density and incubated with and without indicated. The cells were pulsed with 50 pM ^{125}I-bFGF for 2 h at 4°C in binding buffer, washed three times with 20 mM HEPES (pH 7.4) containing 150 mM NaCl and 0.2% BSA at 4°C. Low-affinity HSPG-binding sites were detected by two collected 1-ml washes of 20 mM HEPES (pH 7.4) containing 2 M NaCl and 0.2% BSA at 4°C. High-affinity FGFR complex binding sites were detected by two collected 1-ml washes of 20 mM sodium acetate (pH 4.0) containing 2 M NaCl and 0.2% BSA at 4°C. Collected washes were counted in a Cobra 5003 counter (Packard/Perkin Elmer, Waltham, MA, USA). Control experiments were performed with unlabeled FGF2 to determine non-specific binding. Results were reported as the relative binding of experimental condition compared with untreated controls.

Generation of GFP- and RFP-labeled melanoma cells

Cells were incubated with lentiviral particles for GFP and RFP expression (Biogenova, Potomac, MD, USA) and were selected with FACS before the inoculation to obtain the cells homogenously expressing RFP or GFP. FACS cell sorting was performed by a FACS Caliburs (BD Biosciences). *In vivo* imaging of RFP- and GFP-labeled tumor cells were carried out by *in vivo* imaging system (IVIS).

Tumor growth assay

BALB/c athymic (nu/nu) mice were inoculated in the right flank with C8161 cells with or without Epac1 shRNA deletions (10^6 cells/0.1 ml culture medium) (n = 6/group). In another series of experiments, prelabeled SK-Mel-2 cells (MM, high Epac1 expression) and WM1552C cells (RGP, low Epac1 expression) were used: (a) SK-Mel-2-GFP + SK-Mel-2-RFP injected mice n = 8/group; (b) SK-Mel-2-GFP+WM-1552C-RFP cells injected mice n = 8/group; c. WM-1552C-GFP+ WM-1552C-RFP cells injected mice, n = 4/group. Tumor growth was assessed twice a week by caliper measurement of tumor diameter in the longest dimension (L) and at right angles to that axis (W) (De Lorenzo et al., 2011). Tumor volumes were estimated using the formula, $L \times W \times W \times \pi/6$. At the end of the experiment, half of each tumor was fixed by immersion in 10% phosphate-buffered formalin, dehydrated, and embedded in paraffin. Major organs were subjected to gross pathology and histology analysis to determine metastases. Studies were approved by the Animal Care and Use Committee of New Jersey Medical School.

Immunofluorescent staining

The paraffin-embedded slides of melanomas from BALB/c mice were subjected to deparaffinization in xylene, followed by treatment with a graded series of alcohols (100%, 95%, and 80% ethanol [v/v] in double-distilled H_2O) and rehydration in PBS (pH 7.5). For antigen retrieval, the sections were submerged in a boiling temperature citrate buffer (pH 6.0) for 15 m. The samples were blocked with the Image-iT FX signal enhancer (Invitrogen) to prevent non-specific staining and incubated with primary antibodies and respective secondary antibodies. Alexa Fluor 488– and 594-conjugated goat anti-rabbit and anti-mouse antibodies (Molecular Probes, Life Technologies) were used as secondary antibodies. The slides were mounted using Prolong Gold mounting media with 4′, 6-diamidino-2-phenylindole (DAPI).

For the study of RFP- and GFP-labeled cells in tumors, tissue sections from tumors were immunostained with rabbit antibody against GFP (dilution 1:100, Abcam), mouse antibody against RFP (dilution 1:200; Abcam). Negative controls without the primary antibody were performed to show specificity of the antibody.

Immunohistochemical staining

Tumor angiogenesis was evaluated by immunostaining for CD31 (dilution1:250, Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Tissue sections were cut and immunostained with the primary antibody for CD31 using the standard VectaStain ABC kit (Vector Laboratories, Burlingame, CA, USA). Microvessel density was assessed by counting the number of microvessels positive for CD31 at ×400 magnification. Negative control without the primary antibody was performed at the same time.

Overexpression of Epac1

Adenoviral OE of Epac1 in melanoma cells was performed as we previously described (Baljinnyam et al., 2009).

Data analysis and statistics

Statistical comparisons among groups were performed using one-factor ANOVA with Bonferroni post hoc test. Statistical significance was set at the 0.05 level.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Epac1 overexpression (OE) increases migration of primary melanoma cell lines. (A) Western blot of Epac1 OE in WM115 and WM3248 cells 24 h after adenoviral infection. (B) Epac1 OE increased migration of WM115 and WM3248 cells. *P < 0.05 versus control, n = 4.

Figure S2. Epac1 regulates CM-induced migration of primary melanoma cells. (A) Western blot of C8161 cells with or without Epac1shRNA (Sigma Aldrich) transduction. (B) Epac1 knockdown with Epac1 shRNA (Sigma) in C8161 cells inhibited the CM-induced migration of WM1552C cells.

Figure S3. FGF2 is involved CM-induced migration of primary melanoma. Indicated combinations of cells for the evaluation of migration and CM preparation were examined. The neutralizing FGF2 antibody reduced cell migration in all examined combinations. #, P < 0.05 versus CM, n = 4.

Figure S4. FGF2 is involved in Epac1 OE-mediated CM migration. CM of WM3248 cells with adenoviral Epac1 OE increased migration of SK-Mel-2 cells. The nFGF2 antibody inhibited the Epac1 OE-induced migration, n = 4.

Figure S5. Epac1 inhibitors reduce CM-induced migration. Migration of WM3248 cells was inhibited by CM of SK-Mel-24 cells were treated with indicated Epac inhibitors, n = 4.

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The Prostanoid EP4 Receptor and Its Signaling Pathway

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Abstract 1012
I. Introduction 1012
II. Discovery and Characterization 1013
A. Cloning 1013
B. Structure and Evolution 1014
1. Receptor Structure 1014
2. Gene Structure 1014
3. Evolution 1015
C. Signaling Pathways 1015
1. G Protein 1015
2. Adenylyl Cyclase 1016
3. Protein Kinase A 1017
4. Exchange Protein Directly Activated by cAMP 1017
5. Phosphodiesterase 1018
6. Phosphatidylinositol 3-Kinase 1018
7. Desensitization and Arrestin Signaling 1019
8. Extracellular Signal-Regulated Kinase 1019
9. Compartmentalization 1019
III. Biologic Function and Diseases 1020
A. Cardiovascular System 1020
1. Heart 1020
a. Expression 1020
b. Function 1020
2. Ductus Arteriosus 1022
a. Expression 1022
b. Function 1022
3. Other Vessels 1023
a. Expression 1023
b. Function 1023
4. Atherosclerosis 1024
5. Aneurysm 1024
B. Cancer 1025
1. Colorectal Cancer 1025
a. Expression 1025

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b. Function 1025
2. Lung Cancer 1026
3. Cervical Cancer 1026
4. Breast Cancer 1027
5. Prostate Cancer 1027
6. Ovarian Cancer 1027
7. Other Cancers 1027
C. Immune System 1028
1. Monocytes/Macrophages/Dendritic Cells 1028
a. Expression 1028
b. Function 1028
i. Tumor necrosis factor-α 1028
ii. Monocyte chemoattractant protein-1 1029
iii. Interleukin-6 1029
iv. Matrix metalloproteinase-9 1030
v. Interleukin-12 1030
vi. Migration 1030
2. T Cells 1030
a. Expression 1030
b. Function 1031
3. Other Immune Cells 1031
D. Osteoarticular System 1031
1. Expression 1031
2. Function 1032
a. Osteoblasts 1032
b. Osteoclasts 1032
c. Bone resorption 1032
d. Bone anabolism 1033
e. Bone healing 1034
f. Rheumatoid arthritis and other diseases 1034

ABBREVIATIONS: AC, adenylyl cyclase; AGN205203, [[3-[[(1*R*,2*S*,3*R*)-3-hydroxy-2-[(1*E*,3*S*)-3-hydroxy-4-[3-(methoxymethyl)phenyl]-1-butenyl]-5-oxocyclopentyl]thio]propyl]thio]-acetic acid; AH23848, (*Z*)-7-[(1*R*,2*R*,5*S*)-2-morpholin-4-yl-3-oxo-5-[(4-phenylphenyl)methoxy]cyclopentyl]hept-4-enoic acid; AH23848B, [1α(*z*),2β5α]-(±)-7-[5-[[(1,1′-biphenyl)-4-yl]methoxy]-2-(4-morpholinyl)-3-oxo-cyclopentyl]-4-heptenoic acid; AH6809, 6-isopropoxy-9-oxoxanthene-2-caboxylic acid; AH19437, 1α(*Z*),2β,5α(±)-methyl,7-2-(4-morpholinyl)-3-oxo-5(phenylmethoxy)cyclopentyl-5-heptenoate; AKAP, A-kinase anchor protein; AP, activator protein; bp, base pair; BB94, (2*R*,3*S*)-*N*-hydroxy-*N*′-[(1*S*)-1-(methylcarbamoyl)-2-phenylethyl]-2-(2-methylpropyl)-3-[(thiophen-2-ylsulfanyl)methyl]butanediamide; CAIA, collagen antibody-induced arthritis; CIA, collagen-induced arthritis; CJ-023423, *N*-[({2-[4-(2-ethyl-4,6-dimethyl-1*H*-imidazo [4,5-*c*] pyridin-1-yl) phenyl]ethyl}amino) carbonyl]-4-methylbenzenesulfonamide; CJ-042794, 2-[3-[[(4-*tert*-butylphenyl)methyl-pyridin-3-ylsulfonylamino]methyl]phenoxy]acetic acid; COX, cyclooxygenase; CREB, cAMP-response element-binding protein; DP, prostaglandin D_2; EGFR, epidermal growth factor receptor; EGR-1, early growth response gene-1; Epac, exchange protein activated by cAMP; ER819762, (*S*)-1′-(3,5-dimethylbenzyl)-2-ethyl-7,9-dimethoxy-10-methyl-5,10-dihydrospiro [benzo[e]imidazo [1,5-*a*]azepine-1,4′-piperidin]-3(2*H*)-one; ERK, extracellular signal-regulated kinase; ET-1, endothelin-1; FD1, 6-[*N*-(2-isothiocyanatoethyl) aminocarbonyl]forskolin; FD6, 6-[3-(dimethylamino)propionyl]-14,15-dihydroforskolin; GFB, glomerular filtration barrier; GPCR, G protein–coupled receptor; GRK, G protein–coupled receptor kinase; GSK3, glycogen synthase kinase 3; GW627368, 2-[4-(4,9-diethoxy-3-oxo-1*H*-benzo[f]isoindol-2-yl)phenyl]-*N*-phenylsulfonylacetamide; H-89, *N*-[2-[[3-(4-bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide; IFN-γ, interferon-γ; IL, interleukin; ILK, integrin-linked kinase; IP_3, inositol trisphosphate; JG, juxta-glomerular granular; kb, kilobase; KO, knockout; KT-5720, hexyl (15*R*,16*R*,18*S*)-16-hydroxy-15-methyl-3-oxo-28-oxa-4,14,19-triazaoctacyclo[$12.11.2.1^{15,18}.0^{2,6}.0^{7,27}.0^{8,13}.0^{19,26}.0^{20,25}$]octacosa-1(26),2(6),7(27),8,10,12,20,22,24-nonaene-16-carboxylate; L-161,982, *N*-[2-[4-[[3-butyl-5-oxo-1-[2-(trifluoromethyl)phenyl]-1,2,4-triazol-4-yl]methyl]phenyl]phenyl]sulfonyl-5-methylthiophene-2-carboxamide; LPS, lipopolysaccharide; LY-294002, 2-morpholin-4-yl-8-phenylchromen-4-one; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; MDL-12330A, (±)-*N*-[(1*R**,2*R**)-2-phenylcyclopentyl-azacyclotridec-1-en-2-amine hydrochloride; MEK, MAPK/ERK kinase; MIP-1α, macrophage inflammatory protein-1α; MMP, matrix metalloproteinase; NF-κB, nuclear factor κB; NSAID, nonsteroidal anti-inflammatory drug; ONO-4819, 4-[2-[(1*R*,2*R*,3*R*)-3-hydroxy-2-[(*E*,3*S*)-3-hydroxy-4-[3-(methoxymethyl)phenyl]but-1-enyl]-5-oxocyclopentyl]ethylsulfanyl]butanoate; ONO-AE1-437, deesterified active form of 2-[(4-{[2-((1*R*,2*R*,3*R*)-3-hydroxy-2-[(1*E*,3*S*)-3-hydroxy-4-[3-(methoxymethyl)phenyl]but-1-enyl}-5-oxocyclopentyl)ethyl]sulfanyl}butanoyl)oxy]ethyl nonanoate; ONO-AE1-329, 2-[3-[(1*R*,2*S*,3*R*)-3-hydroxy-2-[(*E*,3*S*)-3-hydroxy-5-[2-(methoxymethyl)phenyl]pent-1-enyl]-5-oxocyclopentyl]sulfanylpropylsulfanyl]acetic acid; ONO-AE1-734, *methyl*-7-[(1*R*,2*R*,3*R*)-3-hydroxy-2-[(*E*)-(3*S*)-3-hydroxy-4-(*m*-methoxymethylphenyl)-1-butenyl]-5-oxocyclopenthl]-5-thiaheptanoate; ONO-AE2-227, 2-[2-[[2-(1-naphtyl)propanoyl]amino]benzyl]benzoic acid; ONO-AE3-208, 4-[4-cyano-2-[2-(4-fluoronaphthalen-1-yl)propanoylamino]phenyl]butanoic acid; PDE, phosphodiesterase; PGE_2, prostaglandin E_2; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; RANKL, receptor activator of NF-κB ligand; Runx2, runt-related transcription factor 2; S-145, 5*Z*-7-(3-endo-phenylsulfonylamino-(2.2.1.)-bicyclohept-2-exo-yl) heptenoic acid; SC-19220, 1-acetyl-2-(8-chloro-10,11-dihydodibenz[*b*,*f*][1,4]oxazepine-10-carbonyl)hydrazine; TNF-α, tumor necrosis factor-α; W-13, *N*-(4-aminobutyl)-5-chloro-2-naphthahydrochloride; VEGF, vascular endothelial growth factor.

g. Chondrocytes 1035
E. Gastrointestinal Tract 1035
1. Expression 1035
2. Function 1035
a. Mucin secretion 1036
b. Mucosal cell injury 1036
c. HCO_3^- secretion 1036
d. Colitis 1036
e. Inflammatory bowel disease 1037
f. Epithelial barrier 1037
F. Renal System 1038
1. Expression 1038
2. Function 1038
G. Reproductive System 1039
1. Expression 1039
2. Function 1039
H. Lungs 1040
1. Expression 1040
2. Function 1040
I. Skin 1041
1. Expression 1041
2. Function 1041
J. Nervous System 1041
1. Expression 1041
2. Function 1041
K. Other Systems 1041
IV. Conclusions 1042
References 1042

***Abstract*——The EP4 prostanoid receptor is one of four receptor subtypes for prostaglandin E_2. It belongs to the family of G protein–coupled receptors. It was originally identified, similar to the EP2 receptor as a $G_s\alpha$-coupled, adenylyl cyclase–stimulating receptor. EP4 signaling plays a variety of roles through cAMP effectors, i.e., protein kinase A and exchange protein activated by cAMP. However, emerging evidence from studies using pharmacological approaches and genetically modified mice suggests that EP4, unlike EP2, can also be coupled to $G_i\alpha$, phosphatidylinositol 3-kinase, β-arrestin, or β-catenin. These signaling pathways constitute unique roles for the EP4 receptor. EP4 is widely distributed in the body and thus plays various physiologic and pathophysiologic roles. In particular, EP4 signaling is closely related to carcinogenesis, cardiac hypertrophy, vasodilation, vascular remodeling, bone remodeling, gastrointestinal homeostasis, renal function, and female reproductive function. In addition to the classic anti-inflammatory action of EP4 on mononuclear cells and T cells, recent evidence has shown that EP4 signaling contributes to proinflammatory action as well. The aim of this review is to present current findings on the biologic functions of the EP4 receptor. In particular, we will discuss its diversity from the standpoint of EP4-mediated signaling.**

I. Introduction

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

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

strong blood pressure–reducing activity of PGE_1 (Bergstrom et al., 1959a,b). PGE_2 and PGE_3 were subsequently found in the prostate glands (Van Dorpd et al., 1964). PGE_1, PGE_2, and PGE_3 are biosynthesized from three fatty acid precursors: dihomo-γ-linolenic acid, arachidonic acid, and timnodonic acid, respectively. The numerals refer to the number of carbon-carbon double bonds present. Since arachidonic acid is the most important precursor in animals, PGE_2 is by far the most abundant.

In the late 1960s, studies on adenylyl cyclase (AC) and second messenger molecules (e.g., cAMP) blossomed, as initiated by Sutherland (Robison et al., 1967). Following the discovery of PGEs, Butcher and Baird (1968) were the first to demonstrate an association between prostaglandins and AC/cAMP. In 1982, Kennedy et al. (1982) described a comprehensive classification of prostanoid receptors based upon response to pharmacological stimulation using prostanoids, TP antagonist (AH19437), and EP1 antagonist (SC-19220). They proposed that prostanoid receptors were to be termed P receptors, and that their ligands, natural prostanoids, were to be indicated by a preceding capital letter. Thus, the receptors sensitive to prostaglandins D_2, E_2, $F_2\alpha$, I_2, and thromboxane A_2 were termed the DP, EP, FP, IP, and TP receptors, respectively. The use of the letter P preceded by another letter was intended to avoid possible confusion with purinergic receptors, because these had also been termed P receptors. Kennedy et al. (1982) also subdivided the PGE-sensitive receptors into EP1 and EP2, which were SC-19220–sensitive and SC-19220–insensitive, respectively. The use of terms such as EP1 and EP2 was intended to avoid any implication that they represent in this case specific receptors for PGE_1 and PGE_2, respectively. In 1987, Coleman et al. (1987) demonstrated that there was another EP receptor, which was insensitive to EP1 antagonist (SC-19220) and EP2 antagonist (AH6809) and thus was termed EP3. EP4 is the most recently identified subtype of the EP receptor, having been discovered in the piglet saphenous vein (Coleman et al., 1994a) as an EP receptor insensitive to the agonists of EP1, EP2, and EP3.

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

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

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

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

II. Discovery and Characterization

A. Cloning

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

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

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

B. Structure and Evolution

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

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

Despite the presence of the above-mentioned conserved motifs and their common response to PGE_2, amino acid identity is limited among the EP receptor family (Narumiya et al., 1999; Sugimoto and Narumiya, 2007). The amino acid identity of EP4 to EP1 is 30%, whereas that of EP4 to EP3 is 37%. EP4 and EP2 have similar signaling pathways in terms of activation of $G_s\alpha$ and subsequent cAMP production, but the amino acid identity of EP4 to EP2 is only 38%. In contrast, among animal species, amino acid identity of EP4 is maintained. Among various mammals, such as monkey, cow, mouse, and rat, the homology ranges from 88 to 99%. The sequence homology between human and mouse EP4 is 88%.

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

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

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

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

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

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

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

C. Signaling Pathways

In terms of signaling pathways, AC/cAMP was the major research focus in the E series of prostaglandins prior to the cloning of EP receptors (Fig. 1). Elevation of cAMP via AC by PGE_1 was first demonstrated in rat tissues involving fat pads, lungs, spleens, and kidneys (Butcher and Baird, 1968). A similar effect of PGEs was observed in the corpora lutea (Marsh, 1971). In contrast, an inhibitory effect of PGEs on cAMP production was demonstrated in isolated fat cells (Butcher and Baird, 1968), suggesting that PGEs can have opposite effects on cAMP signaling. The cloning of EP receptors led to an explanation, at least in part, for these counteracting signal transductions because EP receptors target both stimulatory and inhibitory G proteins. Among the four receptors, i.e., EP1–EP4, there are biochemical similarities between EP2 and EP4, e.g., both subtypes are coupled with stimulatory G protein and thus can activate AC to produce cAMP. The EP3 subtype has been demonstrated to have an inhibitory effect on cAMP production (Sugimoto et al., 1993). In 1997, the EP1 subtype was shown to be linked with the $G_q\alpha$ protein (Nemoto et al., 1997). Recent studies, however, have identified differences between EP2 and EP4 downstream signaling, such as the coupling of EP4 to the inhibitory G protein (Fujino and Regan, 2006) and PI3K (Fujino et al., 2002; Fujino and Regan, 2003; Pozzi et al., 2004; Yao et al., 2009), which results in further differences in the downstream signaling pathways of the two receptors and thus their cellular functions. These findings suggest that EP4 is not just another EP2; rather, it has unique biologic properties as an independent target receptor of PGE_2, which will be further discussed in the following sections.

1. G Protein. EP4 is classified as a member of the prostanoid receptor family, which belongs to GPCRs that consist of approximately 900 receptors (Lappano and Maggiolini, 2011). Heterotrimeric G protein is a direct downstream effector of GPCRs. Upon ligand binding, the inactive, GDP-bound form of G protein is transformed into its active, GTP-bound form followed by the dissociation of the α and $\beta\gamma$ subunits. The $G\alpha$ subunit includes four major subtypes, i.e., the stimulatory ($G_s\alpha$) and inhibitory ($G_{i/o}\alpha$) subtypes, $G_q\alpha$, and G12/13α. $G_s\alpha$ stimulates AC, a membrane-bound cAMP-generating enzyme. The activated $G_{i/o}\alpha$ subunits inhibit AC activity, resulting in a decrease in intracellular cAMP levels. Activation of $G_{i/o}\alpha$ results in the release of relatively high amounts of $\beta\gamma$ subunit, thus activating the $\beta\gamma$-mediated multiple signaling processes (Wettschureck and Offermanns, 2005; Smrcka, 2008). The dissociated $\beta\gamma$ subunit itself stimulates AC subtypes, i.e., AC2, AC4, and AC7, and could eventually increase cAMP as described later in this article. $G_q\alpha$ targets PLC, leading to activation of inositol trisphosphate (IP_3)- or diacylglycerol-mediated signaling pathways.

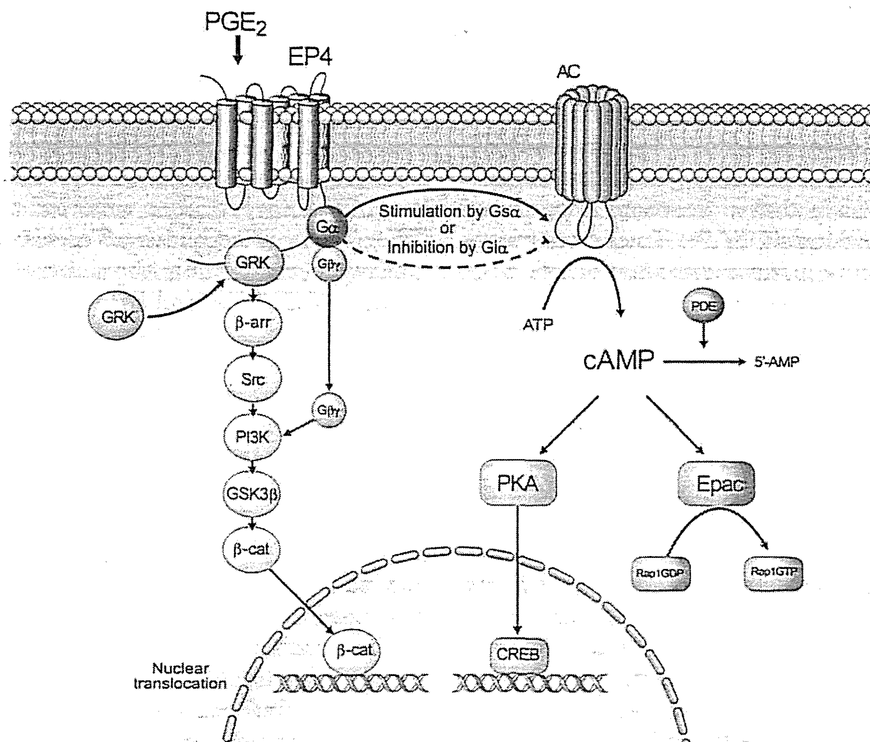

Fig. 1. Upon activation of the EP4 receptor by PGE_2, the Gα subunit is dissociated from the receptor and Gβγ. Conventionally, EP4, similar to EP2, activates $G_s\alpha$, leading to increased AC catalytic activity and thus cAMP production. In some cases, however, EP4, unlike EP2, may activate $G_i\alpha$, leading to decreased cAMP signaling. This may require the presence of a $G_i\alpha$-inhibitable AC isoform(s). Cyclic AMP signaling activates two major target molecules, PKA and Epac. PKA, a cAMP-dependent kinase, phosphorylates multiple downstream molecules including CREB, a major cAMP-regulated transcription factor, and thus regulates their function. Epac is a guanine nucleotide exchange factor for Rap that acts independently from PKA. Cyclic AMP is eventually degraded to 5′-AMP by PDE. EP4 stimulates cAMP-independent signaling as well. This is achieved through activation of the GRK/β-arrestin/Src/PI3K/GSK3 pathway, leading to, for example, nuclear translocation of β-catenin. This pathway may be modified by Gβγ dissociated from Gα via interaction with PI3K upon EP4 activation. Thus, EP4 can elicit multiple signaling pathways within a cell. β-arr, β-arrestin; β-cat, β-catenin.

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

EP4 is coupled not only with $G_s\alpha$ but also with $G_i\alpha$ (Fujino and Regan, 2006), as mentioned earlier. This phenomenon would partially explain why the potency of EP4 to increase cAMP is less than that of EP2 and is reminiscent of the relationship between the β1- and β2-adrenergic receptors; the β1-adrenergic receptor is coupled with only $G_s\alpha$, whereas the β2-adrenergic receptor is coupled with both $G_s\alpha$ and $G_i\alpha$ (Feldman, 1993; Ho et al., 2010). Since several studies, especially those in the cardiovascular field, have demonstrated that the role of the β2-adrenergic receptor is distinct from that of the β1-adrenergic receptor (Ho et al., 2010), this is potentially the case for EP4 as well. In other words, it is possible that the cellular functions evoked by PGE_2 are unique to each EP receptor.

2. Adenylyl Cyclase. AC, a target enzyme of $G_s\alpha$ and $G_i\alpha$, is a 12-transmembrane enzyme that converts ATP to cAMP, a major second messenger (Iwatsubo et al., 2006; Ho et al., 2012). The AC family consists of nine membrane-bound isoforms and one cytosolic, soluble isoform. Membrane-bound ACs are classified according to their tissue expression, amino acid homology, and biochemical properties (Iwatsubo et al., 2003). Historically, membrane-bound ACs were classified into four groups. Group 1 consists of AC1, 3, and 8, originally found to be expressed in the central nervous system and regulated by Ca^{2+}/calmodulin. Group 2 consists of AC2, 4, and 7, ubiquitously expressed isoforms that are regulated by Gβγ subunits. Group 3 consists of the isoforms AC5 and 6, which are mainly expressed in the heart and the brain, and are sensitive to inhibition by $G_i\alpha$ subunit the micromolar range of Ca^{2+}. AC9 is the only member of group 4 and is regulated by calcineurin. The intracellular domains of AC, i.e., the C1a and C2a domains, form a cleft that serves as a catalytic core. Within this catalytic core, ATP is converted into cAMP. Regulators such as G proteins or forskolin, a direct AC stimulator (Iwatsubo et al., 2003), can change the conformation of the catalytic core, resulting in alteration of enzymatic activity.

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

isoforms, mRNA expressions of all AC isoforms with the exception of AC1 and 8 were observed in the ductus arteriosus. Knockdown of AC2, 5, and 6 inhibited 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 $G\beta$ subunit and a $G\gamma$ subunit. The $G\beta$ subunit has five different isoforms in humans and mice, and the $G\gamma$ 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 cardiomyocyte 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