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 PGE_1-induced cAMP elevation; in addition, it is noteworthy that only AC6 knockdown was able to suppress PGE_1-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 $G_s\alpha$ and $G_i\alpha$, whereas EP2 associates with $G_s\alpha$ only, as mentioned earlier. Accordingly, AC5 and AC6 could be targets of EP4 because these group 3 ACs are $G_i\alpha$-sensitive isoforms (Iwatsubo et al., 2003; Oshikawa et al., 2003; Willoughby and Cooper, 2007; Okumura et al., 2009). The other $G_i\alpha$-sensitive isoforms, AC1, AC3, AC8, and AC9, could also be targets of EP4 (Willoughby and Cooper, 2007). Furthermore, from the perspective of PGE_2's effects, AC5 and AC6 are potentially inhibited by the elevation of cytosolic Ca^{2+} evoked by EP1. Capacitive Ca^{2+} elevation also inhibits these isoforms (Willoughby and Cooper, 2007). This cross-talk is another potential explanation for the contradictory effects of PGE_2 on cAMP production under different conditions. Taken together, the evidence suggests that AC is a key molecule that could help explain the divergence of PGE_2'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 PGE_2-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 PGE_2 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 PGE_2'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-κB 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 PGE_2 regulated two independent pathways in the same cell, i.e., the EP2/PKA pathway and EP4/PI3K pathway, suggesting that PGE_2 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 Gβ subunit and a Gγ subunit. The Gβ subunit has five different isoforms in humans and mice, and the Gγ 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 Ca^{2+} 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 PGE_2 and Wnt signaling via PI3K. The EP4-activated PI3K pathway was associated with the initiation of transcription via GSK3β/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. PGE_2 activated GSK3β 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 PGE_2-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 βarrestin-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 βarrestin-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, βarrestin-1 bound to EP4 can activate membrane-bound c-Src, leading to the transactivation of EGFR. Activation of this EP4/βarrestin-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 βarrestin 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, Ca^{2+}, and IP_3 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 tranducer 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/IP_3 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-κB and cytokine production	Ogawa et al., 2009
	Suppressed autoimmune myocarditis	N.D.	Inhibition of T-cell proliferation and MCP-1 production	Ngoc et al., 2011
Ductus arteriosus	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; PKAγ; 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
	Angiogenesis	N.D.	VEGF production; endothelial cell proliferation	Yanni et al., 2009
Atherosclerosis	Increased early atherosclerosis	PI3K; Akt	Inhibition of macrophage apoptosis	Babaev et al., 2008
	Proteolytic activation	N.D.	MMP-2 and MMP-9 activation in macrophages	Cipollone et al., 2005
	Inhibition of inflammation	N.D.	Inhibition of MCP-1 and IFN-γ-inducible protein 10 production	Tang et al., 2011a
Aortic aneurysm	Progression of aortic aneurysm	N.D.	Inhibition of IL-6 production and MMP-2 activation	Yokoyama et al., 2012
	Progression 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 cardiomocyte hypertrophy.

EP4 may play a beneficial role in cardiac ischemia (Table 1). During ischemia, it is well known that PGE_2 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-1β, 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 PGE_2 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. PGE_2 is the most important endogenous prostaglandin involved in the regulation of ductus arteriosus patency in utero (Smith, 1998). The expression of PGE_2 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. PGE_2 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 PGE_2 in utero is to maintain the patency of the ductus arteriosus via vasodilatation. Stimulation of PGE_2 receptors activates $G_s\alpha$/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 PGE_2 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 $O_2^{\cdot-}$ 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 PGE_2 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). PGE_2-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 PGE_2-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 Ca^{2+} sensitivity by phosphorylating myosin light-chain kinase and thereby decreasing its affinity for the Ca^{2+}-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, PGE_2 may play a role in angiogenesis, in particular, in cancer (Chang et al., 2004; Wang et al., 2006b). Such PGE_2-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 PGE_2-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 PGE_2 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 PGE_2 receptor subtype in macrophages from human atheroma, and EP4 signaling inhibits PGE_2-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 PGE_2 (Walton et al., 1999). Studies using COX-2 inhibition (King et al., 2006), genetic deletion of COX-2 (Gitlin et al., 2007), and PGE_2 synthase 1 deletion (Wang et al., 2008) have demonstrated that the inhibition of COX-2–PGE_2 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 PGE_2 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 (Dannenberg and Howe, 2003), breast cancer (Bundred and Barnes, 2005), prostate cancer (Zhang et al., 2012), and esophageal cancer (Altorki, 2004). Because COX-2 produces PGE_2, 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 PGE_2 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 PGE_2 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. PGE_2 and EP4 regulate proliferation of colorectal cancer cells. PGE_2 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 PGE_2 biosynthesis and proliferation of mouse colon adenocarcinoma cells. The inhibition of proliferation was negated by PGE_2 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 PGE_2/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 G_0/G_1 arrest in colon cancer cells, which was reversed by PGE_2. Similarly, an EP4-selective antagonist induced G_0/G_1 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 PGE_2 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. PGE_2 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 PGE_2 increased EGR-1 expression via CREB. They also showed that the EP4 receptor agonist PGE_1-OH phosphorylated ERK in human colon adenocarcinoma cells, whereas the EP4 receptor antagonist L-161,982 blocked PGE_2-induced ERK and CREB phosphorylation. These data suggest that the PGE_2-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
	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
Lung cancer	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
	Production of estrogen	BRCA1	CREB phosphorylation, expression of CYP19	Subbaramaiah et al., 2008
Prostate cancer	Progression of castration-resistant, proliferation	cAMP	ARactivation, 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 PGE_2 increases S100P expression and that this effect is attenuated by knockdown or pharmacological inhibition of EP4. When the *CRE* sequence within the S100P promoter region was deleted or mutated, PGE_2-mediated transcriptional activity was abolished (Chandramouli et al., 2010).

These data suggest that PGE_2-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 PGE_2/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, PGE_2 and EP4 agonists increased phosphorylation of Akt and migration (Yang et al., 2006). Similarly, in human lung cancer cells, PGE_2 enhanced activation of tyrosine kinase c-Src and cell migration. When c-Src was blocked, PGE_2-induced cell migration was also decreased. PGE_2-induced cell migration was blocked by an EP4 receptor antagonist and by EP4 short hairpin RNA. Depletion of either EP4 or βarrestin, a downstream effector of EP4, negated PGE_2-induced cell migration (Kim et al., 2010). These data suggest that PGE_2/EP4/β-arrestin/c-Src signaling enhances lung cancer cell migration.

Zheng et al. (2009) reported that PGE_2 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 PGE_2-induced ILK expression. When ILK was knocked down, the mitogenic effect of PGE_2 was also decreased. PGE_2 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 PGE_2-mediated mitogenic effect (Zheng et al., 2009).

3. Cervical Cancer. Expression of PGE_2/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 PGE_2 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 PGE_2 was significantly higher in cervical carcinoma tissue than in normal cervix. Thus, PGE_2 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 PGE_2 and PGE_2-induced EP4 expression in HeLa (cervical adenocarcinoma) cells (Sales et al., 2002). Further study is required to clarify the roles of PGE_2-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 PGE_2 in breast cancer is its involvement in production of estrogen, a key stimulator for breast cancer progression. Both PGE_2 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. PGE_2 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).

PGE_2 activates cell migration of prostate cancer cells via EP4. These functions were regulated at least in part by transactivation of EGFR and $\beta 3$ 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 PGE_2 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 PGE_2 in human ovarian carcinoma cell lines (Spinella et al., 2004b). Subsequently, they demonstrated that ET-1 increases expression of EP4, and that PGE_2 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 PGE_2 increased colony formation of gallbladder cancer cells probably via increased expression of c-Fos (Asano et al., 2002). George et al. (2007) demonstrated that PGE_2 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). PGE_2 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 PGE_2 mediates the inhibition of MCP-1 and macrophage inflammatory protein-1α (MIP-1α).

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 PGE_2 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. PGE_2 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 PGE_2 production. Released PGE_2 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, $G_s\alpha$ 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-κB complex that includes p105. Similarly, the expression of the gene encoding MIP-1α was also inhibited by the targeting of type II PKA by AKAP to an NF-κB 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-κB	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-1α 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 PGE_2-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 PGE_2 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). PGE_2 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. PGE_2-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). PGE_2 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-κB 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). PGE_2 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-κB 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 PGE_2 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. PGE_2 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 PGE_2. 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 PGE_2, as described in the previous section. Recent studies, however, suggest that PGE_2 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, PGE_2 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 PGE_2. At higher concentrations, PGE_2 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, PGE_2 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-γt. PGE_2 may positively synergize with IL-1 and IL-23 to upregulate retinoic acid receptor–related orphan receptor-γt (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 PGE_2-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 PGE_2 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 PGE_2-EP4 signaling could promote development of the Th1 and Th17 subsets (Table 3). Since the local concentration of PGE_2 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 potently attenuated the chemotaxis of human peripheral blood eosinophils through the PI3K pathway, but not the cAMP pathway (Luschnig-Schratl et al., 2011). PGE_2 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 PGE_2 in B cell lymphoma. Consistently, PGE_2-EP4 signaling promoted B cell receptor–induced G_0/G_1 arrest in the B cell line WEHI 231. Thus, caspase-mediated, B cell receptor–induced apoptosis was induced (Prijatelj 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;). Miyaura 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 PGE_2-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-1β 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. PGE_2 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 PGE_2 stimulates osteoblastic differentiation from bone marrow–derived cells (Shamir et al., 2004; Alander and Raisz, 2006), leading to increased osteoblast numbers and bone formation. PGE_2 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 PGE_2 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. PGE_1 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. PGE_2 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 PGE_2 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 PGE_2 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). PGE_2-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 PGE_2-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 osteoclastgenesis inhibitory factor	Sakuma et al., 2000
	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
Cartilage	Endochondral bone formation	cAMP	Chondrocyte differentiation	Miyamoto et al., 2003b
	Matrix degradation and inhibition of proteoglycan synthesis	N.D.	MMP-13, ADAMTS-5 expression	Attur et al., 2008
Rheumatoid arthritis	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-1β and IL-6 production in macrophages	McCoy et al., 2002
	Development of arthritis	N.D.	Th1 differentiation and Th17 expansion	Chen et al., 2010
	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

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

vitro. Induction of bone resorption by PGE_2 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 PGE_2 and dibutyryl 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 PGE_2. Furthermore, dibutyryl 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 PGE_2-stimulated osteoclast-like cell formation and bone-resorbing activity. These findings suggest that PGE_2 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 PGE_2-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 $G_s\alpha$ 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 PGE_2 contributes to the pathogenesis of rheumatoid arthritis (Akaogi et al., 2006; Hikiji et al., 2008), and inhibitors of PGE_2 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 PGE_2 synthesis in mouse primary osteoblasts from the synovial tissues (Kotake et al., 1999).

The roles of PGE_2 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 PGE_2 synthase 1 are resistant to CIA and CAIA (Myers et al., 2000; Hegen et al., 2003; Trebino et al., 2003; Kamei et al., 2004). PGE_2 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/PGE_2/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, PGE_2 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. PGE_2 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 PGE_2 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. HCO_3^- secretion. Duodenal mucosal HCO_3^- secretion is critical to prevent acid peptic injury. PGE_2 increases HCO_3^- secretion from the rat duodenal mucosa (Takeuchi et al., 1997). It has been suggested that EP4 synergizes with EP3 to promote duodenal mucosal HCO_3^- secretion (Takeuchi et al., 2010a). Studies have demonstrated that duodenal HCO_3^- 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 PGE_2. 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 HCO_3^- response induced by PGE_2 (Aoi et al., 2004).

The stimulatory effect of PGE_2 on duodenal HCO_3^- is mediated intracellularly by cAMP, PI3K, and Ca^{2+} (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 HCO_3^- 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 Ca^{2+} elevation. Tuo et al. (2007) demonstrated that PGE_2-stimulated duodenal HCO_3^- 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 Ca^{2+}-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 nonselective PDE inhibitor 3-isobutyl-1-methylxanthine, the PDE1 inhibitor vinpocetine, and the PDE3 inhibitor cilostamide increased HCO_3^- 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