THE ROLE OF THE MURINE EP3 RECEPTOR VARIANTS ON CELL FUNCTION

By

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Dedicated to my mother, Ines Macias, and the memory of my father, Juan Macias, who taught me an education is our most powerful tool in life.

ORIGINAL PUBLICATIONS

Macias-Perez I, Breyer RM, Breyer MD, Carmosina M, Matrisian LM, Zent R, Pozzi A. Activation of the PGE_2 mEP3 receptor variants enhances cell-cell interaction via Rho. J Biol Chem. 2008 Jan.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
ACF	Aberrant crypt foci
AOM	Azoxymethane
AVP	Arginine-vasopressin
cAMP	Cyclic adenosine monophosphate
COX	Cyclooxygenase
CRC	Colorectal cancer
DMEM	Dulbecco's modified Eagle's media
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FAP	Familial adenomatous polyposis
FCS	Fetal calf serum
GPCR	G protein couple receptor
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MDCK	Madin-Darby canine kidney
MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumor virus
PBS	Phosphate-buffered saline
PGDH	Prostaglandin dehydrogenase

PGE	Prostaglandin E
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PLC	Phospholipase C
SDS	Sodium dodecyl sulfate
VEGF	Vascular endothelial growth factor

CHAPTER I

INTRODUCTION

Cyclooxygenase-2

The cyclooxygenase enzymes, COX-1 and COX-2, catalyze the rate-limiting steps in the biosynthesis of prostaglandins and thromboxanes, collectively known as prostanoids. The cyclooxygenases utilize arachidonic acid (AA) as their substrate and convert it to prostaglandin H_2 (PGH_2) (**Figure 1**). AA is generated by phospholipase A2 and is further oxidized by COX. The downstream isomerases can convert PGH_2 to prostacycline, prostaglandins, or thromboxane A_2, which play a vital role in multiple physiologic and pathologic processes (2).

COX-2 was originally cloned as a primary response gene whose messenger RNA was rapidly induced by treatment with phorbol esters (3). Regulation of expression of COX-1 and COX-2 is distinct: COX-1 is constitutively expressed and responsible for "housekeeping" prostaglandin synthesis, whereas COX-2 is an inducible enzyme whose expression and activity in most cells are upregulated in response to a variety of proinflammatory stimuli (2, 4, 5). Various extracellular stimuli including growth factors, cytokines and tumor promoters can also induce COX-2 expression.

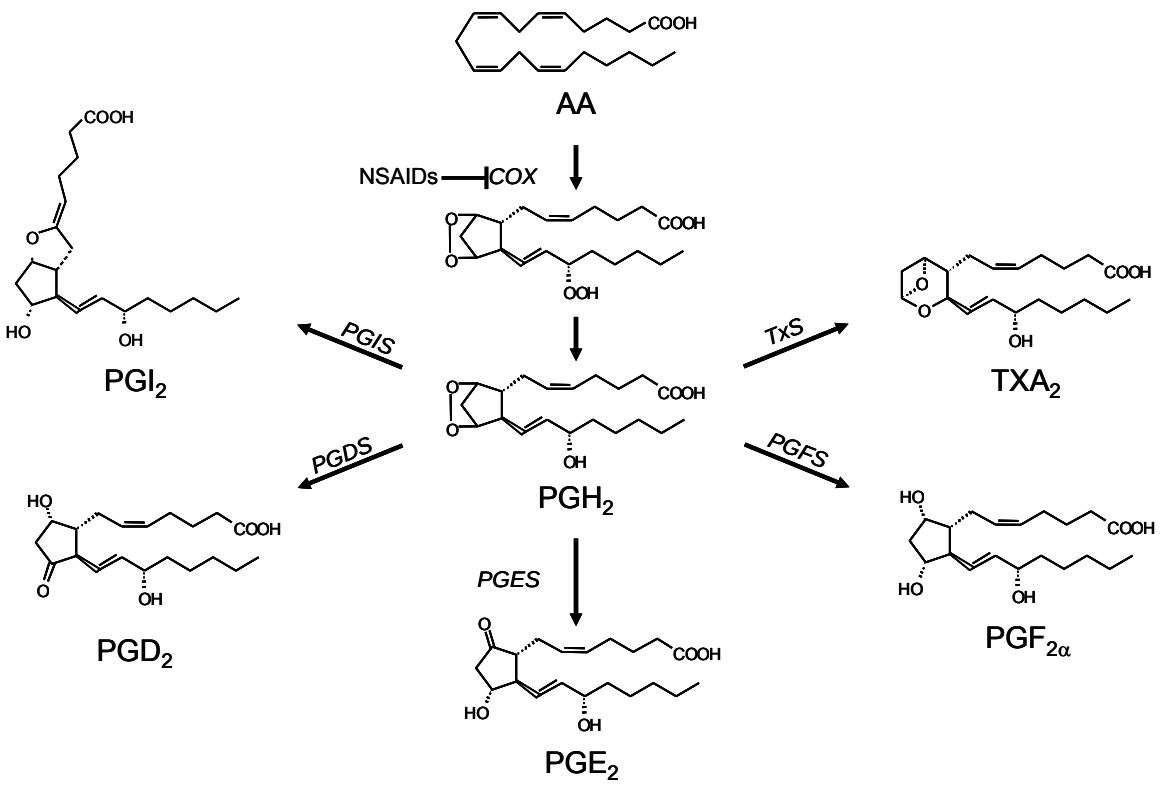

Figure 1. Synthesis of prostanoids by cyclooxygenases.

COX-2 and cancer

A substantial body of research has attributed an important role for COX-2 and its products in cancer development, cancer cell growth and cancer cell survival. Increased amounts of COX-2 are commonly found in both premalignant tissues and malignant tumors. This appears to reflect the effects of oncogenes, growth factors and tumor promoters, known inducers of COX-2 (6, 7). The most specific data supporting the cause-effect relation between overexpression of COX-2 and carcinogenesis come from genetic studies in mice (8, 9). It also has been demonstrated that prostaglandins and other COX-2-generated downstream mediators promote tumor cell proliferation and survival in an autocrine and/or paracrine manner (10-14). Furthermore, knockout of the COX-2 gene suppressed tumorigenesis in mice with a genetic predisposition for polyp formation (8).

Pharmacological evidence also implicates COX-2 in tumorigenesis. Selective inhibitors of COX-2 can reduce the formation of intestinal, breast, lung, skin, bladder, and tongue tumors in rodents (8, 15-22). Nonsteroidal anti-inflammatory drugs (NSAIDs) have also been implicated to have COX-independent mechanisms of inhibiting tumorigenesis (23). However the concentrations used in these studies were so high the pharmacological relevance is questionable. Collectively the genetic and pharmacological evidence strongly suggests COX-2 is a potential therapeutic target for preventing cancer.

NSAIDs and colorectal cancer

Colorectal cancer (CRC) is highly prevalent in the western world where it is the second most frequent cause of cancer-related death. In 2007, an estimated 153,760 cases of colorectal cancer will be diagnosed, and 52,180 people will die from the disease (24). By the age of 70 about 50% of the western population develops an adenomatous polyp and approximately 10% of these polyps will progress to malignancy (25). CRC is a multi-factorial disease. It is a result of a time dependent accumulation of mutations in genes controlling colonic epithelial cell turnover, and its incidence depends on both genetic as well as environmental risk factors, such as diet and lifestyle behavior. It is therefore not surprising to find various differences in prevalence, age of onset and anatomical distribution among various international populations and intra-national ethnic groups.

From a molecular-genetic perspective CRC is likely the best understood solid malignancy, which relates to the accessibility of the tumors and the fact that different stages of the same malignancy can coexist within the same patient. The seminal adenoma-carcinoma sequence of tumor progression integrates the notions that colorectal tumors result from mutational activation of oncogenes combined with inactivation of tumor-suppressor genes, multiple gene mutations are required to produce malignancies and that genetic alterations may occur in a definite sequence and yet the accumulation rather than the chronological order of these changes determines the histopathological characteristics of the colorectal tumor (26). While there is a 90% 5-year survival rate with surgical resection and/or adjuvant therapy of early CRC, only 37% of CRC are diagnosed

at this early stage. In advanced CRC traditional chemotherapy regimens have provided only a modest improvement in 5-year survival rates (27). For this reason, in addition to improved therapeutic options, better cancer prevention strategies must be developed.

Beginning with Waddell's serendipitous finding that sulindac, an NSAID used to treat a desmoid tumor also caused the dramatic regression of rectal polyps (28), a number of studies have shown that regular use of NSAIDs over a 10–15-year period can reduce the relative risk of developing CRC by 40%–50% (29-31). Although these studies were originally developed and substantiated in rodent models (32, 33), subsequent clinical trials confirmed that NSAID use leads to the regression of preexisting adenomas in patients with familial adenomatous polyposis (FAP) (28, 34-37).

In 1994 Eberhart and colleagues were the first to show a positive relationship between COX-2 expression and human CRC (38). Since then subsequent reports have confirmed COX-2 is overexpressed in about 90% of colorectal adenocarcinomas and in 40–90% of colorectal adenomas (39-41). In adenomas, COX-2 expression correlates with size and seems to be unrelated to the degree of dysplasia (38, 42, 43). COX-2 expression is thus observed early during colorectal tumor development in both benign and malignant tumors; however, these studies do not confirm COX-2 overexpression to be a central event. Direct evidence that links COX-2 to colorectal tumor development was obtained using animal models: chemically-induced and genetically engineered animal models of intestinal polyposis. Elevated COX-2 expression is observed in colonic adenomas and carcinomas from multiple intestinal neoplasia (*Min*) mice, which harbor a

mutation in the APC gene, and azoxymethane (AOM)-treated rats (44, 45). Deletion of COX-2 in two *Min* mice models decreased tumor formation (8, 46). Nonselective NSAIDs and COX-2 selective inhibitors had chemopreventive effects in AOM-treated rats (16, 47-50) and reduced the number of intestinal polyps in *Min* mice (15, 17, 51-54). Collectively, these studies suggest COX-2 is a biologic target of NSAIDs *in vivo* and selective COX-2 inhibitors may be efficacious in the prevention and treatment of CRC. Interestingly COX-1 deletion in *Min* mice also decreased polyp number (46) suggesting the actual enzymatic source may not be as important as the overall load of prostaglandins.

In 1971 Vane identified prostaglandins to be the target of NSAID activity (55). NSAIDs are thought to exert their anti-inflammatory, analgesic and anti-pyretic effects mainly by inhibiting the biosynthesis of prostaglandins (**Figure 1**) (55). Prostaglandins are members of a large family of bioactive lipid molecules known as eicosanoids. In humans, prostaglandins are involved in diverse functions including blood clotting, ovulation, initiation of labor, bone metabolism, nerve growth and development, wound healing, kidney function, blood vessel tone and immune responses (56). Given the broad role these bioactive compounds play in normal human physiology, it is not surprising that systemic suppression by NSAIDs can lead to unwanted side effects. These side effects increase with the age of the population and result in an increased risk of gastrointestinal bleeding, even at relatively low doses. Enormous effort has been expended to develop NSAIDs whose specificity of action will enhance the benefits of eicosanoid inhibition yet minimize the harmful effects.

Prostaglandins

Prostaglandins, thromboxanes and leukotrienes, collectively referred to as 'eicosanoids', are the metabolites of AA (**Figure 1**). Discovery of eicosanoids (from Greek *eicosa*=twenty; for twenty carbon fatty acid derivatives), was initiated in 1930 (57-59). First, it was found that exclusion of fat from the diet of rats led to growth retardation, reproductive disturbances, scaly skin, kidney lesions and excessive water consumption, which led to the discovery of essential fatty acids. Second, a factor with fatty acid properties and vasodepressor and smooth muscle-stimulating activity was identified and termed "prostaglandin." Bergström and Samuelsson linked these observations when they elucidated the structures of the "classical" prostaglandins and demonstrated that they were produced from an essential fatty acid, AA (60).

Prostaglandins are produced in most cells in the body and act as autocrine and paracrine mediators of such cell functions as pain generation, vascular permeability, febrile response and uterine contractility (61). The chemistry of eicosanoid biosynthesis is well known. Most eicosanoids are derived from AA that is sequestered in membrane phospholipids. Upon physiologic and pathologic stimulation, the rate-limiting step of prostanoid synthesis is the release of AA from the plasma membrane by phospholipase A_2. Thereafter, AA is metabolized through 1 of 3 major pathways: the cyclooxygenase pathway, the lipoxygenase pathway or the cytochrome P-450 monooxygenase pathway, depending on the availability of enzymes. Prostaglandin H synthase, commonly referred to as cyclooxygenase (COX), as mentioned earlier, is the key regulatory enzyme which catalyzes the conversion of AA to PGG_2 and PGH_2. PGH_2 is subsequently converted by

cell-specific synthases to a variety of eicosanoids that include PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and thromboxane A_2 (TXA_2) (**Figure 1**).

Prostaglandins can signal at or immediately adjacent to their site of synthesis. They are not only key mediators of inflammation and involved in apoptosis, cell differentiation and oncogenesis, but also play critical physiologic roles in tissue homeostasis and function. For example, gastric mucosal protective function, sleep induction and vascular smooth muscles contraction and relaxation are all dependent upon these compounds (61-63). Some actions of prostaglandins are illustrated in **Figure 2**.

Biological functions of PGE_2

Among the prostanoids, prostaglandin E_2 (PGE_2) is the most widely produced in the body, the most widely found in animal species and exhibits the most versatile actions (64). PGE_2 activates a pleiotropic effect on signal transduction and the actual cellular effects exerted depend on the receptor subtypes expressed. PGE_2 is a major COX product in a number of physiological settings. It was first isolated and its structure determined in the 1960s (65). Thereafter, this prostaglandin was found in many different tissues. PGE_2 plays a protective role in maintaining the integrity of the gastric mucosa (66). The production of PGE_2 in the kidney is critical for normal renal function by preserving renal blood flow and glomerular filtration rate in settings of physiological stress, modulating salt and water transport in the distal tubule, and stimulating renin release from the juxtaglomerular apparatus (67). PGE_2 was also shown to play a role in the maintenance of blood pressure, particularly in the setting of salt overload (68). In certain instances,

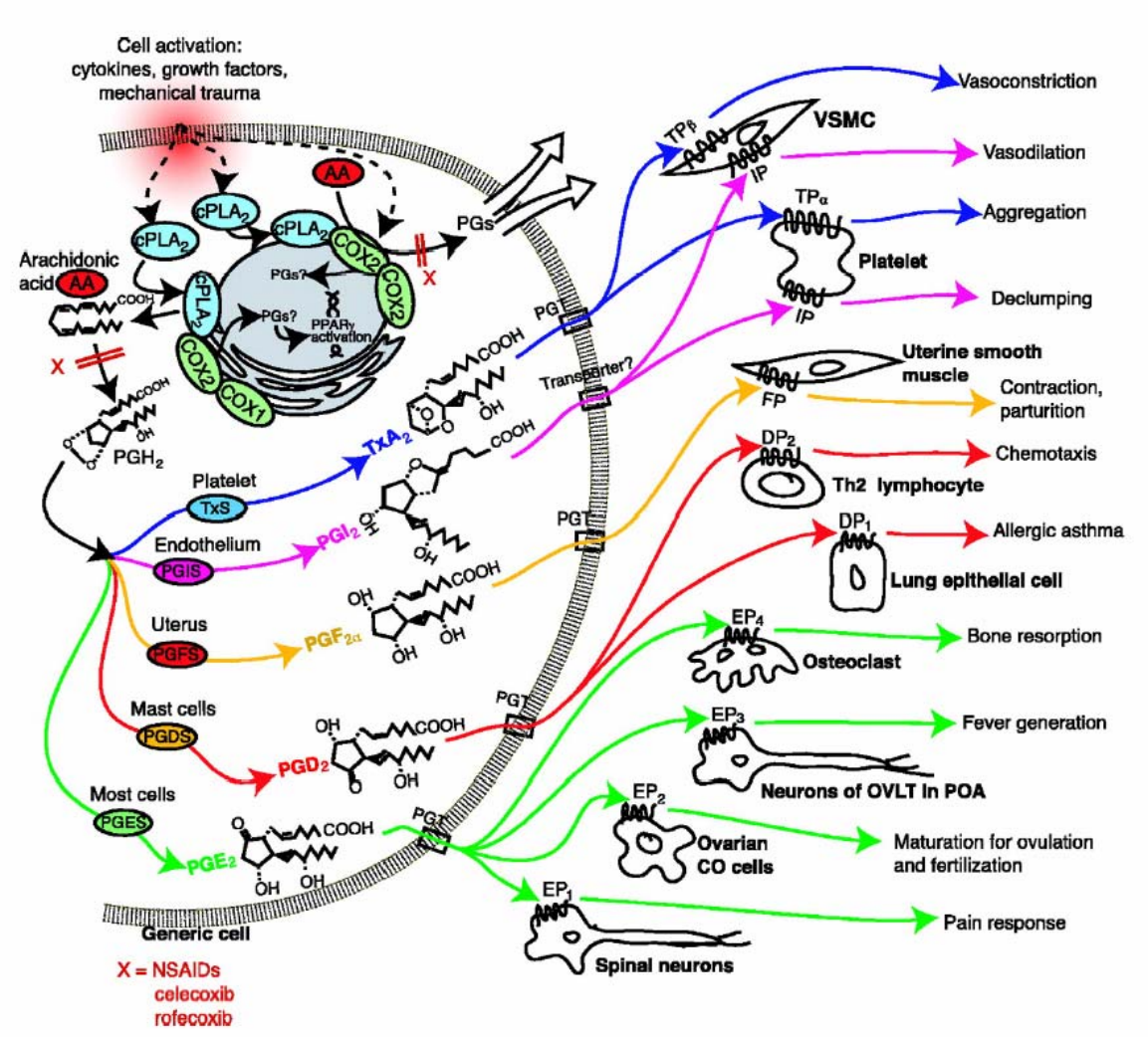

Figure 2. Prostaglandin actions and synthesis. Mechanical trauma, cytokines, growth factors, or various inflammatory stimuli activate cells, triggering signalling, including cytosolic phospholipase ($cPLA_2$) translocation to endoplasmatic reticulum and nuclear membranes, release of arachidonic acid from membrane lipids and production of PGH_2 intermediate by COX-2 or COX-1. Heterogeneous family of PGH_2 metabolizing enzymes can form PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 (prostacyclin) and TxA_2 (thromboxane). These prostaglandins may pass cell through a known prostaglandin transporter (PGT) to exert their actions on a family of prostaglandin receptors named EP1, EP2, EP3, EP4, DP1, DP2, FP, IP, TPα and TPβ. Adapted from (61).

PGE_2 was observed to have multiple and apparently opposing functional effects. For example, PGE_2 elicits both smooth muscle relaxation and constriction (69, 70). Complexity was also observed in modulation of the immune response by PGE_2; it was shown that PGE_2 regulates the function of many cell types including macrophages, dendritic cells, T and B lymphocytes leading to both pro- and anti-inflammatory effects (71). PGE_2 signalling promotes tumor angiogenesis (72), increases cell proliferation and stimulates oncogenesis (73).

PGE_2 and cancer

COX-2-derived PGE_2 is a pro-inflammatory bioactive lipid and the major prostaglandin produced in many human solid tumors (74-76). Among the various downstream prostaglandins, PGE_2 has long been suggested as the key player. TxA_2, which has been shown to promote angiogenesis, is the only other prostaglandin implicated in oncogenesis (77). PGE_2 mediates essential roles in tumor progression, such as tumor cell proliferation, invasion, angiogenesis, and immunosuppression. The stimulatory effect of PGE_2 was first observed in dog kidney cells. These cells required PGE_2 or PGE_1 for growth in serum-free media (78, 79). Several studies since have provided direct evidence in regards to the role of PGE_2 in promoting tumor growth. For example, PGE_2 was shown to reverse NSAID-induced adenoma regression (80) and accelerate intestinal adenoma growth in *Min* mice (81).

While COX-2 selective NSAIDs are thought to inhibit all COX-2-mediated prostaglandin production, it is predominantly PGE_2 that is thought to be responsible for

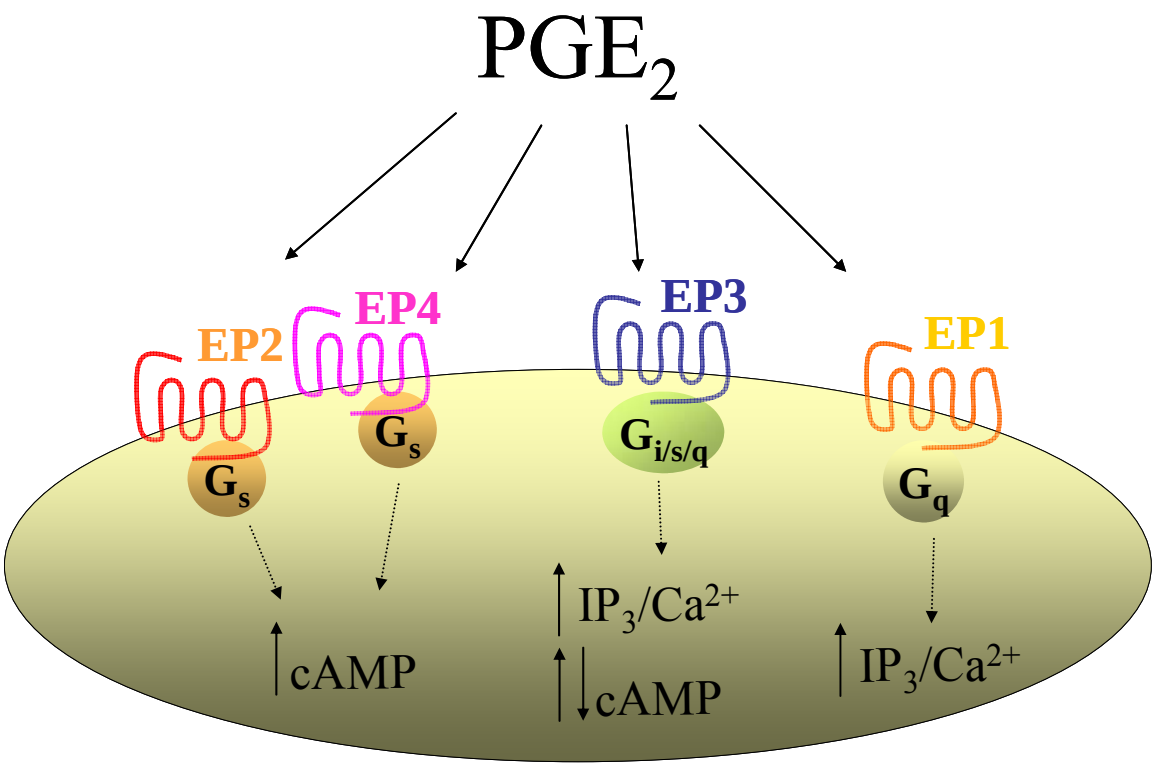

Figure 3. Signal transduction of prostaglandin E_2 receptors. PGE_2 is actively transported out of the cell, where it exerts its effect by binding to its seven transmembrane G-protein coupled receptors, EP1, EP2, EP3 and EP4 to activate second messengers, such as cAMP and inositol (1,4,5)-trisphosphate (IP_3), and intracellular signalling cascades.

promoting colorectal tumorigenesis, with elevated levels reported in benign and malignant human and rodent colorectal tumors *in vivo* (74, 82, 83). There is a body of evidence, beyond the association of a cancer phenotype and increased levels of PGE_2, which suggests that PGE_2 also contributes to the development and progression of colorectal cancer. The level of PGE_2 increases in a size-dependent manner in colorectal adenomas from FAP patients (84). PGE_2 can induce angiogenesis *in vitro* and increases cellular resistance to apoptosis (85), enhancing the survival and motility (86, 87) in colon cancer. Immune surveillance is also inhibited by PGE_2 (88). Cumulative evidence indicated that COX-2-derived PGE_2 provides growth advantage to colorectal carcinomas through transactivation of the epidermal growth factor receptor (EGFR) signalling system (73, 87). Furthermore, PGE_2 exposure induces the expression of VEGF (vascular endothelial growth factor) in colon cancer cells (89). Studies with experimental animals also suggest that PGE_2 promotes tumorigenesis. For instance, treatment with an anti-PGE_2 monoclonal antibody retards the growth of a transplantable tumor *in vivo* (90).

The side effects associated with long term COX-2 inhibition are predominantly due to the fact that as well as inhibiting the production and signalling of pro-tumorigenic PGE_2, <u>all</u> inducible prostaglandin and thromboxane production is inhibited. So while blocking PGE_2 synthesis along with all other prostanoids with NSAIDs confers a protective effect, inactivating PGE_2 *via* its catabolism by prostaglandin dehydrogenase (PGDH) provides a promising COX-2 independent method of targeting PGE_2 in order to inhibit cancer progression. The steady-state cellular levels of PGE_2 depend on the relative rate of synthesis by COX-2/PGE synthases and its degradation by PGDH.

Genetic deletion of PGDH in mice leads to increased tissue levels of PGE_2 (91). Loss of PGDH expression correlates with tumor formation in several types of cancers (92-95). When a subset of colorectal carcinomas were analyzed, PGDH was observed down-regulated (92) and further studies showed EGF is responsible for repressing PGDH by inducing the transcriptional repressor Snail, which binds to the PGDH promoter (96). Interestingly, NSAIDs have been shown to upregulate PGDH expression in colorectal cancer (92). Collectively, these studies suggest that by decreasing PGE_2 levels, PGDH serves a novel tumor suppressive role due to the loss of expression during tumor progression.

EP receptors

The diverse effects of PGE_2 on various tissues suggested the existence of multiple transmembrane receptor subtypes, which was confirmed by molecular cloning. These receptors were named EP1, EP2, EP3, and EP4, which are all products of separate genes and yet highly conserved among mammalian species (97). For example, the sequence homology between human and mouse orthologues for the EP1, EP3 and EP4 is 84%, 84% and 88%, respectively (98). Although the four cloned EP receptor subtypes bind PGE_2 with higher affinity than other prostanoids, they are not closely related when compared to other prostanoid receptors based on amino acid homology. Moreover, stimulation by PGE_2 leads to activation of different G-proteins leading to various intracellular signal transduction pathways depending of the EP receptor subtype engaged (**Figure 3**). Synthesized PGE_2 can be released through a prostaglandin transporter (PGT) (99) out of the cell and then bind to receptors in the vicinity of the site of PGE_2

production. Of the four known EP receptors, EP3 and EP4 receptors bind PGE_2 with the highest affinity (K_d ~ 1 nM), whereas EP1 and EP2 receptors bind with lower affinity (K_d > 10 nM) (100). The EP1 receptor mediates PGE_2-induced elevation of free Ca^{2+} concentration, whereas the EP2 and EP4 receptors are coupled to G_s G-protein and their activation leads to an increase in cyclic AMP (cAMP) levels. The signalling pathway of the EP3 receptor mediates inhibition of adenylate cyclase via G_i (**Figure 3**) (98).

Most prostanoid receptors are G protein–coupled rhodopsin-type receptors with seven transmembrane domains, and each is encoded by different genes. In addition, there are several splice variants of the EP3 receptor, which differ only in their intracellular C-terminal tails. Prostanoid receptors are generally expressed on the cell surface plasma membrane; although recent data suggests some EP receptor subtypes are also associated with the nuclear membrane (101, 102). In particular, endogenous EP1 receptor has been shown to have perinuclear localization in porcine cerebral microvascular endothelial cells, as well as cloned EP1 in Swiss 3T3 fibroblasts (101). EP3 and EP4 have also been suggested to be functionally expressed in the nuclear membranes of neonatal porcine brain and adult rat liver cells (102). Among the four subtypes EP2 and EP4 receptors mediate a cAMP rise and have been termed "relaxant" receptors, whereas the EP1 receptor induces calcium mobilization and constitutes as a "contractile" receptor. The remaining receptor, EP3, induces a decline in cAMP levels and has been termed the "inhibitory" receptor (**Figure 3**). However, these basic classifications do not always apply as the G-protein activated by a specific receptor may differ between cell types. For instance, various G_s-coupled EP receptors may activate G_q in specific cell types.

In the 1990s the functional cDNAs for each EP subtype was isolated and expression of recombinant receptors in mammalian cells allowed for the characterization of their structural and biochemical properties (103-106). In addition, the tissue and cell distribution of the receptor subtypes was studied and correlated with pharmacological studies using COX inhibitors and various PGE_2 analogs having agonistic and antagonistic effects allowing us to define the actions of each subtype. Lastly, targeted disruption of the EP receptor genes has enabled us to examine the physiological and pathophysiological importance of the action of PGE_2 mediated by each EP receptor.

Structure of EP receptors

The EP receptor subtypes have seven hydrophobic putative transmembrane domains, and thus belong to a rhodopsin-type receptor superfamily. Although the four EP receptor subtypes respond to PGE_2, the amino acid homology among EPs is quite limited making it difficult to use sequence comparison among subtypes in order to learn about ligand binding specificity from simply comparing amino acid sequences (**Figure 4**). The mouse EP1, EP2, EP3 and EP4 consist of 405, 362, 366 and 513 amino acids, respectively. The homology of EP1 to EP2, EP3 and EP4 is 30, 33 and 28%, respectively. Even between the two adenylate cyclase-stimulatory subtypes, EP2 and EP4, the amino acid identity is only 31%. The EP2 receptor shares more homology to the other two relaxant receptors IP (40%) and DP (44%) than any other EP. Similarly, the EP1 is more homologous to the other two contractile types of prostanoid receptors, TP

Figure 4. Amino acid sequence alignment for mouse EP receptor subtypes and EP3 variants. Amino acid identities are indicated by *shading*; predicted transmembrane domains are shown with *overlining* and gaps are indicated with *dashes (64)*.

(34%) and FP (35%). The limited homology shared among EPs probably reflects the phylogenetic relationship among prostanoid receptors (64, 107).

Structurally, the EP4 receptor subtype has the longest intracellular C-terminal tail and a long intracellular third loop. The EP1 receptor subtype also has a long third loop while the EP2 and EP3 receptor subtypes have more compact structures. The EP3 receptor is unique from other EP receptors in that it exists as multiple variants generated by alternative splicing. These variants differ only in the carboxy-terminal tails and are capable of inducing a broad range of effects. These variants show similar ligand binding properties but activate different signal transduction pathways further described below.

Properties of EP receptors

Each of the four subtypes of EP receptors display a selective ligand-binding specificity that distinguishes it from one another. In fact, each was identified by their preferential responsiveness to native ligand and subsequently to synthetic PGE_2 analogs synthesized to selectively mimic or inhibit specific effects. For instance, using select regions of the GI tract from farm animals, the EP receptors were first characterized since PGE_2 had the most potent agonistic effect of all prostanoids (108). They were then further subdivided by their sensitivity to anatagonists, for instance EP1 to SC-19220, and EP2 from EP3 on the basis of its different sensitivities to 11-deoxy-PGE_0 and sulprostone (109). Lastly, EP4 was first discovered to have relaxant effects on smooth muscle like EP2 however it differed from EP2 as it displayed weak potency to AH13205 and its antagonism by AH23848 (110). These studies while only qualitative have proven to be

both valid and successful at characterizing the EP receptor subtypes. Bioassay tissues however have several receptor types and therefore the outcome observed by each compound tested demonstrates the sum total effect of all receptors present in the tissue. Important to note were the differences in the degree of responsiveness and the efficacy of actions in different tissues and species.

Cloning of the EP subtypes has allowed homogeneous expression of each subtype from the same species to be evaluated for ligand-binding properties, signal transduction properties as well as the cross-reactivity of compounds for several types of receptors. The cDNA for the mouse EP1 receptor was isolated from the mouse kidney library (106), and its human homologue was subsequently isolated from a human erythroleukemia cell (111). Although sulprostone was first identified as a potent EP1 receptor agonist, it is more potent as an EP3 agonist (112). To date, there is one reported example of a highly selective EP1 receptor agonist, ONO-D1-004. Well-known antagonists for the EP1 receptors are SC-19220 and AH6809 (110).

Initially two cloned EP receptor subtypes, positively coupled to adenylate cyclase, were reported as EP2 (103, 113). The EP2 receptor is pharmacologically defined as being sensitive to butaprost. Butaprost is highly selective for this receptor as it only shows affinity to EP2. Later on, it was shown that the first EP2 subtype cloned was actually EP4 and the latter represents the EP2 subtype. The EP2 subtype can be distinguished from EP4 by its insensitivity to the EP4 agonist PGE_1-OH and insensitivity to the weak EP4 antagonist AH23848. Furthermore, the EP4 receptor undergoes rapid

desensitization after agonist stimulation, whereas EP2 does not (**Table 1**). The mouse EP3 receptor binds to the following EP ligands with an order of affinity of sulprostone, MB28767, PGE_2 PGE_1-OH, 11-deoxy-PGE_1, GR63799X, 16,16-dimethyl-PGE_2, 17-pheynyl-PGE_2 > misoprostol (98). Although EP ligands such as sulprostone and MB28767 bind other receptors, they show the highest affinities for the EP3 receptor. GR63799X, on the other hand, has a very high affinity to only the EP3 receptor subtype indicating it is a highly selective agonist for this receptor. Interestingly, although sulprostone and PGE_2 show equal potency for EP3-G_i activation, sulprostone potency to EP3-G_s is 10 times weaker than PGE_2. Since sulprostone demonstrates a lower affinity to G_s coupled EP3 compared to G_i suggests the ligand-binding properties of prostanoid receptors can depend on the G-protein coupled to the receptor.

PGE_2 can cause the stimulation or suppression of neurotransmitter release, the inhibition or stimulation of sodium and water reabsorption in the kidneys; these opposite actions of PGE_2 are due to the receptor subtypes being coupled to a variety of signal transduction pathways (**Table 1**). Activation of the human EP1 receptor is most likely mediated by G_q activation and leads to IP_3 generation and increased intracellular calcium. Narumiya et. al. suggests however that the increase is not G_q mediated since agonist stimulation in the mouse EP1 causes a strong increase in calcium but only a modest increase in IP_3 (106). The EP2 receptor subtype activates G_s, which in turn stimulates adenylate cyclase and leads to the production of the second messenger cyclic AMP (cAMP) (114) and thus activation of the cAMP-dependent protein kinase termed PKA. Although EP2 and EP4 couple to increases in cAMP and in some processes function

redundantly, there are processes in which EP2 and EP4 play distinct roles. For instance, EP4 selectively couples to PI3K and subsequently activates extracellular signal-regulated kinases (ERK) 1 and 2 (115, 116). The EP3 receptor was originally identified as a smooth muscle constrictor (110) and multiple spliced variants exist defined by their unique C-terminal tails. Multiple spice variants may explain the differences in receptor phosphorylation, receptor desensitization and variations in signal transduction pathways activated by the EP3 receptor. While EP2 and EP4 stimulate adenylate cyclase and generate cAMP, the EP3 receptor generally inhibits cAMP production via activation of the pertussis-toxin sensitive G-protein G_i (117). It is important to note however that splice variants have been described to couple to different signalling pathways. For instance, the EP3 receptor has been shown to signal through the small GTP-binding protein Rho to induce neurite retraction *via* a tyrphostin A25-sensitive tyrosine kinase upstream of Rho (118) and the p160 RhoA-binding kinase ROKα downstream of Rho (119) as well as induce stress fiber formation in MDCK cells *via* Rho (120).

Next, besides differences in actions dependent on the G-proteins to which they are coupled to, the EP receptor subtypes may also vary with respect to the tissue distribution of their expression.

Distribution of EP receptors

Northern blot and *in situ* hybridization analyses have revealed detailed information on the EP subtype distribution in the body and the levels of expression among tissues. The majority of studies on EP receptors have investigated expression at the mRNA level by reverse transcription-PCR (RT-PCR) or *in situ* hybridization due to

TABLE 1.

Subtype	Variant	Amino Acid	Gprotein	Signaling	Desensitization	Constitutive activity
EP2		362	G_s	cAMP↑	No	
EP4		513	G_s	cAMP↑, PI3K	Yes	
EP1		405	G_q ?	Ca_{2+}↑	-	
EP3	EP3α	366	G_i, G_{12}	cAMP↓, IP_3/Ca_{2+}↑, Rho	Yes	++
	EP3β	363	G_i, G_{12}	cAMP↓, IP_3/Ca_{2+}↑, Rho cAMP↓, cAMP↑	No	-
	EP3γ	365	G_i, G_s	IP_3/Ca_{2+}↑, Rho	-	+

Signal transduction properties of EP receptor subtypes and EP3 variants. Data obtained from mouse EP subtypes are summarized, and representative signal transduction pathways for each receptor are shown. PI3K, phosphatidylinositol 3-kinase; ↑, increase; ↓, decrease (64).

the lack of well-characterized antibodies to EP receptors. Among the prostanoid receptors, EP3 and EP4 receptors are the most widely distributed throughout the body. In contrast, the distribution of EP1 is restricted to several organs, such as the kidney, lung, and stomach, and EP2 is the least abundant of the EP receptor subtypes. EP1 receptors mediate the contraction of smooth muscle in various tissues including the gastrointestinal tract, respiratory tract, vas deferens, myometrium and iris sphincter muscle. EP1 receptor mRNA is expressed most highly in the kidney, followed by gastric muscularis mucosae and then adrenal tissue (121). The precise tissue distribution of the EP2 receptor has been revealed to be most abundant in the uterus and only low levels of expression in the kidney (121). Although EP2 mRNA is the least abundant among the EP receptor subtypes, it is effectively induced in response to stimuli and does not undergo desensitization. EP4 receptor mRNA is highly expressed compared to the EP2 receptor and widely distributed in such organs as the thymus, ileum, lung, spleen, adrenal, and kidneys (122). EP4 receptor activation has been shown in venous and arterial beds to have important vasodilator effects (110). When examined in the mouse gastrointestinal tract, EP4 mRNA is highly expressed in the gland of the gastric antrum suggesting this subtype is involved in PGE_2-mediated mucus secretion. Additionally, EP4 is found in epithelial cells of the intestinal villi (123) and since EP4 increases cAMP and PGE_2 stimulates chloride secretion and inhibits salt absorption *via* production of cAMP, the above findings suggest that EP4 is involved in these processes and consequently PGE_2-induced diarrhea (124). Nuclease protection and northern analysis demonstrated relatively high levels of EP3 receptor expression in several tissues including kidney, uterus, adrenal gland, and stomach tissues. EP3 mRNA was most abundantly expressed

in the brain and is widely distributed over the central nervous system (125). In the mouse gastrointestinal tract EP3 mRNA is expressed in smooth muscle cells in the longitudinal but not circular muscle layer as well as enteric nervous system indicating it may regulate smooth muscle contraction both directly and indirectly (123). In addition, the expression of EP3 mRNA was also found in fundic gland epithelial cells, both parietal and chief cells, suggesting its involvement in acid secretion.

Genetic deletion of the EP receptors

Understanding of the pharmacology and physiologic roles of each of the EP receptors has been enhanced greatly by derivation of individual EP receptor "knockout" mouse models (**Table 2**). Experiments using EP1 deficient mice showed PGE_2 acts through EP1 to control impulsive behavior under stress (126), a finding potentially exploitable for development of drugs that attenuate impulsive behavior in humans. Recently the role of the EP1 receptor in modulating urine concentrations was investigated since it is expressed along the renal collecting duct where arginine-vasopressin (AVP) exerts its anti-diuretic activity and in the paraventricular and supraoptic nuclei of the hypothalamus where AVP is synthesized. Data suggests that PGE_2 modulates urine concentration by acting on EP1 receptors, not in the collecting duct, but within the hypothalamus to promote AVP synthesis in response to acute water deprivation (127). The EP1 receptor has also been shown to play a direct role in mediating algesia and in regulating blood pressure (128). Systolic blood pressure is significantly reduced in EP1 receptor–deficient mice and is accompanied by increased renin-angiotensin activity suggesting a role for this receptor in cardiovascular homeostasis.

Functional studies suggest the EP2 receptor plays an important role in uterine implantation (68, 129) and a relaxant role in bronchioles and the vasculature. In addition, recent studies have demonstrated that targeted disruption of the EP2 receptor interferes with fertility and results in salt-sensitive hypertension (68, 129, 130). For instance, upon PGE_2 or butaprost administration intravenously, only wildtype but not EP2 -/- mice became hypotensive. Surprisingly, EP2 -/- mice became hypertensive with PGE_2 indicating the absence of EP2 abolishes the ability of the vasculature to dilate in response to PGE_2 and unmasks the contractile response *via* vasoconstrictor EP receptor(s) (68). In addition, PGE_2-EP2 signaling is also reported to contribute to the spinal inflammatory hyperalgesia in the zymosan A peripheral inflammation model (131). Yeast extract injected subcutaneously into the paw induces inflammatory hyperalgesia. Although thermal and mechanical sensitization in EP2 -/- mice is similar to wildtype mice at 2 hours post injection, from 4 hours onward EP2 -/- mice recovered faster from hyperalgesia. These results suggest a dominant role for PGE_2-EP2 signaling in the generation of inflammatory pain. COX-2 deficient mice have been shown to have reproductive failures in early pregnancy such as in ovulation, fertilization, implantation, etc. suggesting that prostaglandins play a crucial role. Kennedy et. al. found EP2 -/- female mice have consistently fewer pups than wildtype and detected slightly impaired ovulation and a reduction in fertilization concluding the failure in COX-2 deficient mice is due to dysfunction of the EP2 receptor (68). Whether polymorphisms in the EP2 receptor are associated with infertility and/or hypertension in humans remains to be determined.

TABLE 2

Genotypes	Phenotypes
EP1 (−/−)	Defect in concentrating urine
EP2 (−/−)	Impaired ovulation and fertilization
	Salt-sensitive hypertension
	Faster recovery from hyperalgesia
	Vasopressor or impaired vasodepressor response to intravenous PGE_2
	Loss of bronchodilation with PGE_2
	Impaired osteoclastogenesis in vitro
EP3 (−/−)	Impaired febrile response to pyrogens
	Impaired duodenal bicarbonate secretion and mucosal integrity
	Enhanced vasodepressor response to intravenous infusion of PGE_2
	Disappearance of indomethacin-sensitive urine diluting function
EP4 (−/−)	Patent ductus arteriosus
	Impaired vasodepressor response to intravenous infusion of PGE_2
	Decreased inflammation-dependent bone resorption

Major phenotypes of EP receptor knockout mice (132).

PGE_2 is known to have bone-resorptive activity and to mediate bone resorption induced by cytokines and LPS. Several groups found impaired osteoclast formation in cells cultured from EP2- and EP4-deficient mice (133-135). This likely reflects the redundant roles of the two relaxant EP receptor subtypes. In addition to inducing bone resorption, exogenous PGE_2 can also induce bone formation, although the receptor mediating this latter response has not been identified.

Mice with targeted disruption of the EP4 receptor gene have shown EP4 to play a particular role in regulating the peri-natal closure of the pulmonary ductus arteriosus (136, 137). Disruption of the EP4 gene results in death of most homozygous EP4 -/- neonates within 3 days of birth due to pulmonary congestion and heart failure (136, 137). As noted earlier, EP4 mRNA is present in the ductus and works in the dilation of the vessel. EP4 -/- neonates showed full patency of the ductus after birth indicating a critical role of EP4 in the ductus. The EP4 receptor ligands may prove useful in promoting closure or maintaining patency of the ductus arteriosus in newborns with congenital heart disease. Additionally, EP4 deficient mice have shown that PGE_2-EP4 signalling facilitates initiation of skin immune responses by promoting the migration and maturation of Langerhans cells. While PGE_2 is produced substantially in skin exposed to antigen, its role was unclear. Although Langerhans cells express all four EP subtypes, their migration to regional lymph nodes was decreased only in EP4-deficient mice and in wildtype mice treated with an EP4 antagonist (138). Lastly, EP4 deficiency impaired mucosal barrier function and induced epithelial loss, crypt damage, and aggregation of neutrophils and lymphocytes in the colon. Among the eight prostanoid receptor-deficient mice tested,

only EP4-deficient mice and not mice deficient in either DP, EP1, EP2, EP3, FP, IP, or TP developed severe colitis with 3% dextran sodium sulfate treatment, which induced only marginal colitis in wildtype mice. These studies conclude EP4 maintains intestinal homeostasis by keeping mucosal integrity and downregulating immune response (139).

Mice with targeted deletion of the EP3 receptor exhibit an impaired febrile response to PGE_2, suggesting that EP3 receptor antagonists could be effective antipyretic agents (140). EP3 deficient mice were also used to determine the role of PGE_2 in pain perception. EP3 and IP were found to be the major prostaglandin receptors mediating the enhanced pain response in mice pre-treated with LPS to induce COX-2 expression (141). Additionally, EP3 deficient mice were found to have impaired duodenal bicarbonate secretion and mucosal integrity. The presence of EP3 receptors were found to be essential for maintaining duodenal acid secretion and maintaining the mucosal integrity against luminal acid (142). EP3 deficient mice also demonstrated the PGE_2 -EP3 pathway is an important negative modulator of allergic reactions. Only EP3 deficient mice were shown to develop an allergic inflammation that was much more pronounced than that in wildtype mice or mice deficient in other EP receptor subtypes. Conversely, an EP3-selective agonist suppressed the inflammation (126). A recent study has shown the roles of PGE_2 signaling in metabolic regulation have expanded beyond the reported stimulation of leptin release from adipose tissue to also involve actions mediated by the EP3 receptor in the regulation of sleep architecture and feeding behavior (143). EP3 deficient mice exhibit increased frequency of feeding during the light cycle of the day and develop an obese phenotype under a normal fat diet fed *ad libitum*. Although EP3

deficient mice showed an increased motor activity, this did not compensate sufficiently to offset the increased body weight. These findings further indicate there is a link between inflammatory signaling and obesity.

Collectively studies using mice deficient in each EP subtype and subtype-specific EP agonists/antagonists have shown interactions between PGE_2 and its receptor subtypes to be dependent on tissue/cell type and location specific receptor expression, variation in PGE_2 binding affinities and hence differential receptor activation (144, 145). These variables therefore allow PGE_2 the ability to mediate highly varied effects on cell biology in many different tissue types and in some cases diseases such as cancer.

EP receptors and cancer

Overexpression of COX-2 and the upregulation of PGE_2 production is a common finding in many cancers of epithelial origin. Because PGE_2 exerts its effects by signalling through the EP receptors, the activation of specific EP receptors contributes to the importance of PGE_2 signalling in the development of several types of cancers. For instance, EP1, EP2 and EP4 have been implicated to play a role in breast cancer. Recent reports have demonstrated a possible link between EP4 and the formation of breast cancer metastasis (146). Firstly, all four EP subtypes were found expressed in three murine mammary tumor cell lines. Next it was observed that EP4 antagonists were able to reduce tumor metastasis *in vivo*, however antagonists to EP1, EP2 and EP4 could reduce PGE_2-induced migration *in vitro* suggesting selective inhibition of these EP subtypes can reduce the metastatic potential of breast cancer cells. EP3 antagonism had

no effect on tumor metastasis (146). Similar to EP4, the lack of EP2 in *MMTV-COX-2* transgenic mice strongly suppressed COX-2–induced effects such as precocious development of the mammary gland in virgins and the development of mammary hyperplasia in multiparous female mice suggesting disruption of EP2 signalling as a chemopreventive approach to breast cancer (147). EP1 antagonists may also possess chemopreventive effects through the induction of apoptosis without any side effects (148). Administration of an EP1 antagonist significantly decreased carcinogen-induced breast cancer incidence, multiplicity and volume compared with control animals. EP1 expression was present in breast cancer but not in normal tissue. Breast cancer from mice fed the EP1 antagonist showed significantly increased apoptosis suggesting selective disruption of PGE_2-EP1 signalling as an alternative approach to the use of COX inhibitors (148).

Upregulation of COX-2 activity and PGE_2 production is also a common event that occurs during the formation and progression of skin cancer (149). The EP2 receptor was shown to play a significant role in the protumorigenic action of PGE_2 in skin tumor development. Unlike EP3 knockout mice, the EP2 knockout mice produced significantly fewer tumors and reduced tumor incidence compared with wildtype mice in a two-stage skin carcinogenesis model (150). Furthermore, UV-irradiated mice lacking EP2 receptors exhibit decreased proliferation and a poor capacity for epidermal hypertrophy in response to UV injury. Interestingly, in a chronic irradiation model, these animals were protected from tumor formation, developing 50% fewer tumors than wildtype controls. Despite this capacity to protect against tumorigenesis, animals lacking EP2

receptors grew tumors that were larger in size, with a more aggressive phenotype. This was found to be associated with greater quantities of active matrix metalloproteinases than keratinocytes expressing the EP2 receptor, thereby enhancing the invasive potential of EP2-/- cells (151). Drugs designed to block EP1 may also have the potential to be used as a chemopreventive agent that reduces the risk of skin cancer development. Topical treatment with celecoxib or the specific EP1antagonist ONO-8713 decreased inflammation in the skin and significantly reduced the number of tumors after UVB exposure and topical treatment (152).

PGE_2 significantly enhances cell proliferation and reduces apoptosis in colon cancers induced by AOM. PGE_2 increased the incidence and multiplicity of AOM-induced colon tumors in rats (153). All four EP receptors were detected in the tumors and paired mucosa although EP3 to a lesser extent in the cancers compared to mucosa (153). Genes for each of these receptors have been disrupted and corresponding knock-out mice produced (129, 136, 140). EP1, EP2 and EP4 have been implicated to play a role in intestinal tumorigenesis using different animal models. Mice lacking EP4 were partially resistant to AOM-induced aberrant crypt foci (ACF,) putative precursors of colon cancer, while EP3 deficient mice showed no effect (154, 155). Either pharmacological or genetic inhibition of the EP1 receptor results in a significant reduction in colon cancer development, indicating a role for this receptor in colon carcinogenesis (155). Furthermore, one study showed EP2 disruption decreases the size and number of intestinal polyps in $APC^{\Delta 716}$ mice and that EP2-generated cAMP stimulates expression of more COX-2 and vascular endothelial growth factor (VEGF) in

the polyp stroma (156). Interestingly, genetic deletion or inhibition of an EP receptor has shown consistently a 40-60% decrease in intestinal polyps compared to the 80-90% reduction seen with COX deletions using the same animal models. This suggests more than one EP receptor subtype is involved in these models and/or that other prostaglandins *via* their specific receptor may contribute to the promotion of intestinal tumorigenesis.

As PGE_2 is implicated to have a very important role in tumorigenesis, with EP receptors currently being the only class of prostanoid receptor with a proven link to neoplasia, a further chemotherapeutic approach may be to block PGE_2 mediated signalling events through selective EP receptor antagonism. This may have advantages in allowing some PGE_2 signalling while inhibiting specific events more closely associated with tumorigenesis.

EP3 receptor variants

As noted, in addition to the receptor subtypes encoded by different genes, alternative splicing can create several variants of the EP3 receptor which differ in their carboxy-terminal sequence (**Figure 5**). The mouse EP3 receptor has three variants, EP3α, EP3β, and EPγ (157-159), the bovine EP3 receptor has four variants (158), the rabbit EP3 receptor has five variants (160, 161), and the human EP3 receptor has eight variants (162). G_i activation mediated by the mouse EP3 receptor variants has been well investigated.

The mouse EP3 receptor splice variants display identical agonist binding properties, but are functionally different in their efficiency of G protein activation, the specificity of G protein coupling and sensitivity to agonist-induced desensitization. For instance, mouse EP3α and EP3β couple to G_i, but three orders lower concentration of agonists were required for EP3α than EP3β for the activation of the G_i protein. EPα undergoes both short and long term agonist-induced desensitization, whereas EP3β undergoes neither short nor long term desensitization. With regards to their agonist-dependency for G_i activation, the mouse EP3α and EP3γ variants have partially constitutive G_i activity, but the EP3β variant has no constitutive G_i activity (163, 164).

A truncated mutant at the splice site of EP3 was generated in order to determine the role of the C-terminal tail (164). The mutant receptor retained the ability to couple to G_i and showed fully constitutive G_i activity. These results indicate that the C-terminal tail of the EP3 receptor is essential for the regulation of agonist-dependent activation of G protein.

Despite the vast amount of research performed on EP receptors, little is known on the pathological effects of the EP3 splice variants and their signal transduction pathways. The EP3 receptor is the one EP receptor that has consistently *not* been directly involved using intestinal tumorigenesis animal models. Examination of mRNA expression levels in colon tissue of mice, rats and human by RT-PCR and quantitative RT-PCR shows a marked reduction in the EP3 receptor in colon cancer compared to normal mucosa. In contrast EP1 and EP2 are found increased in colon cancers (153, 165). Expression of

EP3 mRNA was detected in only one of eleven colon cancer cell lines tested and treatment of that one cell line with an EP3 agonist decreased viable cell numbers by 30%. Immunohistochemical staining demonstrated rat EP3 protein expressed in normal epithelium and some parts of small carcinomas but absent in large carcinomas of the colon, suggesting EP3 is downregulated during colon cancer progression. While deficiency in EP3 had no effect on ACF development, an increase in AOM-induced colon carcinoma was found in EP3 knockout mice suggesting EP3 may not impact early stages as greatly as later stages of colon carcinogenesis. As noted, EP2 and EP4 stimulate adenylate cyclase whereas EP3 inhibits cAMP formation, suggesting a possible suppressive role against colon carcinogenesis. In addition to CRC, EP1, EP2 and EP4 receptors were elevated, whereas EP3 receptor levels were decreased in mammary tumors from *COX-2/MMTV* mice (166). In contrast to these findings, a recent study reported EP3 receptor signaling to play an important role in tumor-associated angiogenesis (167). In this context, implanted tumor growth (sarcoma-180, Lewis lung carcinoma) and tumor-associated angiogenesis were markedly reduced in EP3 null mice. Moreover, EP3 antagonists reduced tumor size in wildtype but had no effect on EP3 null mice. Using the sponge implantation model, it was shown that VEGF expression is significantly reduced in EP3 null mice compared to wildtype mice. The inconsistency between models highlights the effects of PGE_2 on carcinogenesis ultimately depends on the expression pattern of PGE_2 receptors which is tissue-specific and model-specific. Collectively the inconsistencies of these studies on EP3 prompted the need for further investigation into the role EP3 splice variants and their underlying mechanisms play on tumor cell function.

Aims of Dissertation

The overall aims of this dissertation are:

1) **To characterize the mouse EP3 receptor variants and determine their role on tumor cell function.**

Despite the extensive characterization of the EP3 receptor splice variants in cell culture systems (157-159, 163, 164), the functional significance of these different C-terminal splice variants remains uncertain. The variants are identical over the first 355 amino acids up to the end of the seventh transmembrane helix, but differ in carboxyl tails. The variants may serve different functions and contribute to the diversity of the actions of PGE_2. When expressed in a variety of cell lines, the variants display similar affinity for PGE_2 but differ in coupling to G proteins, agonist-dependent desensitization and in agonist-independent constitutive G_i activity.

To investigate the functional differences among the three mouse EP3 (mEP3) variants on cell function we expressed the myc-tagged mEP3 α, β, and γ in tumor cells and examined downstream signaling pathways activated in these cells utilizing selective EP3 agonists. We focused particularly on calcium mobilization, ERK, Akt and Rho activation, as these pathways have been shown activated upon EP3 stimulation. Furthermore, as prostanoids can induce receptor internalization we determined whether EP3 selective agonists affect the cell surface localization of the mEP3 receptor variants. Finally, since activation of the EP3 receptor has been shown to induce stress fiber formation and cell migration, we analyzed morphological changes in the tumor cells overexpressing the mEP3 receptor variants following agonist stimulation.

2) **To determine the functional role of the mouse EP3 variants on tumor cell growth**.

Several lines of evidence indicate the involvement of EP receptors in tumor progression. The rationale behind this aim is that, although EP1, EP2 and EP4 have been implicated to play a role in several models of tumorigenesis and types of cancer, EP3 has consistently *not* been shown to promote tumorigenesis (146, 150, 153-155). We and others have shown that EP3 expression is downregulated or absent in various tumor cells (165, 166, 168). This suggests the lack of EP3 may confer a growth advantage to tumor cells and restoring EP3 expression may have a growth suppressive role.

To examine the effect of the mEP3 variants on tumor cell growth we overexpressed each variant in human colon cancer cells (HCT116). These human colon cancer cells lack EP3 (165) as well as COX-2 (169) expression, thus allowing us to examine the effects of exogenous EP3 agonists on tumor cell function. We examined populations of mEP3 receptor variant expressing HCT116 for their ability to grow *in vitro* and *in vivo*.

CHAPTER II

MATERIALS AND METHODS

Reagents

Prostaglandins were purchased from Cayman Chemicals (Ann Arbor, MI). MB28767 was a generous gift from Dr. Matthew Breyer (Vanderbilt University), the plasmid of constitutively activated RhoA (G14V) was a gift from Dr. Brent Polk (Vanderbilt University) and the expression plasmid for *Clostridium botulinum* C3 toxin was a gift from Dr. Richard Neubig (University of Michigan). The expression plasmids of constitutively activated and dominant negative G protein subunits α_i, α_{12} and α_{13} were purchased from UMR cDNA Resource Center (University of Missouri, Rolla, MO). [^{3}H]PGE_2 was purchased from Amersham Biosciences (Piscataway, NJ). Pertussis toxin, BAPTA/AM, PD 98059, Y-27632 and wortmannin were purchased from Calbiochem (La Jolla, CA). FBS and DMEM were obtained from Hyclone (Logan, UT). Penicillin/streptomycin, L-glutamine and G418 was purchased from Invitrogen (Carlsbad, CA). Effectene transfection reagent was purchased from Qiagen (Valencia, CA)

Expression of mEP3 receptor variants in HEK293 and HCT 116 cells

The mEP3 variants were subcloned into pcDNA3 (gift from Dr. Ray Harris, Vanderbilt University) using EcoRI and XhoI restriction sites and a myc tag (5'-p CATGGCAGAACAAAAACTTATTTCTGAAGAAGATCTGCATATGG-3') was

added to each at the extracellular N-terminus using KpnI and NheI sites and where the NdeI site is conserved. Cells were maintained at 37ºC in humidified air containing 5% CO_2 in DMEM supplemented with 10% FBS, 2mM L-glutamine, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin. HEK293 cells (gift from Dr. Richard Breyer, Vanderbilt University) and HCT116 cells (gift from Dr. Ray DuBois, Vanderbilt University) were stably transfected with each pcDNA3/mEP3 variant or pcDNA3 using Effectene (Qiagen) and cells expressing mEP3 were selected by addition of medium containing 600 μg ml^{-1} G418 at 48 h post-transfection. Polyclonal populations of each mEP3 variant were sorted for equal expression using flow cytometry.

Flow cytometry

Flow cytometric analysis was performed on vector and mEP3 variant transfected and selected cells harvested with 0.25% trypsin; 1 mM EDTA. Single cells (1×10^6) in normal media were incubated with 1 μg of anti-myc (9E10) from Santa Cruz (sc-40, Santa Cruz, CA) for 30 minutes at room temperature. Cells were washed three times with sterile PBS and incubated with phycoerythrin-conjugated anti-mouse secondary antibody (Jackson Immunoresearch, West Grove, PA) for 30 minutes at room temperature. Cells were washed three times, resuspended in sterile PBS, and analyzed using a FACSAria instrument (BD Biosciences, San Jose, CA). Cells were sorted for equal expression to avoid potential pitfalls related to monoclonal populations.

Preparation of cell membranes

Membranes for binding experiments were harvested once cells were sorted and analyzed for equal expression of each mEP3 variant. Cells were rinsed once with ice-cold PBS containing 1 mM EDTA and lysed by scraping in lysis buffer (15 mM HEPES, pH 7.6, 5 mM EDTA, 5 mM EGTA, and 2 mM phenylmethylsulfonyl fluoride) and passage through a 21-gauge needle five times. To collect membranes, the cell lysate was layered on a 60% sucrose cushion and centrifuged at 150,000*g* for 1 hour at 4°C. The membrane fraction was passed through a 26-gauge needle five times and frozen at –80°C. Protein concentrations were determined by the BCA assay (Pierce).

Radioligand binding assays

For one-point binding experiments, 50 μg membrance protein in the presence of 2 nM of [^{3}H] PGE_2 in the presence or absence of 5 μM sulprostone was incubated at 30°C for 2 h in binding buffer [25 mM HEPES (pH7.4), 1 mM EDTA, 5 mM $MgCl_2$, 140 mM NaCl, 5 mM KCl]. The binding reaction was terminated by the addition of 3 ml of ice-cold binding buffer and rapidly filtered under vacuum over Whatman GF/F filters (Clifton, NJ). Filters were washed three times with 3 ml of ice-cold binding buffer, dried, and counted in 4 ml of CytoScint ES^{TM} liquid scintillation fluid (MP Biomedicals LLC, Solon, OH). For saturation binding experiments 5 μg of membrane protein was incubated in binding buffer for 2 hours at 30°C with various concentrations of [^{3}H] PGE_2. Nonspecific binding was determined in the presence of 5 μM unlabeled PGE_2 and reaction carried out as above. Three independent experiments were performed in triplicates.

Intracellular calcium mobilization assay

HEK293 cells overexpressing mEP3 variants were plated in 96-well plates (50,000 cells/well). Mobilization of intracellular calcium was measured on a FLEXstation system (Molecular Devices, Sunnyvale, CA) using the FLEXstation calcium assay kit according to the manufacturers instructions. Briefly, cells were labeled with calcium assay reagent resuspended in Hanks' balanced salt solution/20 mM HEPES, pH 7.4, for 1 hour at 37°C before measurement. MB28767 was added to parallel wells in a volume equivalent to 10% of the final well volume while fluorescence was monitored, λ_{ex}= 485 nm, λ_{em} = 525 nm. In each case, the experiment was terminated by addition of 10 μM ionomycin to determine maximum Ca^{2+} response. Three independent experiments were performed in triplicates.

Morphology and immunofluorescence analysis

To visualize actin, ZO-1 and mycEP3 variant receptors, cells in normal media were plated at a density of 2 x 10^5 cells into 8-chamber slides (Falcon; Becton Dickinson, Heidelberg, Germany) and cultured 48 hours. Cells were treated for 4 hours with 0.1 μM PGE_2, sulprostone, MB 28767, butaprost or vehicle control then gently washed in PBS, fixed in 4% formaldehyde in PBS for 20 minutes, permeabilized with 0.1% Triton X-100 in PBS for 3 minutes and blocked in PBS/3% BSA for 1 hour at room temperature followed by the addition of rabbit anti-ZO-1 (Zymed Laboratories; 1:100 dilution in PBS/3% BSA) or mouse anti-myc (Santa Cruz; 1:100 dilution in PBS/3% BSA) overnight at 4°C. After two washes in PBS, cells were incubated with rhodamine-phalloidin (Molecular Probes, Eugene, OR; 1:1000 dilution in PBS/3% BSA) and

fluorescein-conjugated anti-rabbit or anti-mouse secondary antibody (Jackson; 1:200 dilution in PBS/3% BSA) in the dark for 1 hour at room temperature. Chamber slides were washed twice in PBS, fixed and mounted using Vectashield mounting medium for fluorescence (Vector Laboratories, Burlingame, CA) and visualized using an epifluorescence microscope (Nikon).

Cell morphology was evaluated whereby 2 x 10^5 cells were plated in normal medium into 24 well plates (Falcon). For replacement experiment, the cell media was treated with 0.1 μM butaprost, MB 28767 or vehicle for 4 hours then replaced with normal media and cells photographed at indicated time points. For the inhibitors study cells were either transfected (Effectene) 48 hours prior to ligand treatment or treated with inhibitors (PD98059 5 μM, Wortmannin 100 nM, IBMX 0.5 mM, Bapta 5 μM, Y-27632 5 μM) overnight prior to treatment with 0.1 μM MB 28767 or vehicle for 4 hours. Cells were gently washed in PBS, fixed in 4% formaldehyde in PBS for 20 minutes and visualized using an inverted microscope (Nikon). Three independent experiments were performed.

Rho GTP pulldown assay

1 x 10^6 serum-starved cells treated with 0.1 μM MB28767 or vehicle for 15 minutes were washed with ice-cold PBS and lysates were prepared using Rho activation assay kit (Upstate Biotechnology, Lake Placid, NY). GTP-bound Rho was precipitated with RBD agarose beads and measured in pellets by Western blot with anti-Rho antibody,

using crude cell extracts for normalization. Densitometry was performed using Labworks Image software (Upland, CA). Three independent experiments were performed.

Western blot analysis

To evaluate the effects of mEP3 activation on ERK, p38, and Akt phosphorylation, semi-confluent HEK cells were serum-starved for 24 hours and then treated with 0.1 μM MB 28767 or vehicle for 15 minutes. The cells were washed with PBS and lysed in cell lysis buffer (#9803; Cell Signaling Technology, Beverly, MA) and centrifuged for 10 minutes at 14,000 rpm. Cell lysates were resolved by SDS/PAGE (10% gels; 30 μg of total protein/lane) and transferred to Immobilon-P membranes. Membranes were incubated with a rabbit antiphospho-ERK, anti-phospho-p38, or antiphospho-Akt antibody (all from Cell Signaling Technology) overnight at 4°C followed by the anti-rabbit HRP-conjugated secondary antibody. Immunoreactive proteins were visualized using Western Ligntning Chemiluminescence reagent plus (PerkinElmer, Boston, MA) according to manufacturers' instructions. Equal loading was verified by stripping the membranes in 50 mM Tris-HCl, pH 6.5, containing 2% SDS and 0.4% β-mercaptoethanol for 1 h at 55 °C, and re-probing with a rabbit anti-p38 (Cell Signaling Technology). Three independent experiments were performed.

To confirm expression of selective G protein alpha subunits, total lysates of cells transfected 48 h prior using Effectene according to manufacturer's instructions were collected, resolved and transferred as described above. Membranes were incubated with

rabbit anti-$G\alpha_{i-2}$ (T-19), anti-$G\alpha_{12}$ (S-20) or anti-$G\alpha_{13}$ (A-20; all from Santa Cruz) overnight at 4°C followed by the anti-rabbit HRP-conjugated secondary antibody. Equal loading was verified by probing with rabbit anti-FAK (C-20; Santa Cruz). Three independent experiments were performed.

Cell Proliferation

Cell proliferation was evaluated by seeding a 96-well plate with 5×10^3 cells/well in DMEM containing 2.5% FCS and left to incubate overnight at 37°C in 5% CO_2. Cells were pulsed for 48 h with [^{3}H] thymidine (1 μCi/well) with or without 0.1 μM of PGE_2, MB-28767, sulprostone or butaprost. For Rho inhibitor study, 10 μM of Y-27632 was added to appropriate wells. The cells were then washed three times with PBS, lysed in 2% SDS (100-μl final volume), and the lysates measured with a beta counter. Four independent experiments were performed in quadruplicates.

Tumor Growth in nude mice

Athymic nude mice were challenged by a single subcutaneous injection with 0.2 ml of 1×10^6 trypsinized cells washed three times in PBS. The growth of tumors was evaluated twice weekly and all mice were sacrificed at 35 days. All mice were anesthetized and euthanized by Institutional Animal Care and Use Committee approved procedures. All tumor tissue was harvested, measured, weighed, embedded in ornithine carbamyl transferase and frozen on dry ice to prepare frozen sections. 15 mice were used in our HEK293 cell studies and 14 mice were used in our HCT116 cell studies. Volume was determined according to the equation $V = [L \times W^2] \times 0.5$, where V = volume, L = length, and W = width.

Statistical analysis

The student's t test was used for comparisons between two groups, and analysis of variance using Sigma-Stat software for statistical difference between multiple groups. $p<0.05$ was considered statistically significant.

CHAPTER III

CHARACTERIZATION OF THE MURINE EP3 RECEPTOR VARIANTS

Introduction

Prostaglandin E_2 exhibits a broad range of biological actions in diverse tissues through its binding to specific transmembrane receptors. PGE_2 receptors are subdivided into four distinct G protein-coupled receptor (GPCR) subtypes designated EP1, EP2, EP3 and EP4. The EP1 receptor couples to phospholipase C, the EP2 and EP4 receptors couple to the stimulation of adenylyl cyclase and the EP3 receptor couples to inhibition of adenylyl cyclase. In mouse, alternative splicing generates three EP3 variants, α, β, and γ, containing carboxyl tails of 30, 26 and 29 amino acids that do not share any structural motifs or hydrophobic features. The variants are identical over the first 355 amino acids up to the end of the seventh transmembrane helix, but differ in carboxyl tails. The variants may serve different functions and contribute to the diversity of the actions of PGE_2 (**Figure 3 and Table 1**).

Although all EP3 splice variants bind PGE_2 and the EP3 selective agonists MB28767 and sulprostone with similar affinity, they are functionally different in their efficiency of G-protein activation, the specificity of G-protein coupling and sensitivity to agonist-induced desensitization. G_i activation mediated by the mouse EP3 receptor variants has been well investigated. For instance, the mouse EP3α and EP3γ variants have constitutive G_i activity, but the EP3β variant has no detectable constitutive G_i

activity (163, 164). While EP3α and EP3β signaling have been shown to inhibit the activation of adenylate cyclase, EP3γ signaling has been shown to have both stimulatory and inhibitory effects of adenylate cyclase(159). Despite extensive studies to elucidate the significance of these spliced tail variants, it is yet unclear their distinct roles on cell function.

Although initially described as coupled to inhibiting cAMP production *via* G_i, splice variants have been described to couple to different signalling pathways. Activation of the mouse EP3α, EP3β and EP3γ receptors are known to lead to intracellular calcium mobilization in a pertussis toxin (PT)-sensitive manner. This calcium mobilization mediated by the EP3 receptor is conducted by the $G_{\beta\gamma}$ subunits from the G_i protein, since the PLCβ isoform is activated by these subunits (170). In addition to calcium, the EP3 variants signal through the small GTP-binding protein Rho to induce neurite retraction in PC-12 cells (118, 119) as well as induce stress fiber formation in MDCK cells (120).

Despite extensive studies, the functional significance of these alternatively spliced variants remains unclear. A truncation mutant at the splice site of EP3 was generated in order to determine the role of the C-terminal tail (164). The mutant receptor retained the ability to couple to G_i and showed fully constitutive G_i activity. These results indicate that the C-terminal tail of the EP3 receptor is essential for the regulation of agonist-dependent activation of G protein. To investigate the functional difference between the three mouse EP3 variants on cell morphology and function we generated populations of HEK293 cells expressing the myc-tagged mouse EP3α, EP3β and EP3γ receptor variants

and examined these populations for activation of signal transduction pathways and morphology. We used HEK293 cells as they are commonly used to study prostanoid receptors, they maintain robust expression of the mEP3 variants over many passages and, importantly for our second aim, HEK293 cells are tumorigenic in nude mice.

Results

The mEP3 receptor variants are expressed at similar levels

To characterize the three mEP3 variants on cell morphology and function, HEK293 cells were stably transfected with pcDNA3 mEP3 α, β, and γ myc-tagged at the extracellular N-termini. The specificity of the myc antibody (9E10) was demonstrated by comparing flow cytometric analyses of mEP3 receptor variant transfected HEK293 cells with cells transfected with only the vector pcDNA3 **(Figure 5A)**. Cell populations expressing comparable levels of the receptor variants were isolated by FACS using the 9E10 anti-myc antibody.

To demonstrate functionality of the mEP3 receptor variants we next confirmed native ligand binding by isolating membrane preparations and incubating equal amounts of membrane protein with radioactive PGE_2 in the absence or presence of saturating levels of the selective EP3 agonist, sulprostone **(Figure 5B)**. PGE_2 binding to the EP3 variants was similar and equally inhibited by cold sulprostone. In contrast, vector-transfected cells exhibited no specific PGE_2 binding activity. Finally to confirm comparable receptor number a saturation isotherm binding assay was performed and

revealed high-affinity specific binding of radioactive PGE_2 of membrane protein and no specific binding on membrane proteins from vector transfected cells **(Figure 5C)**.

Signaling by the mEP3 receptor variants mobilizes intracellular calcium as well as activates ERK and Rho

To characterize the intracellular signaling pathways activated by the mEP3 receptor variants we first examined the PI3K and MAPK pathways in stably transfected HEK293 cells, as PGE_2 has been shown to activate both of these pathways **(Figure 6A)** (171-173). Treatment with MB28767 had no effect on the PI3K pathway as seen by phosphorylation of Akt or the p38 MAPK pathway as seen by phosphorylation of p38. In contrast, we consistently observed activation of ERK in all three mEP3 receptor variant transfected cells within 15 minutes of stimulation with MB28767, but not in vector transfected cells.

As activation of the mEP3 receptor variants leads to increased intracellular calcium (170), the ability of the two selective EP3 agonists MB28767 and sulprostone (not shown) to induce intracellular calcium mobilization was determined. Each of the three mEP3 variants mobilized calcium in a concentration and time dependent manner after stimulation with EP3 agonists (**Figure 6B & 6C**). In contrast, no calcium mobilization was detected in vector-transfected cells. Thus, each of the three mEP3 receptor variants was functionally expressed in HEK293 cells. Lastly, since EP3 receptor variants have been shown to induce neurite retraction in PC-12 cells and mediate stress fibers in MDCK cells via Rho (119, 120, 174), we evaluated the effect of mEP3 receptor variant activation on Rho GTPases.

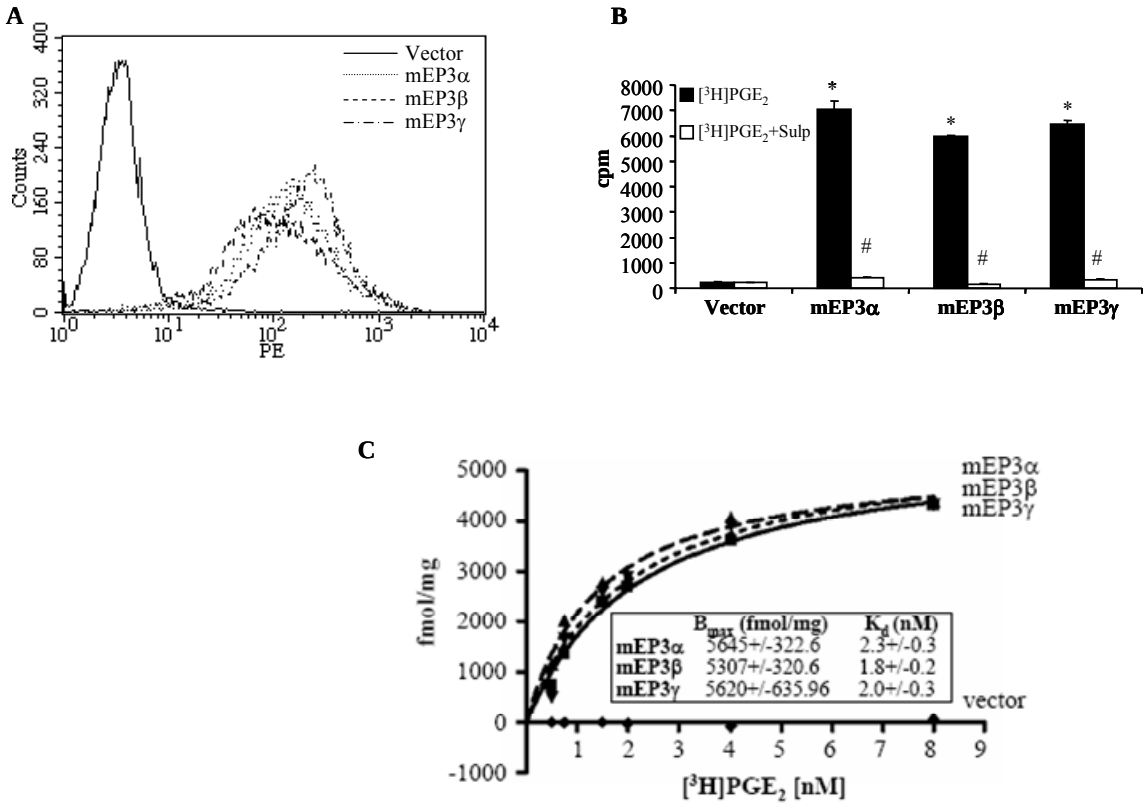

Figure 5. The mEP3 receptor variants are expressed at similar levels. (A) Flow cytometric analysis of stably transfected HEK293 cells with mEP3 receptor variants using fluorescent anti-myc antibody. mEP3 receptor variant expression is displayed by a shift in mean fluorescent intensity compared to vector transfected HEK293 cells. **(B)** One-point ligand binding assay on membranes from HEK cells stably expressing mEP3 receptor variants incubated at 30°C for 2 h with 2 nM $[^3H]PGE_2$ in the absence or presence of 5 μM sulprostone to confirm equal expression on cell membrane; Membrane preparations assayed in triplicates. **p* < 0.05 versus vector control. #*p* < 0.05 versus $[^3H]PGE_2$ only control. **(C)** Saturation isotherm analysis of $[^3H]PGE_2$ binding to mEP3 receptor variants expressed in HEK293 cells. Membranes isolated from HEK293 cells stably expressing mEP3 receptor variants were incubated with varying concentrations of $[^3H]PGE_2$ in the absence (total binding) or presence (nonspecific binding) of 10 μM PGE_2, as described under *Materials and Methods*. Specific binding was determined to be the difference between total and nonspecific binding and is shown above. Values in the insert box represent the mean +/- SD of one representative experiment performed in triplicate.These data are representative of three independent experiments.

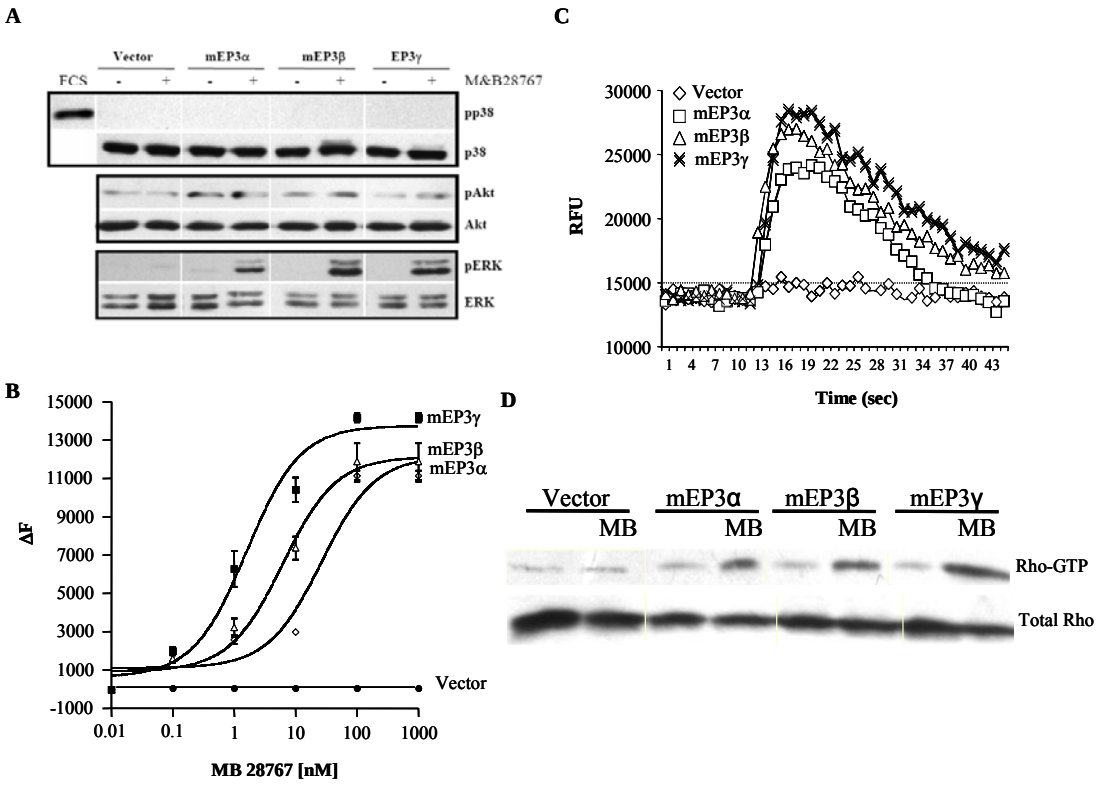

Figure 6. Signaling by the mEP3 recpetor variants mobilizes intracellular calcium as well as activates ERK and Rho. (A) To investigate the signal transduction pathways activated by the mEP3 receptor variants, lysates were collected following overnight serum-starvation and treatment with 0.1 μM MB-28767 for 15 minutes. As described under *Materials and Methods*, western blot analysis was performed using anti-phospho-Akt, anti-phospho-ERK, anti-phospho-p38, and anti-p38 MAPK specific antibodies. The concentration dependence **(B)** and time course **(C)** of MB-28767 (shown) and sulprostone-induced intracellular calcium mobilization for the three mEP3 receptor variants and vector control with stimulation by 0.1 μM selective agonists. **(D)** Rho pulldown assay performed on HEK293 cells stably expressing mEP3 receptor variants or vector alone following overnight serum-starvation and treatment with 0.1 μM MB-28767 or vehicle for 15 minutes. Total Rho levels were determined from whole cell lysates as shown in the bottom panel. Cell lysates were separated by a 15% SDS-polyacrylamide gel followed by immunoblotting with anti-Rho antibody, as described under *Materials and Methods.* Samples for pull-down assay and total Rho were from the same homogenates. The results shown are representative of three independent experiments that yielded similar results.

We used the Rho-binding domain of RhoA effector, rhotekin, to affinity precipitate active RhoA, as a direct readout for RhoA activation **(Figure 6D)**. We also used Rac1- and Cdc42-binding domain of Rac1 and Cdc42 effector, PAK to affinity precipitate active Rac1 and Cdc42 as a direct readout for Rac1 and Cdc42 activation (not shown). HEK293 cells stably transfected with mEP3 receptor variants or vector were stimulated with MB28767 for 15 minutes. Densitometry readings of treated versus untreated variants demonstrated a 3- to 4-fold increase in RhoA activity. However, all mEP3 receptor variants failed to activate Rac1 or Cdc42 suggesting a mechanism to regulate the specificity toward different Rho GTPases.

Differential surface localization by the mEP3 receptor variants

The molecular regulation of EP3 splice variant trafficking is yet unclear. Typically C-tail splice variants show differences in internalization and trafficking. For example the human EP3 receptor comprises eight variants that differ solely in their carboxyl-tail. Upon PGE_2 stimulation EP3.I internalizes, EP3.II, EP3.V, EP3.VI and EP3.f internalize to a lesser extent and EP3.III and EP3.IV do not internalize at all indicating the importance of the C-tails in determining their degree of PGE_2-induced internalization (162). Here we analyze the role of the three mouse C-terminal tails on localization of the mEP3 variants in HEK293 cells before and after treatment with selective EP3 agonists by immunofluorescence and FACS analysis.

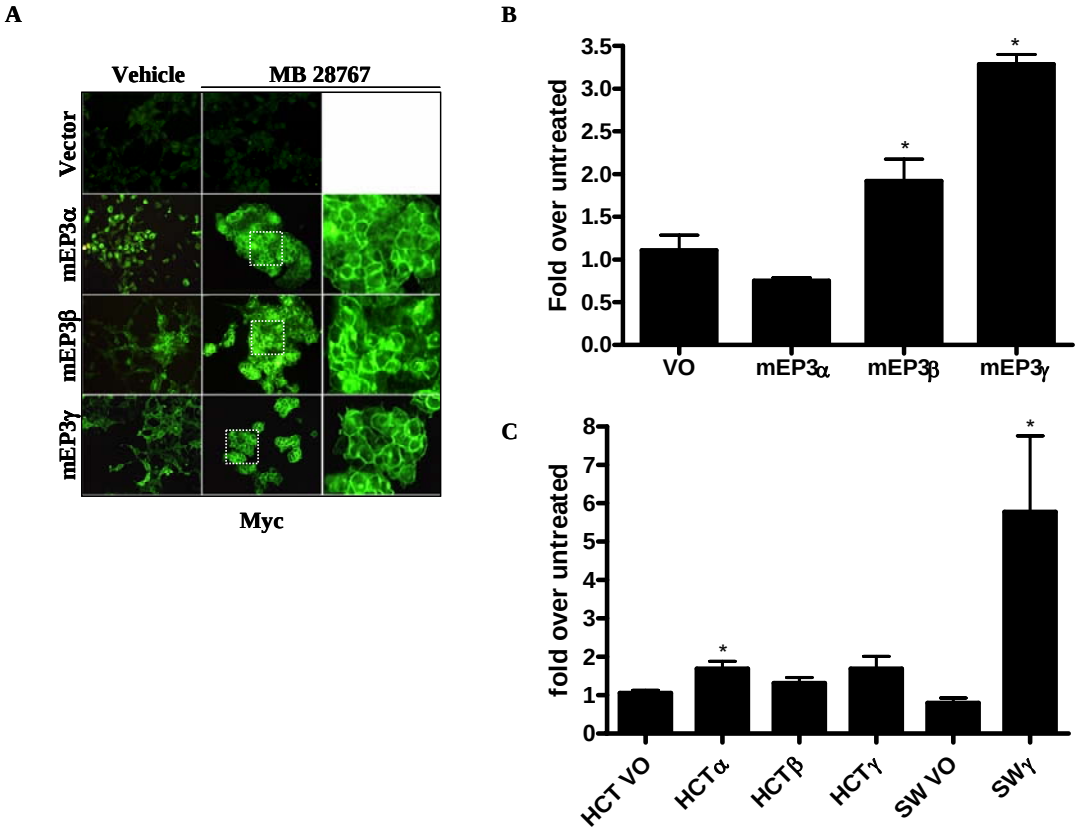

Figure 7. Differential surface localization by the mEP3 receptor variants. (A) To confirm mEP3 receptor variant expression, cells were stained with an anti-myc antibody following 0.1 μM MB28767 treatment or vehicle for 4 h. The results shown are representative of three independent experiments that yielded similar results. The *bar* represents 10 μm. **(B) & (C)** Mean intensity fluorescence of HEK293 cells (**B**), HCT116 cells and SW480 cells (**C**) treated with MB28767 over vehicle for four hours. The results represent the average of three independent experiments. *$p < 0.05$.

In order to confirm EP3 expression and localization in HEK293 cells before and after treatment with selective EP3 agonists we performed immunofluorescence staining on HEK293 cells expressing each of the three mouse variants or vector alone. We observed for changes in EP3 expression on the cell surface before and after treatment with MB28767 by staining with an anti-myc antibody **(Figure 7A).** The specificity of the anti-myc antibody in immunofluorescence studies was apparent from the observation that vector transfected cells showed no fluorescence regardless of treatment while the variants showed intracellular and cell-surface localization. Stimulation with MB28767 increased the level of EP3 variant at the cell surface within four hours of EP3 activation. As this result was not quantitative, we next examined this interesting qualitative observation further using flow cytometry analyses to determine the mean intensity flurescence of EP3 receptors before and after stimulation with selective agonist.

We next examined EP3 surface localization by flow cytometry using the anti-myc antibody before and after stimulation with MB28767 for four hours in HEK293 cells **(Figure 7B)**. Cell surface localization of the EP3α receptor variant decreased after a four hour incubation period with MB28767 stimulation while the levels of membrane-associated EP3β and EP3γ receptors increased by 2- to 3-fold over unstimulated. Interestingly, two colon cancer cell lines we stably transfected with the mEP3 receptor variants, namely HCT116 and SW480 cells, show an increase in plasma membrane localization of the three variants after stimulation with MB28767 for four hours compared to untreated (**Figure 7C**). HCT116 EP3β and HCT116 EP3γ show relatively high expression at baseline compared to HCT116 EP3α over cell passages. Treatment

does not significantly increase EP3 surface localization for EP3β or EP3γ however treatment did significantly increase EP3α plasma membrane localization two fold. This may be due to the fact that EP3α downregulates its cell surface expression unlike EP3β and EP3γ and upon stimulation enhances its downstream cell function with increased cell surface expression. Similarly, SW480 EP3γ cells significantly downregulate their cell-surface expression over several passages. However, MB28767 stimulation led to a dramatic 5- to 6-fold increase of EP3γ receptor to the cell surface **(Figure 7C).** In this study, we examined agonist-induced internalization of the mouse EP3 variants. In conclusion, our results indicate that the C-tail of the mouse EP3 variants is important in determining their degree of internalization mediated by receptor agonists.

Activation of the mEP3 receptor variants induces a morphological change characterized by enhanced cell-cell contact

It has been reported that the formation of stress fibers by the mEP3 receptor variants in MDCK cells involve Rho (120). Since we have found Rho activated by EP3 signaling and RhoA mediates the assembly of stress fibers and the actin cytoskeleton, we next examined the morphology of HEK293 cells stably expressing the mEP3 receptor variants or vector by immunofluorescence microscopy in the absence or presence MB28767. Rhodamine-conjugated phalloidin was used to visualize the actin cytoskeletal changes induced by MB28767 **(Figure 8A)**. All three mEP3 receptor variants stimulated with MB28767 caused HEK293 cells to form clusters within four hours of activation while vector and untreated variant cells were well dispersed with fibroblast-like foot processes evident. The time course of EP3-induced clustering was similar for the three

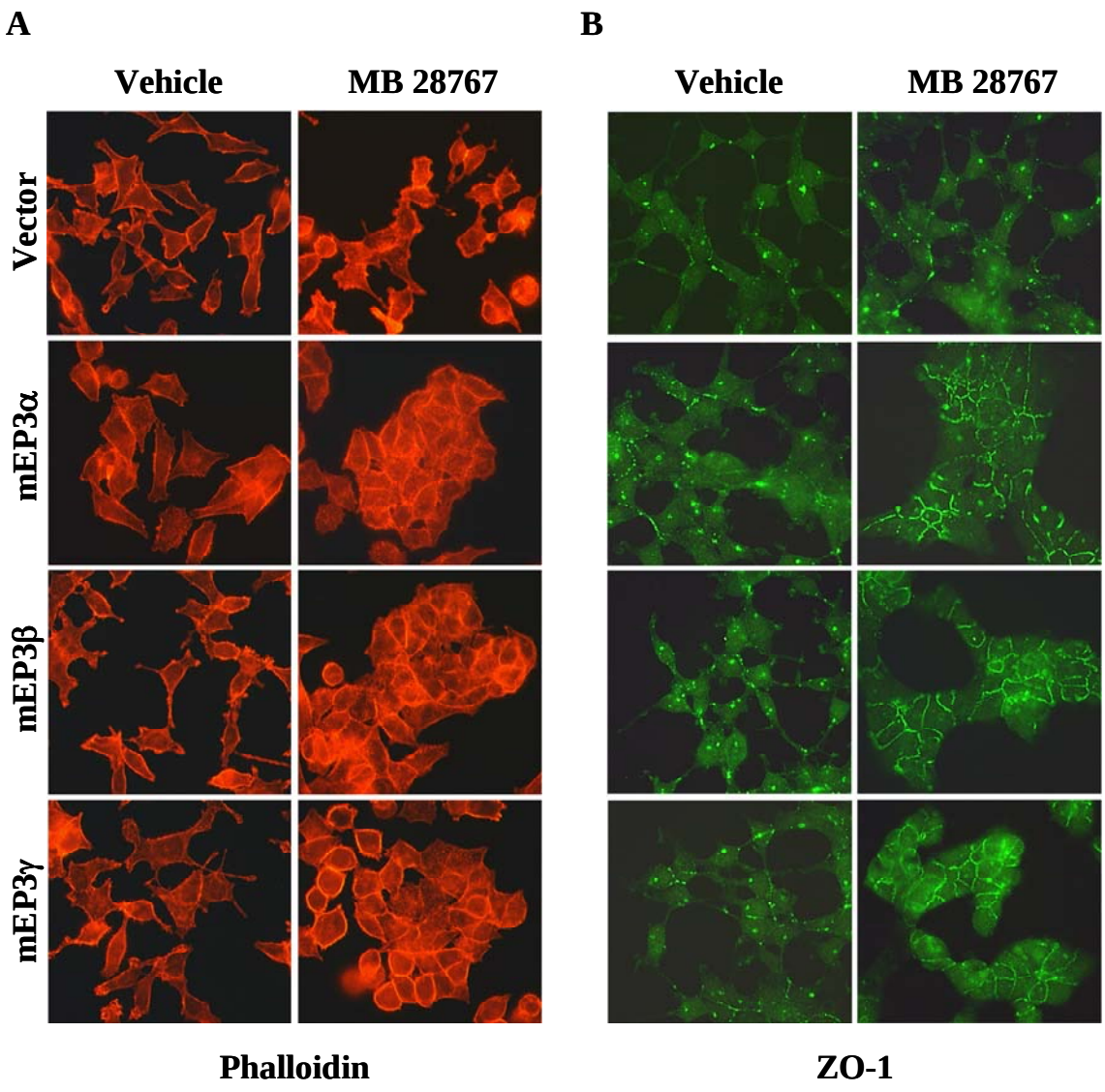

Figure 8. Activation of the mEP3 receptor variants induces a morphological change that enhances cell-cell contact. (**A**) Vector-transfected and mEP3-expressing HEK293 cells were treated with vehicle or 0.1 μM MB28767. After four hours, the cells were stained with rhodamine-phalloidin and **(B)** anti-ZO-1 antibody to visualize the cytoskeleton and tight junctions, respectively. Scale bar, 20 μm.

variants. We next examined whether this morphological change enhanced cell-cell contact by localization of ZO-1, a marker for tight junctions. While vector and untreated variant cells displayed punctate staining for ZO-1, the three variants stimulated with MB28767 led to enhanced membrane-associated ZO-1 within four hours of EP3 activation **(Figure 8B)**. As this experiment takes place over a four hour period and immunoblots for ZO-1 showed no increase in ZO-1 protein levels (not shown), we conclude that the enhanced cell-cell contact in response to EP3 activation is due to an increase in membrane localization and not to an increase expression level of cell adhesion molecules. These results indicate activation of the mEP3 receptor variants equally cause a morphological change which is characterized by enhanced cell-cell contact.

<u>The morphological change induced by the mEP3 receptor variants is both physiologically relevant and specific to EP3 activation</u>

To confirm the EP3-induced morphological change is physiologically relevant we next stimulated HEK293 cells expressing mEP3 variants or vector alone with native ligand, PGE_2 **(Figure 9A)**. In addition, to confirm the morphological change is specific to EP3 activation we stimulated vector and mEP3 expressing HEK293 cells with the EP1/3 selective agonist, sulprostone, or the structurally similar EP2 selective agonist, butaprost **(Figure 9A)**. Although we only show mEP3γ cells, all three variants expressed in HEK293 cells were observed to cluster and enhance cell-cell contact when stimulated with PGE_2 or sulprostone within four hours of EP3 activation similar to MB28767. No changes were observed in vector transfected cells. Butaprost had no effect on morphology after 4, 24 or 48 hours of EP2 activation confirming the enhanced cell-cell contact as an EP3 specific effect.

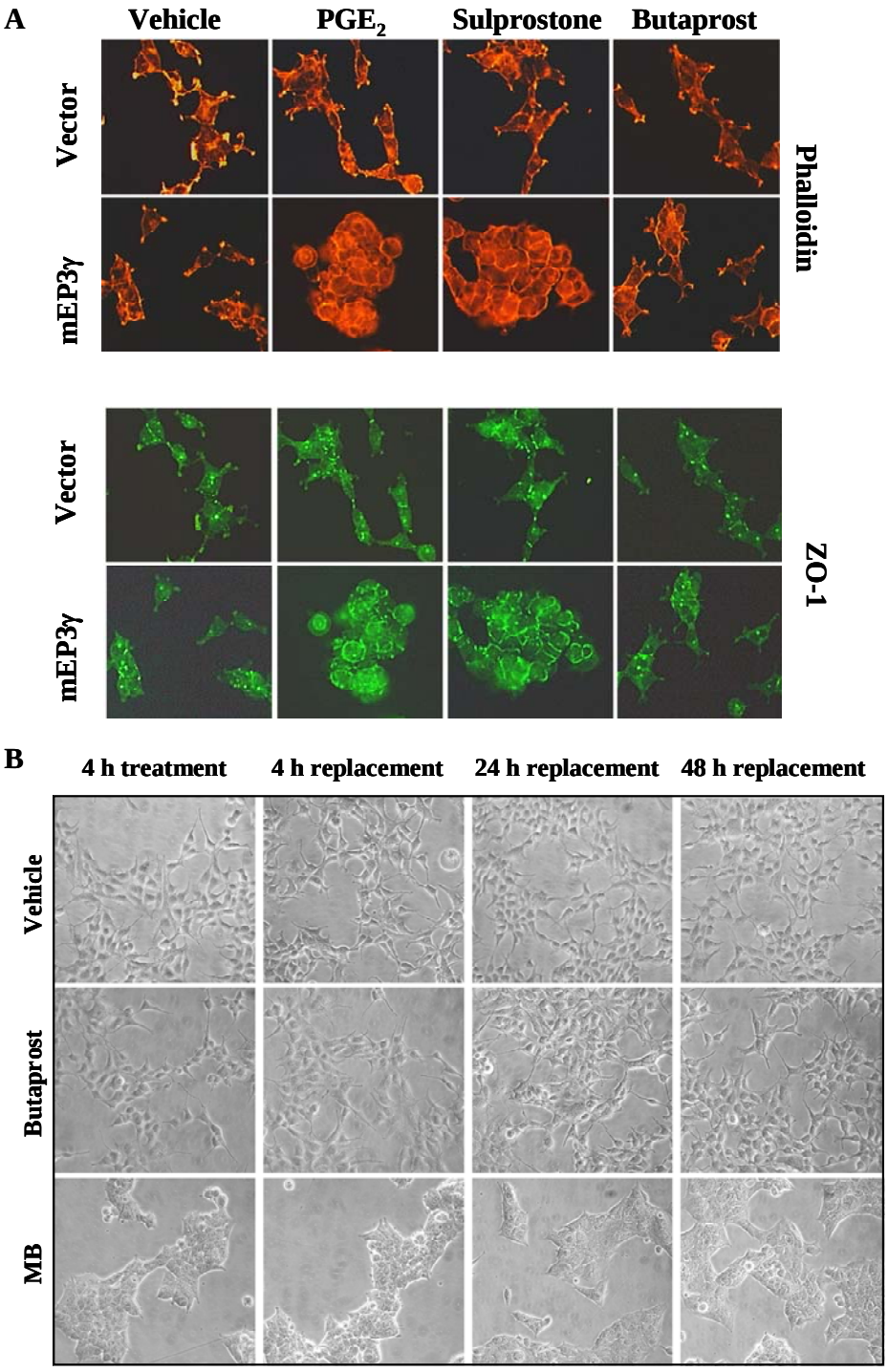

Figure 9. The morphological change induced by the mEP3 receptor variants is both physiologically relevant and specific to EP3 activation. (A) Vector and mEP3 HEK293 cells (mEP3γ shown only) were treated with vehicle or 0.1 μM PGE_2, sulprostone, or butaprost. After four hours the cells were stained with rhodamine-phalloidin and anti-ZO1 antibody to visualize actin and tight junctions, respectively. Scale bar, 20 μm. **(B)** The cells indicated were treated with 0.1 μM MB28767, butaprost or vehicle. After 4 hours the media was replaced with media lacking prostanoid and cells were photographed 4, 24 and 48 hours after medium replacement. The images shown are representative of three independent experiments. Scale bar, 100 μm

We examined whether this morphological change can be reversed by replacing the media of cells stimulated with MB28767 for four hours with normal media for 4, 24 and 48 hours (**Figure 9B**) and 72 hours (not shown). We found the EP3-induced morphological change could not be reversed by the removal of agonist and replacement with normal media. This suggests EP3 activation has a lasting effect on cell morphology even after ligand is degraded and unavailable to activate its receptor.

The morphological change induced by mEP3 is mediated by RhoA

To explore the mechanism by which mEP3 variants induce a morphological change in HEK293 cells, we assessed signaling pathways shown activated by EP3 receptor variants. We used selective pharmacological inhibitors to block EP3-induced morphological changes. We have only shown EP3γ but this experiment was performed with all three variants and empty vector transfected HEK293 cells multiple times **(Figure 10A)**. We used forskolin and IBMX to elevate cAMP levels, H-89 to inhibit PKA, BIM to inhibit PKC, or BAPTA/AM, a calcium chelator, but none of these were able to block the morphological changes induced by EP3 activation. Similarly, although we have shown EP3 activation to stimulate ERK activation, the MEK inhibitor, PD98059, or a PI3K inhibitor, wortmannin were unable to block the morphological change. In contrast, Y-27632, a rho kinase inhibitor or expression of the C3 *Clostridium botulinum* toxin, which inactivates Rho, blocked the mEP3-induced morphological change in all three variants. Expression of the C3 toxin was confirmed by comparing RhoA activation after MB28767 treatment in mEP3 expressing HEK293 cells with and without C3-transfection **(Figure 10B)**. Active Rho and a morphological change were evident exclusively in mEP3

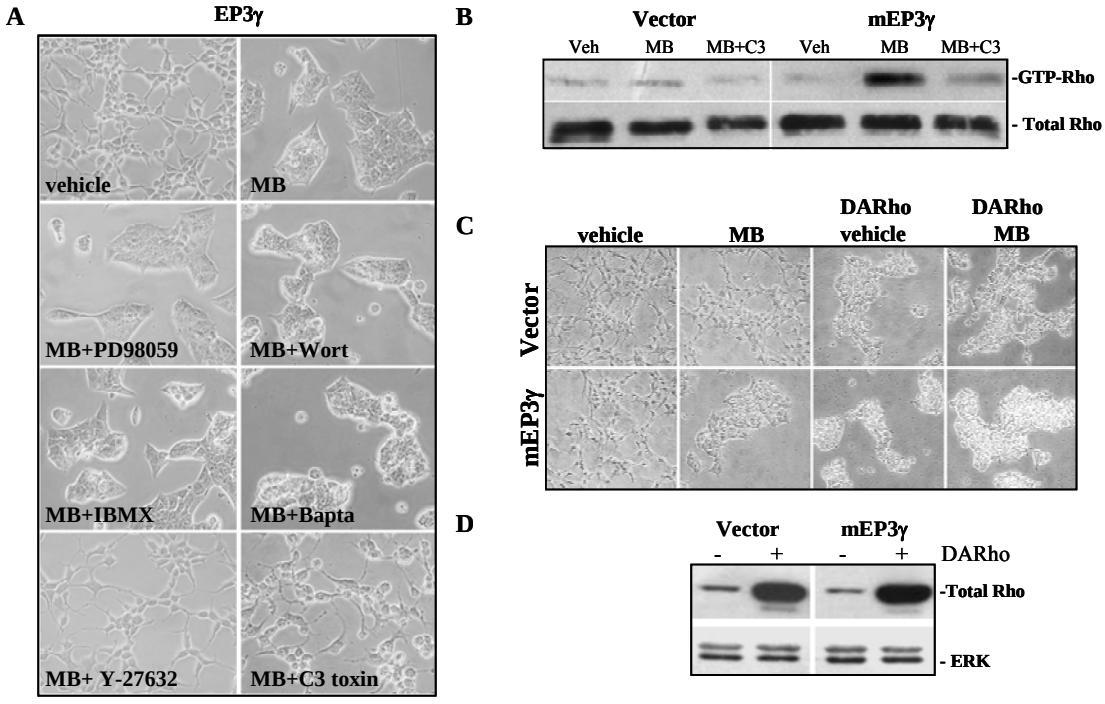

Figure 10. The morphological change induced by mEP3 is mediated by RhoA. (A) mEP3-expressing HEK293 cells (mEP3γ shown only) were serum-starved for 12 hours in the presence or absence of PD-98059 (5 μM), wortmannin (100 nM), IBMX (0.5 mM), 5 μM BaptaAM (5 μM), Y-27632 (5 μM) or transfected for 48 h with the C3 toxin expression plasmid. Cells were subsequently treated with vehicle or 0.1 μM MB28767 and their morphology evaluated 4 hours after treatment**. (B)** Rho pulldown assay was performed on cells lysates of serum-starved vector- and mEP3-expressing HEK293 (mEP3γ shown only) cells untransfected or transfected 48 h prior with C3 toxin and treated for 15m with MB28767. GTP-Rho was detected by immunoblotting with anti-Rho antibody (top). Total cell lysates were used to detect the levels of total Rho (bottom). **(C)** The cells indicated were transfected with vector control or dominant active Rho (DARho) and the levels of Rho were evaluated 48 hours after transfection by Western blot analysis in 30 μg of total cell lysates. **(D)**Membranes were subsequently incubated with anti-ERK antibody to verify equal loading.

variant expressing HEK293 cells after MB28767 treatment yet not in the presence of the C3 toxin. This indicates that the mEP3 receptor variant-induced morphological change is mediated by Rho.

To further examine the Rho pathway as a putative mechanism we used a dominant active construct of Rho (G14V) to determine whether this was sufficient to induce the morphological change in vector transfected cells and in all three variants in the absence of ligand. Although only EP3γ is shown, all three variants responded similarly in that overexpression of dominant active Rho was able to induce the morphological change in both vector and mEP3 variant expressing HEK293 cells in the absence of selective EP3 agonists **(Figure 10C)**. To confirm overexpression of dominant active Rho construct in transfected cells, an immunoblot for total Rho is shown **(Figure 10D)**. These results further suggest the EP3-induced morphological change characterized by enhanced cell-cell contact is mediated by Rho.

G_{12} and G_{13} may regulate the mEP3 receptor variant-induced morphological change.

Among classical second messenger pathways, the EP3 receptor variants are coupled to adenylate cyclase inhibition through G_i. However pertussis toxin (PT) treatment did not suppress the morphological change induced by EP3 agonists (data not shown) indicating that this action is not mediated by G_i. Pretreatment with forskolin or IBMX, both of which drive cAMP levels up in cells, failed to block the EP3-induced morphological change suggesting this action is not mediated by G_s either. These observations suggest that the EP3 receptor variants are not coupled to adenylate cyclase but to a PT-insensitive heterotrimeric G-protein other than G_s or G_q.

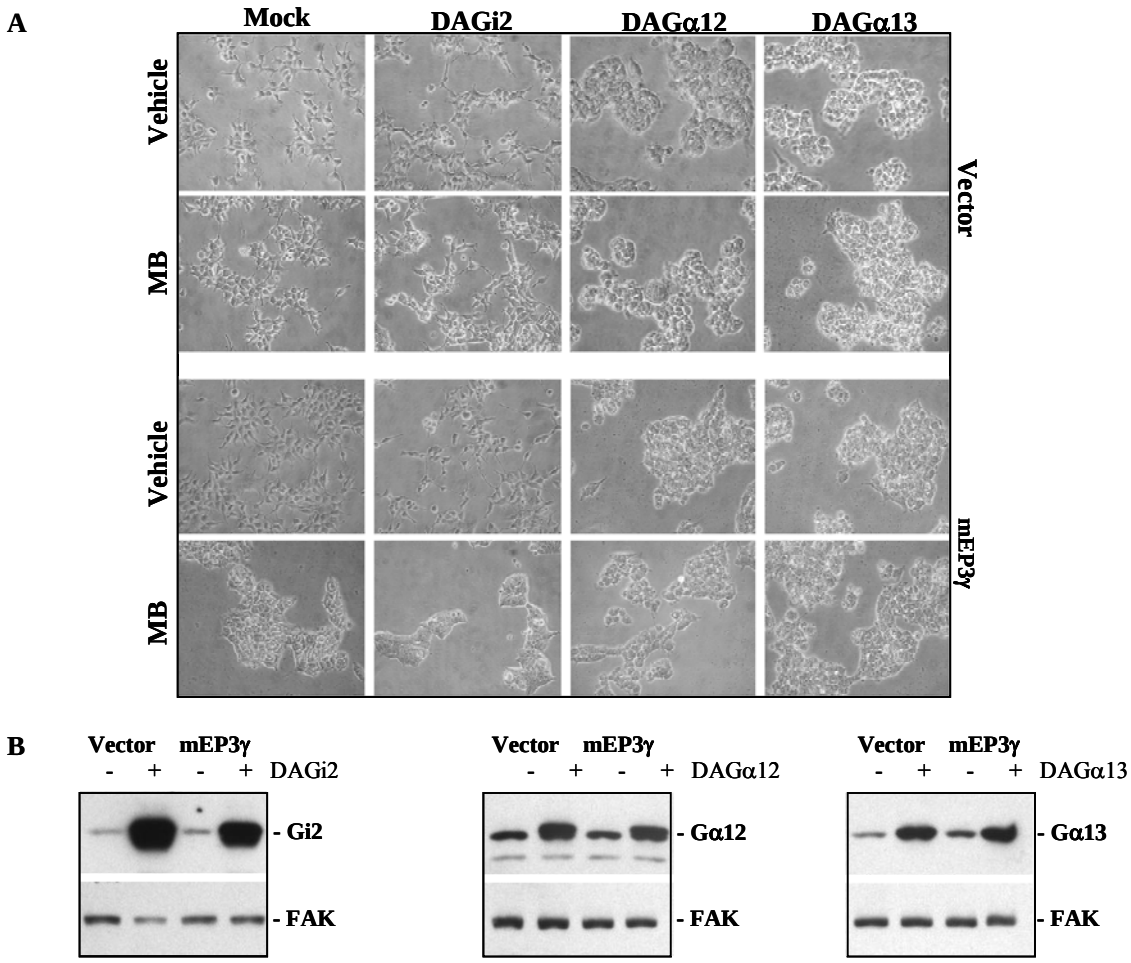

Figure 11. G_{12} and G_{13} may regulate the mEP3 receptor variant-induced morphological change. (**A**) The cells indicated were transfected with vector or dominant active G_i, G_{12} or G_{13}. Forty-eight hours later cell morphology was evaluated in 4 hours vehicle-treated or MB28767-treated (not shown) cells. (**B**) The cells indicated were transfected with the constructs indicated above and the levels of these G protein alpha subunits were evaluated 48 hours after transfection by western blot analysis in 30 μg of total cell lysates. Membranes were subsequently incubated with anti-FAK antibody to verify equal loading.

We have shown thus far that EP3 induces a morphological change dependent on Rho activation. Several reports have shown that constitutively activated G_{12} and G_{13} can stimulate Rho-dependent stress fiber formation and focal adhesion assembly (119, 120, 174). As G_{12} and G_{13} are PT-insensitive G proteins, we postulated that the EP3 receptor variants may regulate morphology through G_{12} and/or G_{13}. To test this hypothesis we transfected constitutively activated G_i as a negative control, G_{12} and G_{13} in HEK293 cells stably transfected with either vector or mEP3 variants. We found that both constitutively activated G_{12} and G_{13} can induce a morphological change in the absence of ligand in both vector and mEP3 receptor variant transfected HEK293 cells similar to MB28767 treated and dominant active Rho transfected HEK293 cells **(Figure 11A)**. To confirm overexpression of constitutively activated G_i, G_{12} and G_{13} constructs, cell lysates were analyzed by western blot for G_i, G_{12} and G_{13} with the appropriate anti-G-protein antibody **(Figure 11B)**. We found robust overexpression consistently in each of our experiments. This suggests that G_{12} and G_{13} may transduce the EP3-mediated Rho activation causing a morphological change which enhances cell-cell contact.

<u>The mEP3 receptor variant-induced morphological change is likely mediated by G_{12}</u>

To determine whether G_{12} and/or G_{13} transduces the EP3-mediated Rho activation, dominant negative constructs of G_i, G_{12} and G_{13} were overexpressed in vector or mEP3 expressing HEK293 cells (mEP3γ shown). Cells were treated with MB28767 (100nM) and their morphology evaluated 4 hours after agonist treatment **(Figure 12A)**. Whereas dominant negative G_i and G_{13} had no effect on blocking the EP3-induced morphological change, expression of the dominant negative G_{12} prevented cells from

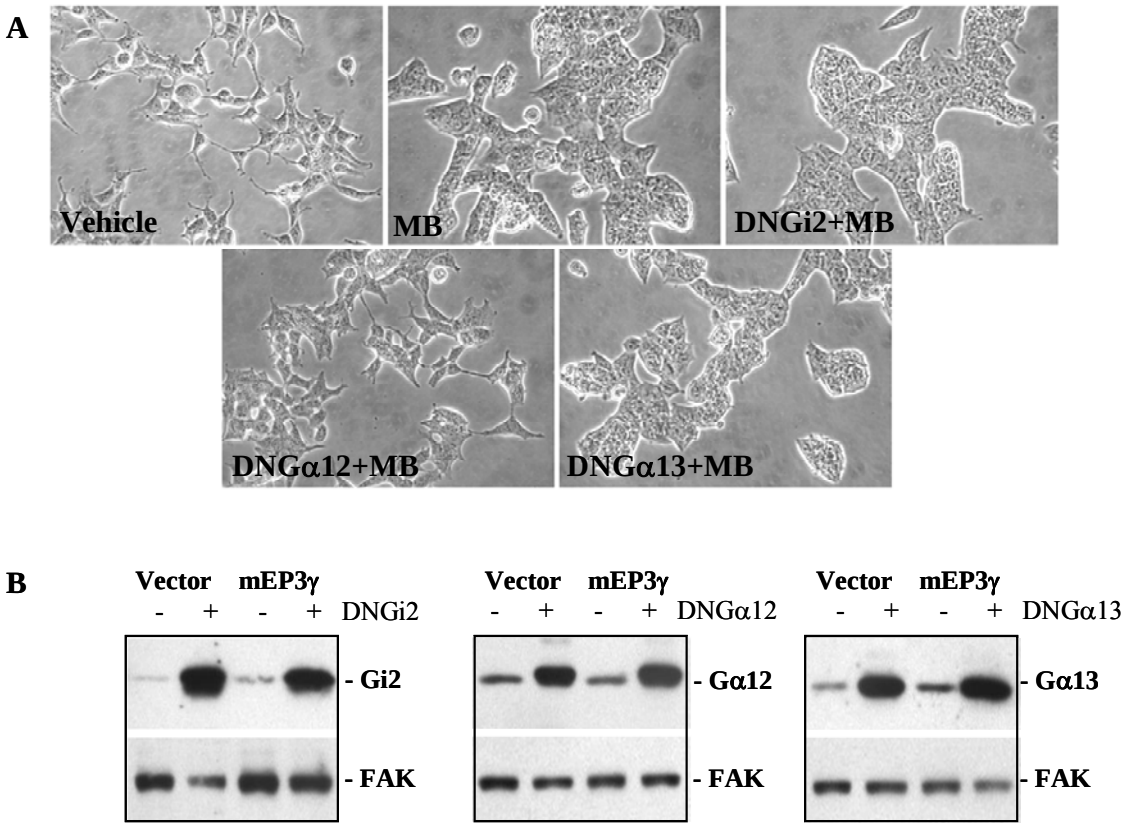

Figure 12. The mEP3 receptor variant-induced morphological change is likely mediated by G_{12}. (**A**) mEP3-expressing HEK293 cells (mEP3γ shown only) were transfected with vector control or dominant negative G_i, G_{12} or G_{13}. Forty-eight hours later the cells were either treated with vehicle or 0.1 μM MB28767 and their morphology was evaluated 4 hours after treatment. Images are representative of three independent experiments. (**B**) mEP3-expressing HEK293 (mEP3γ shown only) were transfected with the constructs indicated above and the levels of these G protein alpha subunits were evaluated 48 hours after transfection by western blot analysis in 30 μg of total cell lysates. Membranes were subsequently incubated with anti-FAK antibody to verify equal loading.

clustering and developing a more epithelial-like morphology. To confirm overexpression of the dominant negative constructs, cell lysates were analyzed by Western blot analysis with the appropriate G protein specific antibody **(Figure 12B)**. These results strongly suggest the mEP3 receptor variants induce a morphological change *via* a G_{12}-Rho pathway in HEK293 cells.

Conclusions

The most distinctive feature of the EP3 receptors is the diversity generated by multiple alternative splice variants that generate alternate C-terminal tails of this receptor subtype. Here we aimed to characterize the mouse EP3 receptor variants and determine their functional differences in HEK293 cells. These cells consistently overexpress robustly our receptor variants and they are widely used to study prostanoid receptors. Cell populations overexpressing each variant were isolated by flow cytometry for equal expression before characterizing variants for their functional significance. Once we confirmed cell populations for equal expression, native ligand binding and equal receptor number, we analyzed variants for their signal transduction usage. The mEP3 receptor variants each mobilized calcium similarly in both a time and dose-dependent manner. Interestingly they all activated the ERK, but had no effect on the PI3K or p38 MAPK pathways after treatment with a selective agonist for EP3. We also observed Rho activation within 15 minutes of EP3 activation in all three variants however they each failed to activate Rac1 or Cdc42 suggesting a mechanism to regulate the specificity toward different Rho GTPases.

When examining HEK293 cells for mEP3 receptor expression by immunofluorescence, interestingly we found an intense staining on the cell surface for mEP3 after activating variants with a selective EP3 agonist for 4 hours. Quantitatively by flow cytometry we found cell surface localization of the EP3α receptor variant decreased while EP3β and EP3γ increased by 2- to 3-fold over unstimulated. Two colon cancer cell lines stably expressing mEP3 variants also showed an increase in plasma membrane localization after stimulation. One colon cancer cell line in particular consistently demonstrated a dramatic 5- to 6-fold increase of EP3γ receptor with stimulation. Our results indicate that the C-tail of the isoforms is important in determining their degree of PGE_2-induced internalization

Lastly we observed EP3 variants to induce a morphological change characterized by enhanced cell-cell contact. We determined this morphological change to be both physiologically significant, EP3 specific and irreversible, hence a long-lasting effect of EP3 activation. We found the mechanism of this morphological change to be likely G_{12} mediated and requiring Rho activation.

CHAPTER IV

FUNCTIONAL ANALYSIS OF THE MURINE EP3 RECEPTOR VARIANTS ON TUMOR CELL GROWTH

Introduction

Pharmacological and genetic inhibition studies have shown EP1, EP2 and EP4 to play a role in tumorigenesis using several mouse models for different types of cancer (146-148, 150-152, 154, 155). In contrast, the EP3 receptor is the only receptor subtype not directly implicated in tumorigenesis (154-156). For instance, EP3 receptor expression was decreased in colon cancer from mice, rats and humans when compared to normal mucosa (165). Similar to colon cancer, EP3 receptor levels were found decreased in mammary tumors suggesting the silencing of EP3 during tumor development is not specific to cancers in the colon (166). Interestingly, deletion of the EP3 receptor does not alter early stages of colon cancer progression, namely aberrant crypt foci development, whereas EP1, EP2, and EP4 receptors were implicated in small intestinal polyp formation (154-156). However, in contrast to the other EP receptors, an increase in carcinogen-induced colon carcinoma was found in EP3-null mice, suggesting EP3 may impact later stages of colon carcinogenesis (165). Furthermore, only one of eleven human colon cancer cell lines was shown to express EP3 and when that cell line was treated with an EP3 agonist its viability decreased by 30% (165). Taken together, these results suggest that EP3 expression confers a growth disadvantage to tumor cells and may play a protective role against cancer progression.

The following aim examines the distinct contribution the murine EP3 variants may have on tumor cell growth *in vitro* and *in vivo* in order to explore their therapeutic potential. Based on the findings that all three variants are downregulated or absent in cancer (165, 166, 168), reduced viability is observed in treated EP3-expressing cancer cells (165) and enhanced incidence of colon carcinomas is evident in mice deficient for EP3 (165), it is conceivable that restoring EP3 expression in tumor cells will suppress growth *in vitro* and reduce their tumorigenic potential *in vivo*. In order to test this hypothesis, we expressed each receptor variant in HCT116 cells, a human colon cancer cell line expressing no detectable levels of endogenous EP3 (165) and HEK293 cells, since they are tumorigenic *in vivo* (175) and are commonly utilized to study prostanoid receptor signaling (151, 176-179). These cells were examined for their ability to proliferate, signal and undergo morphological change.

Results

Activation of the mEP3 receptor variants reduces proliferation *in vitro*

While EP1, EP2 and EP4 receptor signaling have all been implicated in tumorigenesis in different rodent models (154-156), EP3 receptor deletion has no impact on early stages of tumorigenesis but may play a protective role in later stages (154, 156). In contast, EP3 has been implicated in tumor-associated angiogenesis in sarcoma-180 and sponge implantation models (167). In order to evaluate the role the mEP3 receptor variants may have on cell growth we analyzed the mEP3 receptor variants for proliferation. HEK293 cells stably expressing mEP3 receptor variants exhibit a 30-40%

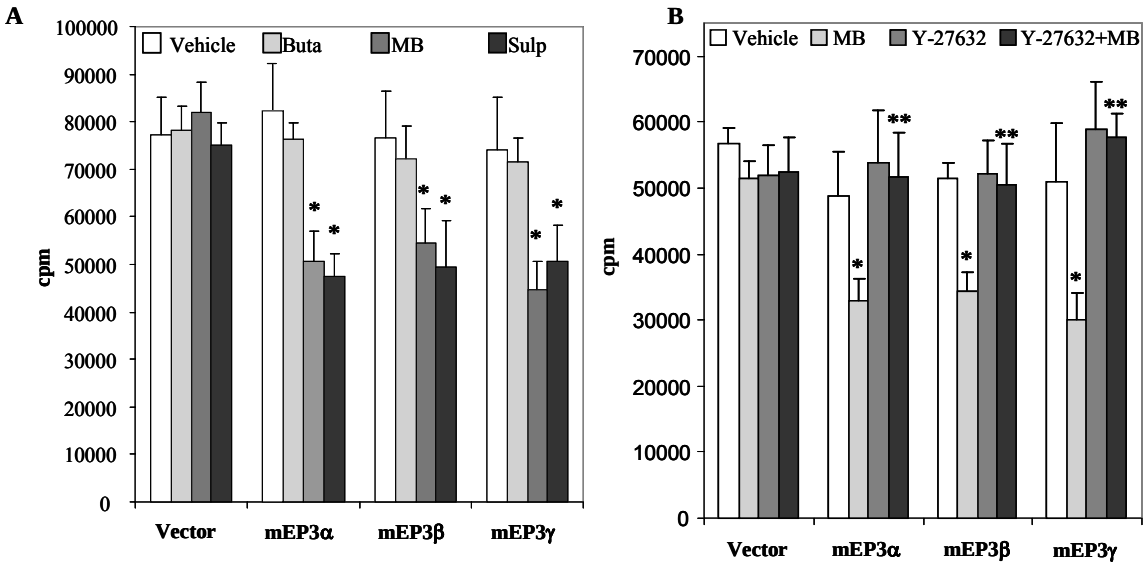

Figure 13. Activation of the mEP3 receptor variants reduces proliferation *in vitro* and this inhibitory effect is mediated by Rho in HEK293 cells. (A). [^{3}H]Thymidine incorporation on vector-transfected and mEP3-expressing HEK293 cells treated with vehicle, MB28767 (MB), sulprostone (Sulp) or butaprost (Buta) was evaluated as described under *Materials and Methods*. (**B**) ^{3}H-thymidine incorporation assay on vector-transfected and mEP3-expressing HEK293 cells incubated in the presence or absence of 10 μM Y-27632 and 0.1 μM MB28767 (MB) was evaluated as described under *Materials and Methods*. The bars and errors represent the mean +/- S.D. of a representative experiment performed in quadruplicates (*) indicates significant differences ($p<0.05$) between vehicle and MB-treated cells. (**) indicates significant differences ($p<0.05$) between MB- and MB+Y-27632 treated cells.

reduction in growth when treated with either the EP3 selective agonists, MB28767 or sulprostone, compared to vector treated or untreated cells **(Figure 13A)**.In contrast, butaprost did not alter proliferation compared to untreated cells, suggesting the cell growth inhibition observed is specific to the EP3 receptor and activation.

The inhibitory effect of mEP3 receptor activation *in vitro* is mediated by Rho in HEK293 cells

To determine whether Rho is also involved in the EP3-mediated cell growth inhibition as observed with the morphological change (**Figure 10**), the effect of the Rho kinase inhibitor Y-27632 was determined on proliferation *in vitro*. As shown in **Figure 13B** Y-27632 reversed the mEP3-mediated cell growth inhibition, and this effect was independent of the mEP3 variant expressed. Thus, these results suggest the EP3-induced reduction in cell growth and changes in morphology observed *in vitro* are both mediated by Rho.

The mEP3 receptor variants inhibit tumor cell growth of HEK293 cells *in vivo*

In order to confirm *in vivo* the protective role of the mEP3 receptor variants seen *in vitro*, we injected 15 male athymic/nude mice with 10^6 HEK293 cells. We found that vector transfected HEK293 cells form tumors more readily than mEP3 receptor variant-expressing cells (13/15 Vector; 1/15 EP3α; 5/15 EP3β; 3/15 EP3γ) **(Figure 14A)**. Further, the tumors isolated from vector-transfected HEK293 cells were larger than those derived from mEP3 receptor variant-expressing cells. Tumors were analyzed for mEP3 receptor variant expression using the anti-myc antibody **(Figure 14B)**.

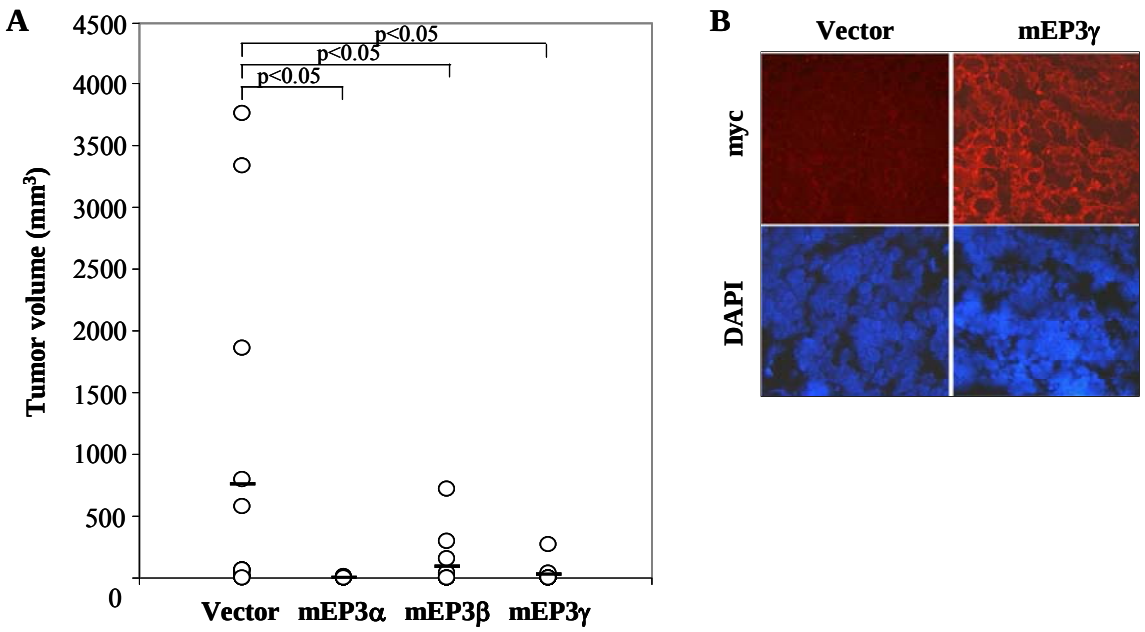

Figure 14. The mEP3 receptor variants inhibit tumor cell growth in HEK293 cells *in vivo*. (B) HEK293 cells stably expressing mEP3 receptor variants exhibit reduced tumor growth 35 days post-s.c. injection of 0.2 ml of 1 x 10^6 trypsinized cells in nude male mice compared to vector control. Volume was determined according to the equation ($V = [L \times W^2] \times 0.5$, where V = volume, L = length, and W = width. **(C)** Frozen sections of mEP3α, mEP3β, mEP3γ (shown) receptor tumors isolated from s.c.-injected nude mice were stained with anti-myc antibody to confirm the expression of the mEP3 receptor variants. DAPI staining was performed to visualize cell nuclei.

All mEP3 variant HEK293 tumors exhibited positive staining for myc (mEP3γ shown) while vector HEK293 tumors were negative for myc staining, indicating HEK293 cells stably transfected with mEP3 receptor variants do not downregulate expression during tumor formation. Together these results demonstrate the mEP3 receptor variants in HEK293 cells induce growth suppression evident both *in vitro* and *in vivo*.

EP3 receptor variant signalling activates Rho and enhances cell-cell contact in human colon cancer cells

Thus far, we have demonstrated that PGE_2 *via* the EP3 receptor activates the G_{12}-Rho pathway causing a morphological change which enhances cell-cell contact and reduced cell proliferation in HEK293 cells (**Figures 12 & 13**). We next sought to investigate the role of the mEP3 receptor variants in a human colon cancer cell line (HCT116) rather than HEK293 cells in order to confirm EP3 signaling can activate Rho and cause a morphological change in a more pathophysiologically relevant cell line. As with the HEK293 cells, HCT116 cells were transfected, selected and sorted for comparable cell-surface expression of the mEP3 receptor variants by flow cytometry **(Figure 15A)**. We then evaluated the effect of mEP3 receptor variant activation on RhoA GTPase **(Figure 15B)**. HCT116 cells expressing variants (mEP3γ shown) and vector were stimulated with MB28767 for 15 minutes and the results demonstrate all three mEP3 receptor variants can induce a robust increase in RhoA activity (mEP3γ shown). Similar results were obtained with a second human colon cancer celline, SW480 (**Figure 15B**). These results confirm mEP3 signaling *via* Rho is not cell-type specific.

Next we examined the ability of selective EP3 agonists to induce a morphological change in vector or mEP3 variant expressing HCT116 cells. All three mEP3 receptor variants expressed in HCT116 cells formed cell clusters with increased cell-cell contact within four hours of MB28767 stimulation as seen in **Figure 15C** by staining with rhodamine-phalloidin for the actin cytoskeleton and anti-ZO-1 for tight junctions. In contrast vector and untreated HCT116 cells remained well dispersed and exhibited punctuate ZO-1 staining (**Figure 15C**) confirming the morphological change is ligand-dependent. The time course of EP3-induced clustering was similar for the three variants. Similar results were obtained with the second human colon cancer cell line, SW480 expressing mEP3γ and its vector counterpart (**Figure 15D**) and using native ligand, PGE_2 (not shown). These results demonstrate human colon cancer cells with EP3 expression restored display a morphological change with EP3 stimulation characterized by enhanced cell-cell contact indicating EP3-induced Rho activation and morphological change are not cell-type specific but importantly, physiologically relevant.

Activation of the mEP3 receptor variants inhibits human colon cancer cell growth *in vitro* and *in vivo*

Previous studies have shown the EP3 receptor to be downregulated or absent in carcinomas of the colon and breast (165, 166). It has been reported HCT116 colon cancer cells to express EP1, EP2 and EP4 but no detectable EP3 (165) indicating the lack of EP3 expression in tumor cells may confer a proliferative advantage. To test this hypothesis we analyzed vector and mEP3 variant HCT116 cells for proliferation following stimulation with MB28767, sulprostone or butaprost. As shown in **Figure**

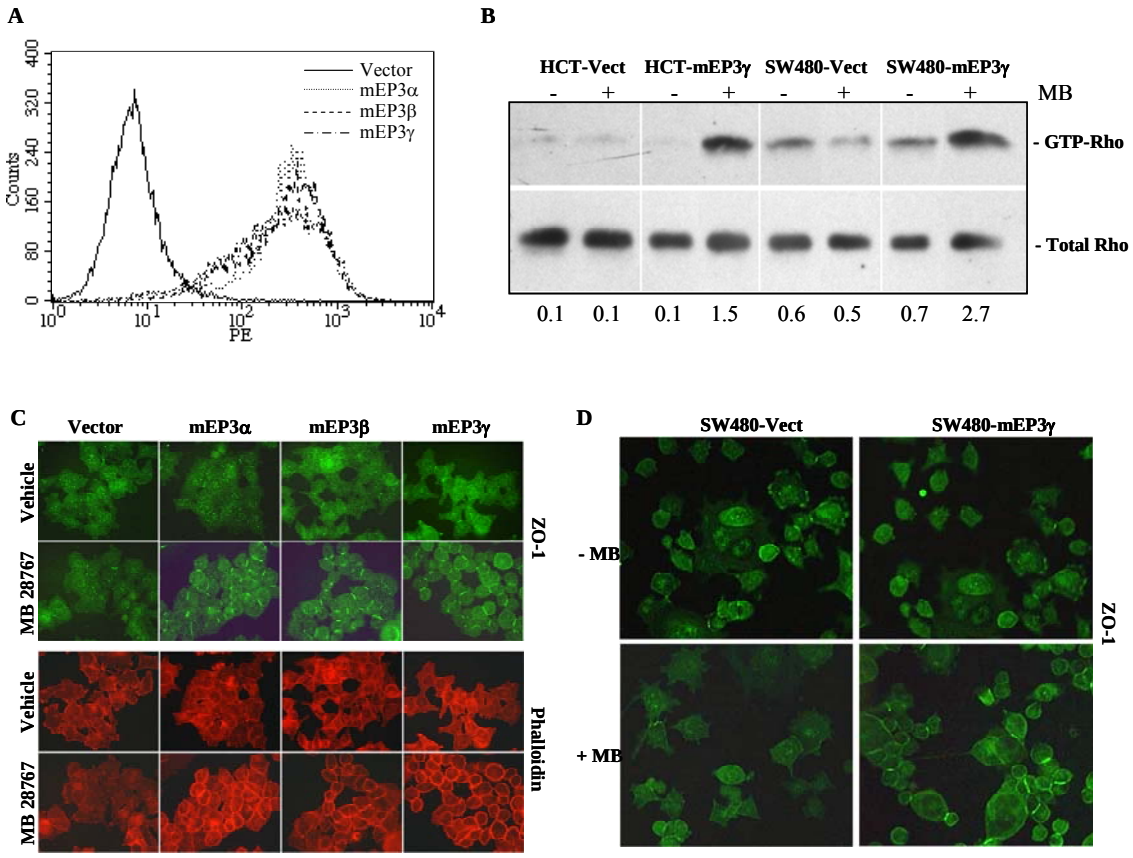

Figure 15. EP3 receptor variant signaling activates Rho and enhances cell-cell contact in human colon cancer cells. (A) Flow cytometry of stably transfected HCT116 cells with mEP3 receptor variants labeled with anti-myc antibody. mEP3 receptor expression is displayed by a shift in mean fluorescent intensity compared to vector-transfected HCT116 cells. The result shown is representative of three independent experiments. **(B)** Rho pulldown assay performed on cells lysates of serum starved cells treated with vehicle or 0.1 μM MB28767 for 15 minutes. Bound GTP-Rho was detected by immunoblotting with anti-Rho antibody (top). Total cell lysates were used to detect the levels of total Rho (bottom). **(C& D)** Vector-transfected and mEP3-expressing HCT116 and SW480 cells were treated with vehicle or 0.1 μM MB28767. After four hours, the cells were stained with rhodamine-phalloidin (HCT116 only) and anti-ZO-1 antibody to visualize the cytoskeleton and tight junctions, respectively.

16A, a 40-50% reduction in proliferation was observed only when cells were treated with the EP3 selective agonists but not with the EP2 agonist, butaprost. These results confirm data obtained with HEK293 cells and suggest the effects on proliferation by the mEP3 variants are not cell-type specific.

To further test the hypothesis that the EP3 receptor can suppress growth of colon cancer cells, we injected mEP3 variant expressing HCT116 cells *in vivo*. We injected 6 male athymic/nude mice with 10^6 vector transfected colon cancer cells and 8 male athymic/nude mice with 10^6 variant expressing colon cancer cells. Although nearly all mice formed tumors within a month (6/6 Vector; 8/8 EP3α; 7/8 EP3β; 8/8 EP3γ) **(Figure 16B)**, the tumors isolated from vector HCT116-injected mice were larger than those tumors isolated from mEP3 receptor variant HCT116-injected mice. Tumors were analyzed for mEP3 receptor variant expression using the anti-myc antibody **(Figure 16C)**. All mEP3 expressing HCT116 tumors exhibited positive staining for myc (mEP3γ shown for simplicity) while empty vector tumors were negative for myc staining, indicating HCT116 cells stably transfected with mEP3 receptor variants do not downregulate expression during tumor formation. Collectively these results indicate human colon cancer cells with either mEP3α, mEP3β or mEP3γ receptor expression restored can suppress growth evident both *in vitro* and *in vivo*.

Conclusions

Although EP3 is widely expressed throughout the body, expression in tumors is found downregulated or absent (165, 166, 168) but the role of the mouse EP3

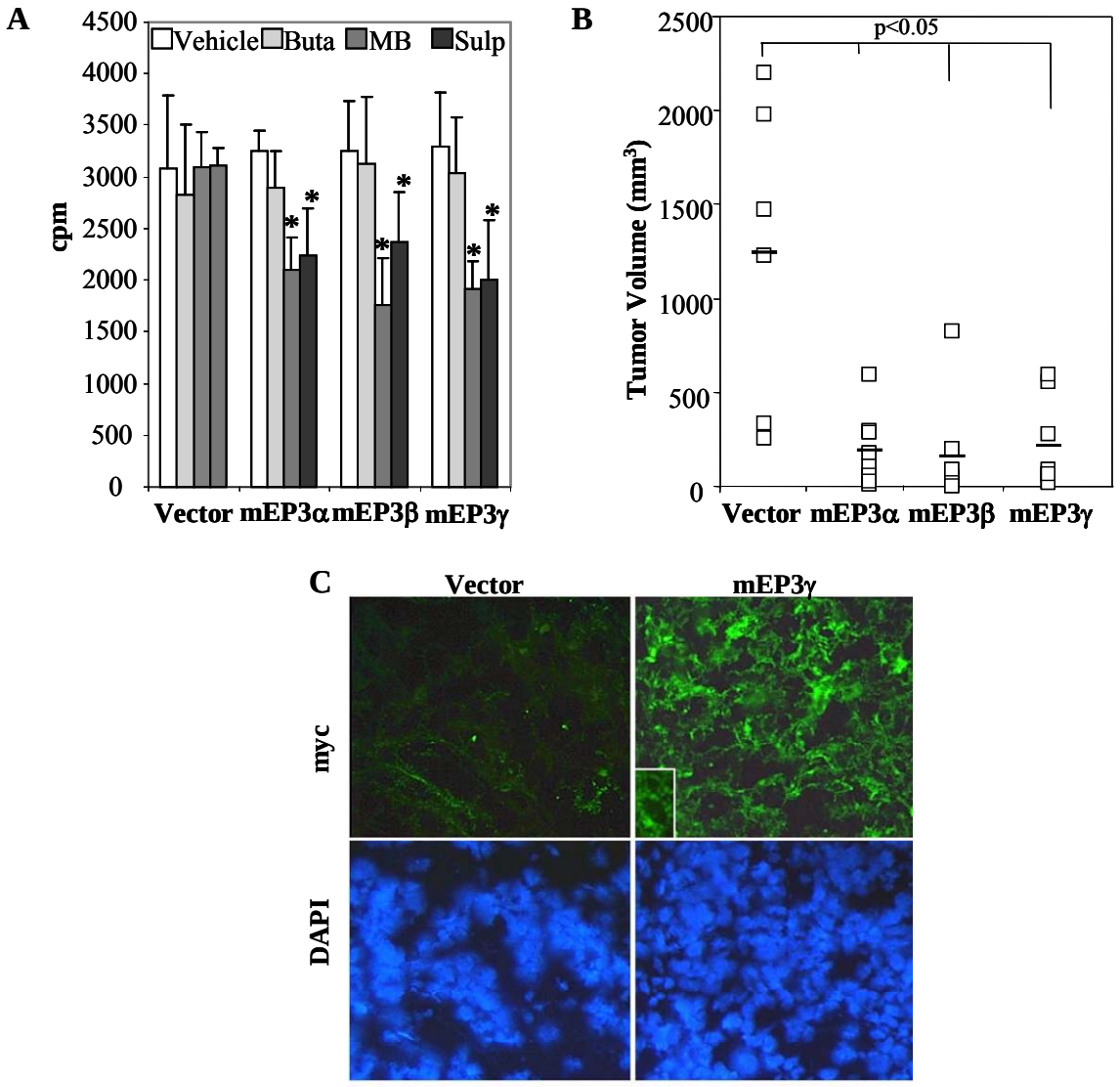

Figure 16. Activation of the mEP3 receptor variants inhibits human colon cancer cell growth *in vitro* and *in vivo*. (A) ^{3}H-Thymidine incorporation assay on HCT116 colon tumor cells stably expressing mEP3 receptor variants pulsed for 48 h with [^{3}H]thymidine (1 μCi/well) with 0.1 μM of MB-28767, sulprostone, butaprost or vehicle. These data are representative of three independent experiments performed in quadruplicates. *$p < 0.05$ **(B)** $1x10^{6}$ vector-transfected and mEP3-expressing HCT116 cells were injected subcutaneously into nude mice (n=10/cell type). Thirty-five days later the mice were sacrificed and tumor volume was evaluated as described under *Materials and Methods*. Open squares represent the volume of single tumors, while the bars represent the mean. **(C)** Frozen sections of tumors derived from the tumors indicated (only mEP3γ shown) were stained with anti-myc antibody to confirm the *in vivo* expression of the mEP3 receptor variants.

receptor variants α, β and γ on tumor cell function is yet unknown. By expressing all three variants in HEK293 cells, which do not demonstrate any measureable membrane associated EP3 receptor (**Figure 5A & 5C**), we have demonstrated all three variants reduce proliferation *in vitro* and that this growth inhibitory effect is mediated by Rho. Furthermore, as HEK293 cells are tumorigenic in nude mice, we examined the contribution of each mEP3 variant on tumor growth *in vivo* and showed all three variants led to the formation of fewer and smaller tumors compared to vector HEK293 cells.

Although HEK293 cells are commonly used to investigate prostanoid receptor function (151, 176-179), the significance of our aforementioned results in this cell line can be addressed by investigating these effects in a more physiologically relevant cell line. Since EPs have been well studied in intestinal tumorigenesis (146-148, 150-152, 154, 155) and EP3 receptor expression is down-regulated in human colon cancer cells (165), we chose to restore mEP3 receptor variant expression in two such cell lines which lack detectable EP3 and investigate their effect on tumor growth. First we demonstrate mEP3 receptor variant signaling can activate Rho in HCT116 and SW480 cells as well as enhance cell-cell contact similar to results observed in HEK293 cells. Activation of the three variants increased Rho GTP levels robustly in human colon cancer cells and the change in morphology was similar for all three variants where cells clustered and increased their cell-cell contact levels. Lastly and most importantly, all three variants led to reduced tumor cell growth *in vitro* and *in vivo* indicating the results observed in HEK293 cells are physiologically relevant and not cell-type specific.

CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

Overview

Despite the vast amount of research performed on EP receptors, little is known on the physiological or pathological effects of the EP3 splice variants and their signal transduction pathways. Overall genetic or pharmacological cancer models have shown the EP3 receptor to not play a direct role in tumor carcinogenesis (146, 150, 153-155). Recently however a number of studies have suggested the EP3 receptor may have a tumor suppressive role as its expression in tumor cells is found absent or downregulated and the loss of the EP3 receptor leads to increased colon carcinoma following chemical treatment (165, 166). Thus far, no study has examined the role of the EP3 variants on tumor cell function. One of the major goals of this thesis was to examine the molecular mechanisms by which the EP3 variants control specific tumor cell functions. We focused on the role of the mouse EP3 variants as they are highly homologous to the human variants (98) and unique to exist with three alternatively spliced cytoplasmic tails. These three C-terminal tails have the potential to signal uniquely and cause alternative effects upon stimulation with their common native ligand, PGE_2. We hypothesized the three mouse EP3 variants would have a suppressive effect on tumor cell growth. We first characterized the three variants for signaling and morphological differences in HEK293 cells. Our results indicate the three variants signal similarly where intracellular calcium is mobilized and the ERK and Rho pathways are activated with selective agonist

stimulation. This suggests the differences in amino acid sequences between the three variants' C-terminal tails do not influence signaling of these pathways. Additionally, we show activation of all three mouse variants causes a morphological change which enhances cell-cell contact and causes cells to appear less fibroblast-like and more epithelial-like. This suggests the mEP3 variants do not promote an invasive phenotype, in contrast, they promote tighter junctions thus reducing the tumorigenic potential of cells. Furthermore, we show the morphological change is mediated by Rho and most likely involves the G_{12} G-protein. Lastly, we examined the role of the mouse C-terminal tails to influence agonist-induced internalization of the EP3 receptor. Our results indicate the C-terminal tails are important in determining the level of EP3 expression at the cell surface. Further investigation is needed in order delineate the regulatory and signaling mechanisms involved in agonist-internalization of the EP3 variants.

In addition to examining the role of mEP3 receptors in HEK293 cells, we examined the functional role of each variant on tumor cell growth *in vitro* and *in vivo*. In contrast to EP1, EP2 and EP4, EP3 receptor expression is decreased in colon cancer in mice, rats and humans when compared to normal mucosa (165). Deletion of the EP3 receptor does not alter early stages of colon cancer progression, namely aberrant crypt foci development, whereas EP1, EP2, and EP4 receptors are involved in small intestinal polyp formation (154-156). However, in contrast to the other EP receptors, an increase in carcinogen-induced colon carcinoma was found in EP3-null mice, suggesting EP3 may impact later stages of colon carcinogenesis (165). Furthermore, only one of eleven colon cancer cell lines was shown to express EP3 and when that cell line was treated with an

EP3 agonist its viability decreased by 30% (165). In order to investigate the role of the three mEP3 variants on tumor cell function we chose to restore EP3 expression in HCT116 cells, a human colon cancer cell line shown to lack both EP3 (165) and COX-2 (169) expression. We demonstrate in both HCT116 and HEK293 cells that the mEP3 variants reduce tumor cell growth *in vitro* and *in vivo*. We further evaluate the mechanism of this inhibitory growth effect by mEP3 in HEK293 cells and show Rho to mediate proliferation *in vitro*. Interestingly, we observed a morphological change in HCT116 tumor cells similar to what was observed in HEK293 cells characterized by enhanced ZO-1 staining, thus cell-cell contact. Furthermore, we analyzed HCT116 cells for EP3-induced Rho activation and found all three variants to increase levels of Rho GTP within 15 minutes of stimulation by selective EP3 agonist. These results suggest the mechanism for the EP3-induced morphological change and growth inhibition is via Rho and thus not cell-type specific.

Discussion and Future Directions

Activation of the mEP3 receptor variants induces a morphological change mediated by a G_{12}-RhoA pathway

Despite the vast amount of research performed on the EP3 receptor, little is known on the signaling mechanisms and physiological significance of the C-terminal splice variants. Functional differences among the murine splice variants have been reported, including coupling to different signal transduction pathways (158), different sensitivities to agonist-induced desensitization(180), different extents of constitutive activity(164), different intracellular trafficking patterns(181), and different agonist-induced internalization patterns(162). We have addressed the fundamental question as to

what is the functional significance of these alternative splice variants, by examining the distinct role of the murine EP3 receptor variants in tumor cell function both *in vitro* and *in vivo*. We provide evidence that all three variants promote intracellular calcium mobilization in a similar dose- and time-dependent manner as described by others (170). Moreover, we show the three variants have indistinguishable effects on tumor cell function: they each can activate ERK and Rho, and cause a long-lasting morphological change characterized by a marked increase in cell-cell contact mediated by a G_{12}-Rho pathway and reduce cell growth *in vivo* and *in vitro*. Therefore, in the context of tumor cell function, all three mEP3 variants can similarly reduce tumorigenic potential.

We further investigated the morphological change for reverse epithelial-to-mesenchymal transition since activation of all three variants caused a morphological change characterized by enhanced cell-cell contact. We observed and show in Figure 8B by immunofluorescent staining for ZO-1, enhanced tight junctions with EP3 activation. EMT is an essential developmental process by which cells of epithelial origin lose epithelial characteristics and polarity, and acquire a mesenchymal phenotype with increased migratory behavior (182-186). Thus, EMT is characterized by loss of intercellular adhesion (E-cadherin and occludins); down-regulation of epithelial makers (cytokeratins); up-regulation of mesenchymal markers [vimentin, fibroblast-specific protein (FSP) and smooth muscle actin (SMA)]; acquisition of fibroblast-like (spindle) morphology with cytoskeleton reorganization; and increase in motility, invasiveness, and metastasic capabilities (182-186). In addition, the process known as "cadherin switching" (down-regulation of E-cadherin and up-regulation of mesenchymal cadherins such as N-

cadherin or cadherin-11) and the accumulation of β-catenin have also been associated with EMT (183, 184). We therefore looked by immunofluorescence and western blot analysis for enhanced E-cadherin and β-catenin and reduced FSP and SNAIL, a transcriptional repressor of E-cadherin. Similar to ZO-1, we observed enhanced staining of E-cadherin and β-catenin at the cell-surface by immunofluorescence but not by western blot indicating they are not upregulated rather localizing to the cell-surface with EP3 activation. Results of FSP and SNAIL were inconclusive due to problems with the FSP antibody and inconsistent western blot results with anti-SNAIL antibodies. Further analysis can be performed looking at cell cycle and proliferation proteins (cyclins, p27, p21, survivin, and Ki67).

Among classical second messenger pathways, the EP3 receptor is coupled to adenylate cyclase inhibition through G_i (163, 164), however the morphological change induced by activation of this receptor in HEK293 cells was not suppressed by pertussis toxin (PT) treatment, indicating that its action was mediated by PT-insensitive heterotrimeric G protein. The EP3 receptor has been shown to activate Rho in various cell lines and constitutively active G_{12} and G_{13} can activate Rho (119, 120, 174), suggesting that one or both of these G proteins may signal to Rho *via* EP3. Our *in vitro* data strongly suggests that all three murine variants mediate Rho activation *via* G_{12}. Our data demonstrates overexpression of dominant negative G_{12}, but not G_{13} prevents the EP3-induced enhanced cell-cell contact. Although dominant negative studies are valid and informative, they present some limitations. Since dominant negative G_{13} could not prevent EP3-mediated cell-cell contact, we can suggest but not unequivocally prove that

G_{13} is not involved in EP3-mediated functions. Presently, the dominant negative approach represents one of the best and most acceptable methods of distinguishing between G_{12}- versus G_{13}-mediated activity. For instance, G_{12} and/or G_{13} coupling have been identified using this method for GPCR receptors such the α_1-adrenoceptor, the vasopressin V_{1a} receptor and the 5-HT_{2C} receptor (187), in addition to our present study for the mEP3 receptor variants. Further investigations utilizing short-interfering RNA (siRNA) may corroborate our results and further confirm the mechanism of the EP3-induced morphological change. By silencing specific Gα subunits, we can downregulate and abrogate G_{12} and/or G_{13} signaling and potentially prevent the morphological change in the presence of selective EP3 agonist. Further studies for this project may also include identifying the link between activation of EP3-G_{12} and the subsequent Rho activation mediating the changes observed in morphology by EP3 activation. Recently, a group of Rho-specific guanine nucleotide exchange factors (RhoGEFs) has been identified which interact with the activated α-subunit of G_{12}/G_{13} and can couple GPCRs to Rho activation. Currently, this group of RhoGEF-proteins comprises three members, p115RhoGEF/Lsc, PDZRhoGEF and Leukaemia-associated Rho guanine nucleotide exchange factor (LARG)(188-190). Recently the siRNA technique was found to be a potent targeting method against RhoGEFs. Also, the three siRNAs targeting LARG, PDZ-RhoGEF, and p115-RhoGEF are able to discriminate the closely related sequences within this RhoGEF gene family (191). With these siRNA sequences we could further clarify the pathway involved in EP3-mediated Rho activation and subsequent changes in morphology.

The mEP3 receptor variants suppress tumor cell growth *in vitro* and *in vivo*

Several studies exploring the role of EP receptor subtypes using genetic deletion or a pharmacologic approach have consistently shown the EP3 receptor to *not* play role on tumorigenesis (154-156). In this context, deficiency in EP3 had no effect on aberrant crypt foci development, putative precursors of colon cancer. In contrast, an increase in azoxymethane-induced colon carcinoma was found in EP3 deficient mice, suggesting EP3 may impact late stages of colon carcinogenesis (165). In agreement with this hypothesis, examination of EP3 mRNA expression in mammary and colon tumors showed a marked reduction in this receptor transcript levels compared to normal mucosa (165, 166). Moreover, EP3 expression was detected in only one of eleven colon cancer cell lines tested and treatment of that one cell line with an EP3 agonist decreased viability by 30%(165). Our *in vivo* findings that over-expression of mEP3 variants decreases the tumorigenic potential of two different tumor cell lines, suggest that EP3 may play a suppressive role in tumor development and its downregulation in tumor cells is advantageous for cancer progression.

The small GTPase Rho plays an important role in processes such as cytoskeletal rearrangement, migration and cell growth (192-195). Rho can be activated by different receptors including extracellular matrix receptors, growth factor receptors and prostanoid receptors. Thus far, only the mouse EP3 α and β variants have been shown to activate Rho and mediate stress fiber formation (120). In this study we report α, β and γ variants

can similarly activate Rho in HEK293 cells and their activation is linked to enhanced cell-cell contact and growth inhibition.

This is the first study to characterize all three variants in HEK293 cells for Rho activation and since all three variants were found to increase Rho-GTP with EP3 agonist treatment we investigated further the role of the mEP3 variant-induced Rho activation on cell morphology and proliferation. Interestingly, these findings demonstrate Rho to have an inverse correlation with cell proliferation; with EP3-induced Rho activation reducing tumor cell growth; and Rho-inhibition restoring tumor cell proliferation. In contrast, others have shown Rho is activated in many tumors types and it plays a positive role in tumorigenesis and metastasis by enhancing cell migration, proliferation, as well as protease synthesis and consequent matrix remodeling (196, 197). The marked increase in cell-cell contact and the mesenchymal-to-epithelial morphological transition observed with EP3 activation results in contact inhibition and cell cycle arrest. A possible explanation for our finding is that EP3-mediated Rho activation leads to increased cell-cell contact, resulting in contact inhibition and cell growth arrest. Contact inhibition is a well known mechanism of controlling cell growth and is thought to be mediated at least in part by blocking the growth factor and integrin-mediated stimuli required for cells to proliferate (198). Furthermore it has been recently shown that Rho activation is necessary to establish complete cell-cell adhesion (199). Thus, as we show that EP3 can induce long lasting cell-cell contact, it is plausible this is a major mechanism whereby this receptor decreases cell proliferation *in vitro* and tumorigenesis *in vivo*. For this reason Rho signaling may not be directly anti-proliferative but indirectly causing the morphological

change which prevents proliferation **(Figure 17)**. The fact that the EP3-induced morphological change is long lasting (**Figure 9B**), even after ligand withdrawl, supports our hypothesis that the Rho-mediated growth inhibitory effects observed upon EP3 activation is most likely the result of prolonged contact inhibition.

In agreement with this possibility, our data demonstrates that restoring and activating the EP3 receptor in tumor cells with a selective agonist is clinically favorable as it was able to fully restore epithelial morphology and reduce growth of tumor cells. The clinical potential of selective EP3 agonists compared with selective inhibitors of COX-2 or with traditional NSAIDs, such as aspirin would seem more favorable knowing the complex and adverse effects of inhibiting multiple prostanoids simultaneously, prostanoids that are known to play a protective effect in some settings. However, EP3 expression is downregulated or absent in tumors of the colon and mammary and the mechanism is yet unclear. Hypermethylation of CpG islands in promoter regions is known to cause silencing of genes in various human cancers (200, 201). Although hypermethylation of the EP3 gene has not been reported, Shoji, et. al. found DNA sequences in the promoter region and exon 1 of the human EP3 gene are GC rich (Genbank AL031429) and demethylation of five human colon cancer cell lines restored EP3 expression (165). These findings suggest that the DNA sequence of the EP3 receptor may be methylated however further investigation is needed to confirm hypermethylation of the EP3 gene occurs and how this is regulated.

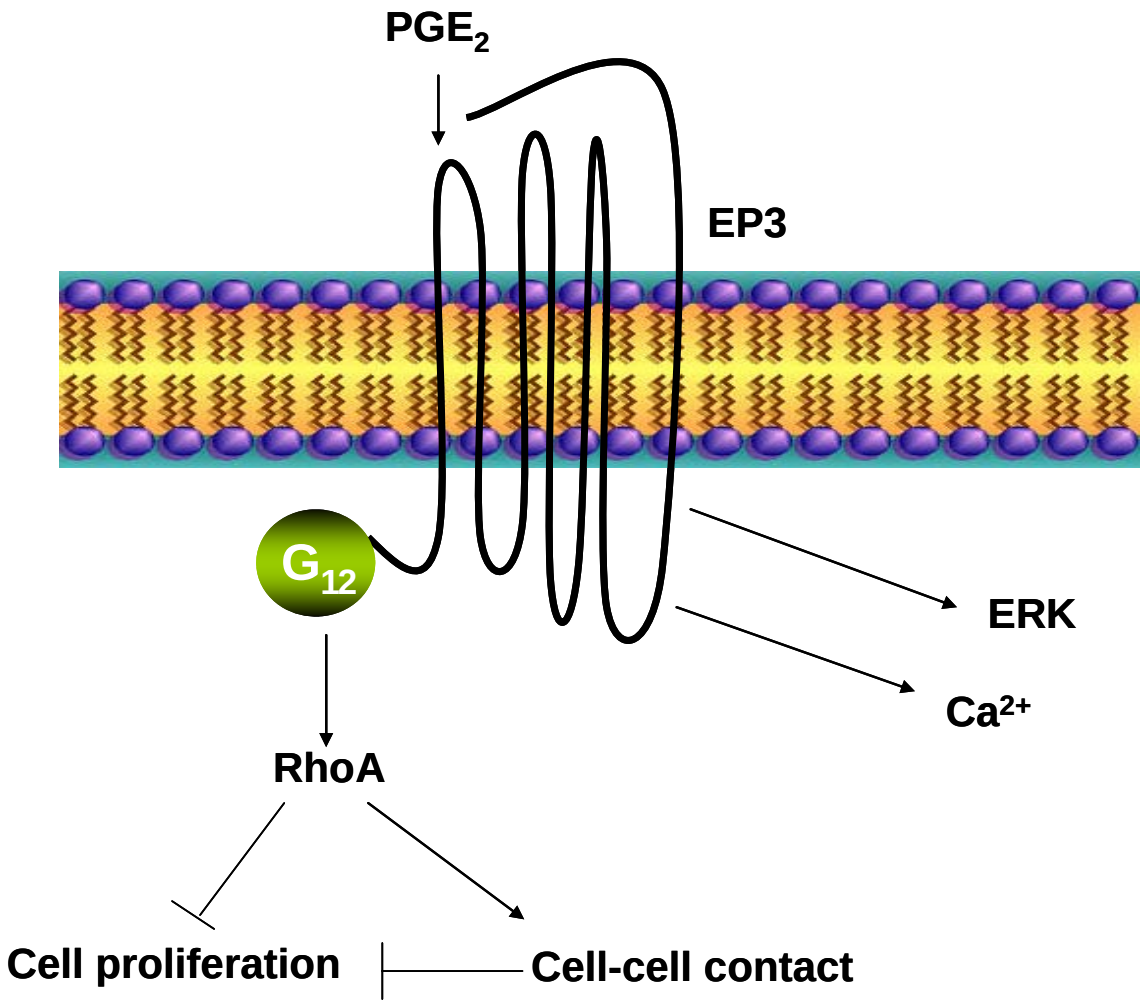

Figure 17. Regulation of cell growth and morphology by the mEP3 receptor variants. Schematic model of how the mEP3 receptor variants may regulate cell morphology and growth. Activation of the mEP3 receptor variants leads to activation of ERK, calcium mobilization and activation of the G protein alpha subunit G_{12}. G_{12} leads to activation of RhoA with consequent increase in cell-cell contact and reduced cell growth.

While our results demonstrate selective EP3 agonists delivered to the tumor cells may be a favorable clinical therapy for cancer, it is important to remember the influence the host microenvironment has on tumor progression. Prostaglandins are important regulators of tumor-host communications. A recent study in EP3 null mice revealed stromal and/or tumor cells release PGE_2 into the tumor microenvironment, which acts on stromal cells that contain EP3 receptors to induce the production of pro-angiogenic factors and consequent angiogenesis (167). This indicates that without EP3 expression on the tumor cells, there is no significant anti-proliferative advantage to administering selective EP3 agonists. Signaling in stromal cells by EP3 receptors has revealed induction of VEGF which has pro-angiogenic action and facilitates tumor growth (167). This is the first study to implicate EP3 as having a pro-tumorigenic role. This highlights the potential tissue and model specificity of EP receptor PGE_2 signaling that is becoming apparent. In the future, it will be important to determine whether COX-2 can modulate EP receptor expression and identify the precise role each receptor signaling pathway plays in regulating angiogenesis and tumor growth. For this reason further investigation is needed into the role the host EP3 receptor expression and activation may have on tumor progression before seriously considering the therapeutic potential of EP3 selective agonists. Further studies that may elucidate the role of EP3 signaling on tumor progression would be to use our human colon cancer cell lines overexpressing each mEP3 variant and injecting these in nude mice deficient in EP3. By injecting these cells either subcutaneously or orthotopically we may delineate the role of EP3 in the tumor versus host EP3 expression/signaling. If we were to demonstrate restoring EP3 expression in tumor cells can reduce tumor growth *in vivo* in both wildtype and EP3

deficient mice this would further support our hypothesis that EP3 acts as a tumor suppressor in tumor cells regardless of host expression of EP3.

Conclusion

From this study, we propose new possible functions of PGE_2 through the EP3 receptor. The major goal of this thesis was to examine the mouse EP3 receptor variants α, β and γ on tumor cell function. The three mouse variants of EP3 have never been investigated for their functional significance in tumor cells before the present study. Our results demonstrated activation of the three mEP3 variants caused a Rho-mediated morphological change never described before characterized by enhanced cell-cell contact. These effects were the same for all three variant, indicating the variants' cytoplasmic tails have no functional difference in respect to tumor cell morphology however we did observe differential cell surface localization depending on the variant activated. This suggests the C-terminal tail is important in intracellular trafficking and agonist-induced internalization. Interestingly, EP3 activation caused a morphological change that was long-lasting even after the removal of agonist which is promising for long-term anti-neoplastic effects by selective EP3 agonists.

In addition we have demonstrated a novel mechanism for the EP3-induced morphological change. Our results indicate EP3 signaling enhances cell-cell contact *via* the small GTPase G_{12}-Rho pathway. In contrast to the published findings that Rho plays a positive role in tumorigenesis, we found Rho activation to have an inverse correlation with proliferation.

In conclusion we have provided new evidence of the involvement of all three variants in reducing tumor cell growth *in vitro* and *in vivo*. Additionally, the effects demonstrated by EP3 activation was evaluated in several tumor cell lines with no difference observed *in vitro* or *in vivo* therefore EP3-induced growth suppression was not cell-specific. Again, there was no difference observed between variants on tumor growth suggesting the C-terminal tail has the same effect on the tumorigenic potential. Importantly, we and others have shown that EP3 expression is reduced or absent in tumor cells (165, 166, 168), this is the first study to show restoring EP3 expression in tumor cells can have an inhibitory effect on tumor development.

Together these studies elucidate the role of the PGE_2 EP3 receptor subtype on tumor cell function. Data obtained in our present studies suggest the EP3 receptor plays an important role in suppression of cell growth. These results along with previous studies suggest its downregulation enhances carcinogenesis. However the exact mechanism regulating to this decrease in EP3 expression warrants further investigation. Taken together, these findings provide a rationale for the development of EP3 agonists. The clinical potential of selective EP3 agonists compared to selective COX-2 inhibitors or traditional NSAIDs is more favorable given that anti-COX therapy prevents the simultaneous synthesis of multiple prostanoids, some of which play a protective effect in many physiological and pathophysiological settings.

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