector were digested using the restriction enzymes Xho-1 and Hind III. EP_3 receptor cDNA fragments were then ligated into the pCEP4 vector using T4 DNA ligase. The plasmids generated were named as follows: pCEP4/ $EP_{3\text{-}Ia}$, pCEP4/ $EP_{3\text{-}II}$ and pCEP4/ $EP_{3\text{-}III}$.

Cell Culture and Generation of Stable EP_3 transfectants.

HEK 293 EBNA cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), 250 μg/ml G418 and 200 μg/ml gentamicin sulfate and incubated at 37°C and 5% CO_2. Transfected cells HEK 293 EBNA cells were grown in the same media as above, but with 200 μg/ml Hygromycin B.

One million HEK 293 cells were seeded in 10 cm plates and the following day transfected with 10-μg pCEP4/ $EP_{3\text{-}Ia}$, pCEP4/ $EP_{3\text{-}II}$ and pCEP4/ $EP_{3\text{-}III}$ plasmid DNA using Fugene 6 transfection reagent. One day after transfection, cells were selected for pCEP4/EP_3 receptor expression using

200 μg/ml hygromycin B. Eight days later, hygromycin B resistant clones for each receptor isoform were transferred into 1 well of a 96-well plate, when the clone reached confluence was transferred into a 48-well plate, then a 6-well plate and finally a 10 cm plate.

Radioligand binding

HEK 293 cells stably expressing pCEP4 plasmid alone, pCEP4/$EP_{3\text{-}Ia,}$ pCEP4/$EP_{3\text{-}II}$ and pCEP4/$EP_{3\text{-}III}$ were grown to 80% confluence in T75 flasks. Cells were detached from the flask using trypsin-EDTA, counted and spun down at 5000 rpm to remove media. Cells were resuspended in 1 ml of MES binding buffer (10 mM MES, pH 6.0, 10 mM $MnCl_2$, 0.4 mM EDTA) at a concentration of ten million cells/ml. Binding assay set up in 12x75 borosilicate glass tubes contained 100 μL cells in MES binding buffer, 2.5 nM [^{3}H] PGE_2, untritiated PGE_2 at 10^{-9}–10^{-5} M and MES binding buffer so that each reaction volume was 200 μL. Binding reactions were incubated at room temperature for one hour. At the end of incubation, reactions were filtered onto Whatman GF/B glass fiber filters using a Brandel cell harvester and ice cold MES binding buffer. Filters containing reactions were washed three times with MES buffer to remove any unbound ligand and vacuum

dried. Each filter was transferred into scintillation vials containing 7 mls of Safety-Solve high flash point scintillation cocktail. Vials of samples were vortexed then radioactivity was detected using a Beckman LS100 scintillation counter. These experiments were conducted in duplicate and data were analyzed using GraphPad Prism software.

Adenosine 3' 5-cyclic monophosphate (cyclic AMP) assay.

HEK 293 cells stably expressing pCEP4 plasmid alone, pCEP4/$EP_{3\text{-}Ia,}$ pCEP4/$EP_{3\text{-}II}$ and pCEP4/$EP_{3\text{-}III}$ were seeded into 6 well plates at a density of one million cells per well in normal culture media and incubated overnight at 37°C, 5% CO_2. In experiments using pertussis toxin (PTX), cells were dosed with 100 ng/ml PTX 8 hours after seeding cells and 16 hours before experiment was conducted using 50 nM PGE_2 and sulprostone. The following day cells were rinsed twice with phosphate buffered saline (PBS) then incubated with 2 mls Opti-MEM reduced serum media containing 0.1 mg/ml of the phosphodiesterase inhibitor 3-Isobutyl-1-methylxanthine (IBMX) for 15 minutes at 37°C, 5% CO_2. After IBMX treatment cells were incubated with DMSO or 10^{-9}–10^{-6}M PGE_2 or sulprostone for 1 hour, followed by treatment with 3 μM forskolin at 37°C, 5% CO_2. Following

drug treatment, media was aspirated and 720 μl cold Tris/EDTA buffer (TE) (50mM Tris/HCl, 4mM EDTA, pH 7.5) was added to each well. Samples were scraped and cell lysates were transferred into labeled screw cap microcentrifuge tubes and boiled for 8 minutes. Samples were then placed on ice for 5 minutes then tubes were centrifuged 12,000 rpm for 2 minutes. A standard curve was prepared to determine the concentration of cyclic AMP in each sample. The standard curve reactions were prepared as follows in microcentrifuge tubes; 50 μl of 0.125-64 picomoles of cyclic AMP diluted in TE or TE alone, 50 μl of 0.9 pmol [^{3}H] cyclic AMP diluted in TE, 100 μl of 0.06 mg.ml protein kinase A (PKA) diluted in 1% BSA or 1% BSA alone. Experimental sample reactions were prepared similarly except 50 μl of cell lysates was added instead of untritiated cyclic AMP. Reactions were incubated for two hours at 4°C. Following reaction incubation, 100 μl of 2.5 mg/ml activated charcoal in 2% BSA was added to each microcentrifuge tube. Samples were vortexed, then centrifuged at room temperature for 1 minute. 200 μl of supernatant was transferred into labeled scintillation vials containing 9 ml of Safety-Solve scintillation cocktail. Vials of samples were vortexed then radioactivity was detected using a Beckman LS100

scintillation counter. These experiments were conducted in duplicate and data were analyzed using GraphPad Prism software.

Inositol phosphate accumulation assay.

HEK 293 cells stably expressing pCEP4 plasmid alone, pCEP4/$EP_{3\text{-}Ia,}$ pCEP4/$EP_{3\text{-}II}$ and pCEP4/$EP_{3\text{-}III}$ were seeded into 24 well plates at density of 200,000 cells/well in normal culture media and incubated overnight at 37°C, 5% CO_2. The following day media was aspirated and replaced with 1 ml of Opti-Mem supplemented with 0.2 μM myo- [2-^{3}H]-inositol. Cells were incubated overnight at normal culture conditions. The following day, cells were treated with 10 mM lithium chloride (LiCl) for 15 minutes to slow hydrolysis of inositol phosphates. Following LiCl treatment, cells were treated with 10^{-9}–10^{-6}M PGE_2 for 1 hour. After PGE_2 treatment, cells were rinsed with 1x PBS and 1ml of ice-cold methanol was added. One milliliter of distilled H_2O was added to cells/methanol and cell were scraped and transferred to glass test tubes. One milliliter of chloroform was added to each tube then solution was vortexed gently. Samples were then centrifuged at 2,100 rpm at 4°C for 10 minutes. Aqueous phase of the samples were carefully added onto anion exchange columns packed with 2.5 mls of AG 1-

X8 resin. Columns were washed three times with distilled H_2O, and then washed twice with 5 ml of 5 mM Na tetraborate/60 mM formic acid solution. Total phosphates were eluted off the column using 2 ml of a 0.2 M-ammonium formate/0.1 M formic acid solution into scintillation vials. Nine milliliters of Safety-Solve scintillation cocktail was added to each vial, then vortexed. Radioactivity was detected using a Beckman LS100 scintillation counter. These experiments were conducted in duplicate and data were analyzed using GraphPad Prism software.

2.3 Results

Competition curves of unlabeled PGE_2 and [^{3}H] PGE_2 in HEK cells expressing EP_3 receptor isoforms. PGE_2 competition-binding experiments were performed on whole cell lysates to identify and characterize functional clones stably expressing EP_3 receptor isoforms as described in the Experimental Procedures. For each EP_3 isoform to be studied a clone was chosen that displayed [^{3}H] PGE_2 binding and followed the laws of mass action showing that ligand is competing for only one binding site. The appropriate clones would have binding curves that descend from 90% to 10% over approximately two log units or 100-fold change in concentration.

Figure 2.1 shows the results of the PGE_2 competitive [^{3}H] PGE_2 radioligand binding experiments. The IC_{50} values of the pCEP4/$EP_{3\text{-}Ia,}$ pCEP4/$EP_{3\text{-}II}$ and pCEP4/$EP_{3\text{-}III}$ clones chosen for use were 23.6 nM, 69.1 nM and 29.8 nM respectively. The specific binding of [^{3}H] PGE_2 to cells expressing $EP_{3\text{-}Ia,}$ $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ were 856.68 fmol/mg protein, 1169.47 fmol/mg protein and 1087.47 fmol/mg protein, respectively.

Inhibition of forskolin induced cyclic AMP production by PGE_2 and sulprostone treatment in HEK cells expressing EP_3 receptor isoforms. The previous whole cell radioligand binding studies indicated that the $EP_{3\text{-}Ia,}$ $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ clones chosen were able to bind PGE_2 but did not allude to the signal transduction capabilities of the clones. The EP_3 receptor is characterized as G_i coupled therefore assessment of cyclic AMP inhibition following PGE_2 treatment was one method utilized to examine if the clones were indeed functional. Cyclic AMP assays were completed as described in Experimental Procedures. EP_3 receptor activation inhibits forskolin induced cyclic AMP production, however the effect is dose dependent. Figure 2.2A shows the effects of PGE_2 on forskolin induced cyclic AMP production. All three cell lines show a biphasic effect on cyclic AMP production where low

concentrations of PGE_2 have little or no effect, mid-range concentrations show inhibition and 1 μM PGE_2 pre-treatment resulted in cyclic AMP levels similar to or greater than forskolin treatment alone. This may be due to a unique cellular environment of HEK cells. In the HEK-Empty Vector (EV) clone, in the presence of forskolin, pre-treatment with increasing concentrations PGE_2 induced a concomitant increase in cyclic AMP production. This effect may contribute to the biphasic effects on cyclic AMP production seen in the EP_3 receptor isoforms. One micromolar and 10 μM PGE_2 treatment alone does increase cyclic AMP levels in HEK-Empty Vector cells. Repeated reverse transcription PCR analysis for expression of prostanoid receptors in the HEK-Empty Vector cells has failed to unveil the presence of any known prostanoid receptors. To resolve the problems associated with co-treatment of HEK cells with PGE_2 and forskolin we used sulprostone an EP_3/EP_1 selective agonist. Figure 2.2B shows that in HEK-EV cells sulprostone had no effect forskolin induced cyclic AMP production. In $EP_{3\text{-}Ia}$ and $EP_{3\text{-}III}$ expressing cells 1 nM-1 μM pretreatment with sulprostone inhibited forskolin induced cyclic AMP accumulation. However, in $EP_{3\text{-}II}$ expressing cells the greatest inhibition of cyclic AMP production by sulprostone was observed at 1 nM and 10 nM. Higher

concentration of sulprostone in $EP_{3\text{-}II}$ expressing cells had minimal reduction of forskolin induced cyclic AMP production (data not shown).

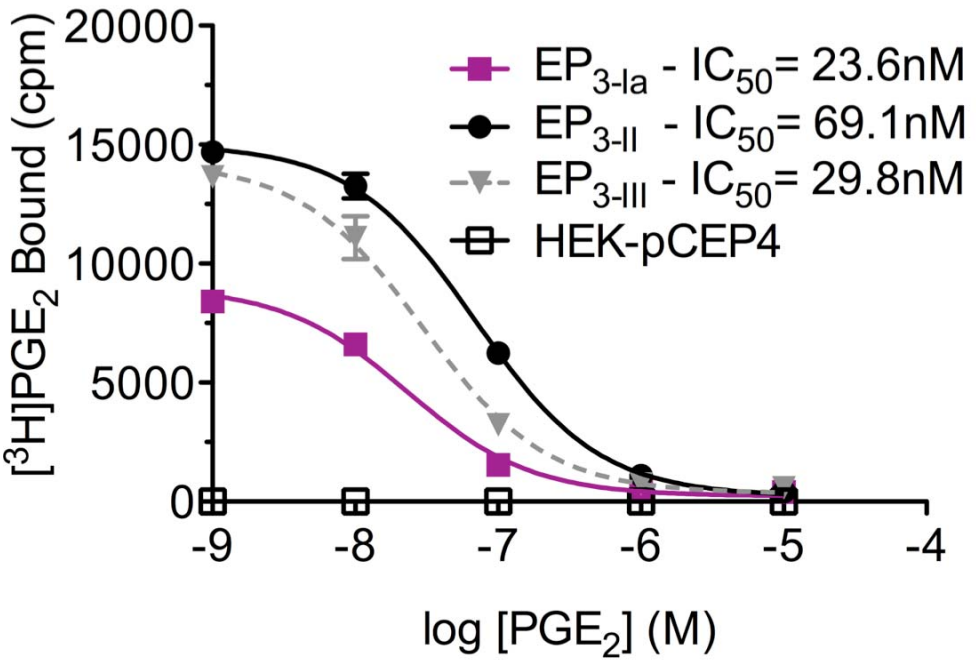


Figure 2.1. Competition curves of unlabeled PGE_2 and [^{3}H] PGE_2 in HEK cells expressing EP_3 receptor isoforms.
HEK cells expressing EP3-$_{Ia}$ (clone 1a), $EP_{3\text{-II}}$ (clone 1a), and $EP_{3\text{-III}}$ (clone 1c) were incubated with DMSO, 1 nM-1μM PGE_2 and assayed for specific binding to [^{3}H] PGE_2 as described in "Experimental Procedures". Data are representative of three independent experiments. Data was analyzed using linear regression analysis in GraphPad Prism software. For all experiments, data are shown as mean ± SEM.

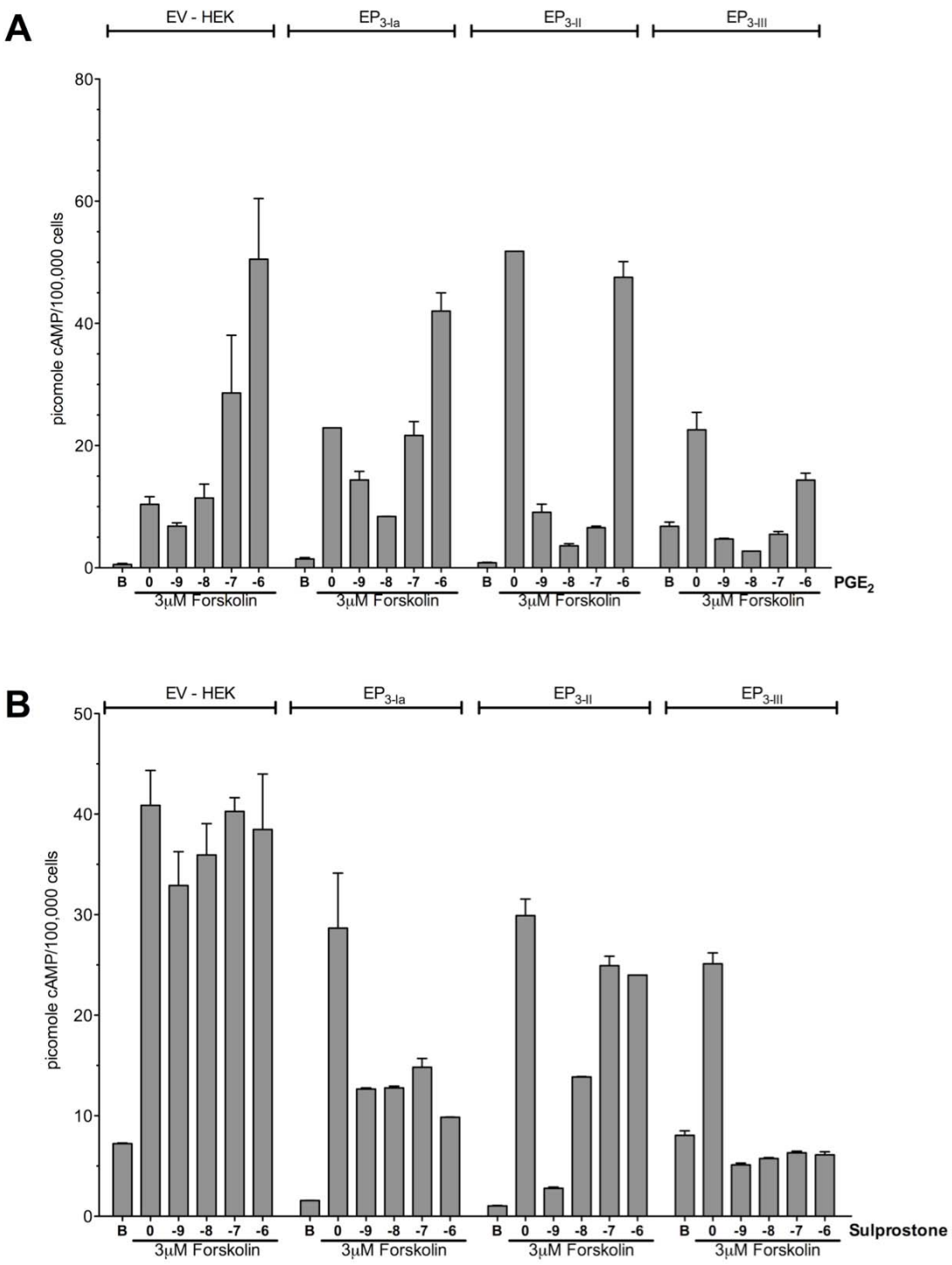

A
EV - HEK
EP3-Ia
EP3-II
EP3-III
picomole cAMP/100,000 cells
80
60
40
20
0
B 0 -9 -8 -7 -6
3μM Forskolin
PGE2
B
EV - HEK
EP3-Ia
EP3-II
EP3-III
picomole cAMP/100,000 cells
50
40
30
20
10
0
B 0 -9 -8 -7 -6
3μM Forskolin
Sulprostone

Figure 2.2. Inhibition of forskolin induced cyclic AMP production by PGE_2 and sulprostone treatment in HEK cells expressing EP_3 receptor isoforms.

$EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and $EP_{3\text{-}III}$ expressing cells were assayed for inhibition of forskolin induced cyclic AMP production following pretreatment with 1nM-1 μM PGE_2 (A) or sulprostone (B) as described in "Experimental Procedures". B is the basal/unstimulated level of cyclic AMP present in the cells. Data is representative of three independent experiments. Data was analyzed using GraphPad Prism software. For all experiments, data are shown as mean ± SEM.

Treatment with pertussis toxin prevents the inhibition of forskolin induced cyclic AMP production by sulprostone treatment in HEK cells expressing EP_3 receptor isoforms. Due to the effects on cyclic AMP production of co-treatment of PGE_2 and forskolin, we used sulprostone to determine if the inhibitory effects EP_3 receptor activation on forskolin induced cyclic AMP production were G_i dependent. HEK cells expressing $EP_{3\text{-}Ia,}$ $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ receptors were pre-treated with pertussis toxin overnight, and then treated with 1 μM sulprostone followed by stimulation with 3 μM forskolin. Figure 2.3 shows that in all cell lines tested we observed the following: 1) sulprostone alone had no effect on cyclic AMP production 2) sulprostone pre-treatment inhibited forskolin induced cyclic AMP production and 3) pertussis toxin pretreatment reversed the effects of sulprostone on forskolin induced cyclic AMP production. In $EP_{3\text{-}II}$ expressing cells, pertussis toxin pretreatment increased cyclic AMP levels above the levels observed in forskolin alone treated cells.

Inositol Phosphate Formation PGE_2 treatment in HEK cells expressing EP_3 receptor isoforms. An additional method of analysis of the functional capabilities of the $EP_{3\text{-}Ia,}$ $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ clones was by using the inositol

triphosphate (IP3) assay. Previous studies have indicated that some isoforms of the EP_3 receptor couple to G_q. Coupling to G_q subunits is known to result in the activation of phospholipase C, the hydrolysis of inositol containing phospholipids and subsequently the production of IP3 and diacylglycerol (DAG). Figure 2.4 illustrates that PGE_2 treatment induces IP3 formation in the $EP_{3\text{-II}}$ and $EP_{3\text{-III}}$ but not in $EP_{3\text{-Ia}}$ expressing cells. In fact, $EP_{3\text{-Ia}}$ expressing cells showed similar EC_{50} value for the formation of IP3 as HEK-EV cells.

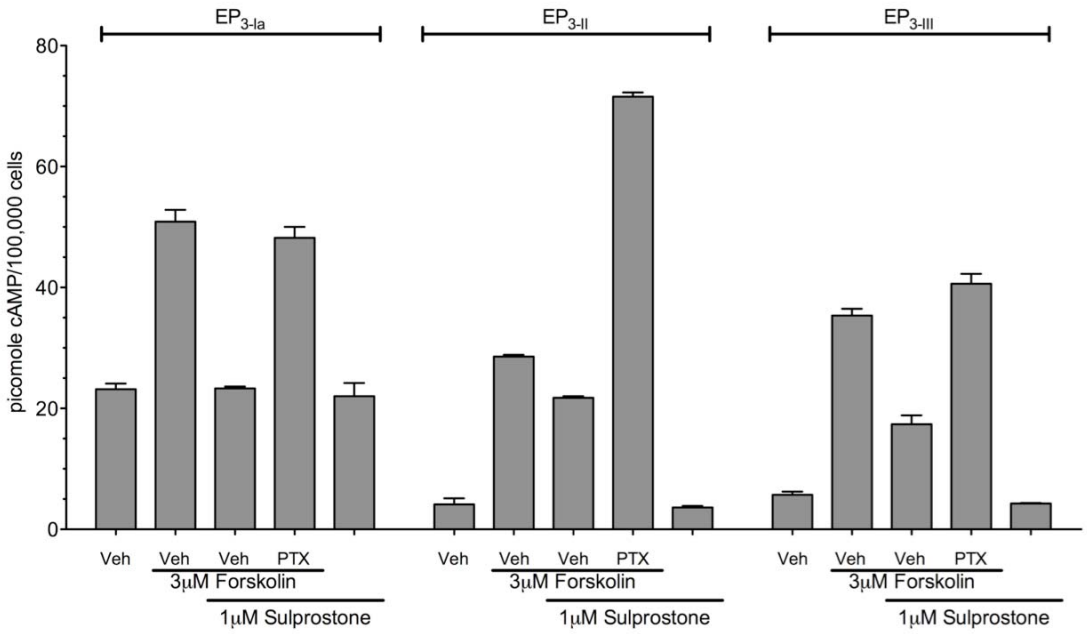


Figure 2.3. Treatment with pertussis toxin prevents the inhibition of forskolin induced cyclic AMP production by sulprostone treatment in HEK cells expressing EP_3 receptor isoforms.

$EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and $EP_{3\text{-}III}$ expressing cells were pretreated with pertussis toxin (PTX) then assayed for inhibition of forskolin induced cyclic AMP production following pretreatment with 1 μM sulprostone as described in "Experimental Procedures". Data is representative of three independent experiments. Data was analyzed using GraphPad Prism software. For all experiments, data are shown as mean ± SEM.

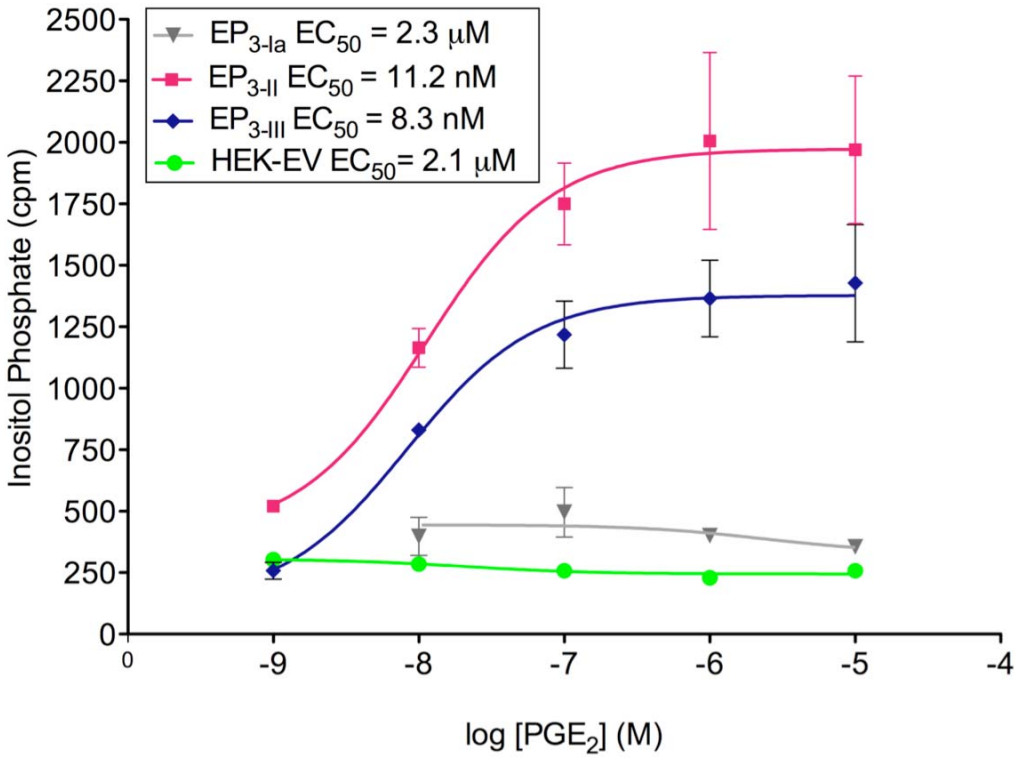


Figure 2.4. Inositol Phosphate Formation PGE_2 treatment in HEK cells expressing EP_3 receptor isoforms.
HEK Cells expressing $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and $EP_{3\text{-}III}$ were treated with 1 nM-1 μM PGE_2 then assayed for the accumulation of myo- [2-^{3}H]-inositol as described in "Experimental Procedures". Data is representative of three independent experiments. Data is shown as mean ± SEM.and was analyzed using GraphPad Prism software.

2.4 Discussion

Stable expression systems provide a way to analyze the functions of proteins of interest in cells expressing nearly identical levels of receptor. Stable expression system thus allow for lower levels of variability within repeated experiments. In this study we generated and characterized HEK 293 cells stably expressing the $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ isoforms. The clones chosen showed characteristics that the cells were able to bind PGE_2 and showed functional characteristics similar to previously published cyclic AMP inhibition and IP3 accumulation studies. EP_3 receptor expressed in HEK 293 EBNA cells had K_d values that followed a similar trend as what we reported in cos-7 cells and An et al. 1994 reported in CHO cells. Values in the HEK 293 cells used here were higher but this may be due to differences in receptor density and/or the cell lines used in previous studies.

Jin et al. 1994 assessed the constitutive activity of EP_3 receptor isoforms expressed in CHO cells. They found that $EP_{3\text{-}III}$ and $EP_{3\text{-}IV}$ receptor expressing CHO cells had greater inhibition of forskolin stimulated adenylate cyclase activity (cyclic AMP accumulation) in the absence of agonist than did $EP_{3\text{-}Ia}$ and $EP_{3\text{-}II}$ expressing cells. Jin et al. 1994 analyzed

their cyclic AMP data by normalizing cyclic AMP data for EP_3 receptor isoforms to forskolin stimulated only HEK-EV values. They concluded that because the level of forskolin stimulated cyclic AMP in $EP_{3\text{-III}}$ and $EP_{3\text{-IV}}$ expressing cells was lower than in $EP_{3\text{-Ia}}$ and $EP_{3\text{-II}}$ cells, this was an indication of higher constitutive activity. If we analyze the data presented in Figure 2.2 in a similar manner, we find that our $EP_{3\text{-Ia}}$, $EP_{3\text{-II}}$ and $EP_{3\text{-III}}$ expressing cells show a similar pattern of forskolin-stimulated cyclic AMP that could be attributed to constitutive activity (data not shown).

There are little data published on inositol phosphate accumulation in human $EP_{3\text{-Ia}}$, $EP_{3\text{-II}}$ and $EP_{3\text{-III}}$ expressing cells. Most studies report EP_3 receptor mediated increases in intracellular calcium ($[Ca^{2+}]_i$) in the absence of extracellular calcium as an indicator of IP3 activity. If we look at studies of the effects of EP_3 receptor activation on calcium concentration, we find that studies by Yang et al. 1994, infer that $EP_{3\text{-Ia}}$ does not induce increased $[Ca^{2+}]_i$ following agonist treatment while both $EP_{3\text{-II}}$ and $EP_{3\text{-III}}$ do. An et al. 1994, concluded that increased $[Ca^{2+}]_i$ is greatest in $EP_{3\text{-II}}$ expressing cells followed by $EP_{3\text{-III}}$ and $EP_{3\text{-IV}}$ and that this calcium is released from intracellular stores rather than brought in from the extracellular environment. This may indicate

that $EP_{3\text{-}II}$, $EP_{3\text{-}III}$ and $EP_{3\text{-}IV}$ receptor stimulation activates phospholipase C, leading to IP3 generation and increased $[Ca^{2+}]_i$. If this is true our studies on IP3 accumulation following PGE_2 treatment being greatest in $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ and non existent in $EP_{3\text{-}Ia}$ cells corroborate data previously reported. We did not assess the effects of PGE_2 on $[Ca^{2+}]_i$ in these studies.

The data attained in these characterization studies indicate that these isoforms expressed in HEK 293 EBNA cells are functional and show similar characteristics reported by others. Differences in cyclic AMP and IP3 accumulation data may be an early indication of potential differential signal transduction capabilities.

CHAPTER THREE

DIFFERENTIAL MECHANISMS FOR INDUCTION OF ERK 1/2 PHOSPHORYLATION AMONG HUMAN $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$ AND $EP_{3\text{-}III}$ RECEPTOR ISOFORMS STABLY EXPRESSED IN HEK 293 EBNA CELLS AND THE EFFECTS ON ELK-1 AND AP-1 GENE REPORTER ACTIVITY AND PROLIFERATION

3.1 Introduction

MAPK pathway activation is often associated with its effects on growth, differentiation and proliferation. The p44/p42 mitogen activated protein kinase (MAPK), also known as ERK 1/2, are serine/threonine kinases activated by extracellular stimulus involving the sequential activation of Ras/Raf/MEK proteins. ERK activities include regulation of cell proliferation, differentiation, and apoptosis [77, 78]. Upstream kinases modulating the activation status of ERK 1/2 include protein kinase C (PKC), phosphoinositide-3 kinase (PI3K), protein kinase A (PKA) and transactivation by receptor tyrosine kinases such as the epidermal growth factor receptor (EGFR). In a previous paper by Burkey and Regan, we described the activation of ERK 1/2 pathway by the $EP_{3\text{-}Ia}$ receptor expressed in COS-7 cells [79]. Regulation of ERK 1/2 by G-protein coupled receptors has proven to be cell-type specific [80]. Therefore, based on our previous observations, we wanted to determine if the $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and $EP_{3\text{-}III}$ receptor isoforms expressed in HEK cells induced ERK 1/2 phosphorylation and the mechanisms by which this was accomplished.

MAPKs mediate their effects on differentiation, cellular growth and proliferation via modulation of downstream effectors such as other kinases and transcription factors. ELK-1 is a transcription factor and AP-1 is a transcription factor complex that's transcriptional and functional activities are regulated by the actions of MAPK. ELK-1 is an Ets-related transcription factor that binds to the serum response element (SRE) of a variety genes regulating growth, including c-fos. AP-1 is a transcription factor complex made up of heterodimers of the Jun (c-jun, JunB and JunD), Fos (*c-fos, fosB,* Fra-1 and FRA-2) and activating transcripition factor (ATF) member proteins. Like ELK-1, AP-1 is regulated by MAPK phosphorylation, however AP-1 binds to the 12-O-tetradecanoylphorbol-13-acetate response element (TRE) to mediate the transcription of proteins important for cell growth and survival [81, 82].

Studies of the activities of EP_3 receptors have uncovered a number of discrepancies related to its effects on proliferation and differentiation. EP_3 signaling mediated inhibition of proliferation has been reported in studies in keratinocytes, colon cancer cell line HCA-7 and growth arrest 3T6 fibroblasts [60, 83-85]. In contrast, EP_3 receptor activation has also been

reported to induce proliferation in hepatocytes, endometrial stromal cells and the adenocarcinoma cell line A549 [62, 63, 86]. The divergent data regarding the influences of EP_3 receptor activation on proliferation indicate that EP_3 receptors may have different effects based on the tissue type in which they are expressed. However, few studies have identified the specific receptor isoforms expressed, therefore the divergent effects attributed to the EP_3 receptor may be due to differences in the expression of the isoforms involved.

First we used immunoblot to examine if, as previously reported, the EP_3 receptor isoforms utilized in these studies induced ERK 1/2 phosphorylation following treatment with PGE_2. Next we utilized pharmacological inhibitors to determine the pathways activated $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and EP_{3III} receptor isoforms and leading to ERK 1/2 phosphorylation.

To assess the potential of $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and EP_{3III} receptor isoforms to differentially regulate gene transcription, we chose to first focus on ERK 1/2 regulated targets. ERK 1/2 regulates the activities of the AP-1 transcriptional factor complex and ELK-1, thus this was a logical first step to identifying if

differences in ERK 1/2 activities resulted in differences in AP-1 and ELK-1 reporter activity.

Due to the plethora of information linking the activities of ERK 1/2 phosphorylation and proliferation, we also assessed the effects of PGE_2 treatment in the HEK $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and EP_{3III} receptor isoform expressing cell lines on cell proliferation. For these studies we used the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. The MTS assay measures the formation of a soluble formazan product, which is directly proportional to the number of live cells in culture.

3.2 Experimental Procedures

Materials.

Dulbecco's Modified Eagle's Medium (DMEM), DMEM without phenol red, G418 and gentamicin sulfate were from Mediatech (Herndon, VA). Fetal Bovine Serum (FBS) and Hygromycin B were from Invitrogen (Carlsbad, CA). Horseradish peroxidase conjugated anti-rabbit IgG antibody and Promega CellTiter 96® AQueous Non-Radioactive Cell Proliferation

Assay kits were from Promega (Madison, WI). Protein assay reagent, horseradish peroxidase-conjugated anti-mouse IgG, and nitrocellulose membranes were from Bio-Rad Laboratories (Hercules, CA). Antibodies against ERK 1/2, and phospho-ERK 1/2, were from Cell Signaling Technology (Waltham, MA). PGE_2 was from Cayman Chemical Company (Ann Arbor, MI). Enhanced chemiluminescence substrate was from Pierce (Rockford, IL). PD98059, AG1478, bisindolylmaleimide I (BIM I), Pertussis Toxin (PTX), PP2 and phorbol 12-myristate 13-acetate (PMA) were from Calbiochem (San Diego, CA).

Cell Culture.

Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 250 μg/ml G418, 200 μg/ml gentamicin sulfate, 200 μg/ml Hygromycin B and incubated at 37°C and 5% CO_2.

Western Blotting.

48 hours prior to experiments ~0.5 x 10^6 HEK cells expressing $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and EP_{3III} were seeded into 10 cm plates and incubated at 37°C and 5% CO_2. On the day of the experiment, cells were treated with vehicle (DMSO),

prostaglandin E_2 (PGE_2) or 100nM phorbol 12-myristate 13-acetate (PMA) for 15 minutes unless otherwise stated differently. Following treatment cells were lysed on ice with 300µl RIPA buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 10 mM sodium fluoride, 10 mM disodium pyropho spate, 0.1% SDS, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM sodium orthovanadate, 10 mg/ml leupeptin and 10 µg/ml aprotonin. Samples were sonicated on ice then centrifuged at 15,000g for 15 minutes. Protein concentrations were determined using the Bio-Rad Protein Assay kit. 50 µg of sample lysates were electrophoresed on 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose membranes. Membranes were blocked for one hour in 5% nonfat milk in TBS and 0.1% Tween 20 (TBS-T) at room temperature. Membranes were then incubated at 4°C overnight with anti-phosphorylated ERK 1/2 primary antibody diluted in 5% non-fat milk in TBS-T. Following incubation in primary antibody, membranes were washed three times with TBS-T then incubated with anti-rabbit secondary antibody conjugated with horseradish peroxidase in 5% nonfat milk TBS-T for one hour at room temperature. After washing membrane three times, immunoreactivity was detected using SuperSignal enhanced chemiluminescence. Membranes were

stripped using 2% SDS, 62.5 mM Tris (ph 7.6) and 100 mM ß-mercaptoethanol for 30 minutes at 55°C, then re-probed with an anti-ERK 1/2 antibody to determine both equal protein loading. Image J software (National Institute of Health) was used for densitometric analysis of autoradiographs.

ELK-1 and AP-1 luciferase assays.

HEK-293 cells stably expressing $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and EP_{3III} isoforms were seeded in triplicate in 6-well plates. Twenty-four hours later cells were transfected with 50 ng ELK-1 hybrid fusion protein consisting of the DNA binding domain of the yeast transcription factor Gal4 plasmid, 1 μg Gal4-Luciferase plasmid and 12.5 ng of renilla (pRL-TK) plasmid. For AP-1 experiments 1 μg AP-1 luciferase reporter plasmid and 10 ng of pRL-TK plasmid were used. Cells were pretreated with either vehicle or MAPK inhibitors for 30 minutes followed by treatment with 50 nM PGE_2 for 16-24 hours. Cells were then lysed with 200 μl Passive lysis buffer, centrifuged at 15,000 g and supernatant transferred to fresh tubes. Samples were analyzed on a Turner Design TD-20 Dual-Luciferase luminometer. In AP-1 and

ELK-1 experiments, renilla luciferase activities for each individual transfection were used to normalize firefly luciferase activity.

MTS cell proliferation assay.

HEK-293 cells stably expressing $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and EP_{3III} receptor isoforms were seeded (5,000 cells/well) in 10% serum and grown for 48 hours in 96-well plates. Cells were then treated with 1 nM-10 μM PGE_2 for 24 hours. To assess PGE_2 mediated changes in proliferation; media was removed and replaced with 100 μl of DMEM without phenol red and 20 μl 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) solution. Cells were then incubated for 4 hours at 37°C and 5% CO_2. Absorbance was measured at 490 nm to determine formazan concentration, which is proportional to the number of live cells.

3.3 Results

Time course of ERK 1/2 phosphorylation in EP_3 receptor isoforms following PGE_2 treatment. Treatment of EP_3 receptor isoforms with 50 nM PGE_2 results in very different temporal patterns of ERK 1/2 phosphorylation. In Figure 3.1, $EP_{3\text{-}Ia}$ showed moderate induction of ERK

1/2 phosphorylation 5 minutes after treatment with PGE_2, but is not sustained. However, at 30 minutes and 1 hour there was a brief increase that is completely abrogated after 3 hours. ERK 1/2 phosphorylation in $EP_{3\text{-II}}$ expressing cells was rapidly and strongly induced 5 minutes post treatment with PGE_2, peaks at 15 minutes then gradually was reduced, but still present even 3 hours after PGE_2 treatment. A similar but slight delayed ERK 1/2-phosphorylation profile was seen in $EP_{3\text{-III}}$ expressing cells.

PGE_2 dose response effects on ERK 1/2 phosphorylation in EP_3 receptor isoforms. $EP_{3\text{-Ia}}$ cells induced minimal ERK 1/2 phosphorylation after 15 minutes of 1 nM-100 nM PGE_2 treatment, but there was a more robust response with 1 μM PGE_2 treatment. $EP_{3\text{-II}}$ and $EP_{3\text{-III}}$ show robust ERK 1/2 phosphorylation even at 1 nM PGE_2 concentrations, although $EP_{3\text{-II}}$ levels are much greater. All three isoforms show robust induction of ERK 1/2 phosphorylation after treatment with 100 nM PMA.

The signal transduction pathways involved in PGE_2 induced ERK 1/2 phosphorylation in $EP_{3\text{-Ia}}$, $EP_{3\text{-II}}$, and $EP_{3\text{-III}}$ receptor isoforms. To examine which pathways activated following treatment with PGE_2 lead to ERK 1/2

phosphorylation among EP_3 receptor isoforms, several known pharmacological inhibitors of ERK 1/2 activation were utilized. In all three isoforms receptor tyrosine kinases, Src and EGFR inhibitors genistein, PP2 and AG1478, respectively, reduced PGE_2 dependent ERK 1/2 phosphorylation. Additionally the $G\alpha_i$ inhibitor pertussis toxin (PTX) completely inhibited PGE_2 mediated ERK 1/2 phosphorylation in all three EP_3 receptor isoforms. The PKC, PI3K inhibitors, bisindolylmaleimide I and wortmannin also inhibited ERK 1/2 phosphorylation but only in HEK cells expressing the $EP_{3\text{-}Ia}$ and $EP_{3\text{-}III}$ isoforms.

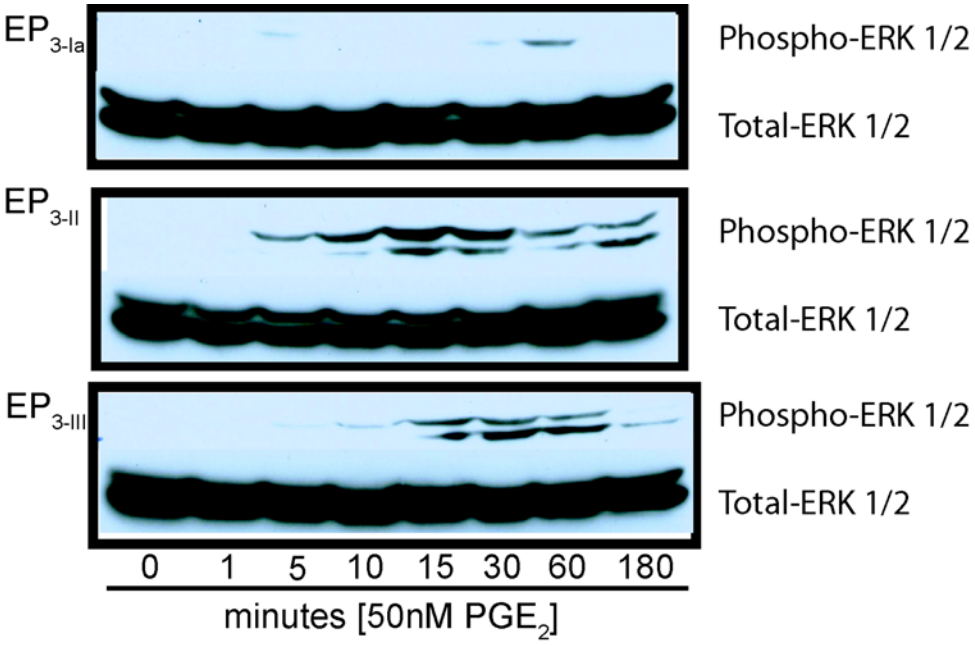


Figure 3.1 Time course of ERK 1/2 phosphorylation in EP_3 receptor isoforms following PGE_2 treatment.
HEK cells expressing $EP_{3\text{-I}}$, $EP_{3\text{-II}}$, and $EP_{3\text{-III}}$ were incubated with DMSO or 50 nM PGE_2 from 0-180 minutes then lysed on ice. Cell lysates were subject to immunoblot as described in "Experimental Procedures" using anti-phosphorylated ERK 1/2 and anti-total ERK 1/2 antibodies. The densities of phosphorylated ERK 1/2 were normalized against the densities of total ERK 1/2 expression. Blots are representative of three independent experiments.

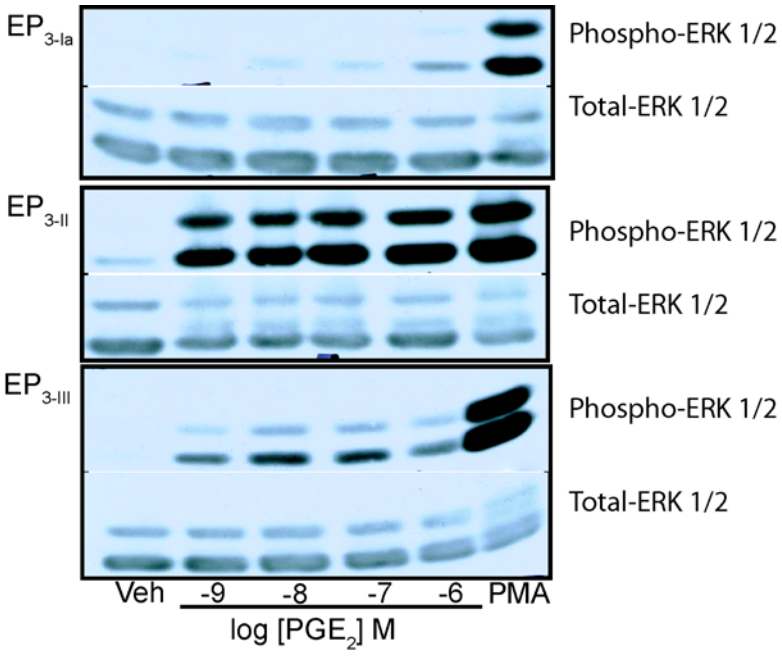


Figure 3.2 PGE_2 dose response effects on ERK 1/2 phosphorylation in EP_3 receptor isoforms.

HEK cells expressing $EP_{3\text{-I}}$, $EP_{3\text{-II}}$, and $EP_{3\text{-III}}$ were incubated with DMSO, 1 nM-1 μM PGE_2 for 15 minutes then lysed on ice. Cell lysates were subject to immunoblot as described in "Experimental Procedures" section using anti-phosphorylated ERK 1/2 and anti-total ERK 1/2 antibodies. The densities of phosphorylated ERK 1/2 were normalized against densities of total ERK 1/2 expression. Blots are representative of three independent experiments.

The PKA inhibitor H-89 did not show significant inhibition of ERK 1/2-phosphorylation in any EP_3 receptor isoform (data not shown). The densities of phosphorylated ERK1/2 were normalized against total ERK 1/2 expression to ensure that differences in loading did not effect interpretations of reductions in ERK 1/2 phosphorylation by inhibitors.

$EP_{3\text{-}II}$, and EP_{3III} receptors mediated ELK-1 luciferase reporter gene activity is MAPK dependent. ELK-1 luciferase reporter activity was stimulated by treatment with PGE_2 in $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ receptor expressing cells. In Figure 3.4, $EP_{3\text{-}Ia}$ receptor activity failed to induce ELK-1 reporter gene activity. The inhibitors PD98059 (MEK), pertussis toxin ($G\alpha_i$), AG1478 (EGFR) and PP2 (src) pre-treatment reduced PGE_2 stimulated ELK-1 luciferase reporter activity in $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ receptor expressing cells. ELK-1 reporter activity was also inhibited by wortmannin and bisindolylmalemeimide I in $EP_{3\text{-}III}$ receptor expressing cells. $EP_{3\text{-}II}$ expressing cells did not show inhibition of ELK-1 luciferase reporter activity following pretreatment with either bisindolylmaleimide I or wortmannin.

$EP_{3\text{-}II}$, and EP_{3III} receptors mediated AP-1 luciferase reporter gene activity is MAPK dependent. PGE_2 induced AP-1 luciferase activity was only seen in $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ expressing cells. Pertussis toxin, AG1478, PP2 and genistein, inhibited AP-1 luciferase reporter activity in both $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ receptor expressing cells. Additionally, wortmannin and bisindolylmaleimide I inhibited AP-1 reporter luciferase activity in $EP_{3\text{-}III}$ receptor expressing cells. Counter intuitively, AP-1 reporter luciferase activity in $EP_{3\text{-}II}$ expressing cells was inhibited following bisindolylmaleimide I pretreatment.

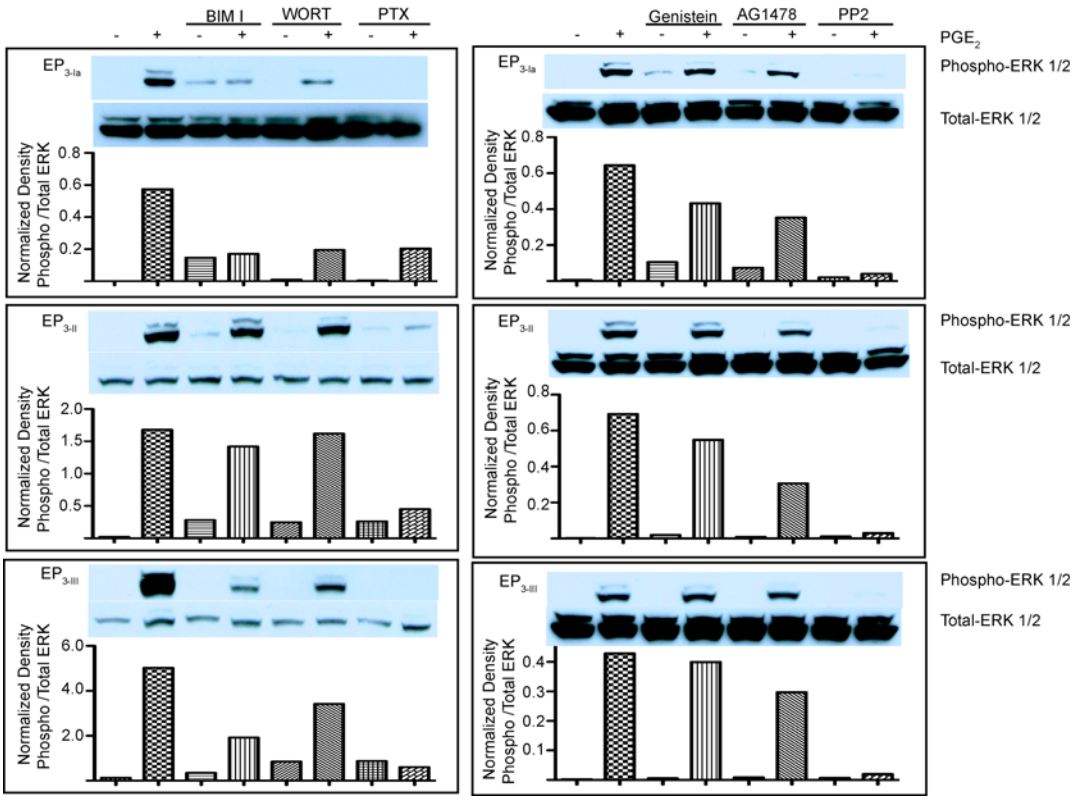


Figure 3.3 The signal transduction pathways involved in PGE_2 induced ERK 1/2 phosphorylation in $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and $EP_{3\text{-}III}$ receptor isoforms. HEK cells expressing $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and $EP_{3\text{-}III}$ were pretreated with either DMSO or Bisindolylmaleimide I (BIM I) (100 nM), Wortmannin (WORT) (100 nM), Genistein (100 nM), AG1478 (100 nM), PP2 (10 μM) for 30 minutes, or Pertussis Toxin (100 ng/ml) for 16 hours. Followed by stimulation with 50 nM PGE_2 for 10 minutes then lysed on ice. Cell lysates were subject to immunoblot as described in "Experimental Procedures" using anti-phosphorylated ERK 1/2 and anti-total ERK 1/2 antibodies. The densities of phosphorylated ERK 1/2 were normalized against densities of total ERK 1/2 expression. $EP_{3\text{-}Ia}$ autoradiographs were exposed for 3-5 minutes, $EP_{3\text{-}II}$, and $EP_{3\text{-}III}$ autoradiographs exposed 15–30 seconds. Blots are representative of three independent experiments.

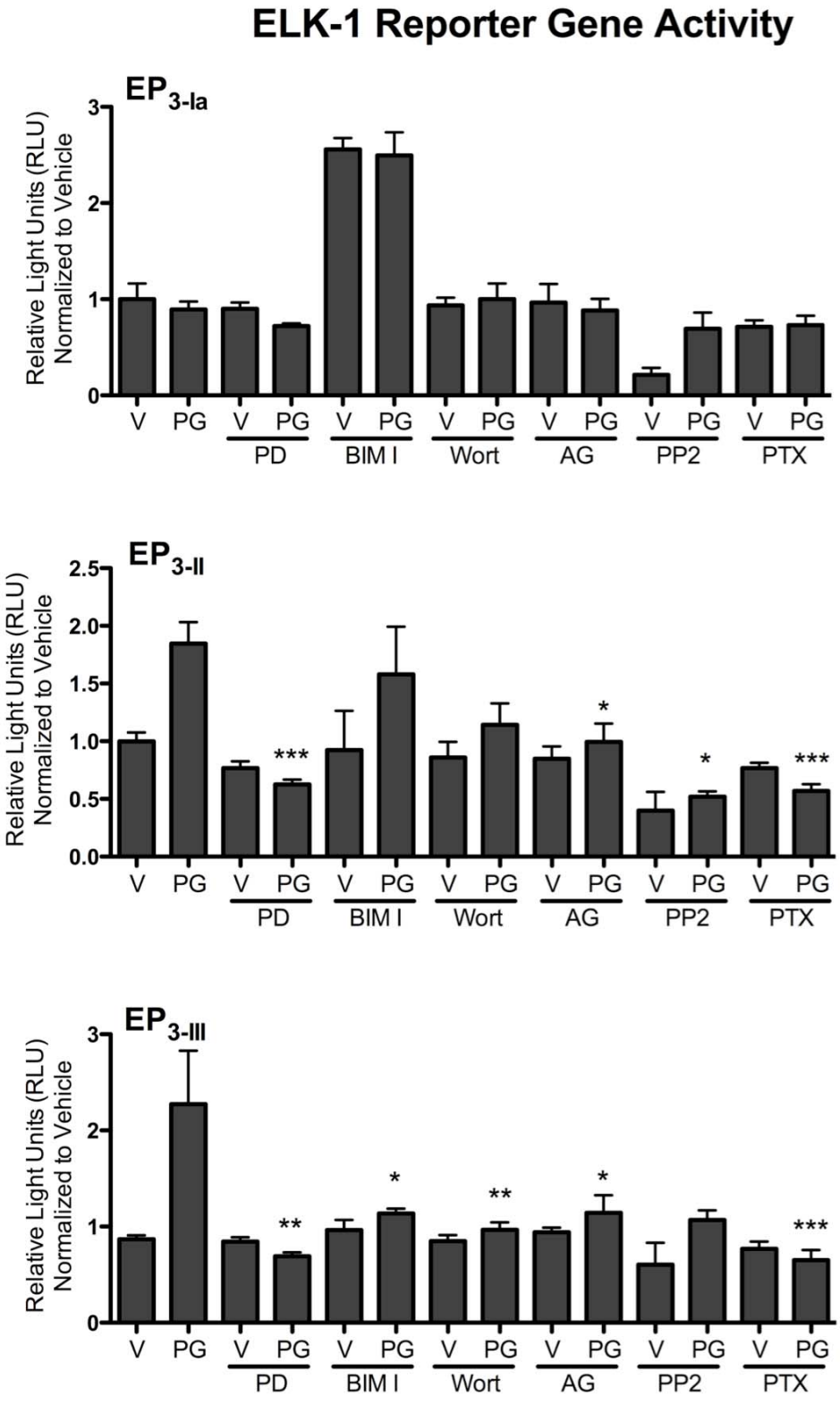

ELK-1 Reporter Gene Activity
EP3-Ia
EP3-II
EP3-III
Relative Light Units (RLU)
Normalized to Vehicle
V
PG
PD
BIM I
Wort
AG
PP2
PTX

Figure 3.4 $EP_{3\text{-}II}$, and EP_{3III} receptors mediated ELK-1 luciferase reporter gene activity is MAPK dependent.
HEK-293 cells stably expressing $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and $EP_{3\text{-}III}$ isoforms were transfected as described in "Experimental Procedures" using ELK-1 plasmid, Gal4-Luciferase plasmid and renilla (pRL-TK) plasmid. After transfection, cells HEK cells expressing $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and $EP_{3\text{-}III}$ were pretreated with either DMSO or Bisindolylmaleimide I (BIM I) (100 nM), Wortmannin (WORT) (100 nM), Genistein (100 nM), AG1478 (100 nM), PP2 (10 μM) for 30 minutes, or Pertussis Toxin (100 ng/ml) for 16 hours followed by stimulation with 50 nM PGE_2 as described in "Experimental Procedures". Following treatment cells were lysed and samples were analyzed on a luminometer. A ratio of the firefly and renilla luciferase activities was used as an indicator of ELK-1 activity. Data was from 3-6 experiments and was normalized to vehicle control. Levels of statistical significance are ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ as compared to PGE_2 treated EP_3 cells. For all experiments, data are shown as mean ± SEM.

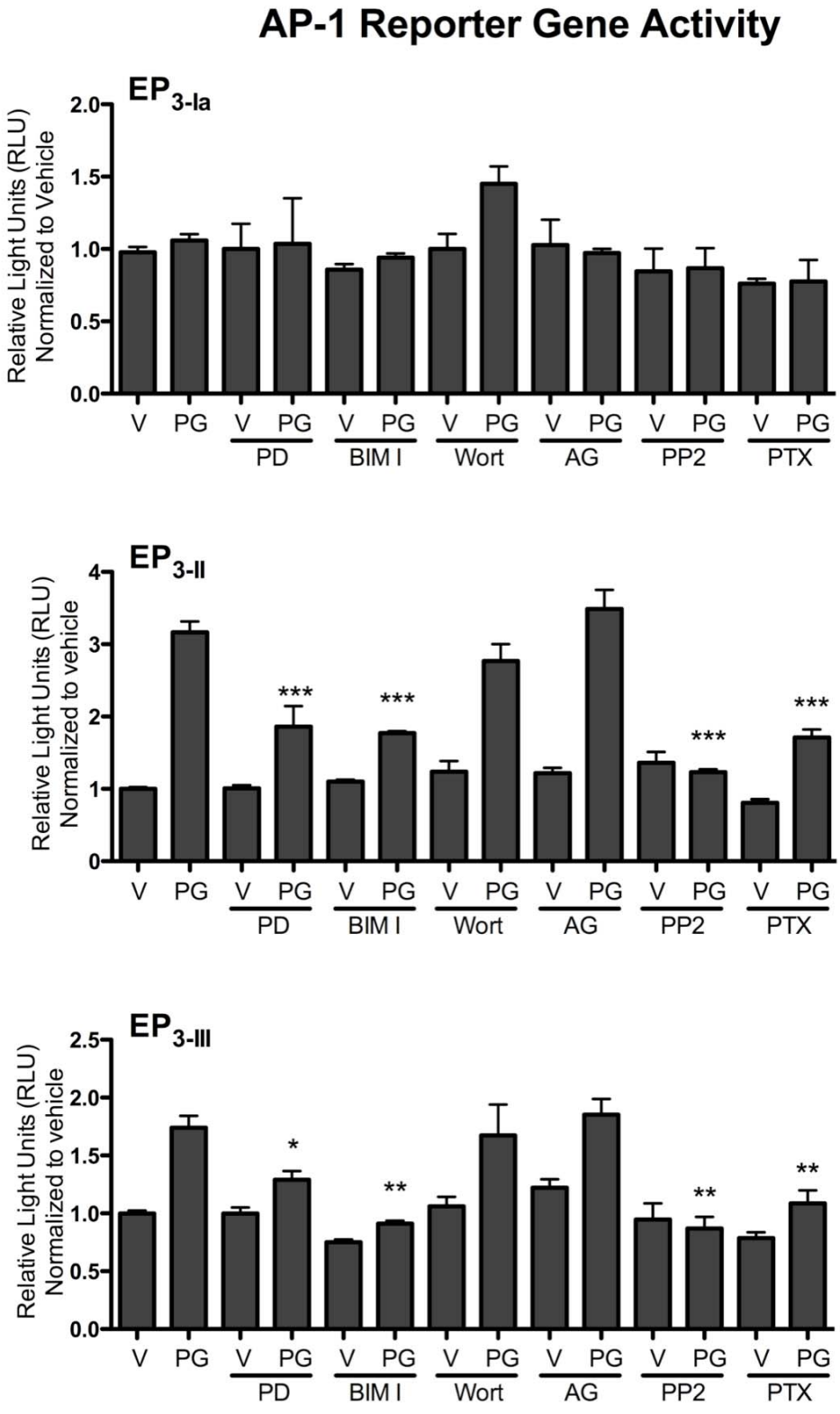

AP-1 Reporter Gene Activity
EP3-Ia
Relative Light Units (RLU)
Normalized to Vehicle
2.0
1.5
1.0
0.5
0.0
V PG V PG V PG V PG V PG V PG V PG
PD BIM I Wort AG PP2 PTX
EP3-II
Relative Light Units (RLU)
Normalized to vehicle
4
3
2
1
0
***
***
***
***
V PG V PG V PG V PG V PG V PG V PG
PD BIM I Wort AG PP2 PTX
EP3-III
Relative Light Units (RLU)
Normalized to vehicle
2.5
2.0
1.5
1.0
0.5
0.0
*
**
**
**
V PG V PG V PG V PG V PG V PG V PG
PD BIM I Wort AG PP2 PTX

Figure 3.5 $EP_{3\text{-}II}$, and EP_{3III} receptors mediated AP-1 luciferase reporter gene activity is MAPK dependent.

HEK-293 cells stably expressing $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and $EP_{3\text{-}III}$ isoforms were transfected with AP-1 luciferase plasmid and renilla (pRL-TK) using Fugene 6. HEK cells expressing $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and $EP_{3\text{-}III}$ were pretreated with either DMSO or Bisindolylmaleimide I (BIM I) (100 nM), Wortmannin (WORT) (100 nM), Genistein (100 nM), AG1478 (100 nM), PP2 (10 μM) for 30 minutes, or Pertussis Toxin (100 ng/ml) for 16 hours, followed by stimulation with 50 nM PGE_2 as described in "Experimental Procedures". Following treatment, cells were lysed and samples were analyzed on a luminometer. A ratio of the firefly and renilla luciferase activities was used as an indicator of AP-1 activity. Data were from 3-6 experiments and were normalized to vehicle control. Levels of statistical significance are ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ as compared to PGE_2 treated EP_3 cells. For all experiments, data are shown as mean ± SEM.

PGE_2 induced effects on cell proliferation in $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ receptor isoforms. 1 nM and 10 nM concentrations of PGE_2 in $EP_{3\text{-}Ia}$ cells significantly increased proliferation compared to vehicle treated cells, but those effects were only observed at lower doses, increasing doses had no effect on cell number. Inhibitors PD98059 and AG1478 combined with PGE_2 treatments appeared to increase cell number compared to the inhibitor with vehicle treatment alone. Pretreatment with pertussis toxin significantly reduced 1 nM and 10 nM PGE_2 effect on cell number in the $EP_{3\text{-}Ia}$ expressing cells. PGE_2 treatment and/or PD98059, pertussis toxin and AG1478 treatment in $EP_{3\text{-}II}$ expressing cells had no effects on proliferation. Statistical significance was not reached in any of the MTS proliferation experiments examining PGE_2 treatment effects in $EP_{3\text{-}II}$ expressing cell lines. PGE_2 treatment of $EP_{3\text{-}III}$ expressing cells resulted in statistically significant increases cell number at all doses. These increases in cell number were inhibited by pretreatment with PD98059, pertussis toxin and AG1478.

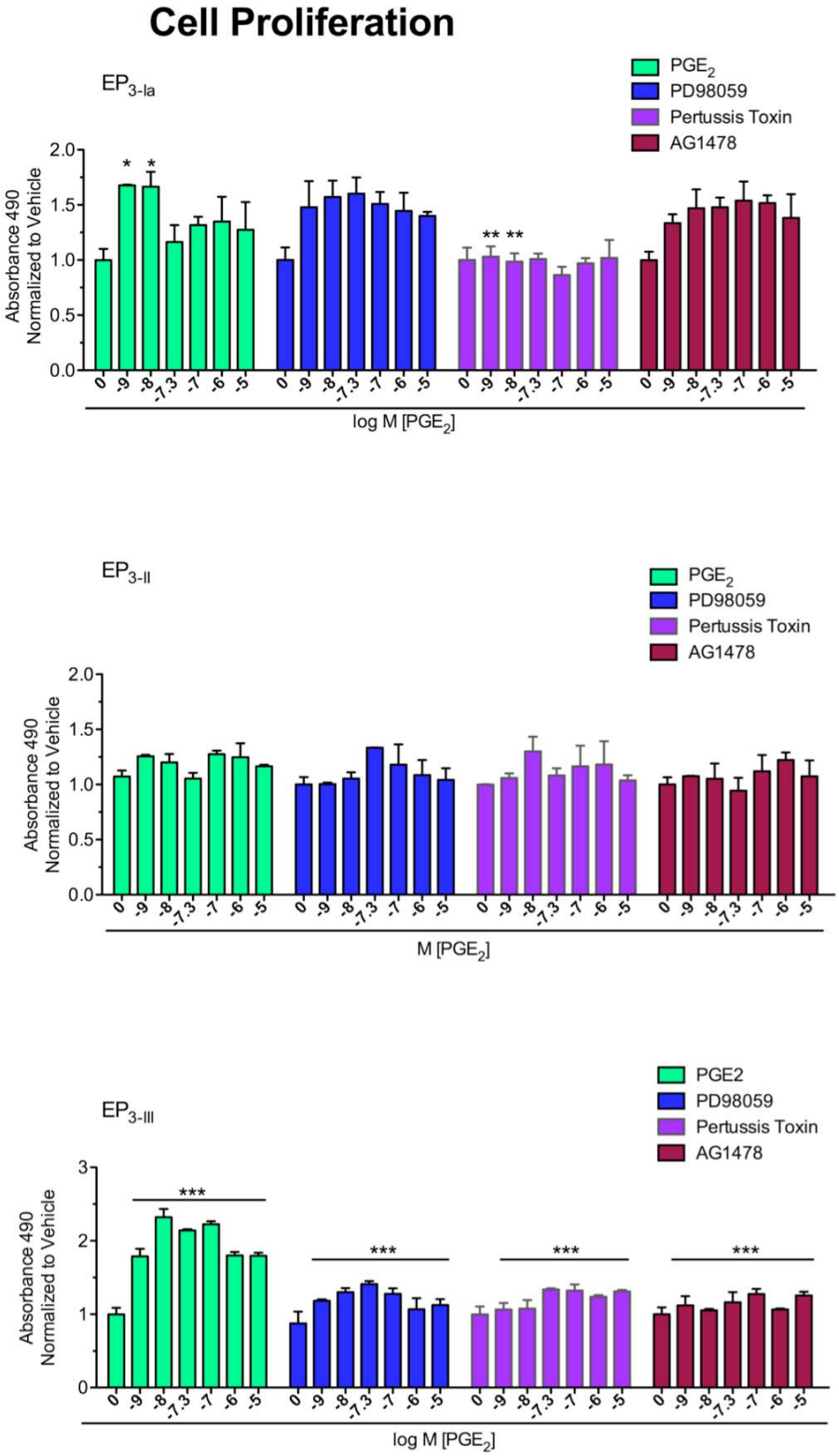

Cell Proliferation
EP3-Ia
PGE2
PD98059
Pertussis Toxin
AG1478
Absorbance 490
Normalized to Vehicle
log M [PGE2]
EP3-II
M [PGE2]
EP3-III
PGE2
log M [PGE2]

Figure 3.6 PGE_2 induced effects on cell proliferation in $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ receptor isoforms.

HEK cells expressing $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and $EP_{3\text{-}III}$ were seeded at 5,000 cells/well in 96 well plates and incubated for 48 hours at 37°C and 5% CO_2. Cells were pretreated with DMSO or PD98059 (100 nM) or AG1478 (100 nM), for 30 minutes, or Pertussis Toxin (100 ng/ml) for 16 hours, followed by stimulation with 1 nM-10 μM PGE_2 for 24 hours. Growth media + drugs was exchanged for phenol-free DMEM + MTS and incubated for 4 hours as described in "Experimental Procedures". Data is representative of 3 experiments and is normalized to vehicle control. Levels of statistical significance are ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; as compared to PGE_2 treated EP_3 expressing cells. For all experiments, data are shown as mean ± SEM.

3.4 Discussion

GPCR mediated MAPK activation has been reported on for many years. Some of the first GPCRs known to activate the ERK 1/2 pathway were the M1 and M2 muscarinic receptors among others [87]. In many cases GPCR mediated ERK 1/2 phosphorylation is dependent on the activation of the $G_{q/11}$ or $G_{12/13}$, PLC, PKC and Ca^{2+}. However, both G_s and G_i activated pathways are capable of MAPK activation by a variety of mechanisms including direct and indirect activation of downstream kinases [88]. Additionally, GPCRs can modulate ERK 1/2 activity via transactivation of receptor tyrosine kinases. EGFR is a common target of GPCR transactivation and subsequent ERK 1/2 phosphorylation.

In Burkey and Regan 1995, we reported on the activation of MAPK by the $EP_{3\text{-}Ia}$ receptor. Along with our own studies, ERK phosphorylation by EP_3 receptor isoforms has been reported by Kotani et al. 2000, who found all isoforms induced ERK 1/2 phosphorylation and found that the $EP_{3\text{-}V}$ and $EP_{3\text{-}VI}$ receptors accomplished this via G_i dependent mechanisms. In addition EP_3 receptor dependent ERK phosphorylation has been linked to cell

growth, differentiation and many other functions in keratinocytes, mast cells and endothelial cells [89-91].

Here we show that in HEK cells expressing $EP_{3\text{-}Ia,}$ $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$, there are distinct differences in the activation of ERK 1/2 phosphorylation as assessed by immunoblot assays. $EP_{3\text{-}Ia}$ dose dependent and time dependent ERK 1/2 activation is less robust as compared to $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ receptor isoforms. Visualization of the proteins on autoradiographs requires 5-10 times longer in experiments with the $EP_{3\text{-}Ia}$ receptor isoform, indicating lower levels of phosphorylated ERK 1/2 in these cells.

The mechanism of ERK 1/2 phosphorylation were different for each isoform. $EP_{3\text{-}Ia}$ and $EP_{3\text{-}III}$ isoforms induced ERK 1/2 phosphorylation was inhibited by the PKC and PI3K inhibitors, bisindolylmaleimide I and wortmannin, respectively. The $EP_{3\text{-}II}$ receptor isoform mediated ERK 1/2 phosphophorylation was not. This indicates that the $EP_{3\text{-}II}$ receptor does not use the PKC and PI3K pathways to induce ERK 1/2 phosphorylation following treatment with PGE_2 in this model system.

ERK 1/2 activation in all three isoforms was dependent on the activities of $G\alpha_i$, the receptor tyrosine kinase epidermal growth factor receptor (EGFR) and the non receptor tyrosine kinase SRC. Inhibitors PTX, AG1478 and PP2 all reduced ERK phosphorylation in the EP_3 receptor isoforms studied here. The thromoboxane A2 (TxA2) utilizes a $G\alpha_i$, PKC, Src and EGFR-dependent pathway to induces ERK 1/2 phosphorylation [92]. $EP_{3\text{-}Ia}$ and $EP_{3\text{-}III}$ receptor isoforms, function similarly but unlike the TxA2 receptor, the activities of PI3K contribute to the activation of ERK 1/2. In these studies, $EP_{3\text{-}II}$ receptor mediated activation of ERK 1/2 appears to be through $G\alpha_i$, Src and EGFR-dependent pathways and independent of PKC and PI3K activity. A similar mechanism of ERK 1/2 activation as seen in $EP_{3\text{-}II}$ receptor expressing HEK 293 EBNA cells is also observed following activation of lysophosphatidic acid receptors [93, 94].

To investigate whether these differences in ERK 1/2-phosphorylation mechanisms translated into differences in downstream targets such as the AP-1 and ELK-1, we utilized luciferase reporter assays and the same pharmacological inhibitors as above. We hypothesized that the mechanisms involved in ERK 1/2 phosphorylation would also be relevant for activation

of the AP-1 and ELK-1 reporter activity. As expected, AP-1 and ELK-1 luciferase reporter activation was dependent on MEK-ERK1/2 activity as evidenced by its inhibition following pre-treatment with PD98059. The relevance of the differential mechanisms involved in ERK 1/2 phosphorylation were substantiated by the fact that the inhibitors which reduced ERK 1/2 phosphorylation also reduced PGE_2 mediated AP-1 and ELK-1 reporter activity. One counterintuitive result of these studies involved the inhibition of AP-1 reporter activity in $EP_{3\text{-II}}$ expressing cells pretreated with bisindolylmaleimide I. The inability of the $EP_{3\text{-Ia}}$ receptor to induce AP-1 and ELK-1 luciferase activity may be due to reduced levels of ERK 1/2 signaling. Previous studies have shown that AP-1 components, such as FRA-1, require sustained, elevated levels of ERK 1/2 activity to stabilize certain AP-1 protein components and prevent proteasomal degradation [95, 96]. Clearly, in these studies $EP_{3\text{-1a}}$ did not show the elevated and sustained levels of ERK 1/2 phosphorylation as was observed in $EP_{3\text{-II}}$ and $EP_{3\text{-III}}$ expressing cells.

The effects of prostaglandins on proliferation are often contradictory. Studies have shown both inhibitory and inductive effects on proliferation by

EP_2, EP_3, and EP_4 receptors but this may be dependent on cell type, context agonist concentration [84, 97-100]. The EP_1 receptor currently appears to be the exception and its activation results in increased proliferation [101].

The proliferative effects of EP_3 receptor activation are contextual. For example studies in the human lung adenocarcinoma A549 cell line found that EP_3 receptor activity increased cell growth in a Gi, src-Stat3 dependent manner [63]. One criticism of the study was that proliferation was only seen at 10 mM concentrations of PGE_2. A better-conducted study in human endometrial stromal cells reported that EP_3 receptor activation induced expression of Fibroblast Growth Factor 9 (FGF9) and increased cell proliferation dependent on PKC, ERK 1/2 and ELK-1 activities [86]. Both studies failed to identify the EP_3 receptor isoforms expressed in the cell lines studied, thus we are unable to conclude if the effects reported were through the three isoforms studied here.

Despite the evidence of a proliferative effect for EP_3 receptors, there are also significant amounts of data indicating that EP_3 also has an inhibitory effect on proliferation. Studies in colon cancer cells and keratinocytes all point to

EP_3 having inhibitory effects on proliferation. Several studies in colon cancer cell lines indicate that loss of EP_3 receptor signaling due to gene hypermethylation increases cell proliferation. When EP_3 receptor expression was restored following treatment with demethylating agent, 5-aza-2'-deoxycytidine, cell proliferation was reduced [59].

The activities of ERK 1/2 play an important role in inducing proliferation in many cell types. Studies in endometrial tissue show that activation of EP_3 receptor and subsequently PKCδ and ERK 1/2 leads to increased proliferation [86]. We performed MTS assays to determine if there are differences in proliferation rates of cells expressing $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ receptor isoforms following PGE_2 treatment and if these differences were ERK 1/2 dependent. Changes in cell number were greatest 24 hours post PGE_2 treatment and were less significant after 48 hours. We found that $EP_{3\text{-}II}$ receptor activation does not affect cell proliferation. However, HEK 293 cells expressing $EP_{3\text{-}Ia}$ and $EP_{3\text{-}III}$ receptors showed increased proliferation but by slightly different mechanisms. Increases in $EP_{3\text{-}Ia}$ expressing cell number following PGE_2 treatment only appeared at 1 nM and 10 nM concentrations, and was also inhibited at these concentrations by pertussis

toxin pretreatment. Interestingly, inhibitors of ERK 1/2 phosphorylation, PD98059 (MEK) and AG1478 (EGFR) did not show any reductions in proliferation induced by $EP_{3\text{-}Ia}$ receptor activation. This leads to the conclusion that $EP_{3\text{-}Ia}$ effects on proliferation may be independent of ERK 1/2. Also of importance was that Bilson et al. reported that the $EP_{3\text{-}Ia}$ receptor isoform when stimulated with PGE_2 undergoes nearly immediate desensitization, whereas $EP_{3\text{-}II}$ takes longer and $EP_{3\text{-}III}$ completely fails to internalize [72]. This immediate internalization may result in reduced ERK 1/2 phosphorylation as seen in these experiments. This may also contribute to the lack of AP-1 and ELK-1 reporter activity in these cells. One interesting possibility is that $EP_{3\text{-}1a}$ induced ERK 1/2 phosphorylation is biphasic, which it appears to be in the time response experiments. Perhaps following PGE_2 treatment there is an immediate internalization, but over time there may be a return of receptor to the membrane and if PGE_2 in the media has not completely degraded there may be a later stimulation of ERK 1/2 phosphorylation. This late phase ERK induction at low PGE_2 concentrations may provide enough stimuli to increase proliferation.

$EP_{3\text{-III}}$ expressing HEK cells showed significant increases in proliferation following PGE_2 treatment that was ERK 1/2 dependent and inhibited by AG1478 and pertussis toxin. It was surprising that PGE_2 treatment increased cell number in $EP_{3\text{-III}}$ expressing cells but not in $EP_{3\text{-II}}$ expressing cells. However, a report by Treinies et al. 1999, reported that activation of the ERK/MAPK pathway could stimulate AP-1 activity in NIH 3T3 cells, but that PI3K was required to stimulate DNA synthesis and proliferation [21]. It is possible that $EP_{3\text{-II}}$ activation does not lead to PI3K activation, thus not leading to proliferation, but this must be further examined. It is clear that PI3K is activated in $EP_{3\text{-III}}$ expressing cells following PGE_2 treatment as wortmannin treatment reduced ERK 1/2 phosphorylation and this may allow for the signaling necessary for increased proliferation. We also examined if PGE_2 treatment effected the expression of the cell cycle regulators, cyclin D1 and $p21^{cip1}$ but did not see expression changes in any of the isoforms (data not shown). Evaluation of changes in cyclin dependent kinases and their inhibitors expression following PGE_2 treatment may provide information regarding the mechanisms utilized by $EP_{3\text{-Ia}}$ and $EP_{3\text{-III}}$ mediated increased proliferation.

ERK 1/2 is able to mediate its diverse activities due to the regulation of effects dependent on signal duration, magnitude and cellular localization. If signal duration is not sustained for sufficient time periods or intensity, it is possible that some downstream targets are not activated. The differences in the magnitude and duration of ERK phosphorylation in $EP_{3\text{-}Ia}$ and $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ expressing cells may account for the differences on the effects on AP-1 and ELK-1 reporter activity as well as on proliferation. These studies detailing the mechanisms of ERK 1/2 phosphorylation may be important for beginning to understand the signaling potential and the diversity of function of $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and $EP_{3\text{-}III}$ receptor isoforms.

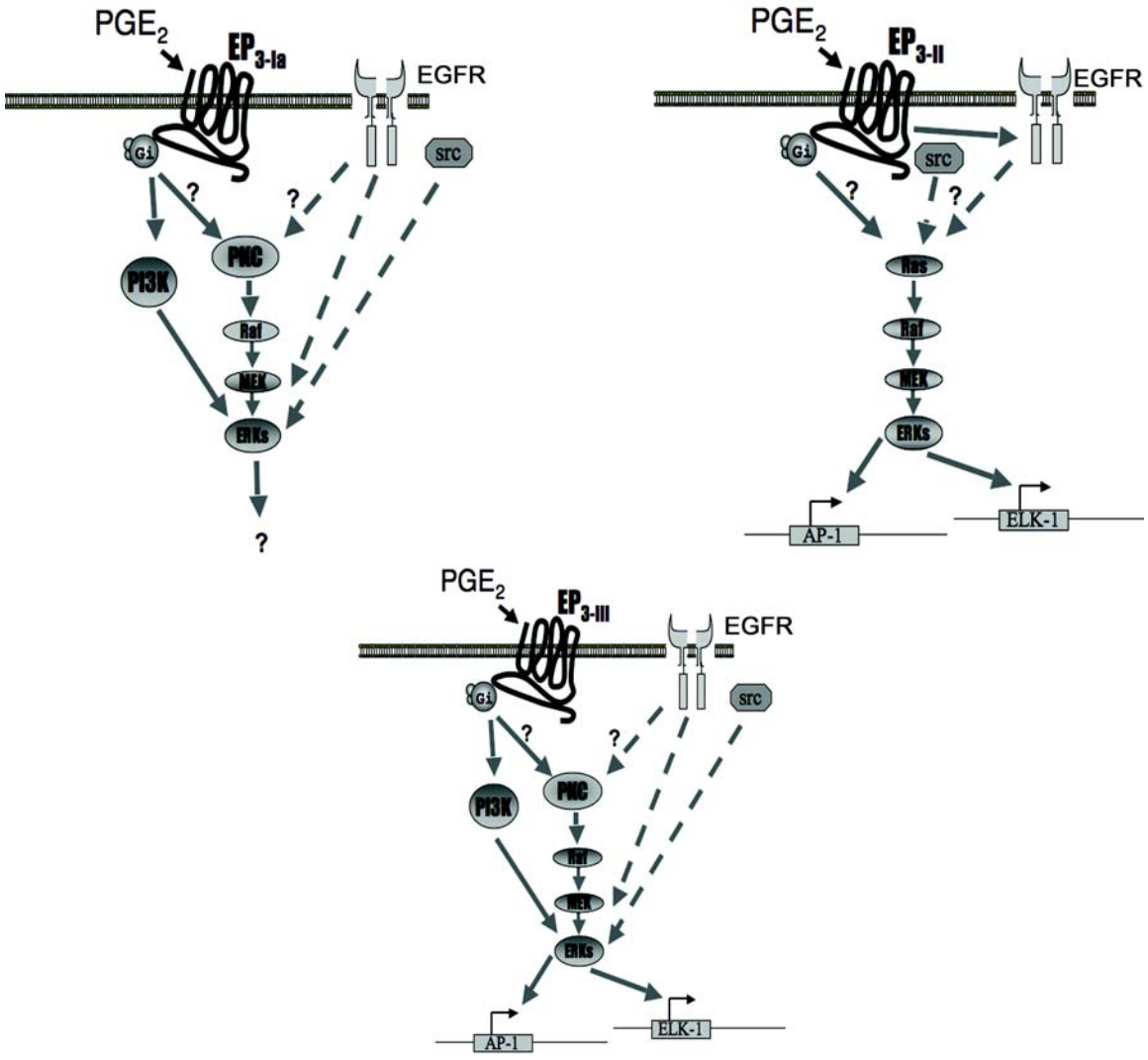


Figure 3.7 Illustration of the signaling pathways activated leading to ERK 1/2 phosphorylation in the $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ receptor isoforms.

CHAPTER FOUR

DIFFERENTIAL GENE REGULATION AMONG HUMAN $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$ AND $EP_{3\text{-}III}$ RECEPTOR ISOFORMS STABLY EXPRESSED IN HEK 293 EBNA CELLS

4.1 Introduction

One of the goals of this research to was to identify the physiological functions of alternative splice variants of the EP_3 receptor isoforms. Our initial studies focused on identifying unique signal transduction pathways activated by the isoforms. Next we examined if these variations led to differential regulation of gene transcription following activation of the $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ isoforms. We utilized Affymetrix Human Genome U133 Plus 2.0 array to identify distinctly regulated genes. The Affymetrix Human Genome U133 Plus 2.0 array features 47,000 transcripts and variants allowing for genome wide analysis of changes gene expression. Following analysis of the microarray data, we identified 15 gene candidates that fulfilled at least two of the following criteria 1) Uniquely regulated by an isoform 2) Greater than 2 fold change over controls 3) Not a secreted protein (due to limitations of the HEK cells) 4) Regulated by MAPK/ERK or other relevant pathways and 5) Genes with unique functions or characteristics which were of interest. These fifteen were then verified using quantitative real time PCR and finally after verification of mRNA changes, immunoblot was used to determine if these transcriptional changes resulted in changes in protein expression levels following activation of the EP_3 receptor isoforms.

4.2 Experimental Procedures

Materials.

Human Genome U133 Plus 2.0 Array was from Affymetrix (Santa Clara, CA). RNeasy kit and Quantitect Reverse Trancription kit were from Qiagen (Venlo, the Netherlands). LightCycler DNA Master HybProbe kit, LightCycler Real 2.0 system and Human Universal ProbeLibrary Set were from Roche (Basel, Switzerland). Dulbecco's Modified Eagle's Medium (DMEM), and gentamicin sulfate were from Mediatech (Herndon, VA). Fetal Bovine Serum (FBS) and Hygromycin B were from Invitrogen (Carlsbad, CA). Horseradish peroxidase conjugated anti-rabbit IgG antibody from Promega (Madison, WI). Protein assay reagent, horseradish peroxidase-conjugated anti-mouse IgG, PVDF and nitrocellulose membranes were from Bio-Rad Laboratories (Hercules, CA). PGE_2 was from Cayman Chemical Company (Ann Arbor, MI). Cell Lysis Buffer 10x was purchased from Cell Signaling (Boston, MA). Anti-decorin was purchased from R&D Systems (Minneapolis, MN). Anti-WT-1, anti-FRA-2, anti-EGR3 and anti-Jagged1 antibodies were obtained from Santa Cruz biotechnology (Santa Cruz, CA). Anti-rabbit IgG conjugated with horseradish peroxidase and vinculin antibody were purchased from Sigma-Aldrich (St. Louis, MO). Enhanced

chemiluminescence substrate was from Pierce (Rockford, IL). PD98059, AG1478, bisindolylmaleimide I (BIM I), Pertussis Toxin (PTX) and BAY 11-0785 were from Calbiochem (San Diego, CA). Bay 43-9006 was provided by Dr. Laurence Hurley at the University of Arizona (Tucson, AZ).

Cell Culture.

Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 250 μg/ml G418, 200 μg/ml gentamicin sulfate, 200 μg/ml Hygromycin B and incubated at 37°C and 5% CO_2.

RNA Extraction

HEK-293 cells stably expressing $EP_{3\text{-}I}$, $EP_{3\text{-}II}$, and $EP_{3\text{-}III}$ isoforms were pretreated with either vehicle or 50 nM PGE_2 for 3 hours. Following treatment, cells were rinsed with PBS and total RNA was extracted using a Qiagen RNeasy kit as per instructions. RNA was resuspended in 50 μl of 0.1% diethylpyrocarbonate (DEPC) water. RNA concentrations were measured using a spectrophotometer and 35 μg of each sample (vehicle and PGE_2 treated) was sent to the Arizona Cancer Center (AZCC) Genomics Core facility for microarray analysis.

Affymetrix Microarray Analysis

Microarray analysis was carried out at the Arizona Cancer Center (AZCC) Genomics Core facility. Expression data was analyzed using GeneSpring Software (Agilent, Palo Alto, CA). We identified differential expressed genes which had raw expression values greater than 150 and were 2 fold above vehicle treatment.

Reverse Trancription

HEK-293 cells stably expressing $EP_{3\text{-}I}$, $EP_{3\text{-}II}$, and $EP_{3\text{-}III}$ isoforms were pretreated with either vehicle or 50 nM PGE_2 for 3 hours. Total RNA was extracted using Qiagen RNeasy kit. RNA concentrations were measured using a spectrophotometer and 1 μg of each sample (vehicle and PGE_2 treated) underwent reverse trancription using the Qiagen Quantitect Reverse Trancription kit

Quantitative Real-Time PCR

After reverse trancription reaction, samples were quantitated. Quantitative real time PCR was carried out using the Roche LightCycler Real 2.0 system.

Primers for WT1, FRA-2, VAV3, Jagged1, Immediate Early Response 3 (IER3), SPRED1, PIM2, Myotrophin, MafB, decorin, Ephrin A2 (EPHA2), tau tubulin kinase 2, EGR3, neuroligin and the reference gene Glucose-6-phosphate dehydrogenase (G6PD) were designed using the Roche Universal Probe Library Assay Design Center. Each PCR run contained 20 ng cDNA, 10 μM of each primer set, 10 μM of appropriate fluorescent probe, and reagents supplied with the Roche DNA Master HybProbe kit. The threshold cycle (Ct) number at which the increase in the signal associated with the exponential growth of the PCR products was detected using the LightCycler Relative Quantification Software version 3.3, according to the manufacturer's instructions, with the housekeeping gene G6PD. The Ct is proportional to the initial number of target molecules and is used in the quantitative analysis. To determine the copy number/proportional representation of the target genes, delta Ct (dCt) was calculated by subtracting the Ct of the target gene from the Ct of the housekeeping gene. Data is representative of three independent experiments each performed in duplicate.

Western Blotting

48 hours prior to experiments ~0.5 x 10^6 HEK cells expressing $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and EP_{3III} were seeded into 60 mm plates and incubated at 37°C and 5% CO_2. On the day of the experiment, cells were treated with vehicle (DMSO) or prostaglandin E_2 (PGE_2) for varying time points unless otherwise stated differently. Following treatment cells were lysed on ice with 100 µl RIPA buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 10 mM sodium fluoride, 10 mM disodium pyrophospate, 0.1% SDS or 1x Cell Lysis Buffer supplemented with the protease inhibitors 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM sodium orthovanadate, 10 mg/ml leupeptin and 10 µg/ml aprotonin. Samples were sonicated on ice then centrifuged at 15,000 g for 15 minutes. Protein concentrations were determined using the Bio-Rad Protein Assay kit. 50 µg of sample lysates were electrophoresed on 10% SDS-polyacrylamide gel and transferred to PVDF membranes. Membranes were blocked for one hour in 5% nonfat milk or 5% BSA in TBS and 0.1% Tween 20 (TBS-T) at room temperature. Membranes were then incubated at 4°C overnight with anti-FRA-2, EGR3, WT-1, decorin or Jagged1 primary antibodies diluted in 5% non-fat milk or 5% BSA in TBS-T. Following incubation in primary

antibody, membranes were washed three times with TBS-T then incubated with anti-rabbit or mouse secondary antibody conjugated with horseradish peroxidase in 5% nonfat milk or 5% BSA TBS-T for one hour at room temperature. After washing membrane three times, immunoreactivity was detected using SuperSignal enhanced chemiluminescence. Membranes were stripped using 2% SDS, 62.5 mM Tris (ph 7.6) and 100 mM ß-mercaptoethanol for 30 minutes at 55°C, then re-probed with an anti-vinculin antibody to determine both equal protein loading. Image J software (National Institute of Health) was used for densitometric analysis of autoradiographs.

4.3 Results

Top 60 genes regulated by $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and EP_{3III} expressing cells following treatment with PGE_2. To identify genes uniquely regulated by activation of $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and EP_{3III} receptors, we used the Affymetrix Human Genome U133 Plus 2.0 array. We treated of $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and EP_{3III} expressing cells with 50nM PGE_2 3 hours as described in Experimental Procedures, then extracted RNA and sent the samples to the AZCC Genomic Core facility for microarray analysis. We chose the 3 hour time point based

on previous results from the laboratory which showed that this time point allowed for identification of immediate early response genes. The top 30 genes uniquely decreased or increased following PGE_2 treatment of cell expressing $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and EP_{3III} receptor isoforms are displayed in Table 4.1, Table 4.2 and Table 4.3 respectively. We identified a total of 306 genes with an expression intensity value of 150 or greater and a 2 fold or greater change in expression after PGE_2 treatment. The $EP_{3\text{-}II}$ receptor isoform had the greatest number of genes identified as uniquely regulated among the isoforms followed by the $EP_{3\text{-}Ia}$ and $EP_{3\text{-}III}$ receptor isoforms.

Functional groupings of genes regulated by $EP_{3I\text{-}a}$, $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$. A pie chart of the functional classification of all the genes identified was created using the Database for Annotation, Visualization and Integrated Discovery (DAVID) through access via the National Institute of Allergy and Infectious Diseases (NIAID) website and is displayed in Figure 4.1 [102]. The largest functional group represented was the phosphoprotein group, which accounted for 14% of the genes identified. Genes regulating or participating in signal transduction, metabolic processes or regulation of DNA-dependent transcription were also highly represented. Genes with

unknown function made up 5% of the genes. Genes involved in other processes make up the remaining 28% genes identified.

These data provides a framework to identify the potential physiologic processes regulated by the activity of different isoforms of the EP_3 receptor. Further characterization of the pathways allowing for differential regulation of specific genes may unveil a reason for the incongruity in studies detailing the functions of the EP_3 receptor.

Table 4.1 Top 60 Genes Differentially Regulated by $EP_{3\text{-}Ia}$ receptor isoform

Decreased Expression Following PGE_2 Treatment		Percent Change Compared to Vehicle		
Genbank	**Gene Title**	**$EP_{3\text{-}Ia}$**	**$EP_{3\text{-}II}$**	**$EP_{3\text{-}III}$**
BC041011	essential meiotic endonuclease 1 homolog 2	-68.7	-8.9	28.2
BF110588	FERM domain containing 3	-64.4	64.0	NC
NM_022747	cyclin K	-64.3	-9.9	7.9
AF209931	synapse defective 1, Rho GTPase, homolog 1	-61.4	-20.2	-39.6
AB051545	cortactin binding protein 2	-60.2	-40.5	-21.2
BE787752	Scavenger receptor class A member 5	-60.0	32.0	-7.0
NM_000565	interleukin 6 receptor	-60.0	-27.6	16.8
AK024269	tight junction protein 4 (peripheral)	-59.2	85.3	49.1
AL042496	Solute carrier family 2, facilitated glucose transporter member 6 (Glucose transporter type 6) (GLUT-6)	-58.2	27.7	58.0
AK097967	dynamin 2	-57.4	NC	61.9
AI806872	zinc finger and BTB domain containing 47	-56.7	27.8	-29.6
AW134535	cyclin G2	-56.3	-19.0	42.7
AB020716	calmodulin binding transcription activator 2	-55.5	13.1	-5.7
AK023115	NADH dehydrogenase [ubiquinone] 1 subunit C1, mitochondrial precursor	-55.4	26.5	-38.8
NM_006289	talin 1	-54.9	5.5	6.6
AW005237	chromosome 9 open reading frame 25	-54.8	48.2	NC
NM_019112	ATP-binding cassette, sub-family A (ABC1), member 7	-54.5	40.4	NC
BC033139	TNF receptor-associated factor 4	-53.8	7.6	87.0
NM_022830	U6 snRNA-specific terminal	-53.6	9.8	-20.0

Decreased Expression Following PGE_2 Treatment		Percent Change Compared to Vehicle		
Genbank	**Gene Title**	**$EP_{3\text{-}Ia}$**	**$EP_{3\text{-}II}$**	**$EP_{3\text{-}III}$**
	uridylyltransferase 1			
NM_006113	vav 3 oncogene	-52.9	-20.5	-30.7
BC011011	ubiquitin-conjugating enzyme E2-like	-52.3	30.1	-37.2
D38169	inositol 1,4,5-trisphosphate 3-kinase C	-52.0	9.4	-19.3
AL022324	Immunoglobulin lambda-like polypeptide 3.	-51.3	24.5	54.8
BF528605	Sorting nexin-22.	-51.0	-11.9	18.4
NM_006946	spectrin, beta, non-erythrocytic 2	-50.9	54.7	NC
AL157851	Serine hydrolase-like protein	-50.8	63.6	10.7
AW575754	phosphoinositide-3-kinase adaptor protein 1	-50.7	NC	5.2
NM_019034	Rho-related GTP-binding protein RhoF precursor (Rho-family GTPase Rif) (Rho in filopodia).	-50.6	-30.9	NC
BF678830	hypothetical protein LOC152485	-50.0	15.8	-31.4
NM_005938	Forkhead box protein O4 (Fork head domain transcription factor AFX1)	-49.3	123.7	-18.1

"NC" means no change in expression following PGE_2 treatment, "-" represents a decrease in expression.

Increased Expression Following PGE_2 Treatment		Percent Change Compared to Vehicle		
Genbank	**Gene Title**	**$EP_{3\text{-}Ia}$**	**$EP_{3\text{-}II}$**	**$EP_{3\text{-}III}$**
NM_013430	gamma-glutamyltransferase 1	128.9	14.7	-9.5
AA524093	F-box only protein 41.	130.0	19.0	13.7
BF984592	High mobility group protein HMG-I/HMG-Y (HMG-I(Y))	131.1	41.8	-16.2
AI829724	Myocyte-specific enhancer factor 2D.	131.5	48.8	36.6

Increased Expression Following PGE_2 Treatment		Percent Change Compared to Vehicle		
Genbank	**Gene Title**	**$EP_{3\text{-}Ia}$**	**$EP_{3\text{-}II}$**	**$EP_{3\text{-}III}$**
NM_003615	solute carrier family 4, sodium bicarbonate cotransporter, member 7	131.6	26.3	-7.6
AB011152	centaurin, delta 1	135.6	11.5	-23.5
AW024420	dual specificity phosphatase 1	136.5	NC	NC
AF130088	myotrophin	137.6	6.5	-46.2
AF132818	Kruppel-like factor 5 (intestinal)	138.0	84.5	69.9
AI742358	Small VCP/p97-interacting protein.	138.8	27.4	58.6
NM_002397	Myocyte-specific enhancer factor 2C.	146.5	NC	NC
AW975728	solute carrier family 16 (monocarboxylic acid transporters), member 7	151.2	7.4	31.7
NM_005826	heterogeneous nuclear ribonucleoprotein R	152.1	10.0	19.0
NM_018275	Similar to CG14977-PA.	155.5	-44.5	125.1
AK000323	Telomere-associated protein RIF1 (Rap1-interacting factor 1 homolog).	156.9	NC	20.4
AK026737	fibronectin 1	163.0	26.0	-20.2
NM_024751	glutathione S-transferase, C-terminal domain containing isoform 2	163.9	NC	-25.7
AK091497	UDP-GalNAc:beta-1,3-N-acetylgalactosaminyltransferase 2	165.8	NC	67.8
BF431902	Electron transfer flavoprotein-ubiquinone oxidoreductase	166.0	6.8	-32.3
AV721563	Uncharacterized protein C11orf71	167.4	NC	-14.6
M81768	solute carrier family 9 (sodium/hydrogen exchanger), isoform 1	180.1	-7.3	1.1
NM_012318	leucine zipper-EF-hand containing transmembrane protein 1	188.1	28.1	19.4
BC001422	placental growth factor, vascular endothelial growth factor-related protein	190.6	-9.2	-8.2
AI923985	GATA like protein-1	195.3	-8.3	-51.3

Increased Expression Following PGE_2 Treatment		**Percent Change Compared to Vehicle**		
Genbank	**Gene Title**	**$EP_{3\text{-}Ia}$**	**$EP_{3\text{-}II}$**	**$EP_{3\text{-}III}$**
AI247494	HIV-1 Rev binding protein-like	202.7	18.1	8.6
AA829635	zinc finger protein 75a	205.1	-18.5	5.3
M10036	Triosephosphate isomerase	234.6	60.5	29.0
AL353759	histone 1, H2bd	276.8	-12.3	63.3
NM_003475	Ras association domain-containing protein 7	285.8	82.7	-25.5
AI817976	SH2 domain-containing adapter protein D	384.4	-31.6	541.6

"NC" means no change in expression following PGE_2 treatment, "-" represents a decrease in expression.

Table 4.2 Top 60 Genes Differentially Regulated by $EP_{3\text{-}II}$ receptor isoform

Decreased Expression Following PGE_2 Treatment		Percent Change Compared to Vehicle		
Genbank	**Gene Title**	**$EP_{3\text{-}Ia}$**	**$EP_{3\text{-}II}$**	**$EP_{3\text{-}III}$**
R41200	Kruppel-like factor 12	27.8	-74.9	30.0
NM_013281	fibronectin leucine rich transmembrane protein 3	33.1	-70.7	-28.2
NM_005346	heat shock 70kDa protein 1B	14.6	-66.6	16.7
NM_001533	Heterogeneous nuclear ribonucleoprotein L (hnRNP L).	20.3	-66.4	126.6
BE779765	Homeobox protein OTX2 (Orthodenticle homolog 2)	44.0	-65.3	49.6
AI382146	Transcription factor SOX-9	0.0	-65.3	-9.0
AF116676	myosin, light polypeptide 4, alkali; atrial, embryonic	-14.1	-64.0	19.2
AI873273	solute carrier family 16 (monocarboxylic acid transporters), member 6	-5.4	-63.5	-15.0
AF230877	TNF receptor-associated factor 3 interacting protein 1	24.6	-63.0	-7.4
NM_014898	likely ortholog of mouse zinc finger protein 30	26.8	-62.9	-19.2
AW291411	zinc finger protein 397	-22.2	-61.6	35.4
AL117381	Apoptosis regulator Bcl-X (Bcl-2-like 1 protein)	-29.7	-61.0	-8.4
BF438227	MAX gene associated	-11.5	-60.6	32.1
AI700476	solute carrier family 39 (zinc transporter), member 10	NC	-60.4	-9.9
AF031469	major histocompatibility complex, class I-related	-13.6	-60.3	-24.1
AJ272212	protein serine kinase H1	40.6	-60.2	26.6
NM_024754	pentatricopeptide repeat domain 2	6.2	-60.0	32.7
NM_021933	invasion inhibitory protein 45	72.4	-59.8	34.5
NM_006472	thioredoxin interacting protein	-9.5	-59.7	-21.0
AK023514	tyrosyl-DNA phosphodiesterase 1	NC	-59.5	53.8

Decreased Expression Following PGE_2 Treatment		Percent Change Compared to Vehicle		
Genbank	**Gene Title**	**$EP_{3\text{-}Ia}$**	**$EP_{3\text{-}II}$**	**$EP_{3\text{-}III}$**
AL136826	repulsive guidance molecule A	NC	-58.3	-37.6
AF005774	CASP8 and FADD-like apoptosis regulator	NC	-58.0	-26.3
AW205418	Roquin (RING finger and C3H zinc finger protein 1) (RING finger protein 198	56.4	-57.6	70.8
NM_002783	pregnancy specific beta-1-glycoprotein 7	-13.1	-56.5	-22.7
AU156755	core 1 UDP-galactose:N-acetylgalactosamine-alpha-R beta 1,3-galactosyltransferase	12.8	-56.3	38.6
BC007524	sperm associated antigen 9	6.6	-56.0	-12.0
AL562152	SH3-domain binding protein 5 (BTK-associated)	22.4	-55.4	NC
AW294133	Zinc finger protein 707	11.4	-55.0	8.6
AB000889	Lipid phosphate phosphohydrolase 3	19.1	-54.4	-36.2
NM_014710	G protein-coupled receptor-associated sorting protein	19.2	-54.4	NC

"NC" means no change in expression following PGE_2 treatment, "-" represents a decrease in expression.

Increased Expression Following PGE_2 Treatment		Percent Change Compared to Vehicle		
Genbank	**Gene Title**	**$EP_{3\text{-}Ia}$**	**$EP_{3\text{-}II}$**	**$EP_{3\text{-}III}$**
NM_006875	pim-2 oncogene	NC	133.8	NC
AI963083	Phosphatidylcholine:ceramide cholinephosphotransferase 2	32.7	137.7	66.9
AB017120	BAI1-associated protein 2	57.0	138.3	8.1
AI972661	tropomyosin 4	20.6	139.6	41.5

Increased Expression Following PGE_2 Treatment		Percent Change Compared to Vehicle		
Genbank	**Gene Title**	**$EP_{3\text{-}Ia}$**	**$EP_{3\text{-}II}$**	**$EP_{3\text{-}III}$**
BF315093	Novel protein	26.2	141.3	-17.2
NM_006254	protein kinase C, delta	26.7	144.4	-11.0
BG151154	ribosomal protein L36	22.0	152.7	-65.3
NM_003811	tumor necrosis factor (ligand) superfamily, member 9	69.2	154.7	-10.2
NM_005261	GTP binding protein overexpressed in skeletal muscle	-16.3	155.9	44.1
NM_001945	diphtheria toxin receptor (heparin-binding epidermal growth factor-like growth factor)	-12.7	157.1	26.9
NM_004417	dual specificity phosphatase 1	56.8	157.5	61.3
U79277	14-3-3 protein zeta/delta (Protein kinase C inhibitor protein 1) (KCIP-1).	NC	168.3	NC
NM_012081	elongation factor, RNA polymerase II, 2	-13.7	171.5	64.7
AI819630	c-Maf-inducing protein	74.8	172.6	84.5
AV725825	Zinc finger CCHC domain-containing protein 12	7.2	173.4	10.9
NM_025195	Tribbles homolog 1 (TRB-1) (SKIP1) (G-protein-coupled receptor-induced protein 2)	51.8	178.8	82.3
NM_020412	guanine nucleotide binding protein (G protein), alpha activating activity polypeptide, olfactory type	22.5	178.9	23.2
NM_001964	early growth response 1	41.1	179.9	72.2
BE550452	homer homolog 1 (Drosophila)	20.4	180.4	19.9
NM_004431	EphA2	70.2	186.8	60.9
AL041747	signal transducer and activator of transcription 3 interacting protein 1	-30.2	194.9	37.9
AI091372	AXIN1 up-regulated 1	-27.7	220.2	-7.0
AU145408	GC-rich promoter binding protein 1	-18.3	228.6	-30.1
AL137725	epiplakin 1	35.7	231.5	20.0

Increased Expression Following PGE_2 Treatment		Percent Change Compared to Vehicle		
Genbank	**Gene Title**	**$EP_{3\text{-}Ia}$**	**$EP_{3\text{-}II}$**	**$EP_{3\text{-}III}$**
AL136840	MCM10 minichromosome maintenance deficient 10 (S. cerevisiae)	62.8	235.0	46.9
NM_003897	immediate early response 3	5.2	239.8	41.6
BC004490	Proto-oncogene protein c-fos (Cellular oncogene fos) (G0/G1 switch regulatory protein	42.3	285.3	46.0
NM_000735	glycoprotein hormones, alpha polypeptide	34.8	295.7	19.1
AL021977	Transcription factor MafF (V-maf musculoaponeurotic fibrosarcoma oncogene homolog F) (U-Maf)	55.8	339.4	81.6
AI670862	FOS-like antigen 2	56.3	351.2	49.1

"NC" means no change in expression following PGE_2 treatment, "-" represents a decrease in expression.

Table 4.3 Top 60 Genes Differentially Regulated by $EP_{3\text{-}III}$ receptor isoform

Decreased Expression Following PGE_2 Treatment		Percent Change Compared to Vehicle		
Genbank	**Gene Title**	**$EP_{3\text{-}Ia}$**	**$EP_{3\text{-}II}$**	**$EP_{3\text{-}III}$**
NM_016473	melanoma ubiquitous mutated protein	NC	-40.3	-71.2
BE645222	SWIM-domain containing Srs2 interacting protein 1	-9.8	-39.8	-70.8
BF216535	nephronophthisis 1 (juvenile)	-42.5	-22.4	-68.6
BC036669	collagen, type XXV, alpha 1	-30.1	10.5	-67.0
AK023821	microtubule-actin crosslinking factor 1	-21.5	24.2	-65.8
AI928387	Cysteine/histidine-rich protein 1 (Fragment)	20.4	18.8	-65.7
BG151154	ribosomal protein L36	22.0	152.7	-65.3
NM_024587	Transmembrane protein 53	-28.0	44.1	-64.8
AI829795	GTP-binding protein Di-Ras1 (Ras-related inhibitor of cell growth)	75.4	68.7	-64.2
AW166925	RALBP1 associated Eps domain containing 1	NC	-8.3	-61.9
AK024480	Novel protein (Fragment)	71.3	35.4	-60.6
AW269335	Lysophosphatidic acid receptor Edg-2 (LPA receptor 1) (LPA-1)	50.4	NC	-59.3
NM_006355	tripartite motif-containing 38	-12.2	NC	-58.7
AA740755	Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and dual- specificity protein phosphatase PTEN	NC	6.9	-58.3
S75264	Wilms tumor 1	-9.7	32.8	-56.7
NM_021184	apolipoprotein M	24.7	-30.3	-56.3
AW298597	CDNA FLJ90508 fis, clone NT2RP3004202 (Chromosome 1 open reading frame 213)	NC	-34.6	-56.2
NM_021173	DNA polymerase subunit delta 4	-5.1	-28.5	-56.1

Decreased Expression Following PGE_2 Treatment		Percent Change Compared to Vehicle		
Genbank	**Gene Title**	**$EP_{3\text{-}Ia}$**	**$EP_{3\text{-}II}$**	**$EP_{3\text{-}III}$**
BF195628	ATPase WRNIP1 (Werner helicase-interacting protein 1)	-16.8	7.0	-56.0
AL558164	Transmembrane protein 143	36.3	61.2	-55.4
NM_002674	pro-melanin-concentrating hormone	37.5	-35.7	-53.5
BC032462	vacuolar protein sorting 29 (yeast)	-31.2	-33.3	-53.2
AK090801	quinolinate phosphoribosyltransferase (nicotinate-nucleotide pyrophosphorylase (carboxylating))	-22.0	19.6	-52.9
AA419275	nuclear factor I/A	28.0	-9.8	-52.7
AL117477	PHD finger protein 19 isoform a	NC	NC	-52.5
AF043899	bridging integrator 1	38.9	35.7	-52.3
NM_024103	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 23	42.6	-22.8	-51.6
AI677902	solute carrier family 26, member 10	6.7	10.4	-51.4
AI923985	GATA like protein-1	195.3	-8.3	-51.3
AF007132	abhydrolase domain containing 5	41.2	30.9	-51.3

"NC" means no change in expression following PGE_2 treatment, "-" represents a decrease in expression.

Increased Expression Following PGE_2 Treatment		Percent Change Compared to Vehicle		
Genbank	**Gene Title**	**$EP_{3\text{-}Ia}$**	**$EP_{3\text{-}II}$**	**$EP_{3\text{-}III}$**
N48266	stromal interaction molecule 2	-8.1	-26.7	127.5
NM_024590	Arylsulfatase J precursor	10.9	-12.4	127.7
AF465407	general transcription factor IIIC, polypeptide 3, 102kDa	18.1	-32.0	129.0
AW511239	Zinc finger protein 207	78.0	-49.4	130.0
AI656232	0	51.2	NC	131.0
AI912122	neuroligin 1	45.5	NC	131.6

Increased Expression Following PGE_2 Treatment		Percent Change Compared to Vehicle		
Genbank	**Gene Title**	**$EP_{3\text{-}Ia}$**	**$EP_{3\text{-}II}$**	**$EP_{3\text{-}III}$**
BC004958	leucine rich repeat (in FLII) interacting protein 1	13.7	36.1	136.3
AA476916	Chromosome 22 open reading frame 9	48.0	61.5	136.5
NM_023067	forkhead box L2	NC	NC	137.2
BG054798	SH3 and PX domains 2B	6.6	34.4	143.9
AF009619	CASP8 and FADD-like apoptosis regulator	-29.1	-5.8	144.5
AW511222	Lipoma-preferred partner (LIM domain-containing preferred translocation partner in lipoma)	20.3	-8.9	146.9
AL040198	hairy/enhancer-of-split related with YRPW motif-like	-12.7	-5.0	149.7
BC034477	Ectonucleoside triphosphate diphosphohydrolase 4	-7.9	-35.6	151.6
AA701657	leukemia inhibitory factor receptor	7.4	-27.2	153.0
AV724216	NDRG family member 4	13.7	22.3	161.9
N63748	zinc finger and BTB domain containing 4	62.6	-19.6	164.4
BE273906	Uncharacterized protein C17orf56	-9.9	25.5	177.4
AK023580	Niban-like protein (Protein FAM129B) (Meg-3)	32.5	-22.8	202.6
AI912696	Melanoma-associated antigen E1 (MAGE-E1 antigen) (Hepatocellular carcinoma-associated protein 1)	-22.8	-18.1	214.5
NM_153042	Flavin-containing amine oxidase domain-containing protein 1	75.2	8.5	218.8
NM_006352	zinc finger protein 238	22.1	8.2	220.1
AA809353	RING finger protein 215	-5.8	99.3	230.2
NM_004223	ubiquitin-conjugating enzyme E2L 6	-30.4	43.2	247.6
AI494500	Mitochondrial import receptor subunit TOM20 homolog (Mitochondrial 20 kDa outer	NC	-12.4	273.3

Increased Expression Following PGE_2 Treatment		Percent Change Compared to Vehicle		
Genbank	**Gene Title**	**$EP_{3\text{-}Ia}$**	**$EP_{3\text{-}II}$**	**$EP_{3\text{-}III}$**
	membrane protein)			
AV650728	PI-3-kinase-related kinase SMG-1 pseudogene	59.9	-28.1	274.4
AF323119	Nuclear factor (erythroid-derived 2)-like 2	NC	82.1	292.6
BC001742	hypothetical protein BC001742	NC	78.4	303.3
AI817976	SH2 domain-containing adapter protein D	384.4	-31.6	541.6
AI827455	B-cell CLL/lymphoma 6 member B protein (Bcl6-associated zinc finger protein)	15.0	22.5	613.3

"NC" means no change in expression following PGE_2 treatment, "-" represents a decrease in expression.

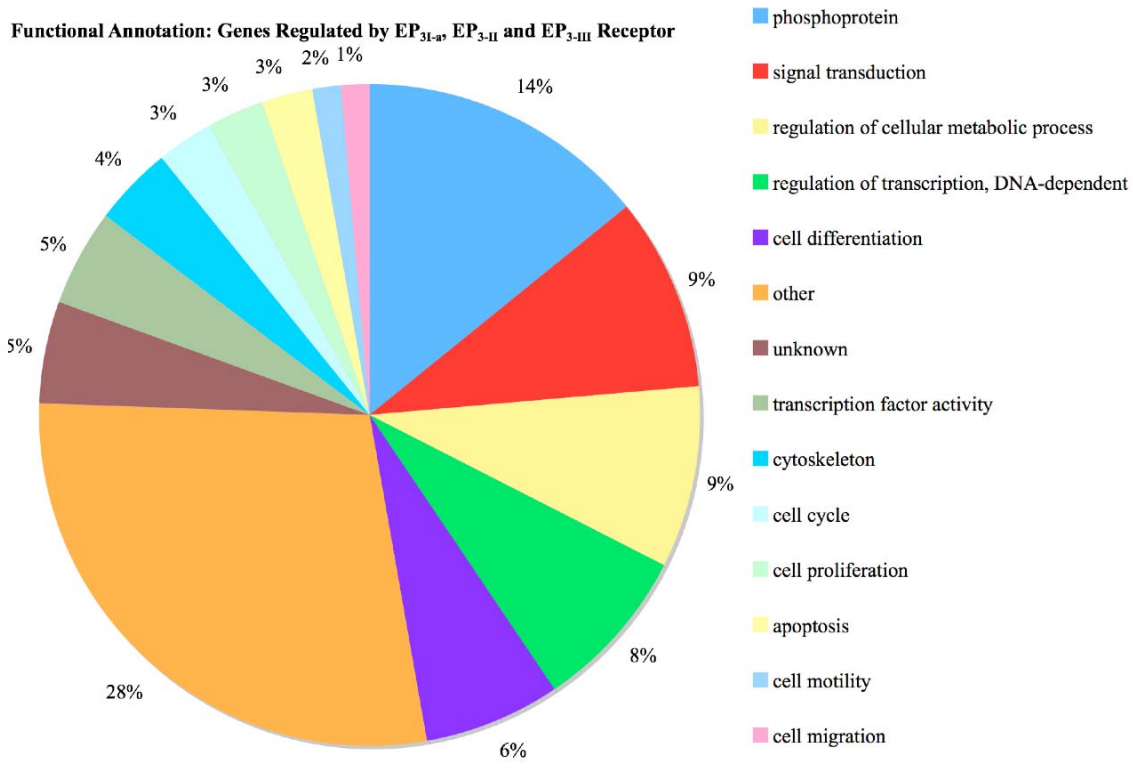


Figure 4.1 Functional groupings of genes regulated by EP_{3I-a}, EP_{3-II} and EP_{3-III}.

Genes identified as upregulated or downregulated by the EP_{3I-a}, EP_{3-II} and EP_{3-III} receptor isoforms in the microarray were grouped according to functional annotation using program DAVID.

Summary of quantitative real-time PCR analysis validation of 15 genes identified as regulated by $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and EP_{3III}. Fifteen genes identified in the microarray as uniquely regulated and/or of interest were chosen for verification by quantitative real-time PCR. Table 4.4 summarizes the results from these experiments. The majority of genes identified were regulated by the $EP_{3\text{-}II}$ isoform, followed by $EP_{3\text{-}Ia}$ then $EP_{3\text{-}III}$ isoforms. Seven of 15 genes verified followed a similar pattern of regulation as seen in the microarray results. Only 2 genes were completely unchanged in all isoforms following PGE_2 treatment (SPRED1 and tau tubulin kinase). We chose to examine the gene VAV3, which was decreased in all isoforms in the microarray to confirm the array correctly identifies genes down regulated following treatment with PGE_2. The quantitative real-time PCR results show that VAV3 increases similarly in all isoforms following PGE_2 treatment.

Some genes chosen for verification were regulated by an isoform not initially identified as the highest regulator in the array. For example, decorin expression was increased over 300% by $EP_{3\text{-}Ia}$ in the microarray but only slightly in the other isoforms. Quantitative real-time PCR studies of decorin mRNA changes following PGE_2 treatment repeatedly showed an increase by

$EP_{3\text{-}III}$ but not by $EP_{3\text{-}Ia}$ or $EP_{3\text{-}II}$. The lack of agreement among these results indicates the importance of validation studies.

Real-Time PCR analysis identified by microarray analysis to be uniquely regulated by $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and EP_{3III} receptor isoforms. Figure 4.2 illustrates the quantitative real-time PCR results from six of the fifteen genes chosen for further validation. Nearly all genes shown have a statistically significant increase in mRNA levels following PGE_2 treatment in at least one isoform and often in more than one isoform. Many genes show greater regulation by the $EP_{3\text{-}II}$ isoform than by the other isoforms. Studies of decorin show that it was the only gene in these studies that was uniquely regulated by one isoform. We failed to identify a gene uniquely regulated by the $EP_{3\text{-}Ia}$ isoform in these studies.

Wilm's Tumor 1 (WT-1) is a transcription factor important for mesodermally derived tissue development. In the microarray PGE_2 treatment increased WT-1 expression in $EP_{3\text{-}II}$, and EP_{3III} expressing cells. Treatment of EP_3 receptor isoforms with 50 nM PGE_2 results increased expression of WT-1 protein in $EP_{3\text{-}II}$ expressing cells, but only slight increased WT-1

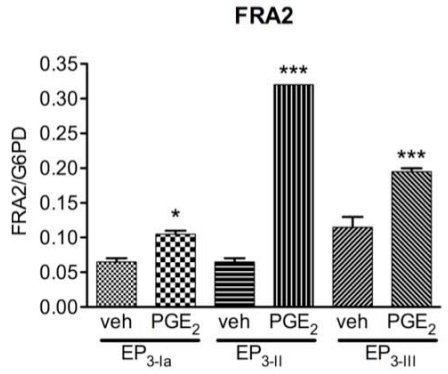
protein in the other isoforms. The agonist-induced pattern of WT-1 lyprotein expression among the EP_3 receptor isoforms followed a comparable trend as seen in the quantitative real-time PCR results.

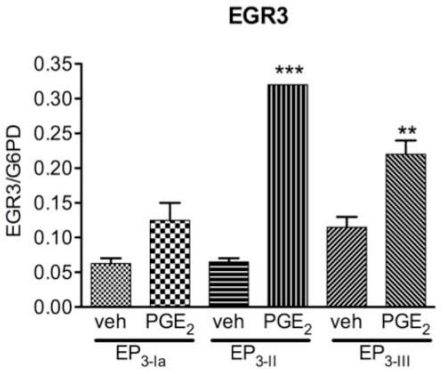
$EP_{3\text{-}II}$ activation induced an increase in WT-1 protein at 1 hour, which peaked at 3 hours and steadily declined to unstimulated levels by 9 hours. $EP_{3\text{-}III}$ mediated increased WT-1 protein expression peaks at 1 hour and reduces to basal levels by 3 hours. There was a further decrease in WT-1 protein levels at 9 and 12 hours followed by a return to unstimulated levels after 24 hours. In $EP_{3\text{-}Ia}$ expressing cells we observe a slight increase in WT-1 protein at 1-6 hours, a decrease at 9 hours followed by a sustained slight increase at 12 hours.

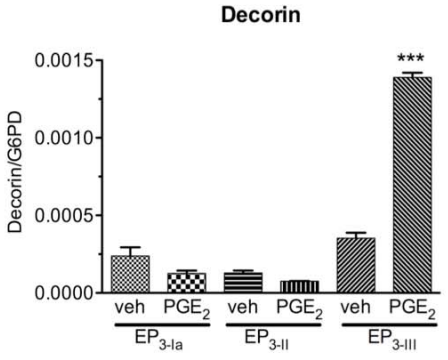
Table 4.4 Summary of quantitative real time PCR validation of 15 genes identified as uniquely regulated by $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and EP_{3III}.

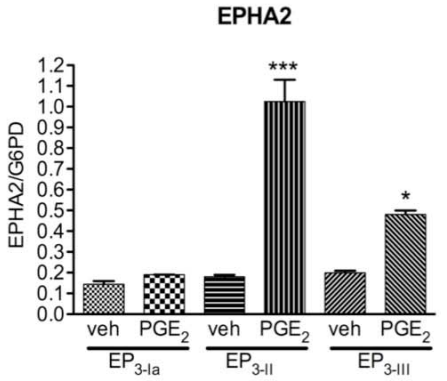
		Microarray Results (% change)			qReal Time Results (% change)		
Genbank	**Gene Title**	**$EP_{3\text{-}Ia}$**	**$EP_{3\text{-}II}$**	**$EP_{3\text{-}III}$**	**$EP_{3\text{-}Ia}$**	**$EP_{3\text{-}II}$**	**$EP_{3\text{-}III}$**
AI453137	Decorin	333	14.3	32.14	-47.2	-40.7	293.8
AI912122	neuroligin 1*	45.5	NC	131.6	NC	NC	251.6
NM_004430	EGR3*	NC	290	60	100.0	392.3	91.3
NM_004431	EPHA2*	70.2	186.8	60.9	31.0	469.4	140.0
AI670862	FRA-2*	56.3	351.2	49.1	61.5	392.3	69.6
NM_003897	Immediate Early Response 3	NC	240	42.8	NC	-70.2	333.3
U73936	Jagged 1*	-30.7	-27.3	83.3	20.0	37.5	50.0
NM_005461	MafB	102.7	45.4	NC	-15.6	-78.6	102.4
AF130088	Myotrophin	137.6	6.5	-46.2	20.4	27.3	10.4
NM_006875	PIM2*	NC	133.8	NC	13.5	100.0	46.5
AW957786	SPRED1	41.5	107.3	37.0	NC	NC	NC
BC041876	Tau Tubulin kinase 2	16.7	NC	57.1	NC	NC	NC
NM_006113	VAV3	-52.9	-20.5	-30.7	3.3	4.4	2.5
NM_024426	WT-1*	-9.7	32.8	-56.7	4.8	39.3	20.0

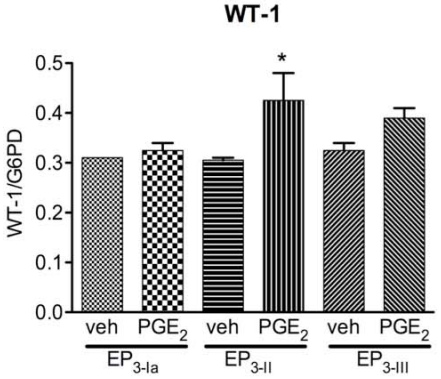
"NC" means no change in expression following PGE_2 treatment, "-" represents a decrease in expression. Bold items are uniquely regulated in microarray. "*" represents quantitative real-time PCR results that validate microarray data. % Change compared to vehicle control


FRA2
FRA2/G6PD
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
***
***
veh PGE2 veh PGE2 veh PGE2
EP3-Ia EP3-II EP3-III


EGR3
EGR3/G6PD
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
***
**
veh PGE2 veh PGE2 veh PGE2
EP3-Ia EP3-II EP3-III


Decorin
Decorin/G6PD
0.0015
0.0010
0.0005
0.0000
***
veh PGE2 veh PGE2 veh PGE2
EP3-Ia EP3-II EP3-III


EPHA2
EPHA2/G6PD
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
***
*
veh PGE2 veh PGE2 veh PGE2
EP3-Ia EP3-II EP3-III


WT-1
WT-1/G6PD
0.5
0.4
0.3
0.2
0.1
0.0
*
veh PGE2 veh PGE2 veh PGE2
EP3-Ia EP3-II EP3-III

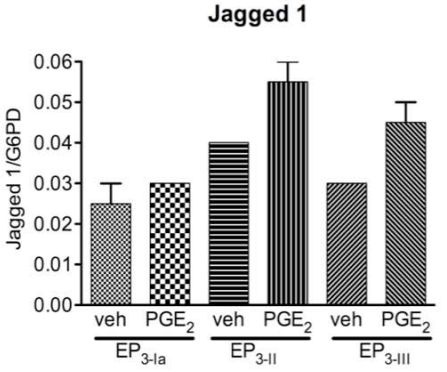

Jagged 1
Jagged 1/G6PD
0.06
0.05
0.04
0.03
0.02
0.01
0.00
veh PGE2 veh PGE2 veh PGE2
EP3-Ia EP3-II EP3-III

Figure 4.2 Real-Time PCR analysis identified by microarray analysis to be uniquely regulated by $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and EP_{3III} receptor isoforms.
HEK-293 cells stably expressing $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and $EP_{3\text{-}III}$ isoforms were pretreated with either vehicle (veh) or 50 nM PGE_2 for 3 hours. Total RNA was extracted and quantitative real time PCR was carried out as described in "Experimental Procedures". Data was normalized to the reference gene G6PD. Data are representative of three independent experiments each performed in duplicate. Levels of statistical significance are ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; as compared to PGE_2 treated EP_3 expressing cells. For all experiments, data are shown as mean ± SEM.

MEK inhibitor reduces $EP_{3\text{-}II}$ dependent increase in WT-1 expression. PGE_2 treatment of HEK cells expressing $EP_{3\text{-}II}$ stimulated an increase in WT-1 protein expression. Pretreatment with the MEK inhibitor, PD98059 increased basal WT-1 protein expression but treatment with PGE_2 reduced this increase. We repeated this experiment ten times and 7 of ten times, regardless of PD98059 concentration used, this occurred.

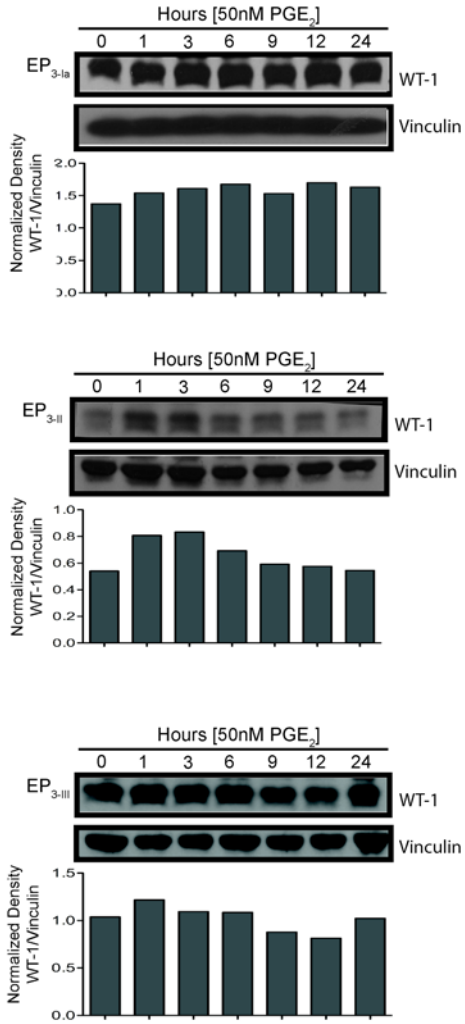


Figure 4.3 Time course of WT-1 expression in $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and EP_{3III} expressing cells following treatment with PGE_2.

HEK cells expressing $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and $EP_{3\text{-}III}$ were incubated with DMSO or 50nM PGE_2 from 0-24 hours then lysed on ice. Cell lysates were subject to immunoblot as described in "Experimental Procedures" using anti-WT-1 and anti-vinculin antibodies. The densities of WT-1 protein were normalized against the densities of vinculin expression. Blots are representative of three independent experiments.

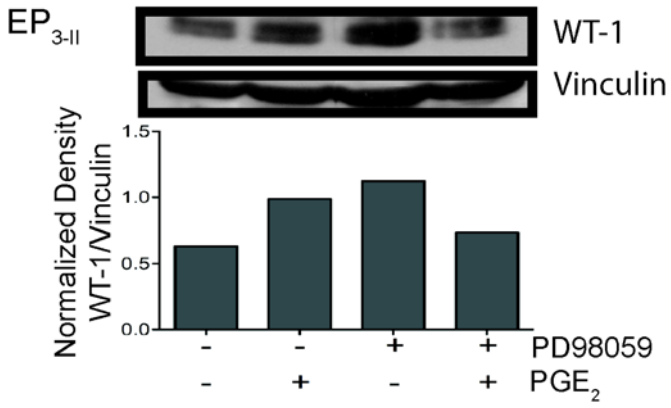


Figure 4.4 MEK inhibitor, PD98059 reduces $EP_{3\text{-II}}$ dependent increase in WT-1 expression.

HEK cells expressing $EP_{3\text{-II}}$ was pretreated with 100 nM PD98059 for 30 minutes followed by stimulation with DMSO or 50 nM PGE_2 3 hours then lysed on ice. Cell lysates were subject to immunoblot as described in "Experimental Procedures" using anti-WT-1 and anti-vinculin antibodies. The densities of WT-1 protein were normalized against the densities of vinculin expression. Blots are representative of three independent experiments.

4.4 Discussion

In these studies we used microarray analysis to identify genes differentially regulated by the $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ receptor isoforms. Over 300 genes that were to varying degrees uniquely regulated by the isoforms were identified. Several genes reported in other studies to be regulated by EP_3 receptor activation, including fibroblast growth factor 9 (FGF9) and vascular endotheial growth factor A (VEGF-A), were upregulated by at least one isoform in these studies [86, 103]. These findings increased our confidence that our microarray results are a relevant representation of the gene regulatory capabilities of EP_3 receptor isoforms.

Micorarray validation studies may reveal results contradictory to what was originally observed in the microarray results. Many of the genes identified as uniquely regulated in one isoform were also regulated to some degree by the other isoforms. This was particularly true for the $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ isoforms. This was not surprising, since MAPK activity or its downstream effectors reportedly transcriptionally regulate many of the genes chosen for verification.

Despite some unexpected results from the quantitative real-time PCR validation studies, it appears the microarray adequately identified genes uniquely regulated by the $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ receptor isoforms. Confirmation at the protein levels for genes regulated by EP_3 receptor isoforms was not as successful as confirmation at the mRNA level. We failed to detect EGR3 and jagged 1 protein expression following PGE_2 treatment in any of the EP_3 receptor isoform cell lines. Basal levels of FRA-2 protein expression were high in all isoforms and no significant increases in expression were seen in these studies. Repeated attempts at optimizing experimental conditions by changing cell density, serum starving/serum reduction, indomethacin treatment, varying antibody dilutions and using different antibodies failed to allow for the detection of these proteins or changes in expression. Finally, we examined WT-1 protein expression and found that $EP_{3\text{-}II}$ isoform could induce its expression in repeated experiments. Basal levels of WT-1 expression were high in all cell lines particularly, $EP_{3\text{-}Ia}$ and $EP_{3\text{-}III}$ expressing cells.

Regulation of Wilm's tumor 1 (WT-1) expression by the EP_3 receptor was of particular interest to us due to studies reporting an increase in the expression

of the EP_3 receptor in the podocytes of patients with the nephropathies, Denys Drash syndrome and glomerulosclerosis [104]. Normally EP_3 receptor expression in the kidney is restricted to the thick ascending limb of the loop of Henle [105].

WT-1 is a zinc finger protein with both transcriptional and translational activities. It was first identified as one of several genes implicated in the pathogenesis of three forms of congenital nephropathy Wilm's tumor, Denys-Drash and Frasier syndrome [106-109]. WT-1 expression is essential for proper urogenital development, as well as olfactory epithelia, retinal ganglia, and epicardial development and hematopoiesis [110-113]. WT-1 expression is found in the urogenital tissues, the central nervous system and hematopoietic tissues in the developing embryo, but is restricted to the podocytes of the kidney in adults.

Determining the mechanisms responsible for upregulation of WT-1 expression by the $EP_{3\text{-II}}$ receptor was not completed in these studies. Preliminary data indicates that one of the common regulators of WT-1 expression, NFκB, does not play a role in $EP_{3\text{-II}}$ receptor mediated increases

in WT-1 expression in HEK 293 cells. WT-1 is a structurally related to early growth response factor 1 (EGR1) and whose expression is increased in a MAPK dependent manner following activation of the FP receptor [114]. Based on this and other reports we analyzed the effects of MAPK inhibitors on $EP_{3\text{-}II}$ induced WT-1 expression. We found that MAPK activity contributes to $EP_{3\text{-}II}$ induced increases in WT-1 expression as the MEK inhibitors PD98059 and UO126 reduced PGE_2 stimulated WT-1 expression but the inhibitor itself spikes WT-1 expression above basal levels. Inhibition of ras, c raf and ERK2 with dominant negative plasmids also produce similar effects as the MEK inhibitors but the results were inconsistent (data not shown). MAPK dependent increases in WT-1 expression have been described in PC12 cells via the activation of the insulin-like growth factor (IGF-1) receptor [115]. However, MAPK activation has also been linked to a reduction in WT-1 expression by activation of this same receptor in the osteosarcoma-derived Saos-2 cells [116]. Whether these discrepancies are cell-type dependent or indicative of multiple unknown signaling pathways converging to create distinct effects on WT-1 expression is not fully understood. It still hasn't been determined whether MAPK pathway is important for WT-1 expression and/or protein stability. Activation of $EP_{3\text{-}II}$

receptor does induce an increase in WT-1 mRNA and protein in HEK 293 cells, but the role members of the MAPK or other pathways play in this process is yet to be determined.

WT-1 expression is also positively regulated by the transcription factors PAX-2 and PAX-8 [117, 118]. PAX proteins are important for the development of the embryonic central nervous system (CNS), kidney and other tissues[118]. The high basal levels of WT-1 expression may be due to the activities of the PAX transcription factors in HEK 293 EBNA cells. PAX-2 not only regulates WT-1 expression, but is also in turn regulated by WT-1. PAX-2 is also regulated by the MAPK, Jun N-terminal kinase (JNK). Studies in human gingival fibroblasts report that EP_3 receptor activation induced JNK activation [119]. Specific isoform expression was not assessed in these studies. In our studies we found that treatment with the MEK inhibtor PD98059 alone, induced WT-1 expression above PGE_2 treatment alone (Figure 4.4). One possible mechanism attributing to this increase in WT-1 expression by PD98059 may be due to a shift in ERK 1/2 and JNK phosphorylation levels. Hotokezaka et al. 2002, reported that treatment with the p38 inhibitors PD169316, SB203580 increased ERK 1/2

phosphorylation in RAW264.7 cells and a similar increase was seen in p38 phosphorylation following treatment with the MEK inhibitors UO126 and PD98059 [120]. There are a few studies indicating that PD98059 has no effect on JNK activity and those indicating the opposite, however several of these studies are undertaken in different cell types [120-122]. We have not examined whether in these EP_3 prostanoid receptor isoform expressing HEK 293 cells PD98059 inhibits JNK activation. A possible mechanism may include JNK activation increasing WT-1 expression following $EP_{3\text{-II}}$ activation and treatment with PD98059 further increasing WT-1 expression due to effects on JNK activity. However this explanation does not address the additive effect of PD98059 and $EP_{3\text{-II}}$ receptor stimulation decreasing WT-1 expression compared to PGE_2 treatment alone (Figure 4.4).

In these studies we show that there are distinct differences in the signaling programs activated by the $EP_{3\text{-Ia}}$, $EP_{3\text{-II}}$ and $EP_{3\text{-III}}$ receptor isoforms. We have identified a number of previously unreported targets of EP_3 receptor signaling which will help to understand the regulatory potential of the EP_3 receptor. Of particular interest, we have identified the transcription factor, WT-1 as a target protein of $EP_{3\text{-II}}$ receptor activities.

CHAPTER FIVE

SUMMARY, CONCLUSIONS AND FUTURE STUDIES

5.1 Summary and Conclusions

In this dissertation I have identified differential signaling pathway activation and gene regulation among the $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ receptor isoforms. EP_3 receptor isoforms differ in the sequence and length of their carboxyl terminal tail and these differences are known to affect G-protein coupling, receptor localization, constitutive activity and regulation of second messenger systems [37, 66, 72]. Currently there are reportedly 10 distinct mRNA splice variants identified for the human EP_3 receptor. We chose to investigate differential signaling and gene regulation among the $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ receptor isoforms over others due to evidence indicating that these are the most highly expressed forms of the receptor in humans [30, 34]. The EP_3 gene consists of 10 exons and all three isoforms express exon 1 which encodes for the extracellular amino terminal tail and all seven transmembrane domains. From here divergence occurs and the $EP_{3\text{-}Ia}$ isoform expresses exons 2a, 3 and 4, $EP_{3\text{-}II}$ expresses exon 2b and $EP_{3\text{-}III}$ expresses 2a. Previous studies show that distinct differences in carboxyl tail expression infer differences in G-protein coupling and second messenger signaling thus examination of these isoforms was logical for this reason as well.

To characterize the signaling and gene regulatory potential of these isoforms we created HEK 293 EBNA cell lines stably expressing the $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ receptor isoforms. We used radioligand binding, cyclic AMP and IP3 accumulation assays to characterize the functional capabilities of the EP_3 isoforms. Comparison of our cell lines to recombinant EP_3 receptor isoform cell lines reported in the literature revealed that our cells behaved similarly in respects in PGE_2 binding, cyclic AMP inhibition and IP3 accumulation [30, 32, 37]. It has been previously been reported that activation of EP_3 receptors inhibits adenylyl cyclase activities through the actions of G_i. Treatment with the EP_3 receptor agonist sulprostone inhibited forskolin induced cyclic AMP production in a pertussis toxin sensitive manner in all three isoforms. $EP_{3\text{-}Ia}$ and $EP_{3\text{-}III}$ were much more effective than $EP_{3\text{-}II}$ at inhibition of forskolin induced cyclic AMP production. EP_3 receptor activation is also reported to stimulate G_q coupling. We used IP3 assays as an indication of this activation of this pathway. $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ activation induced IP3 accumulation but $EP_{3\text{-}Ia}$ activation did not. These initial characterization studies provided evidence for potential differences in signal transduction pathway activation and gene regulation.

After initial characterization of the cell lines we examined for differences in ERK 1/2 phosphorylation following PGE_2 treatment among the cell lines. Burkey and Regan (1994) had previously published a study detailing the activation of the MAPK pathway in the $EP_{3\text{-}Ia}$ isoform, thus we wished to further investigate the other isoforms for their potential to regulate ERK 1/2 phosphorylation. We found that the $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ receptor isoforms utilize distinctly different mechanisms for activating ERK 1/2 phosphorylation. $EP_{3\text{-}Ia}$ and $EP_{3\text{-}III}$ receptors expressed in HEK cells both utilize G_i, src, EGFR, PKC, PI3K dependent mechanisms for ERK 1/2 phosphorylation whereas $EP_{3\text{-}II}$ appears to only utilize G_i, src and EGFR. Also both $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ strongly increase ERK 1/2 phosphorylation following PGE_2 treatment whereas $EP_{3\text{-}II}$ produces a much attenuated and transient increase in ERK 1/2 phosphorylation. Differences in the mechanisms involved in activation as well as differences in ERK 1/2 phosphorylation intensity and duration of signal contribute to variation in the regulation of the downstream effectors ELK-1 and AP-1 as well as to effects on cell proliferation.

Both $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ activated ELK-1 and AP-1 reporter activity in MAPK dependent manner. $EP_{3\text{-}Ia}$ did not stimulate either ELK-1 or AP-1 reporter activity. Interestingly, $EP_{3\text{-}Ia}$ and $EP_{3\text{-}III}$ increased cell number following PGE_2 treatment albeit in differing ways. The $EP_{3\text{-}III}$ isoform increases cell proliferation in a MAPK dependent manner while the $EP_{3\text{-}Ia}$ dose dependently regulates cell proliferation via $G\alpha_i$ and not ERK 1/2. Activation of the $EP_{3\text{-}II}$ receptor had no effects on proliferation. These differences in activation of cell proliferation between $EP_{3\text{-}Ia}$ and $EP_{3\text{-}III}$ versus $EP_{3\text{-}II}$ may be due to the activities of PI3K, which has been reported to be required for stimulation of DNA synthesis and cell proliferation [21]. MAPK activity in $EP_{3\text{-}II}$ cells was not dependent on the actions of PI3K, so we don't know the ability of this receptor to activate this kinase. We did not examine the effects of the PI3K inhibitor wortmannin on cell proliferation in these studies but we hypothesize that it may contribute to the induction of cell proliferation observed in $EP_{3\text{-}Ia}$ and $EP_{3\text{-}III}$ cells but not $EP_{3\text{-}II}$. These studies detailing the mechanisms of ERK 1/2 phosphorylation may be important for understanding the signaling potential and the diversity of function of $EP_{3\text{-}Ia}$, $EP_{3\text{-}II}$, and $EP_{3\text{-}III}$ receptor isoforms.

Investigation of differential gene regulation among the EP_3 receptor isoforms, was accomplished using microarray analysis. We identified over 300 genes that were differentially regulated by the isoforms and used quantitative real-time PCR analysis to validate 15 candidate genes. Of these 15 candidate genes, five were chosen for further analysis using immunoblot but only WT-1 protein expression was significantly increased following PGE_2 treatment. WT-1, a transcription factor important for kidney and heart development, was uniquely upregulated at all levels by the $EP_{3\text{-II}}$ receptor, but only negligible amount in the other isoforms. We have preliminary data indicating a role for MAPK pathway in $EP_{3\text{-II}}$ receptor mediated WT-1 expression but at this time the results are inconclusive.

In the course of preparing this document I learned that as of January 2008 the $EP_{3\text{-Ia}}$ receptor has been re-classified as a nonsense-mediated mRNA decay (NMD) candidate in the National Center for Biotechnology Information (NCBI) website. NMD is a evolutionary conserved mRNA surveillance process that degrades mRNAs that terminate 50 or more nucleotides upstream of a splicing generated exon-exon junction or the presence of a marker downstream of a premature stop codon, termed

premature termination codons (PTCs). These prematurely terminated mRNAs are degraded to prevent the production of potentially toxic truncated proteins generated by errors in mRNA processing. It is believed that 35% of alternative isoforms are candidates for NMD [123]. NMD is also important for the regulation of normal transcript as well and participates in regulation of transcription, cell cycle, cell proliferation and many other biological processes.

Most studies use reverse transcription PCR (RT-PCR) to identify the expression of prostanoid receptors in both tissue and cell lines. During the original characterizations of the EP_3 receptor was found to be located in nearly every tissue. It has been reported that $EP_{3\text{-}Ia}$ and $EP_{3\text{-}II}$ are the most abundantly expressed isoforms [30, 34]. In a recent conversation with Dr. Richard Breyer from Vanderbilt University, he described an observation from his studies where endogenous EP_3 receptor expression in cultured cell lines is often lost. If we accept that $EP_{3\text{-}Ia}$ is a target of NMD, this may partially explain the phenomenon observed by Dr. Breyer. In my own research, I have repeatedly used RT-PCR to confirm EP_3 receptor expression in cell lines reported to express it and not found the receptor expressed.

These finding raises questions about the relevance of these studies with the $EP_{3\text{-}Ia}$ receptor isoform. Due to the use of recombinant DNA technology, we as well as others may have forced the translation of a protein that may not occur naturally. Because $EP_{3\text{-}Ia}$ is still just a candidate and because we lack antibodies that differentiate between EP_3 receptor isoforms we are unable make any conclusions about the validity that this transcript can be translated endogenously into a functional protein.

Another concern is how differential signaling among the isoforms of the EP_3 receptor translates in the native system where isoforms are expressed in multiple rather than singly. There are the several possibilities including modulation of signaling dependent on the isoforms expressed and receptor oligomerization. This is one important question that still remains to be addressed.

In conclusion, these studies show that the EP_3 receptor isoforms are capable of distinct regulation of both signal transduction pathways and gene transcription. Elucidating the differential functions of EP_3 receptor isoforms

may allow for greater understanding of the diverse functions attributed to this receptor and the physiological function for EP_3 receptor isoforms.

5.2 Future Studies

The results detailed in this dissertation raise more questions than they answer. The data generated from the microarray itself is a source for innumerable future studies. Here I will limit the explorations into future studies to five categories: 1) Complete characterization of the pathways responsible for differential effects on cell proliferation in $EP_{3\text{-II}}$ and $EP_{3\text{-III}}$ expressing HEK 293 cells, 2) Generation of new recombinant cell lines to allow for study of proteins of other genes identified in the microarray in conjunction with studies in HEK 293 EBNA cells, 3) Characterization of the pathways activated by $EP_{3\text{-II}}$ receptor and leading to WT-1 expression and 4) Importance of isoform distinct signal transduction and gene regulation in endogenous EP_3 receptor systems.

1) Complete characterization of the pathways responsible for differential effects on cell proliferation in $EP_{3\text{-II}}$ and $EP_{3\text{-III}}$ expressing HEK 293 cells. Cell proliferation is an important pathway in various physiological and

pathological setting. Due to the divergent data available concerning the role the EP_3 receptor plays in proliferation, it is of particular importance to understand why the $EP_{3\text{-II}}$ receptor does not induce cell proliferation following PGE_2 treatment, while the $EP_{3\text{-Ia}}$ and $EP_{3\text{-III}}$ receptor do. I suggest further characterization of the effects of PGE_2 treatment on the expression of $p21^{cip1}$, $p27^{kip1}$ and cyclins D1 and E. It has previously been reported that EP_3 receptor activation induces $p21^{cip1}$ expression and that WT-1 also positively regulates $p21^{cip1}$ expression [83, 85, 124]. Also to consider is that $EP_{3\text{-Ia}}$ and $EP_{3\text{-III}}$ receptor both utilize the activities of PI3K to induce MAPK activation and in the case of the $EP_{3\text{-III}}$ receptor likely contributes to cell proliferation. These observations may be relevant potential mechanisms for differences in $EP_{3\text{-II}}$ versus $EP_{3\text{-III}}$ induction of proliferation.

2) Generation of new recombinant cell lines to allow for study of proteins of other genes identified in the microarray in conjunction with studies in HEK 293 EBNA cells. Studies utilizing EP_3 receptor isoforms expressed in HEK 293 EBNA cells have provided a good model for these studies, but the machinery of the cell line may limit us when exploring other genes identified in the microarray study. For example, we were unable to detect

decorin or jagged 1 protein following PGE_2 treatment but we did see a change in mRNA levels in both the microarray and the validation studies. The EP_3 receptor could possibly regulate these genes in other cell types, but we were unable to detect this regulation in HEK 293 cells. There are over 300 genes identified as regulated by the EP_3 receptor isoforms and having more than one cell line to screen for regulation could an asset for discovering novel signal transduction and gene regulatory differences among the isoforms.

3) Characterization of the pathways activated by $EP_{3\text{-II}}$ receptor and leading to WT-1 expression. EP_3 receptor expression is up regulated in both Denys Drash Syndrome and glomerulosclerosis, both sclerotic conditions in which proper WT-1 expression is critical but lost [104, 125]. Transcriptional regulation of the gene has been attributed to the activities of WT1, SP1, NFκB, MAPK, GATA-1, PAX 2 and 8, via unique DNA binding sites in the promoter region, but the mechanism appears to be cell type specific [107, 117, 118, 126, 127]. Thus far we have examined regulation by NFκB and MAPK pathways. NFκB does not appear to play a role in $EP_{3\text{-II}}$ mediated increase in WT-1 expression. MAPK activation participates in the induction

of WT-1 expression via EP_3 activation but the mechanisms involved were not resolved.

There is currently both “normal” and Denys Drash glomerular podocytes cell lines immortalized by temperature-sensitive SV40 T antigen [128, 129]. We have obtained the normal podocyte cell line from Dr. Moin Saleem, but as expected the cells do not express the EP_3 receptor. It is possible that the EP_3 receptor plays a role in podocyte sclerotic disorders and understanding its function could provide a novel target for therapy.

4) Importance of isoform distinct signal transduction and gene regulation in endogenous EP_3 receptor systems. Reports indicate that EP_3 receptor isoforms are expressed as groups of multiple isoforms rather than singly in cells [30, 36, 39, 72-74]. Therefore, the relevance of studies of differential receptor isoform activities is often questioned. It is unclear if the EP_3 receptor isoform transcripts are all translated into functional proteins at the same time or in the same quantity, but understanding how individual isoforms contribute to activation of multiple signaling pathways and physiologic function.

Studies of adrenergic receptor isoforms indicate evidence of regulation of distinct second messengers, modulation of signal due to ligand competition and receptor oligomerization.[130-132] Heterodimerization of the thromboxane receptor isoforms, TPα and TPβ, induces greater increases in IP3 accumlation and intracellular calcium following isoprostane treatment but not with TxA_2.[132] Of particular importance Einstein et al. (2008) published a report detailing GPCR isoform expression in airway smooth muscle and found 192 GPCRs expressed an average of 5 different receptor isoforms. A number of these GPCRs identified are therapeutic targets for diseases such as asthma and obstructive pulmonary disease [133]. If there are differences in splice variant function, it is particularly important to understand what these are to avoid unanticipated and deleterious side effects when treating disease.

Currently, deCODE Genetics has developed an EP_3 receptor antagonist for use as an anti-platelet drug for the prevention of arterial thrombosis and is in Phase II clinical trials in Iceland. The EP_3 receptor was identified as a potential therapeutic target based on population genetics studies indicating

the expression of EP_3 variants correlates with increased risk of vascular disease. The EP_3 receptor has been identified as a potential therapeutic target for a number of diseases including myocardial infarct, pain and cancer. Due to this increased interest in the EP_3 receptor as a therapeutic target, it is important to understand how isoform differences as well as allelic variances potentially influence therapeutic outcome.

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