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The Prostanoid EP4 Receptor and Its Signaling Pathway

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ABBREVIATIONS: AC, adenylyl cyclase; AGN205203, [[3-[(1*R*,2*S*,3*R*)-3-hydroxy-2-[(1*E*,3*S*)-3-hydroxy-4-[3-(methoxymethyl)phenyl]-1-butenyl]-5-oxocyclopentyl]thio]propyl]thio]-acetic acid; AH23848, (Z)-7-[(1*R*,2*R*,5*S*)-2-morpholin-4-yl-3-oxo-5-[(4-phenylphenyl)methoxy]cyclopentyl]hept-4-enoic acid; AH23848B, [1 α (*z*),2 β 5 α](\pm)-7-[5-[[[1,1'-biphenyl]-4-yl]methoxyl]-2-(4-morpholinyl)-3-oxo-cyclopentyl]-4-heptenoic acid; AH6809, 6-isopropoxy-9-oxoxanthene-2-carboxylic acid; AH19437, 1 α (*Z*),2 β ,5 α (\pm)-methyl-7-2-(4-morpholinyl)-3-oxo-5(phenylmethoxy)cyclopentyl-5-heptenoate; AKAP, A-kinase anchor protein; AP, activator protein; bp, base pair; BB94, (2*R*,3*S*)-*N*-hydroxy-*N'*-[(1*S*)-1-(methylcarbamoyl)-2-phenylethyl]-2-(2-methylpropyl)-3-[(thiophen-2-ylsulfanyl)methyl]butanediamide; CAIA, collagen antibody-induced arthritis; CIA, collagen-induced arthritis; CJ-023423, *N*-[[(2-[4-(2-ethyl-4,6-dimethyl-1*H*-imidazo [4,5-*c*] pyridin-1-yl) phenyl]ethyl)amino] carbonyl]-4-methylbenzenesulfonamide; CJ-042794, 2-[3-[[4-*tert*-butylphenyl)methyl-pyridin-3-ylsulfonamino]methyl]phenoxy]acetic acid; COX, cyclooxygenase; CREB, cAMP-response element-binding protein; DP, prostaglandin D₂; EGFR, epidermal growth factor receptor; EGR-1, early growth response gene-1; Epac, exchange protein activated by cAMP; ER819762, (S)-1'-[(3,5-dimethylbenzyl)-2-ethyl-7,9-dimethoxy-10-methyl-5,10-dihydrospiro [benzo[e]imidazo [1,5-*a*]azepine-1,4'-piperidin]-3(2*H*)-one; ERK, extracellular signal-regulated kinase; ET-1, endothelin-1; FD1, 6-[*N*-(2-isothiocyanatoethyl) aminocarbonyl]forskolin; FD6, 6-[3-(dimethylamino)propionyl]-14,15-dihydroforskolin; GFB, glomerular filtration barrier; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; GSK3, glycogen synthase kinase 3; GW627368, 2-[4-(4,9-diethoxy-3-oxo-1*H*-benzo[f]isoindol-2-yl)phenyl]-*N*-phenylsulfonamide; H-89, *N*-[2-[[3-(4-bromophenyl)-2-propenyl]amino]ethyl]-5-isouquinolinesulfonamide; IFN- γ , interferon- γ ; IL, interleukin; ILK, integrin-linked kinase; IP₃, inositol trisphosphate; JG, juxta-glomerular granular; kb, kilobase; KO, knockout; KT-5720, hexyl (15*R*,16*R*,18*S*)-16-hydroxy-15-methyl-3-oxo-28-oxa-4,14,19-triazaoctacyclo[12.11.2.1^{15,18}.0^{2,6}.0^{7,27}.0^{8,13}.0^{19,26}.0^{20,25}]octacos-1(26),2(6),7(27),8,10,12,20,22,24-nonaene-16-carboxylate; L-161,982, *N*-[2-[4-[[3-butyl-5-oxo-1-[2-(trifluoromethyl)phenyl]-1,2,4-triazol-4-yl]methyl]phenyl]phenyl]sulfonyl-5-methylthiophene-2-carboxamide; LPS, lipopolysaccharide; LY-294002, 2-morpholin-4-yl-8-phenylchromen-4-one; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; MDL-12330A, (\pm)-*N*-[(1*R**,2*R**)-2-phenylcyclopentyl-azacyclotridec-1-en-2-amine hydrochloride; MEK, MAPK/ERK kinase; MIP-1 α , macrophage inflammatory protein-1 α ; MMP, matrix metalloproteinase; NF- κ B, nuclear factor κ B; NSAID, nonsteroidal anti-inflammatory drug; ONO-4819, 4-[2-[(1*R*,2*R*,3*R*)-3-hydroxy-2-[(*E*,3*S*)-3-hydroxy-4-[3-(methoxymethyl)phenyl]but-1-enyl]-5-oxocyclopentyl]ethylsulfanyl]butanoate; ONO-AE1-437, deesterified active form of 2-[[4-[[2-[(1*R*,2*R*,3*R*)-3-hydroxy-2-[(*E*,3*S*)-3-hydroxy-4-[3-(methoxymethyl)phenyl]but-1-enyl]-5-oxocyclopentyl]ethyl]sulfanyl]butanoyl]oxy]ethyl nonanoate; ONO-AE1-329, 2-[3-[(1*R*,2*S*,3*R*)-3-hydroxy-2-[(*E*,3*S*)-3-hydroxy-5-[2-(methoxymethyl)phenyl]pent-1-enyl]-5-oxocyclopentyl]sulfanylpropylsulfanyl]acetic acid; ONO-AE1-734, methyl-7-[(1*R*,2*R*,3*R*)-3-hydroxy-2-[(*E*)-3*S*)-3-hydroxy-4-(*m*-methoxymethylphenyl)-1-butenyl]-5-oxocyclopentyl]-5-thiaheptanoate; ONO-AE2-227, 2-[2-[[2-(1-naphthyl)propanoyl]amino]benzyl]benzoic acid; ONO-AE3-208, 4-[4-cyano-2-[2-(4-fluoronaphthalen-1-yl)propanoylaminol]phenyl]butanoic acid; PDE, phosphodiesterase; PGE₂, prostaglandin E₂; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; RANKL, receptor activator of NF- κ B ligand; Runx2, runt-related transcription factor 2; S-145, 5*Z*-7-(3-endo-phenylsulfonamino-(2.2.1.)-bicyclohept-2-exo-yl) heptenoic acid; SC-19220, 1-acetyl-2-(8-chloro-10,11-dihydrodibenz[*b,f*]1,4[oxazepine-10-carbonyl]hydrazine; TNF- α , tumor necrosis factor- α ; W-13, *N*-(4-aminobutyl)-5-chloro-2-naphthahydrochloride; VEGF, vascular endothelial growth factor.

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Abstract—The EP4 prostanoid receptor is one of four receptor subtypes for prostaglandin E_2 . It belongs to the family of G protein-coupled receptors. It was originally identified, similar to the EP2 receptor as a $\text{G}_s\alpha$ -coupled, adenylyl cyclase-stimulating receptor. EP4 signaling plays a variety of roles through cAMP effectors, i.e., protein kinase A and exchange protein activated by cAMP. However, emerging evidence from studies using pharmacological approaches and genetically modified mice suggests that EP4, unlike EP2, can also be coupled to $\text{G}_i\alpha$, phosphatidylinositol 3-kinase, β -arrestin, or β -catenin. These signaling pathways constitute unique roles for the EP4 receptor. EP4 is

widely distributed in the body and thus plays various physiologic and pathophysiologic roles. In particular, EP4 signaling is closely related to carcinogenesis, cardiac hypertrophy, vasodilation, vascular remodeling, bone remodeling, gastrointestinal homeostasis, renal function, and female reproductive function. In addition to the classic anti-inflammatory action of EP4 on mononuclear cells and T cells, recent evidence has shown that EP4 signaling contributes to proinflammatory action as well. The aim of this review is to present current findings on the biologic functions of the EP4 receptor. In particular, we will discuss its diversity from the standpoint of EP4-mediated signaling.

I. Introduction

Among the prostanoids, prostaglandin E_2 (PGE_2) is most widely produced within the body and most broadly distributed throughout animal species. PGE_2 is involved in a number of physiological and pathophysiological responses (Sugimoto and Narumiya, 2007; Woodward et al., 2011). PGE_2 is one of the major products generated by the actions of cyclooxygenases (COX) on arachidonic acid and is well known to be an important mediator of fever, pain, and inflammation. The discovery of PGE has resulted in the recognition of clinically important targets. For example, COX inhibitors, belonging to nonsteroidal anti-inflammatory drugs

(NSAIDs), are currently the most prescribed medications for treating inflammatory conditions such as arthritis.

Historically, PGE was initially identified as a blood pressure-lowering component from the prostate. The presence of PGE was first suggested by Kurzrok and Lieb (1930). They discovered the pharmacodynamic effects of this lipid-soluble substance in human seminal plasma and male accessory glands (Kurzrok and Lieb, 1930). Subsequently, Goldblatt (1933) and von Euler (1934) independently found that the substance stimulated different smooth muscle organs and lowered blood pressure. A further study by Bergstrom and Sjovall (1957) isolated PGE_1 in crystalline form from the vesicular glands of sheep. They also confirmed the

strong blood pressure-reducing activity of PGE₁ (Bergstrom et al., 1959a,b). PGE₂ and PGE₃ were subsequently found in the prostate glands (Van Dorpd et al., 1964). PGE₁, PGE₂, and PGE₃ are biosynthesized from three fatty acid precursors: dihomo- γ -linolenic acid, arachidonic acid, and timnodonic acid, respectively. The numerals refer to the number of carbon-carbon double bonds present. Since arachidonic acid is the most important precursor in animals, PGE₂ is by far the most abundant.

In the late 1960s, studies on adenylyl cyclase (AC) and second messenger molecules (e.g., cAMP) blossomed, as initiated by Sutherland (Robison et al., 1967). Following the discovery of PGEs, Butcher and Baird (1968) were the first to demonstrate an association between prostaglandins and AC/cAMP. In 1982, Kennedy et al. (1982) described a comprehensive classification of prostanoid receptors based upon response to pharmacological stimulation using prostanoids, TP antagonist (AH19437), and EP1 antagonist (SC-19220). They proposed that prostanoid receptors were to be termed P receptors, and that their ligands, natural prostanoids, were to be indicated by a preceding capital letter. Thus, the receptors sensitive to prostaglandins D₂, E₂, F₂ α , I₂, and thromboxane A₂ were termed the DP, EP, FP, IP, and TP receptors, respectively. The use of the letter P preceded by another letter was intended to avoid possible confusion with purinergic receptors, because these had also been termed P receptors. Kennedy et al. (1982) also subdivided the PGE-sensitive receptors into EP1 and EP2, which were SC-19220-sensitive and SC-19220-insensitive, respectively. The use of terms such as EP1 and EP2 was intended to avoid any implication that they represent in this case specific receptors for PGE₁ and PGE₂, respectively. In 1987, Coleman et al. (1987) demonstrated that there was another EP receptor, which was insensitive to EP1 antagonist (SC-19220) and EP2 antagonist (AH6809) and thus was termed EP3. EP4 is the most recently identified subtype of the EP receptor, having been discovered in the piglet saphenous vein (Coleman et al., 1994a) as an EP receptor insensitive to the agonists of EP1, EP2, and EP3.

Prostanoid receptors are classified into three groups according to molecular evolution, associated primary G proteins, and second messengers (Woodward et al., 2011). Cluster 1 consists of EP2, EP4, IP, and DP1, which are coupled with G_s α proteins and therefore activate AC to increase cAMP. Cluster 2 consists of EP1, FP, and TP. Although only a few studies have suggested the EP1-mediated activation of phospholipase C (PLC)/protein kinase A (PKA) (Nicola et al., 2005; Tang et al., 2005), the members of this cluster are considered to be coupled with G_q α . Cluster 3 consists of the inhibitory receptor, EP3, which is coupled with G_i α .

Both EP2 and EP4 receptors share the classic features of PGE receptors, i.e., coupling to G_s α , stimulation of AC,

and cAMP production (Coleman et al., 1994b; Regan et al., 1994b; Narumiya et al., 1999). EP4, however, has unique signaling pathways and biological functions distinct from those of EP2, as described later in this article. Since the 1990s, many such features have been identified through the use of selective pharmacological tools for each EP subtype as well as through the use of recombinant receptor technology. In addition, gene deletion studies have revealed the roles of EP receptor signaling in vivo in physiology and pathophysiology. Two lines of systemic-null EP4-deficient mice were generated independently (Nguyen et al., 1997; Segi et al., 1998), followed by three lines of EP2-deficient mice in 1999 (Hizaki et al., 1999; Kennedy et al., 1999; Tilley et al., 1999). These extensive studies have contributed significantly to our understanding that EP4 signaling plays a variety of roles not via the cAMP pathway alone but via others as well. It now appears that EP4 signaling is associated not only with G_s α but also with G_i α (Leduc et al., 2009), phosphatidylinositol 3-kinase (PI3K) (Regan, 2003), β -arrestin (Buchanan et al., 2006; Kim et al., 2010), and β -catenin (Banu et al., 2009; Jang et al., 2012), as described in section II.C.

The EP4 receptor, unlike the EP2 receptor, is expressed in a variety of tissues and cells, including the immune, osteoarticular, cardiovascular, gastrointestinal, and respiratory systems, and cancer cells (An et al., 1993; Bastien et al., 1994; Honda et al., 1993; Sando et al., 1994). Recent findings have suggested that the regulation of EP4 signaling could be involved in therapeutic strategies for colon cancer (Mutoh et al., 2002; Yang et al., 2006), aortic aneurysm (Cao et al., 2012; Yokoyama et al., 2012), rheumatoid arthritis (Murase et al., 2008; Okumura et al., 2008; Chen et al., 2010), osteoporosis (Yoshida et al., 2002; Ito et al., 2006; Ke et al., 2006), and autoimmune disease (Yao et al., 2009). Accordingly, the regulation of EP4 signaling has received even greater attention as a potential therapeutic target.

Several excellent and comprehensive reviews of the EP receptors as a group already exist and must be mentioned here (Coleman et al., 1994b; Narumiya et al., 1999; Sugimoto and Narumiya, 2007; Woodward et al., 2011). In this review, we will focus on the properties of EP4 from the perspective of its downstream signaling pathways, not only its conventional AC/cAMP second messenger system but also more recently identified systems. We will also describe its physiologic and pathologic roles, including its therapeutic implications.

II. Discovery and Characterization

A. Cloning

Cloning of the EP receptors was initiated by the identification of the TP receptor. By 1994, all four EP receptors had been identified through TP-homology screening. Ushikubi et al. (1989) purified the human

TP receptor protein from human platelets using the radiolabeled ligand S-145. On the basis of the partial amino acid sequence of the purified protein, cDNA for the TP receptor was isolated in 1991 (Hirata et al., 1991). Subsequently, all EP receptors were identified. The first was EP3 (Sugimoto et al., 1992), first found in a mouse cDNA library through the use of polymerase chain reaction and cross-hybridization, followed by similar cloning of the mouse and human EP1 receptors (Funk et al., 1993; Watabe et al., 1993).

At that time, there was some confusion regarding the nomenclature of the EP2 and EP4 receptors. It was believed that EP2 was the only EP subtype that could stimulate AC/cAMP production. Therefore, the first-cloned EP receptor subtype that stimulated AC was named EP2 (An et al., 1993; Bastien et al., 1994; Honda et al., 1993; Sando et al., 1994). This EP2 was found in humans, mice, and rats. However, it is puzzling that this receptor subtype did not bind to butaprost, an EP2 agonist. This puzzle was solved, at least in part, by Regan et al. (1994b), who identified another EP receptor subtype that could stimulate AC/cAMP formation and was sensitive to butaprost. Concurrently, pharmacological studies suggested the presence of a fourth subtype within the EP receptor family, which appeared to stimulate AC but was insensitive to butaprost (Coleman et al., 1994a). Nishigaki et al. then demonstrated that the receptor initially named "EP2" was sensitive to the EP4-specific antagonist AH23848B (Nishigaki et al., 1995). In contrast, a mouse homolog of the receptor subtype cloned by Regan et al. (1994b) had pharmacological properties of the EP2 receptor (Katsuyama et al., 1995). These results indicated that the receptors which had originally been cloned in mice, humans, and rats and named "EP2" were in fact EP4 (Narumiya et al., 1999; Regan, 2003). As this historical background shows, EP4 is the most recently identified receptor subtype within the EP receptor family. EP2 and EP4 are similar in that both stimulate AC but are different as proven by their specific ligand binding properties. We describe the unique features of this receptor subtype in this review.

B. Structure and Evolution

1. Receptor Structure. The prostanoid receptors belong to the seven-transmembrane G protein-coupled receptor (GPCR) superfamily. Thus, EP4 also shares this membership. The properties of the GPCR superfamily include an aspartate in the second transmembrane domain, which is involved in receptor-ligand interaction (Savarese and Fraser, 1992). Another shared property is a pair of conserved cysteine residues in the second and third extracellular domains, which form a disulfide bond critical for stabilization of receptor conformation and for ligand binding (Savarese and Fraser, 1992). N-Glycosylation of asparagine

residues is also conserved and plays a role in ligand binding in the GPCRs. All of these particular residues or motifs characteristic of the GPCRs are seen in the EP receptors.

In addition to the features preserved among GPCRs, several other motifs are conserved among the prostanoid receptors in the third and seventh transmembrane domains and in the second extracellular loop. In particular, the arginine in the seventh transmembrane domain may be the binding site of the prostanoids (Narumiya et al., 1999). This arginine is also conserved in all EP4 clones from different animal species, i.e., human, mouse, rat, dog, rabbit, chicken, and zebrafish, suggesting its ancestral origin during evolution.

Despite the presence of the above-mentioned conserved motifs and their common response to PGE₂, amino acid identity is limited among the EP receptor family (Narumiya et al., 1999; Sugimoto and Narumiya, 2007). The amino acid identity of EP4 to EP1 is 30%, whereas that of EP4 to EP3 is 37%. EP4 and EP2 have similar signaling pathways in terms of activation of G_sα and subsequent cAMP production, but the amino acid identity of EP4 to EP2 is only 38%. In contrast, among animal species, amino acid identity of EP4 is maintained. Among various mammals, such as monkey, cow, mouse, and rat, the homology ranges from 88 to 99%. The sequence homology between human and mouse EP4 is 88%.

Further comparison of the amino acid sequence homology between EP2 and EP4 was performed by Regan (2003), who identified particular differences in the intracellular domains. The EP4 receptor has a longer serine- and threonine-rich intracellular carboxyl terminus than EP2 (148 vs. 40). In addition, there is an insertion of 25 amino acids in the third intracellular loop in EP4 but not in EP2 (Regan, 2003).

2. Gene Structure. The human and mouse EP4 genes consist of three exons separated by two introns (Arakawa et al., 1996; Foord et al., 1996). A similar exon-intron relationship is present in the other types of prostanoid receptors, such as the DP, EP1, EP2, EP3, FP, and IP receptors (Hirata et al., 1994; Regan et al., 1994a; Batshake et al., 1995; Ogawa et al., 1995; Boie et al., 1997; Hasumoto et al., 1997; Katsuyama et al., 1998b). In the human EP4 receptor, the first exon [530 base pair (bp)] is noncoding. After an intron of 472 bp, the second exon contains a short (43 bp) 5' sequence before a 289-amino acid open reading frame. An 11.5-kilobase (kb) intron is found at the end of the sixth transmembrane, and the rest of the open reading frame is in the third exon.

The deduced initiation site of the human EP4 does not contain a conventional TATA box, but is 70% GC-rich and contains CCAAT boxes (Foord et al., 1996). The promoter region of the mouse EP4 has a TATA box (Arakawa et al., 1996). The ATG start codon is located

16 bp downstream of the translational start site in the mouse EP4 (Arakawa et al., 1996). It is noteworthy that the human EP4 receptor gene contains several motifs responsive to proinflammatory agents such as nuclear factor interleukin (IL) 6, nuclear factor κ B (NF- κ B), and H-apf-1 in addition to a Y box, activated activator protein-1 (AP-1) sites, and AP-2 sites (Foord et al., 1996). The mouse EP4 receptor gene also contains AP-1 sites, AP-2 sites, SP-1 sites, an NF- κ B element, an E box, an nuclear factor interleukin 6 element, a glucocorticoid-responsive element, and Pit-1 sequences (Arakawa et al., 1996). NF- κ B is known to be activated rapidly in response to stress signals and proinflammatory cytokines such as IL-1, resulting in its regulation of immune responses (Li and Verma, 2002). Therefore, EP4 can be upregulated in inflammatory diseases and be involved in inflammatory responses. Indeed, EP4 expression was upregulated in RAW 264.7 macrophage cell lines after stimulation with bacterial lipopolysaccharide (LPS) (Arakawa et al., 1996).

3. Evolution. Phylogenetic studies have shown that the COX pathway was initiated as a system composed of PGE and its receptor. The subtypes of prostanoid receptors later evolved from this ancestral primitive PGE receptor by gene duplication to mediate different signal transduction pathways (Regan et al., 1994b; Boie et al., 1995; Toh et al., 1995; Narumiya et al., 1999; Breyer et al., 2001). The primitive PGE receptor may have mediated signal transduction through cAMP metabolism (Regan et al., 1994b). The primitive receptor was first divided into two subclusters. One was an ancestral receptor for the EP3 subtype, from which an ancestral receptor for the EP1 subtype diverged. The other subcluster included IP, DP, EP4, and EP2. After EP4 and EP2 diverged, IP and DP further diverged from EP2. Hence, the receptors for PGI, PGD, and PGE (the EP2 and EP4 subtypes), all of which share the cAMP signaling pathway, are phylogenetically closer to each other than they are to the other EP receptor subtypes, EP1 and EP3.

Kwok et al. (2008) presented the evolutionary relationships between EP2 and EP4 in different species, including humans, mice, rats, dogs, cattle, chickens, and zebrafish. The phylogenetic tree suggested that the functional divergence between EP4 and EP2 occurred before the divergence of an ancestral bony fish. The unique signaling pathways of the EP4 receptor might have developed during its period of independent evolution.

The genes encoding human, mouse, and rat EP4 have been mapped to chromosomes 5p13.1, 15, and 2q16 (Taketo et al., 1994; Duncan et al., 1995), respectively. The EP4 receptor is also present in nonmammalian vertebrates, such as chickens (Kwok et al., 2008) and zebrafish (Cha et al., 2006).

C. Signaling Pathways

In terms of signaling pathways, AC/cAMP was the major research focus in the E series of prostaglandins prior to the cloning of EP receptors (Fig. 1). Elevation of cAMP via AC by PGE₁ was first demonstrated in rat tissues involving fat pads, lungs, spleens, and kidneys (Butcher and Baird, 1968). A similar effect of PGEs was observed in the corpora lutea (Marsh, 1971). In contrast, an inhibitory effect of PGEs on cAMP production was demonstrated in isolated fat cells (Butcher and Baird, 1968), suggesting that PGEs can have opposite effects on cAMP signaling. The cloning of EP receptors led to an explanation, at least in part, for these counteracting signal transductions because EP receptors target both stimulatory and inhibitory G proteins. Among the four receptors, i.e., EP1–EP4, there are biochemical similarities between EP2 and EP4, e.g., both subtypes are coupled with stimulatory G protein and thus can activate AC to produce cAMP. The EP3 subtype has been demonstrated to have an inhibitory effect on cAMP production (Sugimoto et al., 1993). In 1997, the EP1 subtype was shown to be linked with the G_q α protein (Nemoto et al., 1997). Recent studies, however, have identified differences between EP2 and EP4 downstream signaling, such as the coupling of EP4 to the inhibitory G protein (Fujino and Regan, 2006) and PI3K (Fujino et al., 2002; Fujino and Regan, 2003; Pozzi et al., 2004; Yao et al., 2009), which results in further differences in the downstream signaling pathways of the two receptors and thus their cellular functions. These findings suggest that EP4 is not just another EP2; rather, it has unique biologic properties as an independent target receptor of PGE₂, which will be further discussed in the following sections.

1. G Protein. EP4 is classified as a member of the prostanoid receptor family, which belongs to GPCRs that consist of approximately 900 receptors (Lappano and Maggiolini, 2011). Heterotrimeric G protein is a direct downstream effector of GPCRs. Upon ligand binding, the inactive, GDP-bound form of G protein is transformed into its active, GTP-bound form followed by the dissociation of the α and $\beta\gamma$ subunits. The G α subunit includes four major subtypes, i.e., the stimulatory (G_s α) and inhibitory (G_{i/o} α) subtypes, G_q α , and G_{12/13} α . G_s α stimulates AC, a membrane-bound cAMP-generating enzyme. The activated G_{i/o} α subunits inhibit AC activity, resulting in a decrease in intracellular cAMP levels. Activation of G_{i/o} α results in the release of relatively high amounts of $\beta\gamma$ subunit, thus activating the $\beta\gamma$ -mediated multiple signaling processes (Wettschureck and Offermanns, 2005; Smrcka, 2008). The dissociated $\beta\gamma$ subunit itself stimulates AC subtypes, i.e., AC2, AC4, and AC7, and could eventually increase cAMP as described later in this article. G_q α targets PLC, leading to activation of inositol trisphosphate (IP₃)- or diacylglycerol-mediated signaling pathways.

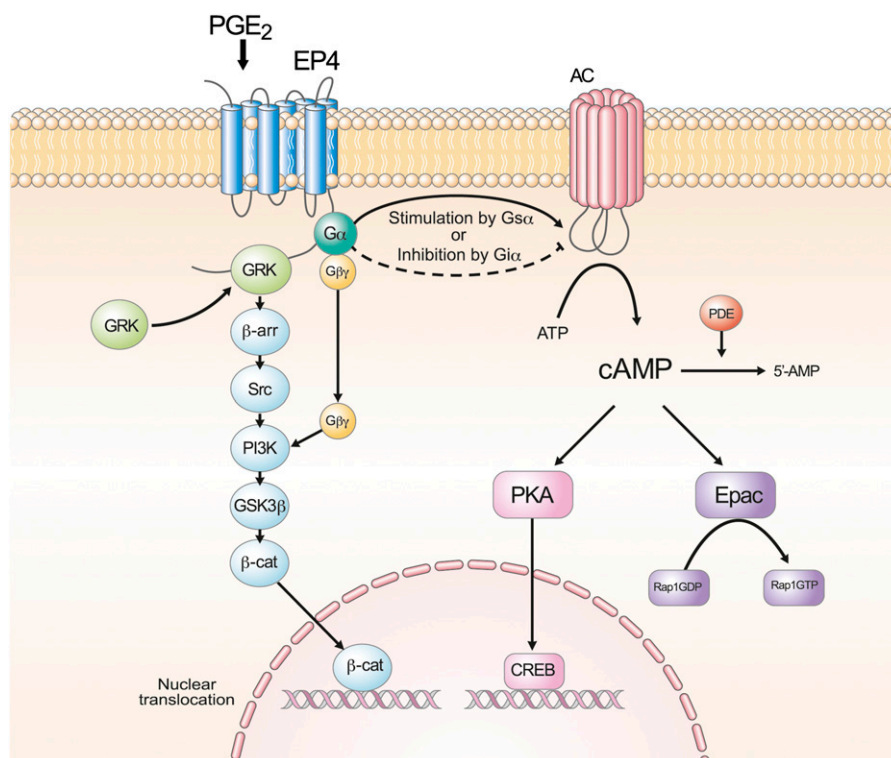


Fig. 1. Upon activation of the EP4 receptor by PGE₂, the G_sα subunit is dissociated from the receptor and Gβγ. Conventionally, EP4, similar to EP2, activates G_sα, leading to increased AC catalytic activity and thus cAMP production. In some cases, however, EP4, unlike EP2, may activate G_iα, leading to decreased cAMP signaling. This may require the presence of a G_iα-inhibitable AC isoform(s). Cyclic AMP signaling activates two major target molecules, PKA and Epac. PKA, a cAMP-dependent kinase, phosphorylates multiple downstream molecules including CREB, a major cAMP-regulated transcription factor, and thus regulates their function. Epac is a guanine nucleotide exchange factor for Rap that acts independently from PKA. Cyclic AMP is eventually degraded to 5'-AMP by PDE. EP4 stimulates cAMP-independent signaling as well. This is achieved through activation of the GRK/β-arrestin/Src/PI3K/GSK3 pathway, leading to, for example, nuclear translocation of β-catenin. This pathway may be modified by Gβγ dissociated from G_sα via interaction with PI3K upon EP4 activation. Thus, EP4 can elicit multiple signaling pathways within a cell. β-arrestin; β-cat, β-catenin.

G12/13α is known to regulate the activity of guanine nucleotide exchange factor for RhoGEF.

EP4 is coupled not only with G_sα but also with G_iα (Fujino and Regan, 2006), as mentioned earlier. This phenomenon would partially explain why the potency of EP4 to increase cAMP is less than that of EP2 and is reminiscent of the relationship between the β1- and β2-adrenergic receptors; the β1-adrenergic receptor is coupled with only G_sα, whereas the β2-adrenergic receptor is coupled with both G_sα and G_iα (Feldman, 1993; Ho et al., 2010). Since several studies, especially those in the cardiovascular field, have demonstrated that the role of the β2-adrenergic receptor is distinct from that of the β1-adrenergic receptor (Ho et al., 2010), this is potentially the case for EP4 as well. In other words, it is possible that the cellular functions evoked by PGE₂ are unique to each EP receptor.

2. Adenylyl Cyclase. AC, a target enzyme of G_sα and G_iα, is a 12-transmembrane enzyme that converts ATP to cAMP, a major second messenger (Iwatsubo et al., 2006; Ho et al., 2012). The AC family consists of nine membrane-bound isoforms and one cytosolic, soluble isoform. Membrane-bound ACs are classified according to their tissue expression, amino acid homology, and biochemical properties (Iwatsubo et al., 2003). Historically, membrane-bound ACs were classified into

four groups. Group 1 consists of AC1, 3, and 8, originally found to be expressed in the central nervous system and regulated by Ca²⁺/calmodulin. Group 2 consists of AC2, 4, and 7, ubiquitously expressed isoforms that are regulated by Gβγ subunits. Group 3 consists of the isoforms AC5 and 6, which are mainly expressed in the heart and the brain, and are sensitive to inhibition by G_iα subunit the micromolar range of Ca²⁺. AC9 is the only member of group 4 and is regulated by calcineurin. The intracellular domains of AC, i.e., the C1a and C2a domains, form a cleft that serves as a catalytic core. Within this catalytic core, ATP is converted into cAMP. Regulators such as G proteins or forskolin, a direct AC stimulator (Iwatsubo et al., 2003), can change the conformation of the catalytic core, resulting in alteration of enzymatic activity.

It is well known that EP4 increases cAMP (An et al., 1993; Coleman et al., 1994a; Nishigaki et al., 1995), indicating an EP4-mediated activation of AC via G_sα. It remains unknown, however, which AC isoform(s) can be preferentially regulated by EP4. An example of such association occurs in the ductus arteriosus. Our group has demonstrated that EP4 is a predominant EP subtype in the rat ductus arteriosus, and activation of EP4 significantly increased hyaluronan production via cAMP signaling (Yokoyama et al., 2006). Regarding AC

isoforms, mRNA expressions of all AC isoforms with the exception of AC1 and 8 were observed in the ductus arteriosus. Knockdown of AC2, 5, and 6 inhibited PGE₁-induced cAMP elevation; in addition, it is noteworthy that only AC6 knockdown was able to suppress PGE₁-induced hyaluronan production (Yokoyama et al., 2010b). Therefore, it is plausible that a functional association between EP4 and AC6 exists in the ductus arteriosus, but further experiments using a combination of knockdown and selective stimulation of EP4/AC6 are necessary to obtain conclusions.

The major difference between EP2 and EP4 is that EP4 associates with both G_sα and G_iα, whereas EP2 associates with G_sα only, as mentioned earlier. Accordingly, AC5 and AC6 could be targets of EP4 because these group 3 ACs are G_iα-sensitive isoforms (Iwatsubo et al., 2003; Oshikawa et al., 2003; Willoughby and Cooper, 2007; Okumura et al., 2009). The other G_iα-sensitive isoforms, AC1, AC3, AC8, and AC9, could also be targets of EP4 (Willoughby and Cooper, 2007). Furthermore, from the perspective of PGE₂'s effects, AC5 and AC6 are potentially inhibited by the elevation of cytosolic Ca²⁺ evoked by EP1. Capacitive Ca²⁺ elevation also inhibits these isoforms (Willoughby and Cooper, 2007). This cross-talk is another potential explanation for the contradictory effects of PGE₂ on cAMP production under different conditions. Taken together, the evidence suggests that AC is a key molecule that could help explain the divergence of PGE₂'s effects and clarify the downstream molecular signaling pathways of the EP receptors. Accordingly, efforts must be made to identify a specific AC isoform(s) that is (are) regulated by a specific EP receptor subtype(s).

3. Protein Kinase A. cAMP has two major targets, cAMP-dependent kinase, also known as PKA, and exchange protein activated by cAMP (Epac), also known as Rap guanine nucleotide exchange factor (Gilman, 1970; de Rooij et al., 1998; Ho et al., 2012). PKA consists of two regulatory and two catalytic subunits. cAMP binds to a regulatory subunit, leading to the dissociation of the catalytic subunit from the regulatory subunit. The released catalytic subunit can then phosphorylate target proteins at their serine or threonine residues, resulting in activation/inhibition of the substrates (Kim et al., 2006). Among them, cAMP-response element-binding protein (CREB), a transcription factor, is one of the major downstream targets of PKA, which controls cellular functions via synthesis of a wide variety of proteins (Shaywitz and Greenberg, 1999). EP4-mediated CREB activation was reported in colon epithelial cells (Srivastava et al., 2012), dorsal root ganglion neurons (Cruz Duarte et al., 2012), Leydig tumor cells (Sirianni et al., 2009), and breast cancer cells (Subbaramaiah et al., 2008). Other signaling pathways in addition to CREB are activated via PKA. In rat ventricular myocytes, the EP4/PKA

pathway significantly increased promoter activity of brain natriuretic peptide, and it was inhibited by mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK), suggesting the involvement of ERK signaling downstream of PKA (Qian et al., 2006). Details of the underlying mechanism were later reported that the EP4/cAMP/PKA pathway activates c-Fos via the Rap1/MEK/ERK pathway, confirming the functional relationship between cAMP/PKA and ERK signaling (He et al., 2010). In human embryonic kidney 293 cells stably expressing EP4, PKA mediated PGE₂-induced phosphorylation of glycogen synthase kinase 3 (GSK3) (Fujino et al., 2002). In addition, using skin of COX-2 knockdown mice, it was demonstrated that PGE₂ phosphorylated the proapoptotic protein Bad via PKA, suggesting the involvement of EP4 as well as EP2 in PKA-mediated apoptosis signaling (Chun et al., 2007). Recently, compartmentalization of cAMP signaling has been proposed as a molecular mechanism by which PKA can adequately control a specific protein(s), although it has a wide variety of targets. A-kinase anchor proteins (AKAPs) bind signaling molecules, including PKA, phosphodiesterases (PDEs), and phosphatases, forming a microdomain that may enable effective initiation/termination of signal transduction (Michel and Scott, 2002). Other reports have demonstrated that PGE₂'s effects are mediated by AKAPs (Schnizler et al., 2008; Schillace et al., 2009; Kim et al., 2011; Lenz et al., 2011), suggesting that AKAPs are additional key proteins in EP4/cAMP signaling and that this relationship should be investigated.

4. Exchange Protein Directly Activated by cAMP. Some Epac-mediated cellular functions are known to be regulated by upstream EP4. Epac, consisting of Epac1 and Epac2, converts the inactive GDP-bound form of Rap1 to an active GTP-bound one, resulting in the initiation of downstream signal transductions. cAMP binds to the cyclic nucleotide-binding domain in the regulatory region of Epac, which causes a conformational change in the catalytic region, resulting in activation of guanine nucleotide exchange factor (Gloerich and Bos, 2010). Epac is not a compensatory molecule for PKA; it is independent and plays even primary roles in certain cell types. One example is renal cell carcinoma cells. Knockdown of Epac or addition of Rap1GAP, a Rap1 inhibitor, suppressed EP4-mediated invasion, but a PKA inhibitor did not, suggesting the superiority of Epac over PKA in EP4 signaling in renal carcinoma cells (Wu et al., 2011). On the other hand, coordination between Epac and PKA in the immune system has been reported. EP4-induced proliferation of Th17 cells, a subset of helper T cells, is PKA-dependent. Furthermore, Epac mediates EP4-induced production of IL-23, which also activates Th17 proliferation, in dendritic cells (Yao et al., 2009). Our group reported that EP4 regulates both PKA- and

Epac-mediated pathways. Activation of EP4, AC, and PKA increased the production of hyaluronan in the ductus arteriosus smooth muscle cells. EP4-induced hyaluronan production was inhibited by a PKA inhibitor, suggesting that the EP4/AC/PKA pathway plays a role in hyaluronan production (Yokoyama et al., 2006). Epac1 increased migration of ductus arteriosus smooth muscle cells, and an EP4 agonist can activate Rap1, suggesting a major role of Epac1 in EP4-mediated migration. Interestingly, EP4-activated Rap1 was observed only under PKA-inhibited conditions, suggesting a feedback mechanism within cAMP signaling, specifically, a PKA-mediated regulation of Epac signaling (Yokoyama et al., 2008).

5. *Phosphodiesterase*. PDEs convert cAMP and cGMP to AMP and GMP, respectively, by degrading the phosphodiester bond. Therefore, PDEs could suppress cAMP-mediated EP4 signaling. Human PDEs have 11 isoforms categorized according to amino acid sequence similarities, tissue distributions, and biochemical properties. They can be classified into three large groups based on substrate specificities: cAMP-sensitive (PDE4, 7, and 8), cGMP sensitive (PDE5, 6, and 9), and both cAMP/cGMP sensitive (PDE1–3, 10, and 11) (Bender and Beavo, 2006). Attempts to use selective inhibitors for PDEs have been highly successful, and some of them are currently used in clinics, such as milrinone, a PDE3 inhibitor used to treat heart failure (Shipley et al., 1996), and sildenafil, a PDE5 inhibitor used to treat erectile dysfunction (Boolell et al., 1996). PDEs also participate in the regulation of EP4 signaling. Osteoblast nodule formation in bone marrow cells was increased by a PDE4 inhibitor, and this increase was altered by an antagonist for EP4. In addition, PDE4 inhibitor-induced cAMP elevation was suppressed by a PKA inhibitor. These data suggest that osteoblast nodule formation is accelerated by the EP4/PKA pathway and that PDE4 negatively regulates this signaling (Miyamoto et al., 2003a). In addition to PDE4, PDE3 is also involved in EP4 signaling. EP4-mediated expression of receptor activator of NF- κ B ligand (RANKL) in osteoblasts was enhanced by inhibitors for PDE3 and PDE4 (Noh et al., 2009). These data indicate that specific PDE isoforms can negatively regulate EP4/cAMP signaling in osteoblasts, whereas the PDE isoforms involved in this signaling in other types of cells remain unidentified.

6. *Phosphatidylinositol 3-Kinase*. Along with AC/cAMP, PI3K is also a major downstream target of EP4. PI3K involvement in EP4 signaling was clearly demonstrated in human embryonic kidney cells stably expressing EP4. Stimulation of EP4 increased phosphorylation of GSK3 and Akt, and this phosphorylation was suppressed by a PI3K inhibitor (Fujino et al., 2002). In this report, it is noteworthy that PGE₂ regulated two independent pathways in the same cell, i.e., the EP2/PKA pathway and EP4/PI3K pathway, suggesting that PGE₂ evokes different functions

by stimulating either EP2 or EP4 independently. Subsequently, multiple reports have demonstrated the involvement of PI3K in EP4 signaling. Fujino et al. (2003a) demonstrated that the EP4/PI3K pathway activates ERK, leading to an increase in the expression of early growth response factor-1. Likewise, in colon carcinoma cells, an EP4-selective agonist activated the PI3K/ERK pathway, which resulted in a rescue of indomethacin- or COX-2 inhibitor-suppressed proliferation (Pozzi et al., 2004). PI3K involvement in EP4 signaling was also reported in cell migration during gastrulation in zebrafish (Cha et al., 2006) and differentiation of helper T cells (Yao et al., 2009).

Despite the above-mentioned evidence of interaction between EP4 and PI3K, the molecular mechanism by which EP4 activates PI3K is largely unknown. One report demonstrated that EP4-induced activation of PI3K was mediated by pertussis toxin-sensitive G protein (Fujino and Regan, 2006), suggesting that inhibitory G protein is involved in this pathway. Considering the effect of the pertussis toxin, it is possible that the other part of the heterotrimeric G protein, the G $\beta\gamma$ complex, mediates EP4-induced PI3K activation. The G $\beta\gamma$ complex is a combination of a G β subunit and a G γ subunit. The G β subunit has five different isoforms in humans and mice, and the G γ subunit has 12. The dissociated G $\beta\gamma$ complex acts as a signaling molecule, leading to regulation of multiple molecules, including G protein-coupled inwardly rectifying potassium channels, PLC, AC, and Ca²⁺ channels (Smrcka, 2008). Reports have suggested that PI3K is the downstream target of the G $\beta\gamma$ complex (Thomason et al., 1994; Hazeki et al., 1998), but further studies are necessary to clarify that this mechanism is the one involved in EP4 signaling. It should be noted that the G $\beta\gamma$ complex itself regulates AC, stimulating AC2 and AC4, inhibiting AC1 and AC8, and having inconsistent effects on AC3, AC5, AC6, and AC7 (Iwatsubo et al., 2006; Willoughby and Cooper, 2007; Halls and Cooper, 2011); this suggests that the G $\beta\gamma$ complex affects not only the PI3K pathway but also the cAMP pathway upon activation of EP4. Unfortunately, little attention has yet been paid to this possibility.

Another possible explanation for the EP4-mediated activation of PI3K is the transactivation mechanism of epidermal growth factor receptor (EGFR) by EP4, which was demonstrated in colorectal cancer cells (Buchanan et al., 2006). Another example of such transactivation occurs in endometriotic cells, showing that activation of EP2 and EP4 evokes transactivation of EGFR, which leads to stimulation of the PI3K/Akt pathway (Banu et al., 2009). This study further demonstrated the presence of cross-talk between PGE₂ and Wnt signaling via PI3K. The EP4-activated PI3K pathway was associated with the initiation of transcription via GSK3 β / β -catenin, a major nuclear import pathway located downstream of Wnt signaling.

Nevertheless, the question of whether EP2 or EP4 plays a more significant role in the cross-talk to Wnt signaling remains unanswered. Further support for cross-talk between EP2/EP4 and β -catenin has been reported. PGE₂ activated GSK3 β and increased nuclear translocation of β -catenin in osteocytes (Kitase et al., 2010). In addition, activation of Akt and increased nuclear translocation of β -catenin under PGE₂-stimulated conditions were reported in colon cancer cells (Kisslov et al., 2012).

7. Desensitization and Arrestin Signaling. Activated GPCRs partially undergo an inactivation process induced by at least two molecules, G protein-coupled receptor kinases (GRKs) and arrestins (DeWire et al., 2007; Gurevich et al., 2012). After the dissociation of G proteins from GPCRs, GRKs phosphorylate the intracellular domains of the GPCRs, and prevent the receptors from rebinding to G proteins. This phenomenon is recognized as the first step in the receptor downregulation. The GRK family consists of seven members, among which the catalytic domain and regulator of the G protein signaling domain are preserved. In addition to desensitizing GPCRs, GRKs also interact with multiple other molecules, which enable GPCRs to regulate pathways other than G protein-associated pathways (Gurevich et al., 2012). Regarding EP4, a few reports are available concerning its GRK-mediated desensitization. In COS-7 cells expressing a rat chimera PGE receptor, i.e., rat EP3 whose C-terminal domain has been replaced with that of human EP4, the basal and agonist-induced phosphorylation of the C-terminal domain was augmented by overexpression of GRK2, 3, and 5. In addition, agonist-induced receptor internalization was increased by overexpression of GRK2. These data suggested that EP4 underwent phosphorylation and thus receptor desensitization mediated by GRKs (Neuschafer-Rube et al., 1999).

Phosphorylation of GPCRs by GRKs leads to the binding of arrestins, another mechanism preventing the reassociation of G proteins with GPCRs. In addition, the binding of arrestins evokes receptor internalization to the intracellular space, one of the important processes in receptor downregulation and recycling. The arrestins are classified into four subtypes, arrestin-1 to arrestin-4; among these, arrestin-2 and -3 are also called β arrestin-1 and -2, respectively (DeWire et al., 2007). In addition to their role in receptor downregulation, arrestins also act as scaffold proteins that accelerate signal transductions in the EP4 pathway. The physical association between EP4 and β arrestin-1 was demonstrated using bioluminescence resonance energy transfer (Leduc et al., 2009). In addition to their desensitization-related functions, arrestins mediate signal transduction of EP4. In colorectal cancer cells, β arrestin-1 bound to EP4 can activate membrane-bound c-Src, leading to the transactivation of EGFR. Activation

of this EP4/ β arrestin-1/c-Src enhanced cell migration as well as cancer metastasis in mice (Buchanan et al., 2006). Similarly, another report demonstrated that EP4 mediated migration of lung cancer cells via the association between β arrestin and the c-Src pathway (Kim et al., 2010).

8. Extracellular Signal-Regulated Kinase. EP4 signaling regulates ERK activity as shown in several reports mentioned earlier (Fujino et al., 2003a; Pozzi et al., 2004; Qian et al., 2006; He et al., 2010) and in other studies. In pulmonary microvascular endothelial cells, EP4 stimulation induced cAMP elevation and ERK activation, leading to capillary formation. This phenomenon was only suppressed by an ERK inhibitor, but not by inhibitors for PKA or PI3K, suggesting that ERK signaling is activated independently from the cAMP and PI3K pathways (Rao et al., 2007). cAMP-independent ERK activation by EP4, which was mediated by transactivation of EGFR, was also demonstrated in rat ventricular myocytes (Mendez and LaPointe, 2005). In contrast, EP4 inhibited ERK in chondrocyte cells, leading to suppression of tumor necrosis factor- α (TNF- α)-induced production of matrix metalloproteinase-1 (MMP-1). ERK was inactivated by EP4 via the phosphorylation of Raf-1 at its Ser259 within the negative regulatory site, which induces inhibition of the Raf-1/MEK/ERK pathway (Fushimi et al., 2007). All of these data indicate an important link between EP4 and ERK; the nature of the resultant effects, i.e., whether they are stimulatory or inhibitory, seems to depend on the type of cells.

9. Compartmentalization. Second messengers such as cAMP, Ca²⁺, and IP₃ are shared by a variety of upstream receptors/channels. It was formerly unknown how a second messenger could find its correct target molecule rather than assuming a random distribution in the cytosolic space. Studies have shown that compartmentalization could provide an answer to this question (Steinberg and Brunton, 2001). Compartmentalization mechanisms consist of multiple structural and trafficking proteins that enable signaling molecules to gather and begin the activation of transduction. One example of a trafficking protein is AKAP, which is involved in cAMP signaling and was mentioned in the previous section. AKAP can bind to cAMP-related regulatory proteins and then anchor them to a specific location associated with the membrane or move them into the cytosolic space (Michel and Scott, 2002). Upstream of the second messenger, e.g., at the receptor level, the caveola plays a significant role in compartmentalization. The caveola is a small pit in the plasma membrane formed by caveolin, an integral membrane protein. Caveolin acts as a scaffolding protein that enables specific effector molecules to assemble into the caveola, resulting in more effective signal transduction (Ishikawa et al., 2005). The caveola's role with regard to EP was

previously reported, as was the expression of EP2 in the caveolar fraction of COS-7 cells (Yamaoka et al., 2009). Another report, however, demonstrated that EP2 was also found in the noncaveolar fraction in rat cardiac myocytes (Ostrom et al., 2001), suggesting that the distribution of EP2 receptor differs among cell types. It is worth noting, however, that the role of the caveola and raft scaffold with regard to EP4 signaling remains largely unknown; studies concerning EP4 compartmentalization are therefore desired. To summarize, although EP4 cloning was completed in the early 1990s, much more work must be done to unveil the molecular signaling mechanism responsible for the EP4 signaling pathway. Such efforts will lead not only to the accumulation of research data, but also to the transmission of such findings from the bench to the bedside in the near future. Importantly, numerous physiologic/pathologic functions of EP4 have been reported in various tissues/organs, as described in detail later.

III. Biologic Function and Diseases

A. Cardiovascular System

1. Heart.

a. Expression. The EP receptor subtypes are expressed in various cell types in the cardiovascular system, including cardiomyocytes (Miyatake et al., 2007; Birkenmeier et al., 2008), noncardiomyocytes, including fibroblasts (Xiao et al., 2004), smooth muscle cells (Purdy and Arendshorst, 2000; Yokoyama et al., 2006, 2012; Foudi et al., 2008), and endothelial cells (Rao et al., 2007). EP4 mRNA is abundant in the hearts of several species, including humans (An et al., 1993), mice (Honda et al., 1993), rats (Sando et al., 1994; Jovanovic et al., 2006), and canines (Castleberry et al., 2001), although the expression levels of each EP subtype may vary among these species. Xiao et al. (2004) demonstrated that, among the EP subtypes, EP4 mRNA was the most strongly expressed in the cardiac ventricles of adult mice. In addition, EP4 mRNA expression was greater in noncardiomyocytes than in cardiomyocytes in mice. Overall, the ratio of EP4 mRNA in cardiomyocytes to that in noncardiomyocytes was approximately 1:3 (Xiao et al., 2004). The capacities for cAMP production in these cell types were comparable: an EP4 agonist induced cAMP production in both cardiomyocytes and noncardiomyocytes but at a higher rate in noncardiomyocytes (Xiao et al., 2004). Cyclic AMP production in the heart is mostly performed by cardiac AC isoforms, i.e., types 5 and 6 (Katsushika et al., 1992; Okumura et al., 2003), as well as by other isoforms (Iwatsubo et al., 2003) via $G_{s\alpha}$ activation. However, functional coupling to a specific AC isoform(s) in the heart has not been fully characterized.

b. Function. EP4 receptor signaling contributes to cardiomyocyte hypertrophy (Table 1). Its contribution to noncardiomyocytes, however, is not well understood. Activation of the EP4 receptor increased protein synthesis and cell surface area via activation of ERK1/2 in rat neonatal cardiomyocytes (Mendez and LaPointe, 2005; Frias et al., 2007; He et al., 2010). Hypertrophic markers, such as atrial natriuretic peptide (Mendez and LaPointe, 2005) and brain natriuretic peptide (Qian et al., 2006; He et al., 2010), were also increased through EP4-ERK1/2 signaling. The transcriptional factor known as signal transducer and activator of transcription 3 was activated, and its expression was correlated with both EP4-mediated cardiomyocyte hypertrophy in vitro and in vivo (Frias et al., 2007; Qian et al., 2008).

EP4 receptor-mediated hypertrophy is more pronounced in the injured heart, where it may play a beneficial role. Qian et al. (2008) demonstrated that cardio-specific EP4 receptor deletion attenuated infarction-induced cardiac hypertrophy and worsened cardiac ejection fraction. Under basal conditions, however, no difference was found in ejection fraction or myocyte cross-sectional area between EP4-null and wild-type mice (Qian et al., 2008). The global EP4-null mouse was likewise not different from the wild type in heart weight, morphology, or diastolic pressure under basal conditions (Xiao et al., 2004).

It is well known that cAMP-producing β -adrenergic stimulation induces cardiomyocyte hypertrophy (Metrich et al., 2010a). Similar findings are seen when $G_{s\alpha}$ protein is overexpressed in the heart (Geng et al., 1999). Thus, enhanced cAMP signaling via EP4 appears to induce cardiac hypertrophy. A downstream effector of cAMP Epac1 was found to contribute to cardiomyocyte hypertrophy via activation of H-Ras in a manner dependent on PLC/IP₃ and two prohypertrophic transcription factors, nuclear factor of activated T cells (Morel et al., 2005) and myocyte enhancer factor 2 (Metrich et al., 2008, 2010b). Ulucan et al. (2007) also suggested that Epac1 activation correlated with the development of cardiac hypertrophy through ERK1/2. Epac may play a role in EP4-mediated cardiomyocyte hypertrophy as a downstream effector of cAMP. In contrast to Epac, PKA, the other downstream effector, inhibited cardiac fetal gene expression and cardiomyocyte hypertrophy through activation of histone deacetylase 5 (Ha et al., 2010). In the heart, AC5 and AC6 are major AC isoforms (Ishikawa et al., 2005; Swaney et al., 2006; Gao et al., 2011). However, studies using genetically engineered mice have shown that neither AC5 nor AC6 promotes cardiomyocyte hypertrophy (Ishikawa et al., 2005; Gao et al., 2011; Sugano et al., 2011). The AC subtypes involved in EP4-mediated cardiomyocyte hypertrophy have not yet been identified. From these data, cAMP and Epac are speculated to be involved in EP4-mediated cardiomyocyte

TABLE 1
Reported roles of EP4-mediated signaling pathways in the cardiovascular system

Tissues/Cells	Reported Functions	Proposed Effector Pathway(s)	Pathophysiologic Process	References
Heart	Cardiomyocyte hypertrophy	EGFR activation; ERK1/2	Protein synthesis	Mendez and LaPointe, 2005
	Cardiomyocyte hypertrophy	ERK1/2; STAT3	Protein synthesis	Frias et al., 2007; Qian et al., 2008
	Cardiomyocyte hypertrophy	PKA; Rap; ERK1/2; p90RSK	c-Fos and BNP expression	He et al., 2010
	Reduced infarct size	N.D.	N.D.	Xiao et al., 2004
	Reduced infarct size	N.D.	Inhibition of MCP-1 production and recruitment of macrophages	Hishikari et al., 2009
	Prolonged graft survival	N.D.	Inhibition of NF- κ B and cytokine production	Ogawa et al., 2009
Ductus arteriosus	Suppressed autoimmune myocarditis	N.D.	Inhibition of T-cell proliferation and MCP-1 production	Ngoc et al., 2011
	Vasodilation	cAMP; AC6	Phosphorylation of vasodilator-stimulated phosphoprotein	Yokoyama et al., 2010b
	Vasodilation	N.D.	N.D.	Smith et al., 1994; Kajino et al., 2004; Momma et al., 2005a,b
	Intimal thickening	cAMP; AC6; PKA; p38 MAPK	Hyaluronan-mediated smooth muscle cell migration	Yokoyama et al., 2006, 2010a
Renal artery	Vasodilation	cAMP	Decreased intracellular calcium concentration	Purdy and Arendshorst, 2000
Saphenous vein	Vasodilation	N.D.	N.D.	Coleman et al., 1994a; Rouaud et al., 1999; Jones and Chan, 2001; Wilson and Giles, 2005
Pulmonary vein	Vasodilation	N.D.	N.D.	Walch et al., 1999; Foudi et al., 2008
Cerebral artery	Vasodilation	N.D.	N.D.	Davis et al., 2004; Maubach et al., 2009
Uterine artery	Vasodilation	N.D.	N.D.	Baxter et al., 1995
Endothelial cells	Angiogenesis	cAMP; PKA; Rap1A	eNOS activation	Zhang and Daaka, 2011
	Angiogenesis	N.D.	eNOS activation; AMP activated protein kinase activation	Zhu et al., 2011
	Angiogenesis	cAMP; ERK1/2	Endothelial cell migration	Rao et al., 2007
	Angiogenesis	N.D.	Endothelial cell migration	Kuwano et al., 2004
Atherosclerosis	Increased early atherosclerosis	PI3K; Akt	VEGF production; endothelial cell proliferation	Yanni et al., 2009
	Proteolytic activation	N.D.	Inhibition of macrophage apoptosis	Babaev et al., 2008
	Inhibition of inflammation	N.D.	MMP-2 and MMP-9 activation in macrophages	Cipollone et al., 2005
Aortic aneurysm	Progression of aortic aneurysm	N.D.	Inhibition of MCP-1 and IFN- γ -inducible protein 10 production	Tang et al., 2011a
	Progression of aortic aneurysm	N.D.	Inhibition of IL-6 production and MMP-2 activation	Yokoyama et al., 2012
	Inhibition of aortic aneurysm	N.D.	Inhibition of MMP-2 and MMP-9 activation	Cao et al., 2012
	Inhibition of aortic aneurysm	N.D.	Inhibition of MCP-1 in macrophages	Tang et al., 2011b

BNP, brain natriuretic peptide; eNOS, endothelial nitric-oxide synthase; N.D., not determined.

hypertrophy. Currently, however, there is no direct evidence regarding second messenger and subsequent downstream signaling in EP4-mediated cardiomyocyte hypertrophy.

EP4 may play a beneficial role in cardiac ischemia (Table 1). During ischemia, it is well known that PGE₂ is increased significantly in the heart, which may activate EP4 (Berger et al., 1976; Calabresi et al., 2003). Thus, the roles of the EP4 receptor in the ischemic heart have been studied using genetically engineered mice and EP4-specific agonists. Deletion of EP4 enhanced infarct size following ischemia/reperfusion injury (Xiao et al., 2004), whereas EP4 agonists reduced infarct size when administered before

ischemia/reperfusion injury (Xiao et al., 2004; Hishikari et al., 2009), suggesting that EP4 is cardioprotective in the ischemic heart. The downstream signaling pathway of EP4 in the ischemic heart has not been reported. As potential downstream pathways, the cAMP-PKA and PI3K signaling pathways themselves seem to be cardioprotective against ischemia/reperfusion injury in the heart (Sanada et al., 2001; Nagoshi et al., 2005), although the role of Epac in ischemia/reperfusion injury has not been reported.

The beneficial effect of EP4 in ischemia may involve inflammatory cells (Table 1). During the development of myocardial infarction and ischemia/reperfusion injury, inflammatory cells migrate into the myocardium,

and inflammatory cytokines and chemokines are produced. Hishikari et al. (2009) demonstrated that an EP4 agonist inhibited infarct size as well as production of ischemia-induced MMP-2, MMP-9, monocyte chemoattractant protein-1 (MCP-1), IL-1 β , TNF- α , and macrophage infiltration in the heart, suggesting that the cardioprotective effect of EP4 is dependent on inflammatory cells. A similar anti-inflammatory effect of EP4 signaling in the heart has been shown in other mouse models. Activation of the EP4 receptor prolonged graft survival (Ogawa et al., 2009) and suppressed experimental autoimmune myocarditis development (Ngoc et al., 2011).

Noncardiomyocytes may also play a role. EP4 is functionally expressed in noncardiomyocytes including cardiac fibroblasts, which are primary cell types in terms of cell number (Xiao et al., 2004). Experimental ischemia/reperfusion injury exacerbated infarct size in global EP4 knockout (KO) mice (Xiao et al., 2004), but not in myocyte-specific EP4 KO mice (Qian et al., 2008). By use of Langendorff hearts, Xiao et al. (2004) demonstrated that the cardioprotective action of EP4 signaling is independent of its actions on blood constituents and the coronary artery. The effect of EP4 on nonmyocyte cell types, such as cardiac fibroblasts and inflammatory cells, appears to contribute to reduction in infarct size.

2. Ductus Arteriosus. The ductus arteriosus is a peculiar structure that exists only in the fetus, undergoing dynamic change in the form of ductal closure upon birth (Yokoyama et al., 2010b). The ductus arteriosus is a bypass artery from the pulmonary artery to the aorta, present in the fetus because the pulmonary artery is not required due to the lack of lung respiration. The ductus arteriosus closes immediately after birth; this is necessary for the establishment of neonatal lung circulation/respiration. Because PGE₂ is abundantly produced by the placenta during pregnancy, but immediately withdrawn upon birth, EP4 receptor expression also changes dynamically during development (Yokoyama et al., 2006).

a. Expression. PGE₂ is the most important endogenous prostaglandin involved in the regulation of ductus arteriosus patency in utero (Smith, 1998). The expression of PGE₂ receptors in ductus arteriosus tissue has been extensively studied in humans (Leonhardt et al., 2003), pigs (Bhattacharya et al., 1999; Bouayad et al., 2001a), lambs (Smith et al., 2001; Waleh et al., 2004), baboons (Waleh et al., 2004), and rats (Yokoyama et al., 2006). These studies suggest that the EP2, EP3, and EP4 receptors are all expressed in the ductus arteriosus. Among the EP receptors, however, EP4 is the most strongly expressed in the smooth muscle layer in both fetuses and neonates in humans (Leonhardt et al., 2003; Rheinlaender et al., 2006) and in mice (Nguyen et al., 1997; Segi et al., 1998; Gruzdev et al., 2012). In contrast, in endothelial

cells, EP4 receptor expression is detectable in mice (Gruzdev et al., 2012) but scattered or absent in humans (Leonhardt et al., 2003; Rheinlaender et al., 2006). Thus, smooth muscle cells appear to be the major cell type expressing EP4.

Closure of the ductus arteriosus occurs in two phases. One is the functional closure of the ductal lumen that occurs within hours after birth; this is classically triggered by smooth muscle constriction. The other is the anatomic occlusion of the lumen over the next several days; this is due to extensive neointimal thickening (Smith, 1998; Clyman, 2006; Yokoyama et al., 2010a). The EP4 receptor is abundantly expressed in the smooth muscle cells that contribute to intimal thickening in humans (Rheinlaender et al., 2006) and mice (Nguyen et al., 1997). Ductal occlusion by EP4 is relevant in most mammalian species. EP4 receptor expression increases as pregnancy nears its end and decreases during the neonatal period in humans (Rheinlaender et al., 2006), mice (Chen et al., 2012), rats (Yokoyama et al., 2006), and pigs (Bhattacharya et al., 1999), although these changes are absent in lambs and baboons (Smith et al., 2001; Waleh et al., 2004).

b. Function. PGE₂ is produced in the placenta (Smith, 1998) as well as in the ductus arteriosus (Clyman et al., 1978; Coceani et al., 1978). The classic function of PGE₂ in utero is to maintain the patency of the ductus arteriosus via vasodilatation. Stimulation of PGE₂ receptors activates G_s α /ACs (Bouayad et al., 2001b). The increased intracellular cAMP inhibits myosin light-chain kinase, resulting in relaxation of the ductus arteriosus (Smith, 1998). Yokoyama et al. (2010b) reported AC isoform-dependent relaxation of the ductus arteriosus; AC6, rather than AC2, was responsible for this vasodilatation as demonstrated through experiments using AC isoform-specific small interference RNA and agonists that were developed by us.

Another line of evidence supports the possibility that this vasodilatation occurs through EP4. The dilatory effect of PGE₂ was mediated by EP4 in rabbits (Smith et al., 1994). The EP4 agonists ONO-AE1-437 and ONO-4819 exhibited a potent dilatory effect on the rat fetal ductus arteriosus against O₂⁻ or indomethacin-induced contractions in a concentration-dependent manner both in vivo and in vitro (Kajino et al., 2004). Another EP4 agonist, ONO-AE1-329, inhibited rat neonatal ductus arteriosus contractions (Momma et al., 2005b), whereas its antagonist, ONO-AE3-208, promoted rat fetal and neonatal ductus arteriosus contractions in vivo (Momma et al., 2005a). Thus, the EP4 receptor maintains the opening of the ductus arteriosus.

In contrast to these pharmacological findings, however, genetic loss of the EP4 receptor paradoxically opens the ductus arteriosus (Nguyen et al., 1997; Segi et al., 1998). When the EP4 gene was globally

disrupted in mice, nearly all (95%) homozygous mice exhibited patent ductus arteriosus and died soon after birth (Nguyen et al., 1997; Segi et al., 1998), indicating that the EP4 receptor was, unexpectedly, required for ductal occlusion. In support of these genetic findings, double-mutant mice in which COX-1 and COX-2 were disrupted and which thus lacked PGE₂ synthesis also exhibited patent ductus arteriosus (Loftin et al., 2001).

As an explanation for this seemingly contradictory evidence, Yokoyama et al. (2006, 2010a) suggested that EP4 plays an additional role in regulating the patency of the ductus arteriosus. EP4 signaling, in fact, induces intimal thickening of the ductus arteriosus, in addition to vasodilatation, during the fetal period and after birth (Yokoyama et al., 2010a; Yokoyama et al., 2006). PGE₂-mediated activation of EP4 led to increased cAMP production and thus PKA signaling, leading to robustly increased hyaluronan synthase activity in smooth muscle cells. Hyaluronan then promoted smooth muscle cell migration into the subendothelial layer, leading to intimal thickening formation in the ductus arteriosus (Yokoyama et al., 2006). Indeed, intimal thickening was completely absent in the ductus arteriosus of EP4-disrupted neonatal mice (Nguyen et al., 1997; Yokoyama et al., 2006).

A study in mice suggested that EP4 in smooth muscle cells may play a more important role than that in endothelial cells in this process. Gruzdev et al. (2012) used mouse lines with EP4 loss restricted to the smooth muscle cells or endothelial cells. They found that mice with EP4 loss in smooth muscle cells, but not in endothelial cells, died in the perinatal period from patent ductus arteriosus, which was indistinguishable from that observed in global EP4 KO mice (Gruzdev et al., 2012). Thus, EP4 signaling in smooth muscle cells has a critical effect on ductus arteriosus tone and remodeling.

Hyaluronan-mediated intimal thickening most likely involves AC6 via PKA and p38 MAPK. A study using genetic disruption of AC6 and AC isoform-specific agonists, 6-[N-(2-isothiocyanatoethyl) aminocarbonyl]forskolin (FD1) and 6-[3-(dimethylamino)propionyl]-14,15-dihydroforskolin (FD6) (Onda et al., 2001), demonstrated that PGE₂-EP4-AC6 was responsible (Yokoyama et al., 2010b). Epac, a relatively new target of cAMP (de Rooij et al., 1998), was also upregulated during the perinatal period in the ductus arteriosus. An EP4 agonist increased activation of Rap1, one of the downstream molecules of Epac, in smooth muscle cells of the ductus arteriosus, suggesting that EP4 activates Epac in the ductus arteriosus (Yokoyama et al., 2008). In this study, Epac itself stimulated smooth muscle cell migration and thus intimal thickening in the ductus arteriosus (Yokoyama et al., 2008), although there is no direct evidence of the involvement of Epac in EP4-mediated migration and intimal thickening. Together, these

findings show that the two phases of closure of the ductus arteriosus, i.e., immediate functional closure and chronic anatomic occlusion, are both regulated by EP4 signaling in smooth muscle cells.

Impaired elastogenesis is a hallmark of the vascular remodeling of the ductus arteriosus. The ductus arteriosus undergoes disassembly and fragmentation of the internal elastic lamina and sparse elastic fibers in the middle layer compared with its connecting arteries, although they are exposed to essentially the same hemodynamics (Jager and Wollenman, 1942; Ho and Anderson, 1979; Toda et al., 1980). We examined the contribution of EP4 signaling to elastogenesis in the ductus arteriosus and found that activation of the EP4 receptor inhibited elastic fiber formation via degradation of lysyl oxidase, which cross-links elastin (unpublished data).

These data suggest that EP4 signaling in smooth muscle cells has a critical effect on tone and remodeling in the ductus arteriosus (Table 1).

3. Other Vessels.

a. Expression. The EP4 receptor is expressed in a variety of arteries and veins. Expression has been shown in the great artery, e.g., the adult aorta in humans (Cao et al., 2012; Yokoyama et al., 2012), mice (Rutkai et al., 2009), and rats (Tang et al., 2008). Small arteries and veins also express EP4, including the cerebral artery (Davis et al., 2004; Maubach et al., 2009), renal arteriole (Purdy and Arendshorst, 2000), pulmonary vein (Foudi et al., 2008), and saphenous vein (Coleman et al., 1994a). Expression is not restricted to smooth muscle cells but is also found in endothelial cells (Rao et al., 2007).

EP4 receptor expression may vary across disease states and across animal models. Yokoyama et al. (2012) demonstrated that EP4 expression in aortic smooth muscle cells was greater in aortic aneurysm patients than in normal subjects. However, Cao et al. (2012) reported no differences between normal aorta and aortic aneurysm tissues in mice. Similarly, there were no differences in the aorta between spontaneously hypertensive rats and Wistar Kyoto rats (Tang et al., 2008) or between mice with type 2 diabetes and wild-type mice (Rutkai et al., 2009).

b. Function. cAMP signaling is a major pathway mediating vasodilation under physiologic conditions via several downstream molecules (Morgado et al., 2012). For example, an increase in cAMP reduces myofilament Ca²⁺ sensitivity by phosphorylating myosin light-chain kinase and thereby decreasing its affinity for the Ca²⁺-calmodulin complex, leading to vasodilation (Conti and Adelstein, 1981). After Coleman et al. (1994a) pharmacologically characterized the EP4 receptor in the piglet saphenous vein, the vasodilatory effect of the EP4 receptor was demonstrated in various parts of the vasculature, such as the saphenous vein (Rouaud et al., 1999; Jones and Chan, 2001;

Wilson and Giles, 2005), pulmonary vein (Walch et al., 1999; Foudi et al., 2008), cerebral artery (Davis et al., 2004; Maubach et al., 2009), renal artery (Purdy and Arendshorst, 2000), and uterine artery (Baxter et al., 1995), as well as the ductus arteriosus (Table 1). Thus, EP4 was originally discovered as a vasodilatory signal and has been shown to play that role elsewhere in the cardiovascular system.

In vascular endothelial cells, PGE₂ may play a role in angiogenesis, in particular, in cancer (Chang et al., 2004; Wang et al., 2006b). Such PGE₂-mediated angiogenesis was recently reported in non-cancer cells as well. Activation of the EP4 receptor promoted in vitro tube formation of human dermal microvascular endothelial cells through PKA catalytic subunit γ -mediated upregulation of endothelial nitric oxide synthase (Zhang and Daaka, 2011). Similar findings were seen in retinal microvascular endothelial cells resulting from vascular endothelial growth factor (VEGF) production (Yanni et al., 2009). In vitro tube formation of mouse pulmonary microvascular endothelial cells was also promoted through EP4-mediated migration (Rao et al., 2007). EP4 also promoted endothelial differentiation from endothelial progenitor cells (Zhu et al., 2011). In vivo experiments have shown EP4-mediated angiogenesis as well (Kuwano et al., 2004; Rao et al., 2007; Zhang and Daaka, 2011). It has been suggested that Src, Epac/Rap1/Akt, and PKA signaling are involved in EP4- or PGE₂-mediated VEGF expression in HeLa cells and mesenchymal stem cells (Liu et al., 2011; Jang et al., 2012). Taking this evidence together, it appears that EP4 receptor signaling contributes to vascular angiogenesis in both normal and cancer cells (Table 1).

4. Atherosclerosis. Atherosclerosis and aneurysm are preeminent medical problems in most countries. The biosynthesis of PGE₂ is increased in human atherosclerotic plaques (Cipollone et al., 2005) and has been implicated in atherosclerotic plaque rupture as well (Linton and Fazio, 2008). Thus, it is not surprising that EP4 signaling plays an important role in this disease process. However, the downstream signaling pathways that regulate atherosclerosis formation, especially the pathway involving cAMP, have not been well discussed. Fantidis (2010) reviewed the beneficial effects of cAMP signaling on atherosclerosis through its modulation of vascular endothelium function, production of reactive oxygen species, recruitment of inflammatory cells, and regulation of triglyceride and cholesterol serum levels. Nevertheless, the role of cAMP in atherosclerosis is not widely accepted and has not yet been thoroughly characterized.

The role of EP4 signaling in atherosclerosis regulation has been examined in macrophages (Table 1), but remains controversial. EP4 is the predominant PGE₂ receptor subtype in macrophages from human

atheroma, and EP4 signaling inhibits PGE₂-induced inflammatory response in macrophages in vitro (Cipollone et al., 2005). In vivo, however, the role of the EP4 receptor has been controversial. Babaev et al. (2008) showed that genetic deletion of EP4 on hematopoietic cells increased macrophage apoptosis and decreased early atherosclerosis. On the other hand, Tang et al. (2011a) reported that similar genetic deletion of EP4 had little effect on plaque size or morphology, but promoted local inflammation, including MCP-1 production and infiltration of macrophages and T cells. Cao et al. (2012) showed that pharmacological inhibition of the EP4 receptor did not affect angiotensin II-induced atherosclerotic lesions of the aortic root. The roles of EP4 in atherosclerosis should be examined in future studies.

5. Aneurysm. Inhibition of EP4 signaling may be a means of preventing aortic aneurysm formation (Table 1). In aneurysm walls, COX-2 is widely expressed in macrophages and smooth muscle cells, along with locally synthesized PGE₂ (Walton et al., 1999). Studies using COX-2 inhibition (King et al., 2006), genetic deletion of COX-2 (Gitlin et al., 2007), and PGE₂ synthase 1 deletion (Wang et al., 2008) have demonstrated that the inhibition of COX-2-PGE₂ decreased angiotensin II-induced abdominal aortic aneurysm. It has been reported that the EP4 receptor is highly expressed in the aortic walls of patients with abdominal aortic aneurysm, contributing to IL-6 production (Bayston et al., 2003). In these contexts, two groups independently examined the role of EP4 signaling in abdominal aortic aneurysm in animal models. They demonstrated that pharmacological inhibition of the EP4 receptor with ONO-AE3-208 or global gene deletion of the EP4 receptor significantly decreased the rate of angiotensin II- or calcium chloride-induced abdominal aortic aneurysm. They also showed that the severity of hallmarks of the inflammatory phenotype, including activation of MMPs and IL-6 production, was also decreased in the vessel wall (Cao et al., 2012; Yokoyama et al., 2012).

When EP4 signaling was inhibited only in bone marrow-derived cells, however, inflammation and angiotensin II-induced abdominal aortic aneurysm formation were enhanced (Table 1). This occurred most likely because PGE₂ in blood cells had an anti-inflammatory effect, especially through reduced MCP-1 production (Tang et al., 2011b). Thus, the systemic inhibition of EP4 signaling may be protective, especially in vascular smooth muscle cells (Yokoyama et al., 2012), whereas the inhibition of macrophage recruitment may have a deteriorative effect (Tang et al., 2011b; Cao et al., 2012) on aneurysm formation. Further studies will be required to clarify the possibility of systemic administration of an EP4 antagonist as a pharmacological therapeutic strategy in abdominal aortic aneurysm.

B. Cancer

Cancer is a common cause of death in advanced countries. The association between PGEs and cancer was first demonstrated in the early 1990s through the discovery that aspirin, a COX inhibitor, protects against colon cancer (Thun et al., 1991) and has been confirmed in recent studies as well (Bastiaannet et al., 2012). Several lines of evidence have indicated that inhibition of COX-2 exerts anticancer effects, which have been reviewed for colorectal cancer (Wang and Dubois, 2010), lung cancer (Krysan et al., 2006), cervical cancer (Dannenbergh and Howe, 2003), breast cancer (Bundred and Barnes, 2005), prostate cancer (Zhang et al., 2012), and esophageal cancer (Altorki, 2004). Because COX-2 produces PGE₂, this association strongly suggests that EP4 may play a significant role in cancer progression, and that its inhibition is a potential strategy for cancer therapy. It is also known that the expression levels of both COX-2 and PGE₂ are elevated in cancer patients, a correlation that was reviewed by Fujino and Regan (2003) focusing on EP4. Reported function of EP4 signaling in cancer is summarized in Table 2, and the following sections will describe the expression of EP4 in cancer as well as its function and downstream signaling pathways.

1. Colorectal Cancer.

a. Expression. The importance of EP4 has been most clearly shown in colorectal cancer. It has been reported that EP4 is the predominant PGE₂ receptor subtype in HT-29 and HCA-7 human colon cancer cell lines (Cherukuri et al., 2007; Doherty et al., 2009). Mutoh et al. (2002) have reported similar results in colon cancer tissues in mice. Immunohistochemical experiments revealed that EP4 protein expression was greater in colorectal cancer and adenoma than in normal colonic epithelium (Chell et al., 2006). EP4 expression was observed in human colorectal cancer tissues (Wu et al., 2010), and it was most abundant among other EP receptor subtypes (Doherty et al., 2009). Another report suggested that EP4 expression was increased during colorectal carcinogenesis (Hawcroft et al., 2007).

b. Function. PGE₂ and EP4 regulate proliferation of colorectal cancer cells. PGE₂ stimulates cell proliferation in colorectal cancer cells via cAMP signaling (Loffler et al., 2008). EP4 involvement in proliferation of colorectal cancer cells was also demonstrated in several reports (Sheng et al., 2001; Mutoh et al., 2002; Cherukuri et al., 2007). Pozzi et al. (2004) demonstrated that indomethacin, a nonspecific COX inhibitor, and COX-1 or -2 selective inhibitors prevented PGE₂ biosynthesis and proliferation of mouse colon adenocarcinoma cells. The inhibition of proliferation was negated by PGE₂ or an EP4 receptor-selective agonist via activation of PI3K/ERK signaling. Indomethacin or COX-2 inhibitors, but not COX-1

inhibitors, decreased the size and the number of CT26-derived tumors in vivo. Accordingly, colon carcinoma cell proliferation is regulated by PGE₂/EP4 receptor-mediated PI3K/ERK activation (Pozzi et al., 2004). Sheng et al. (2001) also reported that the EP4 receptor promoted cell proliferation through the PI3K pathway in colorectal carcinoma cells. EP4 overexpression in human colorectal cancer cells exhibited anchorage-independent growth and resistance to spontaneous apoptosis but no changes in proliferation (Hawcroft et al., 2007).

Formation of aberrant crypt foci, which is recognized as a precancerous condition, is potentially induced by EP4 signaling. EP4 KO mice showed decreased formation of aberrant crypt foci. Similarly, the EP4 receptor-selective antagonist ONO-AE2-227 decreased the number of aberrant crypt foci induced by azoxymethane in mice. In another in vitro study, the EP4-selective agonist ONO-AE1-329 increased colony formation of human colon cancer cells in vitro (Mutoh et al., 2002).

EP4 also participates in abnormal cell cycle control, a key step in malignant transformation. Doherty et al. (2009) demonstrated that a COX-2 inhibitor induced G₀/G₁ arrest in colon cancer cells, which was reversed by PGE₂. Similarly, an EP4-selective antagonist induced G₀/G₁ arrest, which was reproduced by an EGFR tyrosine kinase inhibitor, indicating transactivation of EGFR by EP4. The EP4 antagonist increased expression of p21, a potent cyclin-dependent kinase inhibitor, suggesting the involvement of p21 in EP4-induced cell cycle deregulation (Doherty et al., 2009).

Cell migration and the resultant metastasis of colorectal cancer are regulated by EP4. Sheng et al. (2001) reported that PGE₂ increased cell migration of human colorectal carcinoma cells via the PI3K/Akt pathway. Another in vivo study demonstrated that the EP4 receptor antagonist ONO-AE3-208 inhibited metastasis of colon cancer cells from the spleen to the liver (Yang et al., 2006).

Expressions of multiple molecules that promote cell growth are regulated by EP4 in colorectal cancer cells. PGE₂ increased expression of early growth response gene-1 (*EGR-1*), a downstream molecule of ERK, at the level of transcription. Cherukuri et al. (2007) demonstrated that PGE₂ increased EGR-1 expression via CREB. They also showed that the EP4 receptor agonist PGE₁-OH phosphorylated ERK in human colon adenocarcinoma cells, whereas the EP4 receptor antagonist L-161,982 blocked PGE₂-induced ERK and CREB phosphorylation. These data suggest that the PGE₂-induced transcriptional regulation of EGR-1 likely occurs via the EP4-ERK signaling pathway (Cherukuri et al., 2007). A similar elevation of EGR-1 expression by EP4, but not EP2, has been demonstrated in another study (Fujino et al., 2003a). EP4-mediated activation of CREB can regulate the expression level of

TABLE 2
Reported roles of EP4-mediated signaling pathways in cancer

	Reported Functions	Proposed Effector Pathway(s)	Pathophysiologic Process	References
Colorectal cancer	Proliferation	PI3K; ERK; p38	N.D.	Pozzi et al., 2004
	Proliferation	PI3K; ERK; Akt	N.D.	Sheng et al., 2001
	Proliferation	N.D.	N.D.	Chell et al., 2006
	Proliferation	ERK	CREB phosphorylation, expression of EGR-1	Cherukuri et al., 2007
	Proliferation, apoptosis, cyst formation	cAMP; ERK2	CREB phosphorylation	Hawcroft et al., 2006
	G ₀ /G ₁ cell cycle arrest	EGFR transactivation; cAMP	Expression of p21	Doherty et al., 2009
	Polyp formation	N.D.	N.D.	Mutoh et al., 2002
Lung cancer	Invadopodia formation, colony growth, cell motility	ERK	CREB phosphorylation; expression of S100P	Chandramouli et al., 2010
	Metastasis	N.D.	N.D.	Yang et al., 2006
	Proliferation	ERK; Akt; PI3K; Sp1	Expression of ILK	Zheng et al., 2009
	Migration	β -Arrestin 1	Activation of c-Src	Kim et al., 2010
	Migration, adhesion, invasion, colony formation	Akt	N.D.	Yang et al., 2006
Breast cancer	Proliferation, invasion	N.D.	N.D.	Robertson et al., 2008
	Lymphangiogenesis, angiogenesis, metastasis	Akt	Reduced VEGF-C and VEGF-D production	Xin et al., 2012
	Metastasis	cAMP	N.D.	Ma et al., 2006
	Metastasis	cAMP; ERK	N.D.	Ma et al., 2012
Prostate cancer	Production of estrogen	BRCA1	CREB phosphorylation, expression of CYP19	Subbaramaiah et al., 2008
	Progression of castration-resistant, proliferation	cAMP	AR activation, PSA production	Terada et al., 2010
Ovarian cancer	Migration	N.D.	PGE ₂ production, VEGF production	Spinella et al., 2004a,b
Gallbladder cancer	Colony formation	N.D.	Expression of c-Fos	Asano et al., 2002
T-cell leukemia	Apoptosis	PI3K; Akt	Activation of caspase	George, 2007
Renal cancer	Proliferation, adhesion	Rap1GAP	Decreased E-cadherin expression	Wu et al., 2011
	Invasion	RGC2; Akt	RalA activation	Li et al., 2013

AR, androgen receptor; BRCA1, breast cancer susceptibility gene 1; N.D., not determined; PSA, prostate-specific antigen; RGC2, Ral GTPase activation protein (GAP) complex 2.

S100P, a calcium-binding protein, as well. Chandramouli et al. (2010) reported that PGE₂ increases S100P expression and that this effect is attenuated by knock-down or pharmacological inhibition of EP4. When the *CRE* sequence within the S100P promoter region was deleted or mutated, PGE₂-mediated transcriptional activity was abolished (Chandramouli et al., 2010).

These data suggest that PGE₂-EP4 signaling promotes cancer growth via multiple pathways, i.e., PI3K, ERK, and transactivation of EGFR.

2. Lung Cancer. Expression of EP4 was shown in human lung adenocarcinoma cells (Yano et al., 2002). In non-small-cell lung cancer cells, EP4 was detectable in both plasma and the mitochondrial membrane (Fang et al., 2004). In the same cell line, it was reported that EP4 expression was dependent on COX-2 (Dohadwala et al., 2002).

Both in vitro and in vivo studies have demonstrated that PGE₂/EP4 regulates processes in the progression of lung cancer. Yang et al. (2006) reported that EP4 receptor antagonists inhibited cell migration, adhesion, invasion, and colony formation via inhibition of Akt phosphorylation. In contrast, PGE₂ and EP4 agonists increased phosphorylation of Akt and migration (Yang et al., 2006). Similarly, in human lung cancer cells, PGE₂ enhanced activation of tyrosine kinase c-Src and cell migration. When c-Src was

blocked, PGE₂-induced cell migration was also decreased. PGE₂-induced cell migration was blocked by an EP4 receptor antagonist and by EP4 short hairpin RNA. Depletion of either EP4 or β arrestin, a downstream effector of EP4, negated PGE₂-induced cell migration (Kim et al., 2010). These data suggest that PGE₂/EP4/ β -arrestin/c-Src signaling enhances lung cancer cell migration.

Zheng et al. (2009) reported that PGE₂ increased the expression of integrin-linked kinase (ILK), a serine-threonine protein kinase that mediates diverse cellular functions. An EP4 receptor antagonist or EP4 siRNA inhibited PGE₂-induced ILK expression. When ILK was knocked down, the mitogenic effect of PGE₂ was also decreased. PGE₂ increased Sp1 protein and thus Sp1 DNA-binding activity in the ILK promoter, leading to increased ILK expression and thus a mitogenic effect. These data suggest that activation of EP4 may contribute to a PGE₂-mediated mitogenic effect (Zheng et al., 2009).

3. Cervical Cancer. Expression of PGE₂/EP4 is increased in cervical cancer compared with corresponding normal tissues. Sales et al. (2001, 2002) reported that EP4 expression levels were significantly higher in carcinoma tissues than in normal cervix. COX-2 expression was also observed in cervical cancer tissues, whereas in normal cervical tissue, in contrast, COX-2

expression was not detected. COX-2 and PGE₂ synthesis was detected in endothelial cells of squamous cell carcinoma, and columnar and glandular epithelium of adenocarcinoma, but not in normal cervical tissues. In vitro culture of cervical cancer biopsies demonstrated that the induction of cAMP by PGE₂ was significantly higher in cervical carcinoma tissue than in normal cervix. Thus, PGE₂ potentially regulates neoplastic cellular functions in cervical carcinoma in an autocrine/paracrine manner via EP4 (Sales et al., 2001). The same group also demonstrated COX-1-induced expressions of EP1–4 as well as enhanced cAMP production in response to PGE₂ and PGE₂-induced EP4 expression in HeLa (cervical adenocarcinoma) cells (Sales et al., 2002). Further study is required to clarify the roles of PGE₂-EP4 signaling in cervical cancer.

4. Breast Cancer. Different groups similarly demonstrated expression of EP4 in MCF-7, a breast cancer cell line (Renò et al., 2004; Pan et al., 2008), and in murine breast cancer cell lines (Ma et al., 2010). EP4 expression was detected in human breast cancer specimens, and it was positively correlated with expression of COX-2 (Pan et al., 2008). Immunohistochemical analysis in COX-2-induced breast tumors in mice detected EP4 expression in stromal cells, adipocytes, and hematopoietic cells but no expression in ductal and alveolar epithelial cells, whereas EP2 was expressed in ductal and alveolar epithelial cells, suggesting the role of EP4 in mesenchymal cells (Chang et al., 2004).

EP4 regulates tumor growth and metastasis of breast cancer cells. EP4 antagonists inhibited proliferation of breast cancer cells (Ma et al., 2006). Stimulation of EP4 resulted in increased cell proliferation and invasion of inflammatory breast cancer, an aggressive form of breast cancer, but not of noninflammatory breast cancer. In contrast, EP2 had no such effect on either of the two cell lines. It has also been demonstrated that knockdown of EP4 abolished EP4-mediated cellular proliferation and invasion (Robertson et al., 2008). An attempt to inhibit EP4-mediated breast cancer progression was successful, at least in animal studies. Continuous oral administration of the EP4 receptor antagonist ONO-AE3-208 decreased tumor growth, lymphangiogenesis, angiogenesis, and metastasis to the lymph nodes and the lungs of xenografted human breast cancer cells in mice (Xin et al., 2012). In addition, antagonism of EP4 receptor with either AH23848 or ONO-AE3-208 reduced metastasis of murine breast cancer cells (Ma et al., 2006). Aside from synthetic compounds, Frondoside A, a triterpenoid glycoside derived from the sea cucumber *Cucumaria frondosa*, was found to antagonize EP4 and to inhibit cAMP production and ERK activation. Frondoside A also inhibited spontaneous tumor metastasis to the lungs in vivo, and may be used as an

EP4 antagonist to prevent metastasis in breast cancer (Ma et al., 2012). As described earlier, EP4 is expressed in mesenchymal cells rather than ductal and alveolar epithelial cells (Chang et al., 2004). In that study, it was demonstrated that a COX-2 inhibitor suppressed angiogenesis in xenografted tumors, suggesting a potential role of EP4 in angiogenesis, although a selective inhibitor for EP4 was not tested. An interesting feature of PGE₂ in breast cancer is its involvement in production of estrogen, a key stimulator for breast cancer progression. Both PGE₂ and agonists for EP4 can enhance CYP19 transcription and thereby induce the expression of aromatase, which is critical for the local synthesis of estrogen, through the cAMP/PKA/CREB pathway. PGE₂ also suppressed expression of breast cancer susceptibility gene 1, a major tumor suppressor gene in breast cancer (Subbaramaiah et al., 2008).

5. Prostate Cancer. EP4 expression was detected in human prostate cancer cell lines (Chen and Hughes-Fulford, 2000; Wang and Klein, 2007; Swami et al., 2009) and primary cancer-derived cell culture as well as normal prostate cell culture (Swami et al., 2009). In castration-resistant prostate cancer, an advanced form of androgen-insensitive prostate cancer, expression of EP4 was upregulated during progression of castration resistance (Terada et al., 2010).

PGE₂ activates cell migration of prostate cancer cells via EP4. These functions were regulated at least in part by transactivation of EGFR and β 3 integrin in prostate cancer (Jain et al., 2008). When EP4 was overexpressed in LNCaP human prostate cancer cells, progression of tumors occurred through androgen receptor activation. In castration-resistant prostate cancer cells, the EP4 receptor antagonist ONO-AE3-208 decreased intracellular cAMP levels in vitro and tumor growth in vivo (Terada et al., 2010). Expression of S100A8, a calcium-binding protein which is highly expressed in prostate cancer, was enhanced by PGE₂ and inhibited by both EP4-specific inhibitors. Increased promoter activity of S100A8 via EP4 was potentially mediated by PKA (Miao et al., 2012).

6. Ovarian Cancer. In ovarian cancer cell lines, expression of EP4 was demonstrated in relation to endothelin signaling. Spinella et al. (2004a,b) reported that endothelin-1 (ET-1) induced COX-1 and COX-2 expression through endothelin receptor. Both enzymes contributed to production of PGE₂ in human ovarian carcinoma cell lines (Spinella et al., 2004b). Subsequently, they demonstrated that ET-1 increases expression of EP4, and that PGE₂ induced by ET-1 activates cell invasion and production of vascular endothelial growth factor via EP4 (Spinella et al., 2004a).

7. Other Cancers. The role of EP4 has been reported in other cancer types, such as gallbladder carcinoma, T-cell leukemia cells, renal cancer, and upper urinary carcinoma. Expression of EP4 has been detected in

human gallbladder carcinoma specimens. An EP4 receptor agonist and PGE₂ increased colony formation of gallbladder cancer cells probably via increased expression of c-Fos (Asano et al., 2002). George et al. (2007) demonstrated that PGE₂ decreased camptothecin-induced apoptosis in Jurkat human T-cell leukemia cells. This effect occurred through the EP4/PI3K/Akt pathway, but not through the EP4/PKA pathway (George et al., 2007). PGE₂ increased the invasion of RCC7, a human renal cancer cell line that expresses abundant EP4. Inhibiting EP4 with its antagonists AH23848 and GW627368 or shRNA-mediated EP4 disruption similarly inhibited RCC cell invasion. Rap1 was identified as a potential molecule involved in this process; its activation was blocked by AH23848 (Wu et al.). Another report showed that EP4 involvement in invasion of renal cell carcinoma cells via the Akt/Ral GTPase-activating protein complex 2/RalA small GTPase (Li et al., 2013). Finally, EP4 is a potential cancer biomarker. An immunohistochemical study suggested that analyzing the coexpression of COX-2 and EP4 was useful for evaluating progression of nonmetastatic transitional cell carcinoma of the upper urinary tract (Miyata et al., 2005).

C. Immune System

1. Monocytes/Macrophages/Dendritic Cells.

a. Expression. Early studies demonstrated that EP4 mRNA was abundant in human peripheral blood leukocytes (An et al., 1993; Mori et al., 1996) as well as in the spleens of humans (An et al., 1993), mice (Honda et al., 1993), and rats (Sando et al., 1994). Thus, the EP4 receptor may play an important role in leukocytes and macrophages (Woodward et al., 2011). More recently, abundant expression of EP4 has also been demonstrated in mononuclear cells and macrophages, specifically in mouse macrophages (Ikegami et al., 2001; Akaogi et al., 2004; Pavlovic et al., 2006), the mouse macrophage cell line J774.1 (Katsuyama et al., 1998a), the mouse macrophage-like cell line RAW 264.7 (Tajima et al., 2008; Khan et al., 2012), and human macrophages (Bayston et al., 2003; Iwasaki et al., 2003; Kubo et al., 2004; Cipollone et al., 2005; Wu et al., 2005). Takayama et al. (2002) have demonstrated that EP4 is a primary receptor expressed in macrophages, and therefore suggested that PGE₂ mediates the inhibition of MCP-1 and macrophage inflammatory protein-1 α (MIP-1 α).

In the past decade, emerging evidence has shown that chronic inflammation may increase EP4 expression. An example is the development of atherosclerosis, in which not only T cells but also mononuclear cells and macrophages play a role (Ross, 1999). Cipollone et al. (2005) characterized EP4 expression in plaques from symptomatic and asymptomatic patients undergoing carotid endarterectomy. They found a correlation with the extent of inflammatory infiltration: EP4 was

present in asymptomatic patients but was increased in symptomatic patients, leading to an enhanced inflammatory reaction in response to atherosclerotic plaques (Cipollone et al., 2005). This pattern was most obvious in EP4; the expression of EP2 was very low, whereas EP1 and EP3 were not detectable.

b. Function. EP4 signaling regulates a variety of cytokines and chemokines in macrophages, and plays roles in their anti- and proinflammatory activity (Woodward et al., 2011; Tang et al., 2012) (Table 3). In macrophages, most studies suggest that EP4 is anti-inflammatory because it is through EP4 that PGE₂ suppresses the production of cytokines and chemokines, such as TNF- α , IL-12, and MCP-1. Nevertheless, accumulating evidence shows that EP4 receptor signaling also promotes proinflammatory cytokine IL-6 in macrophages

i. Tumor necrosis factor- α . PGE₂ acts as a major feedback inhibitor in TNF- α production from macrophages (Zhong et al., 1995). LPS treatment of macrophages can induce TNF- α production and results in a concomitant increase in PGE₂ production. Released PGE₂ then acts on the macrophages as an inhibitor of TNF- α production in an autocrine manner (Zhong et al., 1995). Through experiments with the genetic deletion of EP4 in macrophages, Nataraj et al. (2001) demonstrated that suppression of TNF- α production was mediated by EP4. In vitro pharmacological studies also demonstrated that EP4 inhibited TNF- α production in monocytes/macrophages in humans (Meja et al., 1997; Ratcliffe et al., 2007), mice (Katsuyama et al., 1998a; Yamane et al., 2000; Ikegami et al., 2001; Akaogi et al., 2004; Nakatani et al., 2004), and rats (Treffkorn et al., 2004; Aronoff et al., 2008).

Furthermore, TNF- α gene expression is negatively regulated by cyclic AMP-elevating agents, including β -adrenoreceptor agonist, G_s α stimulator cholera toxin, cAMP analogs, and PDE IV inhibitor (Severn et al., 1992; Prabhakar et al., 1994; Seldon et al., 1995; Aronoff et al., 2008). Some studies have used analogs of cAMP to address the relative contributions of the cAMP effectors PKA and Epac. LPS-induced TNF- α production was only inhibited through the PKA pathway in monocytes and macrophages, although Epac was present in these cells (Aronoff et al., 2005; Bryn et al., 2006). Wall et al. (2009) demonstrated that suppression of the expression of the gene encoding TNF- α involved the targeting of type II PKA by AKAP95 to an NF- κ B complex that includes p105. Similarly, the expression of the gene encoding MIP-1 α was also inhibited by the targeting of type II PKA by AKAP to an NF- κ B complex (Wall et al., 2009). This body of evidence suggests that, in general, cAMP-PKA signaling suppresses TNF- α production. Most studies have demonstrated that not only EP4 but also EP2 signaling suppresses TNF- α production (Meja et al., 1997; Katsuyama et al., 1998a; Yamane et al., 2000; Ikegami et al., 2001; Nataraj et al., 2001; Akaogi et al.,

TABLE 3
Reported roles of EP4-mediated signaling pathways in the immune system

Tissues/Cells	Reported Functions	Proposed Effector Pathway(s)	References
Macrophages	Inhibition of TNF- α production	EP4 receptor-associated protein	Takayama et al., 2006
Monocytes	Inhibition of TNF- α production	N.D.	Meja et al., 1997; Katsuyama et al., 1998a; Yamane et al., 2000; Ikegami et al., 2001; Nataraj et al., 2001; Akaogi et al., 2004; Nakatani et al., 2004; Treffkorn et al., 2004; Ratcliffe et al., 2007; Aronoff et al., 2008
	Inhibition of MCP-1 production	EP4 receptor-associated protein	Takayama et al., 2006
	Inhibition of MCP-1 production	N.D.	Hishikari et al., 2009; Tang et al., 2011a; Tang et al., 2011b
	IL-6 production	cAMP; PKA; PKC; p38 MAPK; NF- κ B	Chen et al., 2006
	IL-6 production	PKC	Ma and Quirion, 2005
	IL-6 production	N.D.	Bayston et al., 2003; Akaogi et al., 2004; Treffkorn et al., 2004
	MMP-9 production	ERK1/2	Steenport et al., 2009
	MMP-9 production	N.D.	Cipollone et al., 2005; Pavlovic et al., 2006
	Inhibition of IL-12 production	cAMP	Iwasaki et al., 2003
	Inhibition of IL-12 production	N.D.	Ikegami et al., 2001; Nataraj et al., 2001; Kuroda and Yamashita, 2003
Dendritic cells	Inhibition of IL-12p70, IL-6, and IL-10 production	cAMP	Kubo et al., 2004
	IL-23 production	cAMP; Epac	Yao et al., 2009; Sakata et al., 2010b
	Migration	cAMP; PKA; p38 MAPK	Scandella et al., 2002; Cote et al., 2009
	Migration	cAMP	Luft et al., 2002
	Migration	N.D.	Legler et al., 2006
Langerhans cells	Migration	N.D.	Kabashima et al., 2003
T cells	Inhibition of IL-12, IFN- γ , MIP-1 α production	N.D.	Ogawa et al., 2009
	Th17 expansion	cAMP; PKA	Yao et al., 2009; Sakata et al., 2010a,b
	Th17 expansion	N.D.	Gagliardi et al., 2010
	Th1 differentiation	PI3K	Yao et al., 2009
Eosinophils	Inhibition of migration	PI3K; PKC	Luschnig-Schratl et al., 2011
	Inhibition of migration	N.D.	Konya et al., 2011
Neutrophils	Inhibition of TNF- α production; IL-6 production	N.D.	Yamane et al., 2000
	Inhibition of aggregation	N.D.	Wise, 1998

N.D., not determined.

2004; Treffkorn et al., 2004; Ratcliffe et al., 2007; Aronoff et al., 2008). These data also support the concept that cyclic AMP-PKA is involved in PGE₂-mediated suppression of TNF- α production, because both EP4 and EP2 are positively coupled to ACs and increase intracellular cAMP production.

ii. *Monocyte chemoattractant protein-1*. MCP-1 is known as a chemotactic factor and plays essential roles in the recruitment of inflammatory cells into tissues. Takayama et al. (2002) suggested that PGE₂ suppressed the production of MCP-1 in LPS-activated human macrophages via EP4. The structure of EP4 itself was associated with suppression of MCP-1 production in human monocyte-derived macrophages (Takayama et al., 2006). Lack of EP4 in bone marrow-derived cells accelerated local inflammation in atherosclerotic and aneurysm lesions, leading to increased aneurysm formation (Tang et al., 2011ab). Similarly, when EP4 was activated, it decreased MCP-1 production, as in ischemia/reperfusion injury, as well as inflammatory cell infiltration in mouse ventricles (Hishikari et al., 2009). Given that targeted disruption

of the MCP-1 gene or its receptor C-C chemokine receptor type 2 significantly decreased atherogenesis in mice (Boring et al., 1998; Gu et al., 1998), inhibition of MCP-1 by EP4 may play a beneficial role, especially in cardiovascular diseases. It has been demonstrated that MCP-1 expression was suppressed via the cAMP-PKA pathway in multiple cell types (Iwamoto et al., 2003; Wuyts et al., 2003), including mononuclear cells (Yang et al., 2012). Thus, cAMP-PKA signaling may be a potential downstream signaling pathway for the EP4-mediated inhibition of MCP-1 production.

iii. *Interleukin-6*. IL-6 is a multifunctional cytokine that plays a central role in both innate and acquired immune responses. IL-6 is the predominant mediator of the acute phase response, an innate immune mechanism triggered by infection and inflammation (Graeve et al., 1993). PGE₂ has been shown to increase IL-6 production in peritoneal macrophages in vitro (Williams and Shacter, 1997). Both in vitro and in vivo studies have suggested that EP4 is a potent stimulant of IL-6 production. PGE₂-induced IL-6 production via EP4 has been demonstrated in mouse and rat

macrophages (Akaogi et al., 2004; Treffkorn et al., 2004; Ma and Quirion, 2005; Chen et al., 2006) and in various cell types, including neutrophils (Yamane et al., 2000), smooth muscle cells (Yokoyama et al., 2012), and fibroblasts (Inoue et al., 2002). PGE₂ also increased IL-6 production via EP4 in macrophages in human aortic aneurysms (Bayston et al., 2003). Intracellular signaling events involving p38 MAPK and NF- κ B as well as PKA and protein kinase C (PKC) are responsible for IL-6 induction by EP4 stimulation (Chen et al., 2006). Ma and Quirion (2005) also demonstrated the involvement of PKC signaling in EP4-induced IL-6 production in macrophages in a rat model of nerve injury.

iv. Matrix metalloproteinase-9. Macrophages produce MMP-9, which plays a principal role in the degradation of extracellular matrix components. It also modifies the activities of cytokines and chemokines, growth factors, and proteinase inhibitors (Parks et al., 2004). MMP-9 expression is low or absent in most normal tissues, but is markedly upregulated during chronic inflammation and cancer (Parks et al., 2004). MMP-9 expression was markedly reduced in macrophages isolated from COX-2-deficient mice and in wild-type macrophages treated with COX-2 inhibitors (Khan et al., 2004). Consistent with these results, pharmacological EP4 activation increased MMP-9 expression and activity in human monocytes and macrophages derived from atherosclerotic plaque (Cipollone et al., 2005). Similar results were shown in murine macrophages (Pavlovic et al., 2006; Steenport et al., 2009; Khan et al., 2012). Although studies using murine macrophages have suggested the involvement of cAMP and ERK1/2 in EP4-induced MMP-9 activation, further studies are needed to elucidate the precise signaling mechanisms involved in the process.

v. Interleukin-12. IL-12 plays critical roles in the induction of Th1 response by regulating the differentiation of Th0 to Th1 cells while suppressing Th2 cytokine development (Hsieh et al., 1993; Seder et al., 1993; Manetti et al., 1994). PGE₂ has been suggested to suppress LPS-induced IL-12 production by macrophages and dendritic cells (van der Pouw Kraan et al., 1995; Harizi et al., 2002). In keeping with these results, EP4 activation inhibited IL-12 production in human monocyte-derived dendritic cells (Kubo et al., 2004), human monocytes (Iwasaki et al., 2003), and mouse macrophages (Ikegami et al., 2001; Nataraj et al., 2001; Kuroda and Yamashita, 2003; Ogawa et al., 2009) *in vitro*. When T cells were stimulated with matured monocyte-derived dendritic cells induced by an EP4 agonist, they exhibited decreased Th1 cytokine interferon- γ (IFN- γ) production and increased Th2 cytokine IL-4 and IL-10 production (Kubo et al., 2004).

Cyclic AMP elevators, such as cholera toxin or forskolin, also mimicked the inhibition of IL-12 production by EP4 activation (Ikegami et al., 2001;

Iwasaki et al., 2003; Kubo et al., 2004; Schnurr et al., 2005; Kelschenbach et al., 2008; la Sala et al., 2009). Recent studies have demonstrated that negative regulation of NF- κ B and interferon regulatory factor 8 were mechanisms of cAMP-mediated downregulation of IL-12 (Kelschenbach et al., 2008; la Sala et al., 2009). In addition, Wall et al. (2009) demonstrated a direct positive effect of cAMP-PKA signaling on the expression of IL-10 through CREB. These findings suggest that PGE₂ induces Th2 polarization through EP4-cAMP signaling-mediated IL-12 suppression.

vi. Migration. Migration of dendritic cells from peripheral tissues to the T-cell areas of draining lymph nodes is crucial for the priming of T lymphocytes. PGE₂ is responsible for facilitating chemotaxis in human monocyte-derived dendritic cells (Luft et al., 2002; Legler et al., 2006); this activity has been ascribed to EP4 (Table 3). Indeed, in human blood monocytes or monocyte-derived dendritic cells, EP4 stimulation upregulated C-C chemokine receptor 7 mRNA, which is essential for migration to secondary lymphoid tissues (Scandella et al., 2002; Cote et al., 2009). Kabashima et al. (2003) demonstrated that the migration of Langerhans cells in the skin to draining lymph nodes on hapten application was impaired in EP4-deficient mice, resulting in suppressed contact hypersensitivity to the hapten.

2. T Cells

a. Expression. Although the EP4 receptor is abundantly expressed in the thymus, only a few reports are available on EP4 function in the thymus and T cells. Villablanca et al. (2007) outlined the role of EP4 in the early developmental stages. They used zebrafish because it was difficult to examine the role of EP4 in mammals due to placental secretion of PGE₂. In zebrafish, EP4 was expressed at 26 hours postfertilization in the dorsal aorta-posterior cardinal vein joint region, where definitive hemopoiesis arises. EP4 was then expressed in the presumptive thymus rudiment by 48 hours postfertilization, suggesting that the EP4 receptor is the earliest thymus marker, regulating T-cell precursor development via rag1 (Villablanca et al., 2007).

Messenger RNAs for EP2, EP3, and EP4, but not EP1, were detected in antigen-specific CD4⁺ human T cells (Okano et al., 2006). A recent study demonstrated that CD46 activation induced EP4 expression and caused a decrease in the IL-10/IFN- γ ratio in T cells, and that an EP4 antagonist reversed this effect on cytokine production after CD46 stimulation (Kickler et al., 2012). Nataraj et al. (2001) showed mRNA expression of EP1, EP2, and EP4, but not EP3, on splenic T cells and B cells in mice. Mori et al. (1996) found that EP4 in T-cell lines was downregulated by phorbol 12-myristate 13-acetate, an activator of PKC. In contrast, Raji and THP-1 monocytoid cell lines

showed marked upregulation of EP4 by phorbol 12-myristate 13-acetate, whereas levels of other PGE receptors remained unchanged (Mori et al., 1996).

b. Function. CD4⁺ T cells differentiate into three subsets of effector T cells, termed Th1, Th2, and Th17 (Zhu and Paul, 2008). Th17 was recently identified, and the involvement of Th17 cells in immune diseases, such as rheumatoid arthritis, multiple sclerosis, and Crohn's disease, has been suggested. IL-6 and transforming growth factor- β initially differentiate Th0 cells to Th17 cells. IL-23 then expands Th17 cells and stabilizes their phenotype (Korn et al., 2009).

There are many reports suggesting a Th1-suppressive action of PGE₂, as described in the previous section. Recent studies, however, suggest that PGE₂ could induce Th1 differentiation under certain conditions through EP4 (Yao et al., 2009; Sakata et al., 2010a,b). Yao et al. (2009) demonstrated that, under strengthened T-cell receptor stimulation, PGE₂ facilitated IL-12-induced Th1 differentiation at nanomolar concentrations. T-cell proliferation was not affected. This differentiation occurred through EP4 via the PI3K pathway, but not via the cAMP pathway. A possible explanation for this paradox may be the different concentrations of PGE₂. At higher concentrations, PGE₂ exerts its well known Th1-suppressive effect, whereas its Th1 differentiation-promoting activity, which is robust at lower concentrations, is masked. Furthermore, these studies demonstrated that EP4-cAMP-Epac signaling promoted IL-23 production in dendritic cells, and that, in the presence of IL-23, PGE₂ facilitates the expansion of the Th17 subset of both human and mouse T cells via the EP4-cAMP-PKA pathway (Yao et al., 2009; Sakata et al., 2010b). Other types of studies also demonstrated EP4-mediated Th17 expansion (Gagliardi et al., 2010; Sakata et al., 2010a). Th17 cell differentiation was also controlled by the retinoic acid receptor-related orphan receptor- γ t. PGE₂ may positively synergize with IL-1 and IL-23 to upregulate retinoic acid receptor-related orphan receptor- γ t (Boniface et al., 2009; Napolitani et al., 2009).

Studies using animal models of Th1- and Th17-related diseases, such as autoimmune encephalomyelitis, contact hypersensitivity, colitis, and arthritis, also demonstrated that PGE₂-EP4 signaling promoted Th1 differentiation and Th17 expansion. Yao et al. (2009) demonstrated that an EP4 antagonist reduced disease severity and decreased accumulation of antigen-specific Th1 and Th17 cells in regional lymph nodes in animal models of autoimmune encephalomyelitis and contact hypersensitivity. Based on these results, the same group further demonstrated that PGE₂ exerted dual functions in experimental autoimmune encephalomyelitis. It facilitated Th1 and Th17 cell generation through EP4 during immunization, and it limited the invasion of these cells into the brain by protecting the blood-brain barrier through EP4 (Esaki et al.,

2010). Sheibanie et al. (2007a,b) have reported that stimulation of EP4 with misoprostol exacerbated both 2,4,6-trinitrobenzene sulfonic acid-induced colitis and collagen-induced arthritis in mice. It also upregulated the expression of IL-23 and IL-17 in lesions (Sheibanie et al., 2007a,b). These *in vivo* studies suggested that PGE₂-EP4 signaling could promote development of the Th1 and Th17 subsets (Table 3). Since the local concentration of PGE₂ may vary with disease type and stage, EP4 signaling cannot be simply classified as anti-inflammatory or proinflammatory.

3. Other Immune Cells

Eosinophil extravasation across the endothelium and its accumulation in tissues are hallmarks of allergic inflammation. EP4 may inhibit migration and adhesion of eosinophils (Table 3). In human eosinophils, EP4 mRNA was highly expressed (Mita et al., 2002; Luschnig-Schratl et al., 2011). The selective EP4 agonist ONO-AE1-329 potentially attenuated the chemotaxis of human peripheral blood eosinophils through the PI3K pathway, but not the cAMP pathway (Luschnig-Schratl et al., 2011). PGE₂ and the EP4 agonist ONO-AE1-329 significantly reduced eotaxin-induced eosinophil adhesion to fibronectin and the formation of filamentous actin- and gelsolin-rich adhesive structures. They also reduced eosinophil transmigration across thrombin- and TNF- α -activated endothelial cells (Konya et al., 2011).

EP4 receptors are present in various B cell lines at different maturation stages (Fedyk et al., 1996). B cell receptor signaling contributes to the pathogenesis of B cell malignancies, and most B cell lymphomas depend on B cell receptor signals for survival. Murn et al. (2008) demonstrated that the EP4 receptor was downregulated in human B cell lymphoma. Stable knockdown of the EP4 receptor in B cell lymphoma markedly accelerated tumor spread in mice, whereas EP4 overexpression yielded protection against tumor spread (Murn et al., 2008). These data suggest that EP4 plays a principal role in the growth-suppressive effect of PGE₂ in B cell lymphoma. Consistently, PGE₂-EP4 signaling promoted B cell receptor-induced G₀/G₁ arrest in the B cell line WEHI 231. Thus, caspase-mediated, B cell receptor-induced apoptosis was induced (Priatelj et al., 2011). In mouse peritoneal neutrophils, not only did EP4 suppress growth, it also suppressed TNF- α production (Yamane et al., 2000). EP4 also prevented *N*-formyl methionyl leucyl phenylalanine-stimulated aggregation of rat neutrophils (Wise, 1998).

D. Osteoarticular System

1. Expression. EP4 receptors have been found in rats (Sarrazin et al., 2001) and mice (Miyaura et al., 2000; Suzawa et al., 2000) through primary culture of osteoblasts and various osteoblastic cell lines, including MCT3T3-E1, and bone marrow stromal cell

cultures (Suda et al., 1996; Weinreb et al., 1999, 2001; Sarrazin et al., 2001;). Miyaoura et al. (2000) demonstrated that mouse osteoblasts isolated from calvariae expressed transcripts of all four receptors, ranked according to expression level as follows: EP4 > EP1 > EP2 > EP3. EP4 receptors are also strongly expressed in primary cultures of human osteoblasts (Sarrazin et al., 2001). In human osteoblasts, only EP4 and EP3 were observed immunohistochemically (Fortier et al., 2004).

Osteoclasts have also been shown to express EP4 abundantly (Mano et al., 2000). Although there are convincing *in vitro* and *in vivo* collective data indicating that EP2 receptors may have a role in PGE₂-mediated RANKL expression and anabolic effects on bone, to date no functional EP2 receptors have been identified on human osteoblasts or osteoclasts (Akhter et al., 2001; Li et al., 2002; Woodward et al., 2011). In human chondrocytes, EP4, EP1, and EP2 were detected (Watanabe et al., 2009).

In inflammatory bone diseases, expression of the EP4 receptor appears to be increased. In human rheumatoid synovial fibroblasts, EP4 receptors together with EP2 receptors are consistently expressed (Yoshida et al., 2001; Mathieu et al., 2008; Kojima et al., 2009). It has been reported that the EP4 receptor was upregulated in human osteoarthritis cartilage (Attur et al., 2008). The inflammatory cytokine IL-1 β also increased the expression of COX-2 and the EP4 receptor in cultured human (Watanabe et al., 2009) and rabbit chondrocytes (Alvarez-Soria et al., 2007).

2. Function. Bone remodeling, comprising the resorption of existing bone by osteoclasts and *de novo* bone formation by osteoblasts, is required for bone homeostasis. PGE₂ is known to promote both bone resorption and bone formation (Flanagan and Chambers, 1992; Scutt et al., 1995). Such effects are suggested to be mediated by EP4 signaling (Table 4).

a. Osteoblasts. It has been shown that PGE₂ stimulates osteoblastic differentiation from bone marrow-derived cells (Shamir et al., 2004; Alander and Raisz, 2006), leading to increased osteoblast numbers and bone formation. PGE₂ also induced the expression of Runt-related transcription factor 2 (Runx2) and enhanced the formation of mineralized nodules in a culture of bone marrow cells from wild-type mice but not in a culture of cells from EP4-deficient mice (Yoshida et al., 2002). This positive effect of EP4 on osteoblast differentiation was supported by several subsequent pharmacological studies. Studies using EP4 agonists (ONO-AE1-329 or ONO-4819) or EP4 antagonists (L-161,982) have shown that EP4 activation induces osteoblast differentiation of murine calvarial osteoblastic cells (Alander and Raisz, 2006), rat and mouse bone marrow stromal cells (Keila et al., 2001; Shamir et al., 2004; Nakagawa et al., 2007), and

the multipotent mesenchymal cell line C3H10T1/2 (Ninomiya et al., 2011).

A number of studies have suggested that the anabolic effect of PGE₂ is linked to an elevated level of cAMP (Hakeda et al., 1986; Graham et al., 2009), and that EP4 activation increases cAMP in osteoblasts (Sakuma et al., 2004). Runx2 and osterix are osteoblast-specific transcription factors essential for the development of osteoblastic cells and bone formation. Forskolin, an AC activator, also enhanced Runx2 and osterix transcription, but the stimulatory effects of forskolin were blocked by pretreatment of the cells with H-89, a PKA inhibitor (Wang et al., 2006a). Alkaline phosphatase activity is also a marker of osteoblast differentiation. PGE₁ increases alkaline phosphatase activity through the cAMP-PKA-p38 MAPK pathway in osteoblasts (Kakita et al., 2004). Nakagawa et al. (2007) demonstrated that the EP4 agonist ONO-4819 accelerated bone morphogenetic protein-induced osteoblast differentiation of bone marrow stromal cells. Acceleration of bone morphogenetic protein activity by the EP4 agonist was abolished by pretreatment with PKA inhibitor, but not with PKC, MAPK, or PI3K inhibitors (Nakagawa et al., 2007). These data support the concept that PGE-EP4 signaling promotes osteoblast differentiation primarily through cAMP-PKA signaling.

b. Osteoclasts. PGE₂ is a strong stimulator of osteoclast differentiation in marrow cultures and of bone resorption in bone organ culture (Blackwell et al., 2010). The major effect of PGE₂ on resorption is generally considered to occur indirectly via upregulation of RANKL expression and by inhibition of osteoprotegrin expression in osteoblastic cells (Blackwell et al., 2010). It has been suggested that PGE₂ enhances osteoclast formation through EP4 receptor activation on osteoblasts (Mano et al., 2000; Sakuma et al., 2000). Osteoclast formation was enhanced by the presence of an EP4 agonist in coculture of mouse primary osteoblastic cells and bone marrow cells, but not in cocultures of primary osteoblastic cells from EP4 KO mice (Sakuma et al., 2000). When osteoblasts are absent, EP4 agonists have an inhibitory effect on osteoclast formation and subsequently on bone resorption (Mano et al., 2000). PGE₂-induced RANKL expression was significantly reduced in primary osteoblastic cells derived from EP4 KO mice compared with those from wild-type mice (Li et al., 2002). An EP4 antagonist inhibited PGE₂-induced RANKL expression in osteoblasts, thereby increasing osteoclast differentiation *in vitro* (Tomita et al., 2002). Taken together, these data indicate that osteoblast-mediated osteoclast activation may occur through the EP4-induced activation of RANKL.

c. Bone resorption. In keeping with the data on EP4-mediated osteoclastogenesis, several reports suggest that EP4 signaling induces bone resorption in

TABLE 4
Reported roles of EP4-mediated signaling pathways in the osteoarticular system

Tissues/Diseases	Reported Functions	Proposed Effector Pathway(s)	Pathophysiologic Process	References
Bone	Osteoblast differentiation	N.D.	Runx2 expression	Yoshida et al., 2002
	Osteoblast differentiation	PKA	N.D.	Nakagawa et al., 2007
	Osteoblast differentiation	N.D.	N.D.	Keila et al., 2001; Shamir et al., 2004; Alander and Raisz, 2006 Gao et al., 2009; Ninomiya et al., 2011
	Osteoclast differentiation	cAMP	RANKL expression	Tomita et al., 2002
	Osteoclast differentiation	N.D.	N.D.	Ono et al., 1998
	Bone formation	N.D.	N.D.	Machwate et al., 2001; Hagino et al., 2005; Li et al., 2005; Toyoda et al., 2005 Akhter et al., 2006; Ito et al., 2006; Ke et al., 2006; Onishi et al., 2008
	Bone resorption	cAMP	Osteoclast differentiation factor expression	Suzawa et al., 2000
	Bone resorption	cAMP	MMP-2, MMP-13 expression	Miyaura et al., 2000
	Bone resorption	cAMP	N.D.	Mano et al., 2000
	Bone resorption	N.D.	Osteoclast differentiation factor expression Inhibition of osteoclastogenesis inhibitory factor	Sakuma et al., 2000
Cartilage	Bone resorption	N.D.	RANKL expression	Li et al., 2002
	Bone resorption	N.D.	N.D.	Zhan et al., 2005
	Bone healing	N.D.	MMP-9 expression	Xie et al., 2009
	Bone healing	N.D.	N.D.	Tanaka et al., 2004; Li et al., 2005; Marui et al., 2006 Miyamoto et al., 2003b
	Endochondral bone formation	cAMP	Chondrocyte differentiation	Attur et al., 2008
	Matrix degradation and inhibition of proteoglycan synthesis	N.D.	MMP-13, ADAMTS-5 expression	
	Development of arthritis	PKA; p38 MAPK	COX-2 expression in synovial fibroblasts	Faour et al., 2001
	Development of arthritis	N.D.	PTH-related peptide production in synovial fibroblasts	Yoshida et al., 2001
	Development of arthritis	N.D.	IL-1 β and IL-6 production in macrophages	McCoy et al., 2002
	Development of arthritis	N.D.	Th1 differentiation and Th17 expansion	Chen et al., 2010
Rheumatoid arthritis	Development of arthritis	N.D.	N.D.	Murase et al., 2008
	Development of arthritis	N.D.	N.D.	Okumura et al., 2008
	Development of arthritis	N.D.	N.D.	Honda et al., 2006
	Development of arthritis	N.D.	N.D.	

ADAMTS-5, a disintegrin-like and metalloproteinase (reprolysin type) with thrombospondin type 1 motif, 5; N.D., not determined; PTH, parathyroid hormone.

vitro. Induction of bone resorption by PGE₂ was greatly impaired in cultured calvariae from EP4-deficient mice, but not in those from EP1-, EP2-, or EP3-deficient mice (Miyaura et al., 2000; Zhan et al., 2005). Similar results have been obtained in studies using pharmacological approaches. An EP4 agonist stimulated bone resorption in organ culture of rodent calvaria (Suzawa et al., 2000; Raisz and Woodiel, 2003), and the EP4 antagonist AH23848B inhibited osteoclastogenesis in mouse marrow culture (Ono et al., 1998). These in vitro data suggest that EP4 activation has a resorptive effect, associated with increased osteoclastic differentiation and osteoclastogenesis.

Although reports on the signaling pathway used by EP4 in osteoclast activation are limited, Miyaura et al. (2000) demonstrated that both PGE₂ and dibutyl cAMP stimulated bone resorption and induced MMP-2 and MMP-13 in cultured calvariae, and that the

addition of H-89, an inhibitor of PKA, or BB94, an inhibitor of MMPs, significantly suppressed the bone-resorbing activity induced by PGE₂. Furthermore, dibutyl cAMP greatly stimulated bone resorption and MMP-2 and MMP-13 in both wild-type and EP4-knockout mice (Miyaura et al., 2000). Kaji et al. (1996) also reported that the activation of PKA is linked to PGE₂-stimulated osteoclast-like cell formation and bone-resorbing activity. These findings suggest that PGE₂ stimulates bone resorption by a cAMP-PKA-dependent mechanism via the EP4 receptor.

d. Bone anabolism. Since EP4 plays a role in both osteoblast differentiation and osteoclast activation in vitro, the bone-anabolic and bone-resorptive effects of EP4 have been extensively studied in vivo. There is a disagreement among studies concerning the effects of EP4 deletion. Akhter et al. (2006) reported that aged EP4-deficient female mice had small distal femur and

vertebral bone volumes and exhibited reduced structural and apparent material strength in the femoral shaft and vertebral body. Similarly, bone mass was reduced in aged EP4-deficient male mice (Li et al., 2005). On the other hand, a study using mice with global or osteoblast-targeted deletion of the EP4 receptor suggested that EP4 activation induced osteoblast differentiation in vitro, although its activation is not essential for bone volume or bone formation in living animals (Gao et al., 2009). The mechanisms underlying this disagreement are not clear, but the loss of EP4 may be differentially compensated in various strains of genetically modified mice.

Contrary to this discrepancy between studies using genetically modified mice, most studies using pharmacological approaches have suggested that EP4 activation has anabolic effects in bone. EP4 agonists (ONO-4819) delivered via local or systemic administration increased bone mass and strength in intact rats (Ninomiya et al., 2011) and in ovariectomized rats as an osteoporosis model (Yoshida et al., 2002; Ito et al., 2006; Ke et al., 2006). In a different model of osteoporosis induced through immobilization, ONO-4819 also completely blocked bone loss in rats when infused systemically (Yoshida et al., 2002). ONO-4819 in rats enhanced mechanical loading-induced cortical bone formation (Hagino et al., 2005). Pharmacological inactivation of EP4 in rats suppressed PGE₂-induced bone formation (Machwate et al., 2001; Shamir et al., 2004). Another study showed that an EP4 agonist enhanced bone formation around titanium plates, suggesting that the EP4 agonist promotes early bone formation in the bone-implant interface (Onishi et al., 2008). It is worth noting that recent studies have suggested that attenuated G_sα coupling to AC contributed to age-related decreases in bone formation (Kessler and Delany, 2007; Genetos et al., 2012). Osteoporosis is a systemic skeletal disorder characterized by a reduction in bone mineral density and disruption in bone microarchitecture. Since there are a large number of affected patients, many people would benefit from a potential treatment of osteoporosis incorporating an EP4 agonist.

e. Bone healing. Several in vivo studies have also investigated the potential therapeutic effect of EP4 agonists in facilitating bone healing (Tanaka et al., 2004; Marui et al., 2006). EP4 receptor knockout mice have been shown to have impaired fracture healing (Li et al., 2005). Administration of an EP4 agonist promoted healing of the sternum filled with regenerated bone tissue and increased bone mineral content and density in diabetic rats (Marui et al., 2006). Similarly, the EP4 agonist ONO-4819 dose-dependently accelerated the healing of a cortical bone defect at the drill-hole site by stimulating local bone resorption and formation (Tanaka et al., 2004). Toyoda et al. (2005) used a local drug delivery system and found that the

EP4 agonist ONO-4819 in a carrier polymer enhances bone morphogenetic protein-induced bone formation in mice. Furthermore, an EP4 agonist accelerated delayed fracture healing in aged mice and compensated for the reduced fracture healing observed in COX-2 KO mice (Xie et al., 2009). These data collectively suggest that bone formation is more likely than bone resorption to be promoted by EP4 in vivo. EP4 activation appears to play beneficial roles in osteoporosis and fracture healing through cAMP signaling.

f. Rheumatoid arthritis and other diseases. A substantial body of evidence suggests that PGE₂ contributes to the pathogenesis of rheumatoid arthritis (Akaogi et al., 2006; Hikiji et al., 2008), and inhibitors of PGE₂ synthesis are currently used in the treatment of this disease. Rheumatoid arthritis is characterized by chronic joint inflammation comprising synovial fibroblasts, T cells, and macrophages (Sato and Takayanagi, 2006). T cells activate synovial macrophages that release multiple cytokines, resulting in the amplification of synovial inflammation and the destruction of cartilage and bone. Macrophage-derived cytokines such as IL-1β and TNF-α induce COX-2 expression in human articular chondrocytes and synovial fibroblasts (Martel-Pelletier et al., 2003). IL-17 also stimulates COX-2-dependent PGE₂ synthesis in mouse primary osteoblasts from the synovial tissues (Kotake et al., 1999).

The roles of PGE₂ and EP4 receptor signaling in rheumatoid arthritis have been extensively examined using multiple animal models, such as collagen-induced arthritis (CIA), collagen antibody-induced arthritis (CAIA), and adjuvant-induced arthritis (Table 4). Mice deficient in COX-2 or PGE₂ synthase 1 are resistant to CIA and CAIA (Myers et al., 2000; Hegen et al., 2003; Trebino et al., 2003; Kamei et al., 2004). PGE₂ has also been shown to exacerbate symptoms in CIA through the inflammatory Th17/IL-17 axis (Sheibanie et al., 2007a). EP4-deficient mice, but not EP1-, EP2-, or EP3-deficient mice, showed decreased inflammation, as evidenced by decreased circulating IL-6 and serum amyloid A levels, as well as by the reduced incidence and severity of disease in CAIA (McCoy et al., 2002). In this study, macrophages isolated from EP4 KO animals produced significantly less IL-1β and IL-6 than control samples did, suggesting that EP4 signaling in macrophages contributes to the exacerbation of rheumatoid arthritis (McCoy et al., 2002).

In keeping with this study, pharmacological blockade of EP4 signaling ameliorates rodent models of rheumatoid arthritis. Administration of EP4 antagonists (ER819762, CJ-042794, or CJ-023423) effectively suppressed disease in CIA and adjuvant-induced arthritis (Murase et al., 2008; Okumura et al., 2008; Chen et al., 2010). Chen et al. (2010) suggested that the EP4 antagonist used in their study suppressed the ability of lymph node T cells to produce IFN-γ and IL-17 ex vivo in response to stimulation with bovine type

II collagen/Complete Freund's adjuvant. Significant suppression of CIA is achieved in mice by the simultaneous inhibition of EP2 and EP4 receptors, although the EP4 antagonist ONO-AE3-208 alone did not alter CIA in wild-type mice (Honda et al., 2006). Several studies have shown the involvement of EP4 signaling in cytokine-related action in human synovial fibroblasts (Faour et al., 2001; Yoshida et al., 2001; Mathieu et al., 2008). Taken together, most studies suggest that EP4 antagonists ameliorate rheumatoid arthritis in multiple cell types, including T cells, macrophages, and synovial fibroblasts, and are potentially beneficial in the treatment of rheumatoid arthritis.

Periprosthetic osteolysis is the most common cause of aseptic loosening in total joint arthroplasty. Synovial fibroblasts respond directly to titanium particles, increasing RANKL expression through a COX-2/PGE₂/EP4/PKA signaling pathway and enhancing osteoclast formation (Wei et al., 2005). Tsutsumi et al. (2009) examined the role of EP4 in periprosthetic fibroblasts using mice with conditionally deleted EP4 in FSP1-positive fibroblasts and found that polyethylene-bead-induced osteolysis is impaired in conditional EP4 KO mice. They concluded that fibroblasts rather than osteoblasts are the predominant source of RANKL (Tsutsumi et al., 2009). These data suggested that EP4 signaling in synovial fibroblasts plays a role in osteolysis after joint arthroplasty.

g. Chondrocytes. Chondrocytes are involved in endochondral bone formation as well as coordinated bone formation and mineralized matrix resorption by osteoblasts and osteoclasts. Reports on the role of the EP4 receptor in chondrocytes are limited (Table 4). In the growth plate, chondrocytes undergo a maturation process, in which resting chondrocytes transition into proliferating chondrocytes, which express type II collagen mRNA and synthesize proteoglycan. It has been reported that EP4 receptor stimulation accelerates the maturation of mouse and rat chondrocytes (Miyamoto et al., 2003b; Clark et al., 2005). In contrast, PGE₂ inhibits proteoglycan synthesis and stimulates matrix degradation via the EP4 receptor in chondrocytes from patients with osteoarthritis (Attur et al., 2008).

E. Gastrointestinal Tract

1. Expression. The EP receptors have distinct cellular localizations in the mouse gastrointestinal tract (Morimoto et al., 1997). The EP4 receptor is moderately expressed in the mouse and rat stomach (Honda et al., 1993; Sando et al., 1994; Sugimoto and Narumiya, 2007) and in the rat gastric mucosal cell line RGM1 (Suetsugu et al., 2000). Expression of EP4 mRNA was localized in mouse (Morimoto et al., 1997) and rabbit (Takahashi et al., 1999) epithelial cells of

the corpus and in glands from the surface to the base of the gastric antrum.

In the ileum, the EP4 receptor is highly expressed in humans, mice, and rats (An et al., 1993; Honda et al., 1993; Bastien et al., 1994; Sando et al., 1994; Ding et al., 1997). The EP4 receptor is also highly expressed in the human, mouse, and rat colon (An et al., 1993; Ding et al., 1997; Kabashima et al., 2002; Olsen Hult et al., 2011). A recent study examined the precise distribution of the EP receptors in the intestinal epithelium in humans (Olsen Hult et al., 2011). This study demonstrated that the EP4 and EP2 receptors were expressed in normal colon epithelial crypt cells, but not in normal small intestinal epithelium. The inflamed duodenal epithelium from patients with untreated celiac disease expressed EP4 and EP2 in crypt cells. On the other hand, the EP1 and EP3 receptors were not detected in intestinal epithelium (Olsen Hult et al., 2011).

In contrast to its abundant expression in the colon epithelium, the EP4 receptor was not found in the intestinal smooth muscle layer in mice, rats, or humans (Ding et al., 1997; Morimoto et al., 1997; Olsen Hult et al., 2011). Other EP receptors have been found in the muscle layer of the gastrointestinal tract. mRNA expression of the EP1 receptor has been found in the muscularis mucosae layer of the stomach (Watabe et al., 1993; Morimoto et al., 1997) and in colonic longitudinal muscle (Smid and Svensson, 2009). EP3 receptor mRNA has been found in longitudinal smooth muscle cells and in neurons of the myenteric ganglia (Narumiya et al., 1999). These observations suggest that EP4 receptor signals contribute to epithelial function rather than smooth muscle tone.

The EP4 receptor has been found to be upregulated in inflammatory bowel disease. Expression of the EP4 receptor was strongly upregulated in rats with dextran sodium sulfate-induced colitis (Ding et al., 1997; Nitta et al., 2002). Consistently, Lejeune et al. (2010) have reported that, in healthy human colonic mucosa, EP4 receptors were localized on apical plasma membranes of epithelial cells at the tips of mucosal folds; in patients with inflammatory bowel disease and in rats with dextran sodium sulfate-induced colitis, on the other hand, they were diffusely overexpressed throughout the mucosa.

2. Function. NSAIDs have been widely used to achieve analgesic and anti-inflammatory effects, but a major limitation to their use is their potential to cause damage to the gastrointestinal mucosa (Levi et al., 1990; Tanaka et al., 2002). The healing-impairing effect of the NSAIDs is due to their inhibition of COX, especially COX-2 (Tanaka et al., 2002; Takeuchi et al., 2010a). EP4 activation has been found to attenuate indomethacin-induced intestinal ulcers in rats (Kunikata et al., 2002; Hatazawa et al., 2006; Takeuchi et al., 2010b). A substantial body of evidence

suggests that EP4 plays a role in maintaining gastrointestinal integrity.

a. Mucin secretion. PGE₂ increases gastric mucin secretion and suppression of motor action, and these activities are quite effective in the prevention of gastric mucosal injury (Takeuchi et al., 2010a). It has been reported that EP4 activation promotes mucin secretion from gastric epithelial cells (Takahashi et al., 1999). Similarly, EP4-mediated increases in mucus secretion and intestinal fluid secretion have been found to contribute to the process of intestinal ulcer healing (Kunikata et al., 2002). It has also been reported that cAMP contributes to gastrointestinal mucin secretion, including its principal functional component, mucus glycoprotein (Keates and Hanson, 1990; Słomiany and Słomiany, 2005). Słomiany and Słomiany (2005) demonstrated that β -adrenergic GPCR activation and subsequent AC activation triggered mucin secretion via both the PKA and PI3K pathways. From these data, it is speculated that EP4 activation promotes gastrointestinal mucin secretion through the cAMP pathway.

b. Mucosal cell injury. Several in vivo studies suggested that EP4 activation plays a protective role in rodent models of gastric ulcer via different mechanisms, such as VEGF induction, venous relaxation, and inhibition of apoptosis (Hatazawa et al., 2007; Hattori et al., 2008; Jiang et al., 2009), although there are reports that EP4 did not contribute to ulcer healing (Kunikata et al., 2001; Takeuchi et al., 2003).

Takeuchi et al. (2010b) found that an EP4 agonist reversed indomethacin-induced downregulation of VEGF expression and angiogenesis, and suggested that endogenous PGE₂ promotes the healing of small intestinal lesions by stimulating angiogenesis through the upregulation of VEGF expression mediated by the activation of EP4 receptors. It has also been reported that EP4 activation promotes VEGF production from gastric fibroblasts (Takahashi et al., 1996; Hatazawa et al., 2007). A study using human airway smooth muscle cells revealed that EP4 signaling increases VEGF transcriptionally and involves the Sp-1 binding site via a cAMP-dependent mechanism (Bradbury et al., 2005).

Hoshino et al. (2003) demonstrated that EP4 activation inhibited ethanol-induced apoptosis in primary culture of guinea pig gastric mucosa cells through the cAMP-PKA pathway, but not the PI3K pathway. Similar results were shown in indomethacin-induced apoptosis in human gastric mucosa cells (Jiang et al., 2009). An antiapoptotic effect of cAMP has also been demonstrated in the T84 intestinal epithelial cell line (Nishihara et al., 2003; Rudolph et al., 2004, 2007). By use of pharmacological activators and inhibitors of PKA as well as siRNA, Rudolph et al. (2007) demonstrated that cAMP binding to the PKAII regulatory subunit leads to the subsequent phosphorylation of

ERK1/2, resulting in inhibition of epithelial apoptosis. EP4-cAMP signaling appears to play protective roles in mucosal cell injury via induction of VEGF and inhibition of apoptosis.

c. HCO₃⁻ secretion. Duodenal mucosal HCO₃⁻ secretion is critical to prevent acid peptic injury. PGE₂ increases HCO₃⁻ secretion from the rat duodenal mucosa (Takeuchi et al., 1997). It has been suggested that EP4 synergizes with EP3 to promote duodenal mucosal HCO₃⁻ secretion (Takeuchi et al., 2010a). Studies have demonstrated that duodenal HCO₃⁻ secretion is stimulated by the EP4 agonist ONO-AE1-329 under HCl-induced mucosal acidification conditions (Aoi et al., 2004; Aihara et al., 2007). The maximal response of the EP4 agonist was equivalent to that induced by PGE₂. Coadministration of the EP1/3 agonist sulprostone and ONO-AE1-329 caused a greater secretory response than either agent alone, suggesting that EP4 receptors together with EP3 receptors are involved in the duodenal HCO₃⁻ response induced by PGE₂ (Aoi et al., 2004).

The stimulatory effect of PGE₂ on duodenal HCO₃⁻ is mediated intracellularly by cAMP, PI3K, and Ca²⁺ (Tuo et al., 2007; Takeuchi et al., 2011). Although there is no direct evidence that a downstream signaling mechanism of EP4 is involved in duodenal HCO₃⁻ response, several studies have suggested that EP4 receptor signaling and EP3 receptor signaling could act in coordination to maintain duodenal mucosal integrity against acid through intracellular cAMP and PI3K and Ca²⁺ elevation. Tuo et al. (2007) demonstrated that PGE₂-stimulated duodenal HCO₃⁻ secretion was reduced by the cAMP-dependent signaling pathway inhibitors MDL-12330A (an AC inhibitor) and KT-5720 (a PKA inhibitor) by 23 and 20%, respectively; reduced by the Ca²⁺-influx inhibitor verapamil by 26%; reduced by the calmodulin antagonist W-13 by 24%; and reduced by the PI3K inhibitors wortmannin and LY-294002 by 51 and 47%, respectively. Similarly, elevation of intracellular cAMP levels by the non-selective PDE inhibitor 3-isobutyl-1-methylxanthine, the PDE1 inhibitor vinpocetine, and the PDE3 inhibitor cilostamide increased HCO₃⁻ secretion in duodenal mucosa (Hayashi et al., 2007; Takeuchi et al., 2011, 1997).

d. Colitis. The protective effect of EP4 on the colon has been shown in in vivo studies using mouse and rat dextran sodium sulfate-induced colitis. Kabashima et al. (2002) used mice deficient in EP1, EP2, EP3, or EP4 and found that only EP4-deficient mice suffered from exacerbated 3% dextran sodium sulfate-induced colitis with impaired mucosal barrier function, epithelial loss, crypt damage, and accumulation of neutrophils and CD4⁺ T cells. Consistent with this observation, administration of an EP4-selective agonist (ONO-AE1-734) in wild-type mice ameliorated severe colitis induced with 7% dextran sodium sulfate. This study

focused on immune response induced by EP4 signaling and demonstrated in vitro that the EP4 antagonist ONO-AE3-208 enhanced, whereas the EP4 agonist ONO-AE1-734 suppressed, the proliferation of and Th1 cytokine production by lamina propria mononuclear cells from the colon (Kabashima et al., 2002). Similarly, Nitta et al. (2002) reported that the EP4 agonist ONO-AE1-329 suppressed rat dextran sodium sulfate-induced colitis through upregulation of the anti-inflammatory cytokine IL-10. Jiang et al. (2007) demonstrated that treatment with the EP4 agonists ONO-AE1-329 or AGN205203 ameliorated a murine model of colitis, and that EP4 activation decreased colon epithelial apoptosis, prevented goblet cell depletion, and promoted epithelial regeneration in vitro. These studies suggest that EP4 maintains gastrointestinal homeostasis by preserving mucosal integrity and downregulating inflammatory immune response.

e. Inflammatory bowel disease. In contrast to its protective effect on gastrointestinal mucosal integrity, more recent studies have suggested that the EP4 receptor plays proinflammatory roles in inflammatory bowel disease. Inflammatory bowel disease, including Crohn's disease and ulcerative colitis, is chronic and relapsing and is characterized by inflammation in the large and/or small intestine associated with diarrhea, occult blood, and abdominal pain. Studies in humans have implicated impaired mucosal barrier function, production of proinflammatory cytokines, and activation of CD4⁺ T cells in the pathogenesis of inflammatory bowel disease (Fiocchi, 1998). It is known that PGE₂ is produced abundantly and that EP4 is upregulated in the affected intestine (Hommes et al., 1996; Lejeune et al., 2010). Furthermore, in a genome-wide association study of Caucasian patients with Crohn's disease, Libioulle et al. (2007) identified a region of approximately 250 kb on chromosome 5p13.1. Studies in lymphoblastoid cell lines revealed that genetic variants in the Crohn's disease-associated region influence the expression levels of the closest known gene, PTGER4, located 270 kb away in the direction of the centromere. This study suggested that genetic variants associated with Crohn's disease on chromosome 5p13.1 could modulate *cis*-acting regulatory elements of PTGER4 (Libioulle et al., 2007). From these observations, EP4 is likely to regulate Crohn's disease initiation and progression.

Th17 cells have emerged recently as central players in various inflammatory/autoimmune conditions, including inflammatory bowel disease (Yen et al., 2006). Crohn's disease patients have increased levels of IL-17 in serum and intestinal mucosa (Fujino et al., 2003b; Nielsen et al., 2003; Fuss et al., 2006). In accordance with these data, Sheibanie et al. (2007b) demonstrated that the PGE analog misoprostol exacerbated 2,4,6-trinitrobenzene sulfonic acid-induced colitis and that

this exacerbation was correlated with an increase in IL-23 and IL-17, a decrease in IL-12p35 expression in the colon and mesenteric lymph nodes, and a substantial increase in the numbers of infiltrating neutrophils and Th17 cells in the colonic tissue. The study demonstrated that PGE₂ promotes IL-23 and inhibits IL-12 and IL-27 expression through EP4 in dendritic cells in vitro, suggesting that PGE₂ exacerbates the inflammatory process through the release of dendritic cell-derived IL-23 and the subsequent support of the autoreactive/inflammatory Th17 phenotype (Sheibanie et al., 2007b). Taken together, these data suggest that increased expression and subsequent activation of EP4 contribute to exacerbation of Crohn's disease through enhancement of the Th17 immune response.

IL-8 is a potent neutrophil chemoattractant and activator, and its levels correspond to the active grade of inflammatory bowel disease, including ulcerative colitis and Crohn's disease (Mazzucchelli et al., 1994). Srivastava et al. (2012) examined the mechanisms of differential regulation of IL-8 production by EP4 and demonstrated that PGE₂-EP4 signaling activated CREB through both the PKA and PI3K pathways. Interestingly, they also demonstrated that EP2 activated the transcription factor-inducible cAMP early repressor (Srivastava et al., 2012). Because inducible cAMP early repressor lacks the transactivation domain, it functions as a transcription repressor, unlike CREB. These data suggest that PGE₂ coupling through EP4 and EP2 receptors can therefore act in an opposing manner to either promote (EP4) or decrease (EP2) IL-8 expression, even though both receptors use the same second messenger, cAMP. Since Chadee's laboratory has reported that PGE₂ promotes IL-8 production in the human colonic epithelial cell lines Caco-2 and T84 through both the PKA and PI3K pathways (Dey and Chadee, 2008; Dey et al., 2009; Srivastava et al., 2012), it appears that EP4 contributes to IL-8 production.

f. Epithelial barrier. More recent data have suggested that colonic epithelial barrier function is disrupted by EP4 signaling in Caco-2 and T84 cells (Lejeune et al., 2010, 2011; Rodriguez-Lagunas et al., 2010). It has been reported that EP4 signaling increased intracellular Ca²⁺ concentration through the cAMP-PKA pathway, resulting in disruption of the colonic epithelial barrier in vitro (Rodriguez-Lagunas et al., 2010). Recently, several authors have described the cross-talk relationship between IP₃ receptors/Ca²⁺ release and the cAMP-PKA signaling pathway. Phosphorylation of IP₃ receptors by PKA results in a significant enhancement of IP₃-induced intracellular Ca²⁺ and is involved in diverse Ca²⁺-regulated physiologic processes (Bruce et al., 2002; Tang et al., 2003; Chaloux et al., 2007). Wagner et al. (2008) demonstrated that PKA phosphorylation increases the

sensitivity of the IP₃ receptor to IP₃. Intestinal epithelial barrier function may be regulated by these mechanisms.

Collectively, the evidence indicates that EP4 signaling maintains gastrointestinal homeostasis by preserving mucosal function in physiologic settings. However, in chronic inflammatory bowel disease such as Crohn's disease, EP4 signaling is suggested to promote intestinal inflammation.

F. Renal System

1. *Expression.* Northern blot analysis demonstrated moderate but significant expression of the EP4 receptor in the kidneys of humans (An et al., 1993; Bastien et al., 1994), mice (Sugimoto and Narumiya, 2007), rats (Sando et al., 1994), and rabbits (Breyer et al., 1996b). In situ hybridization studies have revealed that the EP4 receptor is highly expressed in the glomerulus (Sugimoto et al., 1994). The EP3 receptor, on the other hand, is expressed in the tubular epithelium, the thick ascending limb, and the cortical collecting ducts in the outer medulla. The EP1 receptor is expressed in the papillary collecting ducts. Similarly, EP4 receptor mRNA is predominantly expressed in the glomerulus in humans (Breyer et al., 1996b) and rabbits (Breyer et al., 1996a; Morath et al., 1999), suggesting that EP4 contributes to the regulation of glomerular hemodynamics and renin release (Breyer and Breyer, 2001). In the normal and ischemic adult human kidney, vascular COX-2 was colocalized with EP4 receptors (Therland et al., 2004). In a study in rats, the EP4 receptor was strongly expressed in the glomeruli, renin-secreting juxta-glomerular granular (JG) granular cells (Jensen et al., 1999), glomerular epithelial cells (Aoudjit et al., 2006), distal convoluted tubules, cortical collecting ducts (Jensen et al., 2001), and developing renal tubules (Yamamoto et al., 2011). When rats were given low-NaCl diets, the EP4 transcripts in glomeruli were significantly increased, implicating its role in regulating NaCl homeostasis (Jensen et al., 1999).

2. *Function.* The dominant expression of EP4 in the glomerulus suggests that EP4 may regulate glomerular filtration. Albuminuria is a useful marker for evaluation of glomerular filtration barrier (GFB) damage. Conventionally, nonsteroidal anti-inflammatory drugs have been reported to reduce proteinuria (Vriesendorp et al., 1986), suggesting that prostanoids, derived from COX-1 or COX-2, may worsen GFB damage. The effect of EP4 receptor signaling on glomerular function has remained controversial, however.

Animal studies using podocyte-specific EP4 receptor-overexpressing or EP4 receptor-deficient mice were performed by Stitt-Cavanagh et al. (2010). They induced renal ablation by 5/6 nephrectomy, and found increased proteinuria and mortality in mice overexpressing

the EP4 receptor (Stitt-Cavanagh et al., 2010). In EP4-deficient mice, however, proteinuria was decreased and glomerular lesions became milder. A COX-2 inhibitor also decreased proteinuria. They also found that EP4 receptor overexpression in cultured podocytes resulted in enhanced susceptibility to mechanical stretch-induced detachment from culture dishes, which may be a potential mechanism leading to the pathogenesis of proteinuria. Thus, PGE₂, acting via EP4 receptors, may progress podocyte injury and GFB damage, leading to proteinuria.

In contrast to the previous findings, Aoudjit et al. (2006) reported that an EP4 receptor antagonist worsened proteinuria and glomerular apoptosis in a rat model of podocyte injury. Similarly, Nagamatsu et al. (2006) demonstrated that an EP4 receptor agonist was protective in antglomerulus antiserum-induced glomerulonephritis in mice. It was most likely that EP4/cAMP signaling enhanced clearance of aggregated protein from the glomeruli (Nagamatsu et al., 2006). Thus, it seems that the EP4 receptor is, at the least, involved in the development of glomerular diseases, but further studies are required to reveal its mechanistic role.

Meanwhile, EP4 may regulate renal circulation, and thus may contribute to glomerular injury. PGE₂ mediates vasodilatory effects in the preglomerular circulation; this is the mechanism by which NSAIDs reduce glomerular filtration rate and renal blood flow (Schnermann and Weber, 1982; Chaudhari et al., 1990). Edwards (1985) reported that PGE₂ exerted a vasorelaxing effect on the afferent arteriole, but not on the efferent arteriole of rabbit glomeruli.

EP4 may play a role in renin secretion. PGE₂-induced renin release through EP4 receptors in mice was demonstrated in the isolated perfused kidney (Schweda et al., 2004) and in isolated JG cells (Friis et al., 2005). These studies found that EP4 stimulation caused PKA-mediated exocytotic fusion and release of renin granules in rat JG cells. In accordance with these findings, plasma renin concentrations were significantly lower in EP4 receptor-deficient mice than in wild-type mice. Moreover, a low dose of PGE₂ failed to induce renin secretion in the isolated kidneys of EP4 receptor-deficient mice, whereas the same dose of PGE₂ enhanced renin secretion in wild-type and other EP receptor-deficient kidneys. These findings indicate that the EP4 receptor may play a critical role in the regulation of renin secretion under normal conditions. Cyclic AMP has been recognized as an important regulator of renin secretion (Hackenthal et al., 1990). Aldehni et al. (2011) reported that AC5 and AC6, which are G_iα- and Ca²⁺-inhibitable AC isoforms, are involved in the stimulatory effect of catecholamines and that PGE₂-mediated signaling is involved the secretion of renin. Isoproterenol- and PGE₂-induced renin secretion was attenuated in isolated perfused kidneys

from AC5- and AC6-deficient mice. Furthermore, EP2 stimulation caused PKA-mediated release of renin granules in rat JG cells (Friis et al., 2005). These studies suggest that renin secretion might be related to cAMP downstream of EP4 signaling.

EP4 is also expressed in the distal convoluted tubule and the cortical collecting duct (Olesen et al., 2011). A recent report demonstrated that EP4 receptor agonists increase aquaporin-2 phosphorylation and trafficking. EP4 may be involved in the regulation of water homeostasis via the regulation of water transport in the collecting duct. cAMP has been demonstrated to play an important role in the regulation of water transport in the collecting duct. Vasopressin exerts its antidiuretic effect through vasopressin 2 receptors coupled to Gs protein, which activates AC to form cAMP from ATP. Increased cAMP activates PKA, which phosphorylates aquaporin-2 water channels, thereby promoting water reabsorption (Nielsen et al., 1999). Further studies are required to determine whether EP4 is involved in the regulation of water homeostasis in the collecting duct.

G. Reproductive System

1. *Expression.* PGE₂ regulates various uterine functions, such as contraction and relaxation of the uterine smooth muscles, cervical ripening and labor induction, elevation of endometrial vascular permeability, and induction of decidualization (Murdoch et al., 1993). In female reproductive organs such as the ovary and uterus, hormonal exposure induces expression of the EP subtypes in a cell type-specific manner. EP4 is expressed in the mouse ovary (Segi et al., 2003) and in the human (Milne et al., 2001; Astle et al., 2005), baboon (Smith et al., 1998), mouse (Katsuyama et al., 1997; Yang et al., 1997), rat (Blesson et al., 2012), and guinea pig (Terry et al., 2008) uterus, although Arosh et al. (2003, 2004) reported that EP4 mRNA was undetectable in the bovine uterus.

In the mouse ovary, EP4 expression was found in oocytes in the preantral follicles. Upon gonadotropin stimulation, however, it disappeared, reappearing in both cumulus and granulosa cells 3 hours after gonadotropin stimulation. EP4 mRNA was detected in the epithelium throughout the oviduct, the tube extending from the periovarial space to the uterine horns (Segi et al., 2003).

Milne et al. (2001) examined menstrual cyclical variation in endometrial human EP receptor mRNA expression. They demonstrated that EP4 receptor expression was significantly higher in the late proliferative stage than in the early, middle, and late secretory stages. Both EP4 and EP2 receptor expression were found in endometrial glandular epithelial and vascular cells, with no notable spatial or temporal variation (Milne et al., 2001). The expression change in

pseudopregnancy was also demonstrated in the mouse uterus. The EP4 receptor transcripts were expressed mainly in the luminal epithelium during peri-implantation; they were increased in endometrial stromal cells and the glandular epithelium after pharmacological induction of pseudopregnancy (Katsuyama et al., 1997; Yang et al., 1997). EP4 transcripts were also present in the myometrium and remained unchanged throughout gestation in pregnant humans (Astle et al., 2005) and guinea pigs (Terry et al., 2008). EP3 receptor mRNA was predominantly expressed in the myometrium (Katsuyama et al., 1997; Yang et al., 1997).

The uterine cervix also plays a crucial role in pregnancy; it must remain closed during gestation, then soften and dilate during labor. PGE₂ has been used to induce cervical ripening for many years (Woodward and Chen, 2004). EP4 receptor expression has been found in smooth muscle cells and epithelial cells in the cervix, and at its highest concentration at parturition in goats (Gu et al., 2012) and rats (Chien and Macgregor, 2003; Hinton et al., 2010), suggesting that increased EP4 receptor expression may regulate cervical relaxation. EP4 receptor expression is also increased in cervical inflammation. *Chlamydia trachomatis* LGV2 selectively upregulated COX-2 and EP4 in cervical epithelial HeLa 229 cells (Fukuda et al., 2005). Similarly, EP4 receptor was increased in LPS-treated rabbit interstitial cells in the cervix (Fukuda et al., 2007) and in IL-1 β -treated human cervical fibroblasts (Schmitz et al., 2003).

EP4 receptor may be involved in the male reproductive organs as well. Moderate expression of EP4 was demonstrated in the human (An et al., 1993), bovine (Arosh et al., 2003), and chicken (Kwok et al., 2008) testis, although no EP4 expression was detectable in the mouse (Honda et al., 1993) or rat (Sando et al., 1994) testis.

2. *Function.* Myometrial relaxation is mediated by EP4 as well as by EP2 receptors (Senior et al., 1993; Negishi et al., 1995). The localization and expression of these receptors are thus involved in the onset and maintenance of labor. However, no significant pregnancy- or labor-associated changes in EP4 receptor expression were reported in the human uterus (Astle et al., 2005). The EP4 receptor is similarly expressed in the upper and lower segments of the uterus. It is most likely that EP4 does not play a major role in PGE₂-mediated regulation of myometrial tone during pregnancy and labor. Cyclic AMP may play an important role in myometrial quiescence (Yuan and Lopez Bernal, 2007). In general, PKA phosphorylates cellular proteins that may cause smooth muscle relaxation, including myosin light-chain kinase (Nishikawa et al., 1984), PDE4 (Murthy et al., 2002), and PLC. EP4 signaling may be involved in the enhancement of such phosphorylation, and it remains as yet a possibility

that EP4 is involved in other relevant processes during pregnancy and delivery. Glycosaminoglycan redistribution is an important process involved in cervical ripening; Schmitz et al. (2001) demonstrated that, of the four subtypes of PGE₂ receptors, only EP4 mediated PGE₂-induced glycosaminoglycan synthesis in human cervical fibroblasts in a PKA-independent manner.

The EP4 receptor may regulate endometrial function. PGE₂ promotes the survival of human endometrial cells through the EP4 receptors by activating ERK, Akt, NF- κ B, and the β -catenin signaling pathway (Banu et al., 2009). Inhibition of EP4 may suppress proliferation and induce apoptosis of human endometrial cells. In addition, Lee et al. (2012) found that EP4 was expressed in the ovine endometrium, especially during pregnancy. Interferon- τ , a pregnancy recognition signal in ruminants, increased EP4 receptors in the endometrium.

H. Lungs

1. Expression. The lung is an organ in which the EP4 receptor is abundantly expressed in many species, including humans, mice, rats, and rabbits (An et al., 1993; Honda et al., 1993; Bastien et al., 1994; Sando et al., 1994; Breyer et al., 1996b). Anatomically, the lung is composed of the bronchial tree, the alveoli, and a dense vascular network, including a variety of cell types. EP4 is highly expressed in airway smooth muscle cells, pulmonary fibroblasts, and smooth muscle cells of the pulmonary vein. In particular, together with the EP2 receptor, EP4 transcripts and proteins are abundantly expressed in human airway smooth muscle cells (Bradbury et al., 2005; Clarke et al., 2005; Mori et al., 2011; Benyahia et al., 2012). It is also known that EP4 activation causes potent relaxation in human and rat bronchial preparations (Lydford and McKechnie, 1994; Benyahia et al., 2012).

Pulmonary fibroblasts are important in the development and maintenance of lung structure and function. Their proliferation and phenotypic changes play critical roles in normal tissue repair as well as the development of pulmonary fibrosis (Ramos et al., 2001). The EP4 receptor is expressed in both fetal (Choung et al., 1998; Li et al., 2011) and adult lung fibroblasts (Huang et al., 2007; Nikam et al., 2011) in humans. Togo et al. (2008) demonstrated that the EP2 and EP4 receptors were expressed in normal pulmonary fibroblasts and that these receptors were increased in fibroblasts from patients with chronic obstructive pulmonary disease where they contribute to the pathogenesis of emphysema.

In the human pulmonary vasculature, the EP4 receptor is mostly expressed in the smooth muscle layer of the vein and only weakly in the artery (Walch et al., 1999; Foudi et al., 2008), suggesting that EP4 induces relaxation of the vein. This expression pattern

may change under disease conditions. Lai et al. (2008) reported that EP4 expression in the artery is readily detectable in pulmonary arterial hypertension in human and rat models. In other cell types in the lung, the EP4 receptor is found in human pulmonary microvascular endothelial cells (Aso et al., 2012), the human bronchial epithelial cell line BEAS-2B (N'Guesan et al., 2007), and human alveolar macrophages (Ratcliffe et al., 2007).

2. Function. EP4 may induce relaxation of the airway and inhibit smooth muscle cell proliferation. Buckley et al. (2011) reported that PGE₂-induced relaxation of the airway was mediated through EP4 in humans and rats. Mori et al. (2011) demonstrated that PGE₂ inhibited fetal bovine serum-induced proliferation of human airway smooth muscle cells via EP4 receptor activation. Taken together, these findings suggest that the EP4 receptor could potentially be a therapeutic target in treating pulmonary diseases such as asthma and chronic obstructive pulmonary disease. As they potentially occur downstream of EP4/cAMP signaling, both PKA and Epac are involved in anti-inflammatory (Oldenburger et al., 2012) and relaxation (Zieba et al., 2011) signaling in airway smooth muscle cells. It was also demonstrated that PGE₂ inhibits Platelet-derived growth factor-induced phenotype switching of tracheal smooth muscle cells, from a contractile to a proliferative phenotype, through the activation of the cAMP effectors PKA and Epac (Roscioni et al., 2011).

In fibroblasts, PGE₂ inhibits proliferation and collagen synthesis in human lungs (Huang et al., 2008). Proliferation and collagen synthesis were likewise attenuated by the activation of the cAMP effectors PKA and Epac, respectively. The accumulation of cAMP was promoted by EP4 receptor activation. In addition, chemotaxis of human lung fibroblasts was inhibited by EP4 (Li et al., 2011). Subtype-specific modulation of EP receptor activity could potentially be a new therapy for fibrotic lung disease.

An interesting vasodilatory effect of EP4-mediated signaling has been reported. Cyclic AMP accumulation in vascular smooth muscle cells is thought to be the main mechanism of prostanoid-induced vasorelaxation. Lai et al. (2008) demonstrated that iloprost, a stable analog of PGI₂, increased cAMP via the EP4 receptor in pulmonary arterial smooth muscle cells isolated from rats with pulmonary hypertension. Similarly, Foudi et al. (2008) reported that PGE₂-induced vasorelaxation of the human pulmonary vein was also mediated by the EP4 receptor. It has been demonstrated that IP receptors are downregulated in human pulmonary artery hypertension, whereas the EP4 receptor is stably expressed. The EP4 receptor could thus be a novel effective therapeutic target for the treatment of pulmonary artery hypertension.

I. Skin

1. Expression. Tober et al. (2007) reported that the EP4 receptor was abundant in epidermal keratinocytes, dermal leukocytes, and vascular endothelium in murine skin. UV-B exposure induced EP4 relocalization to the plasma membranes of keratinocytes, whereas its diffuse cytoplasmic staining pattern was unchanged in the rest of the epidermis. EP4 expression was also detected in sebocytes (Chen et al., 2009), hair follicles (Colombe et al., 2008), melanoma cells (Singh and Katiyar, 2011), and squamous cell carcinoma (Lee et al., 2005) in humans. Kabashima et al. (2003) reported the expression of EP4 receptor transcripts in Langerhans cells prepared from epidermis. Li et al. (2000) reported that EP4 receptor mRNA was upregulated in fetal rabbit skin wounds, yet downregulated in adult rabbit skin wounds.

2. Function. EP4 is involved in skin inflammation. It has been suggested that PGE₂ is upregulated within antigen-exposed skin (Ruzicka and Printz, 1982; Eberhard et al., 2002). Kabashima et al. (2003) demonstrated that PGE₂ promoted skin immune responses by enhancing the migration and maturation of Langerhans cells through EP4 signaling. Although the transcripts of all four PGE₂ receptor subtypes were detected in Langerhans cells, only EP4 deletion inhibited Langerhans cell accumulation in regional lymph nodes after application of fluorescein isothiocyanate to the skin. In addition, the immune responses in a dinitrofluorobenzene-induced contact hypersensitivity model were significantly attenuated in EP4 receptor KO mice and in EP4 antagonist-treated wild-type mice (Kabashima et al., 2007). Chun et al. (2007) suggested that PGE₂ exerted an antiapoptotic effect in UV-B-exposed mouse skin through EP4/PKA/Akt signaling. Thus, EP4 is suggested to promote immune response in skin, although the downstream signaling process of EP4 remains largely unknown.

J. Nervous System

1. Expression. Zhang and Rivest (1999) reported the distribution of EP4 receptor transcripts in the rat brain. The localization of the EP4 receptor was distinct from that of the EP2 receptor. EP4 receptors were mainly expressed in regions involved in the regulation of neuroendocrine and autonomic activities. Southall and Vasko (2001) demonstrated EP4 receptor expression in embryonic rat sensory neurons and adult rat dorsal root ganglia cells.

2. Function. Traditional NSAIDs exert their antinociceptive effects through the inhibition of prostaglandin. Accordingly, prostaglandin-mediated signaling has been thought to be involved in the development of inflammatory pain. Thermal and mechanical hyperalgesia, mechanical allodynia, and joint pain were suppressed by EP4 antagonists (Lin et al., 2006;

Kassuya et al., 2007; Nakao et al., 2007; Clark et al., 2008; Murase et al., 2008). Southall and Vasko (2001) demonstrated that EP4 receptors mediated the PGE₂-induced sensitization of sensory neurons. PGE₂-induced accumulation of cAMP and release of immunoreactive substance P and calcitonin gene-related peptide, all of which play important roles in the development of pain and hyperalgesia, were blocked by downregulation of EP4 receptors. Because the cAMP-PKA pathway is involved in the development of hyperalgesia after injury in the dorsal root ganglion (Song et al., 2006), EP4 may activate this pathway to regulate hyperalgesia.

Fever production may involve EP4 signaling. Oka et al. (2000) demonstrated that the EP4 receptor was expressed in regions that are involved in PGE₂-induced fever responses, including the organum vasculosum of the lamina terminalis and the adjacent preoptic area. Several reports have indicated that the EP4 receptor may contribute to PGE₂-induced changes in body temperature (Oka et al., 2000, 2003).

EP4 may also play a role in neuronal degeneration and regeneration. Hoshino et al. (2007) reported that PGE₂ enhanced the production of amyloid- β through the EP4 receptors in human neuroblastoma cells. Moreover, they observed that cognitive function of mice in an Alzheimer's disease model was improved by genetic and pharmacological inhibition of EP4 (Hoshino et al., 2012). In contrast, Liang et al. (2011) reported that an EP4 receptor agonist exerted a protective effect against cerebral ischemia injury in mice. Deletion of neuronal EP4 increased the severity of cerebral injury, as did endothelial deletion of EP4. The effect of EP4 on cerebral perfusion via endothelial nitric-oxide synthase function may be involved in such beneficial roles. A neuroprotective effect of EP4 signaling has been reported in various other models, e.g., a mouse multiple sclerosis model (Esaki et al., 2010), a rat spinal cord injury model (Umemura et al., 2010), and a mouse *N*-methyl-D-aspartate-mediated acute brain damage model (Ahmad et al., 2005). Taken together, these data suggest that EP4 contributes to hyperalgesia and fever production and plays protective roles in neuronal degeneration and regeneration, although its precise downstream signaling pathways have not been reported.

K. Other Systems

Prostanoids play a critical role in the regulation of platelet function. Several reports have indicated that EP4 mediates antithrombotic signaling (Iyu et al., 2010; Kuriyama et al., 2010; Philipose et al., 2010). Philipose et al. (2010) have reported that an EP4 agonist inhibited platelet aggregation, adhesion of platelets to fibrinogen, and thrombus formation in vitro. EP4 receptor activation could thus be a novel target for antithrombotic therapy. EP4 is also

expressed in the cochleae (Hori et al., 2009). Hori et al. (2009) reported that local EP4 agonist treatment improved noise-induced hearing loss in guinea pigs. Finally, Woodward et al. (2009) reported that an EP4 receptor agonist potentially decreased intraocular pressure in laser-induced ocular hypertensive monkeys. EP4 could potentially be a new therapeutic target for antiglaucoma therapy.

IV. Conclusions

This article presents an overview of the functions of EP4 and its intracellular signaling pathways in physiologic and pathologic conditions. EP4 was originally identified as a G_s -coupled receptor and has been recognized to produce cAMP. Recent emerging evidence has revealed that, in addition to cAMP and its downstream signaling, EP4 also modulates a variety of signaling pathways, such as PI3K, β -arrestin, and transactivation of EGFR. The roles of these EP4-mediated pathways in physiologic and pathologic processes continue to be discovered.

Among the EP receptors, EP4 is reported to be most abundantly expressed in the heart, the ductus arteriosus, monocytes/macrophages, bone, and the colon. It maintains the physiologic functions of these organs through protein synthesis, a vasodilatory effect, regulation of immune response, anabolic effect, and mucosal barrier function, respectively. EP4 is also highly expressed in pathologic conditions, such as colorectal cancer, inflammatory bowel disease, rheumatoid arthritis, atherosclerotic plaque, and aortic aneurysm. Studies using mouse lines devoid of each of the four EP receptors further support the concept that EP4, but not the other EP receptors, plays a primary role in bone metabolism, osteoarthritis, and immune response in the skin. Therefore, the EP4 receptor appeared to be an attractive target by which to affect manifestations of various pathologic states by application of either agonists or antagonists of the receptor. In particular, EP4 agonists have drawn much attention for their promotion of osteogenesis and their suppression of colitis, and the potential usefulness of an EP4 agonist as a treatment of bone diseases or inflammatory bowel disease has been examined in clinical trials. EP4 antagonists may be suitable for use in the treatment of rheumatoid arthritis and osteoarthritis, where continuous dosing demands a drug with a superior safety profile. Traditional NSAIDs and COX inhibitors affect a number of other related prostaglandins and can cause serious side effects. The potential of an EP4 antagonist to improve prognosis in colon cancer, myocardial infarction, aortic aneurysm, neovascularization, and autoimmune encephalomyelitis is also of great interest.

Interestingly, modulating EP4 signaling could work on more than one mechanism, because EP4 is distributed

in various organs and circulating immune cells. For instance, inhibition of EP4 signaling has been expected to be useful as a treatment of migraine due to its cerebral vasoconstrictive and immunosuppressive effects. The possibilities for such dual mechanisms of action of EP4 signaling in pathologic conditions of various organs should be explored. In particular, it will be important to further clarify the intracellular signaling pathways and the precise molecular mechanisms involved in EP4-mediated pathophysiologic actions. These additional studies should lead to significant opportunities for new pharmacological therapies.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Yokoyama, Iwatsubo, Umemura, Fujita, Ishikawa.

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