Epac1 increases migration of endothelial cells and melanoma cells via FGF2-mediated paracrine signaling

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Summary

Fibroblast growth factor (FGF2) regulates endothelial and melanoma cell migration. The binding of FGF2 to its receptor requires N-sulfated heparan sulfate (HS) glycosamine. We have previously reported that Epac1, an exchange protein activated by cAMP, increases N-sulfation of HS in melanoma. Therefore, we examined whether Epac1 regulates FGF2-mediated cell–cell communication. Conditioned medium (CM) of melanoma cells with abundant expression of Epac1 increased migration of human umbilical endothelial cells (HUVEC) and melanoma cells with poor expression of Epac1. CM-induced increase in migration was inhibited by antagonizing FGF2, by the removal of HS and by the knockdown of Epac1. In addition, knockdown of Epac1 suppressed the binding of FGF2 to FGF receptor in HUVEC, and *in vivo* angiogenesis in melanoma. Furthermore, knockdown of Epac1 reduced N-sulfation of HS chains attached to perlecan, a major secreted type of HS proteoglycan that mediates the binding of FGF2 to FGF receptor. These data suggested that Epac1 in melanoma cells regulates melanoma progression via the HS–FGF2-mediated cell–cell communication.

Introduction

Despite recent advances in melanoma therapies utilizing inhibitors of the ERK-signaling pathway, prognosis of advanced melanoma is still poor. In addition, acquired resistance becomes a critical problem with those inhibitors (Little et al., 2012; Maurer et al., 2011). Therefore, the development of a novel therapeutic strategy is an urgent demand for this life-threatening disease. cAMP signaling controls a variety of cellular functions in cancer cells. Exchange protein activated by cAMP (Epac), a guanine nucleotide exchange factor, was found as an additional target of cAMP apart from the conventional one, that is, protein kinase A (De Rooij et al., 1998). Two isoforms of Epac, Epac1 and Epac2, mediate cAMP signaling by the activation of a small-molecular-weight G protein, Rap1 (Bos, 2006). In cancer cells, reports have demonstrated following functions of Epacs such as cell adhesion in human ovarian carcinoma Ovcar3 cells (Quilliam et al., 2002), apoptosis (Tiwari et al., 2004)

Significance

There is an emerging need for elucidating the mechanism of cell–cell interaction in melanoma progression. Our study provides information regarding FGF2-related cell–cell interaction between melanoma/endothelial and melanoma/melanoma cells which is regulated by melanoma cells with the higher expression of Epac1.

and growth arrest (Grandoch et al., 2009a) in B lymphoma cells, formation of embryonic vasculogenic networks in melanoma cells (Lissitzky et al., 2009), and proliferation of prostate carcinoma cells (Grandoch et al., 2009b). We have previously reported that Epac1 is expressed in various melanoma cell lines (Baljinnyam et al., 2011) and plays a role in cell migration via modification of heparan sulfate (HS) glycosaminoglycan (HSPG) chains. The increased migration by Epac1-enhanced metastasis to the lungs in mice (Baljinnyam et al., 2009). Recently, we have also found that, in addition to this HS-related mechanism, a Ca^{2+}-dependent mechanism is also involved in Epac1-induced melanoma cell migration. Epac1 releases cytosolic Ca^{2+} from the endoplasmic reticulum (ER) via the phospholipase C (PLC)/inositol triphosphate (IP3)/IP3 receptor pathway (Baljinnyam et al., 2010). These data suggested that Epac1 plays a critical role in melanoma cell migration via at least two independent mechanisms, that is, the HS-related and the Ca^{2+}-dependent mechanisms.

Fibroblast growth factor-2 (FGF2) is known to increase tumor growth and metastasis by the activation of migration of cancer and vascular endothelial cells (Hibino et al., 2005; Meier et al., 2003; Montesano et al., 1986; Moscatelli et al., 1986; Nugent et al., 2000; Ponta et al., 1998; Sola et al., 1997; Taylor et al., 1993). Binding of FGF2 to FGF receptor requires coordination with N-sulfated glucosamine (Faham et al., 1996; Kreuger et al., 1999; Maccarana et al., 1993; Schlessinger et al., 2000), a component of HS chain (Iozzo and San Antonio, 2001). In addition, perlecan, one of the HSPGs, attaches to FGF2 for its binding to FGF receptors (Knox et al., 2002; Sharma et al., 1998). We have previously reported that, in a human melanoma cell line, Epac1 increases NDST-1, which converts N-acetylated glucosamine into N-sulfated form (Baljinnyam et al., 2009). In addition, it was suggested that Epac1 overexpression increases N-sulfation of HS chain (Baljinnyam et al., 2009).These data led us to examine the hypothesis that Epac1 can control FGF2 signaling by modification of N-sulfation of HS, most probably on perlecan. Further, as secreted FGF2 can act in a paracrine fashion, it is possible that melanoma cells expressing Epac1 regulate migration of surrounding endothelial or other melanoma cells. In this study, we found that Epac1 in melanoma cells increases N-sulfation of secreted perlecan and activates migration of endothelial/melanoma cells by FGF2/HS-mediated cell-cell interaction. In addition, the Epac1 in melanoma cells activates angiogenesis *in vivo*, which may support the survival of other melanoma cells expressing lower amounts of Epac1. Therefore, in addition to our previous reports showing the role of Epac1 in melanoma cells, this study demonstrated that expression of Epac1 in melanoma cells plays a role in melanoma progression by controlling cell/cell communication with endothelial cells and other melanoma cells.

Results

Epac1 in melanoma cells increases migration of neighboring endothelial cells via cell/cell communication

It was suggested that Epac1-expressing melanoma cells can increase migration of neighboring endothelial cells via N-sulfation of HSPG, and subsequently, the activation of paracrine-acting FGF2 signaling. Therefore, we investigated whether melanoma cells with abundant Epac1 expression can increase migration of those with scarce Epac1 expression. According to our previous report (Baljinnyam et al., 2010, 2011), in this study, we have divided the cell lines into two groups: Epac1-rich cell lines, in which Epac1 expression is of the same or higher level than that in SK-Mel-2 (SK-Mel-2, SK-Mel-24, SK-Mel187, and C8161 cells). Epac1-poor cell lines, in which Epac1 expression is lower than a half of Epac1 expression in SK-Mel-2 (HEMA-LP, WM3248, WM1552C, and WM115 cells). Conditioned medium (CM) of C8161 cells, which expresses abundant Epac1 (Baljinnyam et al., 2011), increased migration of human umbilical vein endothelial cells (HUVEC) (Figure 1A). Both a neutralizing antibody against FGF2 and heparitinase, a HS-cleaving enzyme, inhibited the CM-induced HUVEC migration. Knockdown of Epac1 in C8161 cells (Figure 1B) suppressed the CM-induced HUVEC migration (Figure 1A). Hence, these data suggested that Epac1 in melanoma cells can increase migration of endothelial cells via FGF2- and/or HS-dependent mechanisms.

Epac1 in melanoma cells induces tube formation of endothelial cells via cell/cell communication

As endothelial cell migration is fundamental for angiogenesis (Lamalice et al., 2007), we examined whether Epac1-expressing melanoma cells can stimulate endothelial tube formation, which mimics *in vivo* angiogenesis. As shown in Figure 2A, B, CM of C8161 cells increased tube formation of HUVEC. Similar to migration (Figure 1A), the CM-induced tube formation was inhibited by the neutralizing antibody against FGF2 and by heparitinase. In addition, CM of C8161 cells in which Epac1 was knocked down showed reduced tube formation (Figure 2A, B). *In vivo* angiogenesis assay showed the same effect of Epac1 knockdown (Figure 2C, D). These data suggested that Epac1 in melanoma cells have the ability to induce angiogenesis via FGF2- and/or HS-mediated cell/cell communication.

Epac1 in melanoma cells increases migration of neighboring melanoma cells via cell/cell communication

Based on the increased HUVEC cell migration shown previously, we hypothesized that a similar cell/cell interaction may also exist among melanoma cells. To test this hypothesis, we examined whether CM derived from a melanoma cell line affects migration of other melanocyte/

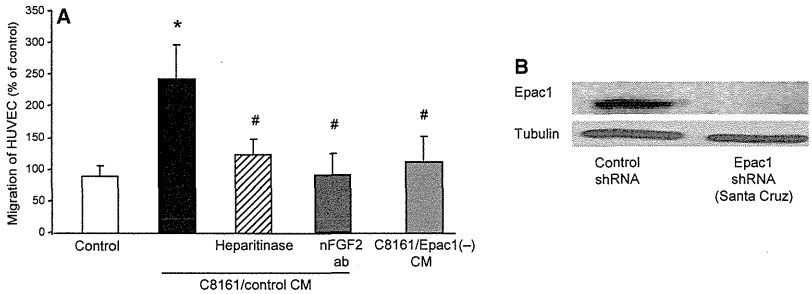

Figure 1. Epac1 in melanoma cells increases migration of endothelial cells via cell/cell communication. (A) CM of C8161 (C8161/control CM) increased migration of human umbilical vein endothelial cells (HUVEC). Epac1 knockdown in C8161 cells (C8161/Epac1(–) CM) inhibited the CM-induced migration. The CM-induced increase in migration was inhibited by the neutralizing antibody against FGF2 [nFGF2 ab (25 μg/ml)], and heparitinase (0.08 U/ml). *P < 0.05 versus control, #P < 0.05 versus C8161/control CM, n = 4. (B) Western blot of C8161 cells with stable knockdown of Epac1 performed with lentivirus-based shRNA induction.

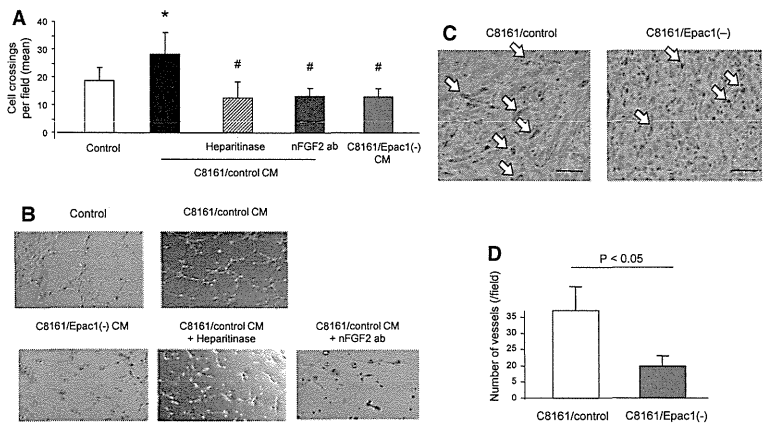

Figure 2. Epac1 in melanoma cells activates angiogenesis. (A) C8161/control CM increased tube formation of human umbilical vein endothelial cells (HUVEC). C8161/Epac1(–) CM showed reduced tube formation compared to C8161/control CM. The C8161/control CM-induced tube formation was inhibited by nFGF2 ab (25 μg/ml), and by heparitinase (0.08 U/ml). C8161/Epac1(–) CM showed reduced tube formation compared with C8161/control CM.*P < 0.01 versus control medium. #P < 0.01 versus C8161/control CM, n = 4. (B) Representative images of HUVEC tube formation described in A. (C and D) Epac1 knockdown reduces angiogenesis *in vivo*. C8161 cells with or without Epac1 knockdown (1×10^6 cells) were inoculated in the interscapular region of BALB/c mice. One week after the inoculation, tumor was removed. (C) Immunohistochemical images with anti-CD31 staining for the detection of endothelial cells are shown. White arrows indicate CD31-positive cells stained brown. Scale bar: 100 μm. (D) The number of microvessels in each mouse was counted with the positively stained cells in 10 different fields, n = 4.

melanoma cells. CM from WM3248 or WM115 cells, both primary melanoma cell lines, did not change cell migration of HEMA-LP melanocyte cells (Figure 3A). In contrast, CM sourced from SK-Mel-2 or C8161 cells, both metastatic melanoma cell lines, increased migration of HEMA-LP. Migration of WM1552C cells, a primary melanoma cell line of the radial growth phase (RGP), was examined next (Figure 3B). CM of WM3248, a melanoma cell line of the vertical growth phase (VGP), SK-Mel-187, SK-Mel-2, or C8161 cells, all metastatic melanoma cell lines, increased WM1552C cell migration (Figure S3). In contrast, migration of the metastatic melanoma cell line, C8161 cells, was not affected by CM of SK-Mel-2. Epac1 overexpression (OE) in Epac1-poor melanoma cells indeed increased cell migration in both WM115 and WM3248 cells (Figure S1), suggesting that Epac1's effect on migration is saturated in Epac1-rich melanoma cells such as C8161 and SK-Mel-2 cells. Epac1 knockdown by two different Epac1 shRNAs (from Santa Cruz Biotechnology and Sigma Aldrich) in C8161 cells inhibited the CM-induced migration of HEMA-LP and WM1552C cells (Figure 3A, B and S2). Similar result was obtained in Epac1 knockdown in SK-Mel-2 cells (Figure 3B). These data suggested the specific role of Epac1 in the CM-induced migration.

The CM-induced migration of HEMA-LP and WM1552C cells were inhibited by heparitinase (Figure 3A and B), and the CM-induced migration of WM1552C cells was suppressed by the neutralizing FGF2 antibody (Figure 3B). The neutralizing FGF2 antibody inhibited CM-induced migration in other combinations of CM and cell lines used for migration (Figure S3). In addition, Epac1 OE in WM3248 cells increased their migration, and it was reduced by neutralizing FGF2 antibody (Figure S4).These

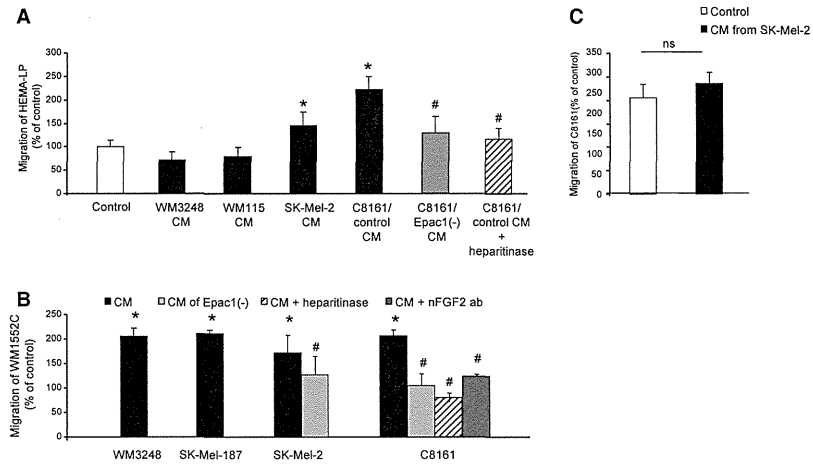

Figure 3. Epac1 in melanoma cells increases migration of melanocytes/other melanoma cells. (A) Conditioned media of indicated melanoma cell lines were used for the Boyden chamber migration assay of HEMA-LP cells. Conditioned media from SK-Mel-2 and C8161 cells, but not those from WM3248 and WM115 cells, increased migration of HEMA-LP cells. Knockdown of Epac1 (C8161/Epac1(−) CM) as well as heparitinase inhibited the CM-induced migration. *P < 0.05 versus control medium, #P < 0.05 versus C8161/control CM, n = 4. (B) Conditioned media of indicated melanoma cell lines were used for the Boyden chamber assay of WM1552C cells. Conditioned media of all cell lines examined increased migration of WM1552C cells. Knockdown of Epac1 inhibited migration induced by CM derived from SK-Mel-2 and C8161 cells. Heparitinase and the nFGF-2 antibody suppressed migration induced by CM of C8161 cells.*P < 0.05 versus control medium, #P < 0.05 versus CM, n = 4. (C) The Boyden chamber assay showed that CM of SK-Mel-2 cells did not increase migration of C8161 cells, n = 4.

data suggested that CM-induced migration was regulated by Epac1, HS and/or FGF2 signaling.

Epac1 augments the binding of FGF2 to FGF receptor

We next investigated the effects of Epac1 on HS including N-sulfation and FGF2 signaling. It has been demonstrated that perlecan interacts with FGF2 via its HS chains (Knox et al., 2002; Sharma et al., 1998). We thus examined perlecan expression of CM by isolation with chromatography. N-sulfated HS chains of perlecan were detected by the anti-HS antibody (clone 10E4) (Figure 4A). The N-sulfation of HS bound to the perlecan was significantly reduced by Epac1 knockdown. In addition, both the amount of N-sulfation and the number of FGF receptors bound to FGF2 were decreased by knockdown of Epac1 (Figure 4B). In contrast, neither the expression of total HS bound to FGF2 nor FGF2 itself in CM were changed by Epac1 knockdown (Figure 4B), suggesting that Epac1 enhances FGF2-binding to FGF receptor via N-sulfation of HS. The binding assay showed that CM from C8161 cells increases FGF2 binding to FGF receptor expressed in HUVEC cells. The CM-induced FGF2 binding was inhibited by the FGF2 antibody and by Epac1 knockdown in C8161 cells (Figure 4C). Taken together, these data demonstrated that Epac1-expressing melanoma cells regulate paracrine-acting FGF2 signaling in neighboring cells such as endothelial and melanoma cells by modification of HS.

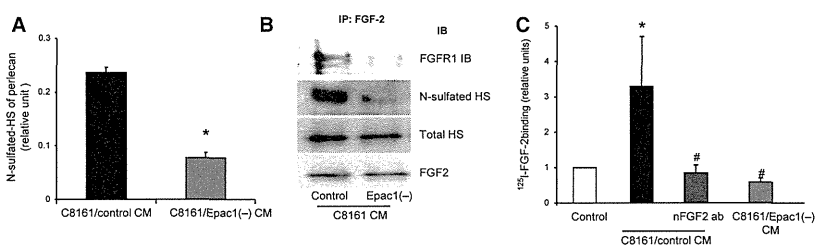

Figure 4. Epac1 enhances the binding of fibroblast growth factor (FGF2) to FGF receptor via N-sulfation of HS. (A) Perlecan was isolated from the DEAE chromatography fractions using a polyclonal antiperlecan antibody. The presence of HS chains on perlecan was detected using an anti-HS-specific antibody (10E4). Epac1 knockdown reduced the amount of N-sulfated HS attached to perlecan. *P < 0.05 versus C8161/control CM, n = 8. (B) CM of C8161 was subjected to immunoprecipitation with the antibody against FGF2 followed by Western blot for indicated antibodies. Both N-sulfated HS and FGF receptor 1 (FGFR1) attached to FGF2 were reduced by Epac1 knockdown whereas the amount of FGF2 in the CM was not different. (C) The binding assay for FGF2 in human umbilical vein endothelial cells (HUVEC) was performed with indicated CM. C8161/control CM increased the binding of FGF2 to HUVEC. The neutralizing antibody for FGF2 (nFGF2 ab) and knockdown of Epac1 inhibited the CM-induced FGF2 binding. *P < 0.05 versus control medium, #P < 0.05 versus C8161/control CM, n = 4.

Epac1-rich melanoma cells support proliferation of Epac1-poor melanoma cells *in vivo*

Increased angiogenesis by Epac1 (Figure 2) suggested that Epac1-rich melanoma cells can support proliferation not only of Epac1-rich melanoma cells themselves but also of Epac1-poor melanoma cells via newly supplied blood flow. If this is the case, melanoma cells expressing low Epac1 that cannot survive *in vivo* are rescued by coexistence of Epac1-rich melanoma cells. Therefore, we examined whether coinoculation of melanoma cells with high Epac1 expression and those with low Epac1 expression enables the second type of cells to survive in mice. To show this, we used SK-Mel-2 cells, which abundantly express Epac1, and WM1552C cells, which poorly express Epac1 (Baljinnyam et al., 2011). In addition, we used green fluorescent protein (GFP) – or red fluorescent protein (RFP) to distinguish WM1552C cells from SK-Mel-2 cells. Our study showed that SK-Mel-2 cells inoculated in athymic nude mice, but not WM1552C cells, formed a tumor (Figure 5A), suggesting that WM1552C cells alone cannot survive in mice. A tumor was formed by WM1552C cells coinoculated with SK-Mel-2 cells, but not with WM1552C cells inoculated alone (Figure 5A–C). The tumor formed by the coinoculation showed both GFP- and RFP-fluorescent signal (Figure 5D). In addition, fluorescence-activated cell sorting (FACS) analysis demonstrated that individual cells isolated from the tumor have either RFP signal or GFP signal (Table 1). These data showed the existence of both WM1552C and SK-Mel-2 cells in the tumor and thus suggested that Epac1-rich melanoma cells can support the survival of Epac1-poor melanoma cells. As the percentages of GFP- and RFP-positive cells are not equal even in the same SK-Mel-2 cells (Table 1) under *in vivo* conditions, it seems that one of the two inoculated cell lines becomes dominant. As CM of SK-Mel-2 cells did not increase proliferation of WM1552C cells (data not shown), these data suggest that SK-Mel-2 cells enable WM-1552C to survive *in vivo* most probably by modification of the extracellular matrix and enhanced angiogenesis.

Discussion

Our previous reports showed that Epac1 increases migration of melanoma cells themselves (Baljinnyam et al., 2009, 2010, 2011). Epac1 in melanoma cells may regulate the cell–cell communication, which could lead to an augmented migration of neighboring endothelial and melanoma cells. Our findings suggest that Epac1-rich melanoma cells play a major role in melanoma progression through migration of the Epac1-rich melanoma cells themselves, but also through increasing migration of neighboring Epac1-poor melanoma cells and more importantly, by the increased migration of neighboring endothelial cells that can accelerate tumor growth via angiogenesis. Therefore, it is plausible that Epac1-rich population in the melanoma tumor critically regulates tumor growth rate.

Although a number of reports demonstrated the role of FGF2 in melanoma progression (Gartside et al., 2009; Hibino et al., 2005; Meier et al., 2000; Ozen et al., 2004), little attention was focused on the role of paracrine-acting FGF2. Using B16F10, an invasive mouse melanoma cell line, CM-activated capillary formation of bovine aortic endothelial cells (Garrido et al., 1995). CM from A375, a

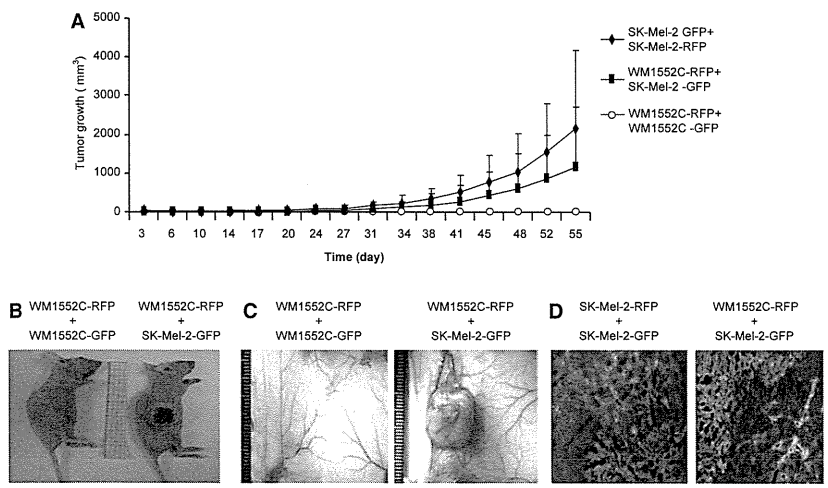

Figure 5. Epac1-rich melanoma cells support survival of Epac1-poor melanoma cells. (A) Tumor growth of WM1552C and SK-Mel-2 cells expressing Red Fluorescent Protein (RFP) and Green Fluorescent Protein (GFP) is shown. A mixture of indicated cells was injected in the right dorsolateral flank region in athymic BALB/c nude mice. Tumor size was measured twice a week to calculate tumor volume. Tumor failed to grow in the mixture of RFP- and GFP-labeled WM1552C. (B and C) Representative images of the tumors in the 12 weeks after the inoculation are shown. The mixture of RFP-labeled WM1552C cells and GFP-labeled SK-Mel-2 cells formed a tumor. (D) Representative images of coimmunostaining for RFP and GFP of the tumors formed by the indicated cell mixtures. Blue indicates 6-diamidino-2-phenylindole (DAPI) staining.

Table 1. Fluorescence activated cell sorting (FACS) analyses for the population of red fluorescent protein (RFP)- and green fluorescent protein (GFP)-positive cells in melanoma tumor

Cell lines coinoculated	Fluorescent signal used	% of total sorted cells in tumor	SD
WM1552C-RFP +	RFP	0.26	0.21
SK-Mel-2-GFP	GFP	85.9	4.72
SK-Mel-2-RFP +	RFP	3.22	1.8
SK-Mel-2-GFP	GFP	42	1.6

Formed tumors with coinoculation of indicated cell lines were isolated, dissected, and subjected to FACS analyses, n = 4.

human melanoma cell line, but not from normal melanocytes, increased migration and invasion of human mesenchymal stem cells. The CM-induced migration was inhibited by neutralization of FGF2 (Watts and Cui, 2012). Our results are consistent with these studies showing that CM of human melanoma cells increased migration of human endothelial cells via FGF2 signaling (Fig. 1). Furthermore, we have demonstrated the role of Epac1 in migration of endothelial cells via paracrine-acting FGF2 signaling, which subsequently results in increased angiogenesis (Figure 2). In addition, our results indicated the existence of FGF2-dependent cell/cell communication not only between melanoma and endothelial cells but also between melanoma and melanoma cells. This melanoma/melanoma cell communication in migration was obvious between Epac1-rich and Epac1-poor melanoma cells, but unclear between Epac1-rich and Epac1-rich melanoma cells (Figure 3C). This lacking of cell/cell communication is probably explained by saturated migration via abundant expression of Epac1 in the same cells as we have previously shown (Baljinnyam et al., 2011) and by the minimal effect of autocrine FGF2 signaling. Regarding WM1552C migration (Figure 3B), although Epac1's expression varies between the cell lines used for the study, the degree of migration did not directly reflect the degree of Epac1 expression. This was attributable, at least in part, to saturation of paracrine-acting FGF2 signaling and is supported by the data showing that FGF2 receptor expression is much higher in WM1552C cells compared with HEMA-LP (data not shown) in which the effects of CM are variable. Altogether, in terms of melanoma progression, Epac1's role in migration affects three types of cells: 1) Epac1-rich melanoma cells themselves, 2) Neighboring endothelial cells, 3) Neighboring Epac1-poor melanoma cells. Accordingly, targeting Epac1 would be an inhibitory mechanism for melanoma progression.

Perlecan is necessary for the binding of FGF2 to FGF receptor in human melanoma cells (Aviezer et al., 1997). N-sulfation of HS chains is critical for this interaction (Faham et al., 1996; Kreuger et al., 1999; Maccarana et al., 1993; Schlessinger et al., 2000). Although N-sulfation is largely regulated by NDSTs, little is known about how the expression/activity of NDSTs is regulated. We have shown that Epac1 can increase NDST-1 expression in melanoma cells (Baljinnyam et al., 2009). In addition, N-sulfation of HS was increased in the mixture of medium and cell lysate (Baljinnyam et al., 2011). In the present study, N-sulfation of secreted perlecan in the CM was reduced by Epac1 knockdown (Figure 4A). Furthermore, FGF2 binding to FGF receptor was inhibited by Epac1 knockdown (Figure 4B, C). Therefore, it is proposed that Epac1-rich melanoma cells can affect FGF2 signaling in neighboring cells via modification of N-sulfation of HS on perlecan. Meanwhile, knockdown of Epac1 reduced the amount of perlecan as demonstrated by Western blot analysis with a perlecan-specific antibody (CCN-1) (data not shown). Interestingly, expression of perlecan is regulated by the cAMP response element (CRE) as its promoter (Furuta et al., 2000). Thus, Epac1 potentially may regulate perlecan expression itself in addition to N-sulfation of HS, suggesting multiple roles of Epac1 on biosynthesis HSPG. However, further studies would be required to confirm this because another study found that Epac1 does not regulate transcription through CREB transcription factors and that the best characterized route for Epac1 to regulate transcription is through C/EBP transcription factors (Yarwood et al., 2008, JBC).

Our data showed that melanomas formed by coinoculation of Epac1-rich and Epac1-poor melanoma cells involved both melanoma populations (Figure 5D and Table 1). These data suggest that cell/cell communication within melanomas may support the survival of melanoma cells with lower malignancy potential. To confirm that Epac1 in Epac1-rich melanoma cells affect proliferation of another Epac1-poor melanoma cells, it is necessary to examine whether Epac1 knockdown decreases the number of Epac1-poor melanoma cells *in vivo*. However, inhibition of Epac1 itself affects angiogenesis as shown in our data (Figure 2), which may result in decreased proliferation of Epac1-rich (SK-Mel-2) cells themselves. Indeed, knockdown of Epac1 reduced tumor growth *in vivo* (data not shown). Therefore, knockdown of Epac1 itself may affect the local blood supply and thus survival and proliferation of Epac1-poor melanoma cells. Therefore, when Epac1 is knocked down in Epac1-rich melanoma cells, multiple factors may affect proliferation of Epac1-poor melanoma cell, suggesting difficulty of interpretation of the acquired data. Recently, specific Epac1 inhibitors have become commercially available. These inhibitors, HJC-0350 and ESI-09, indeed suppressed CM-induced migration in WM3248 cells (Figure S5), suggesting potential usage of these inhibitors for melanoma therapy, which will be addressed in our future study. Finally, HS binds to and regulates the activity of extracellular superoxide dismutase (EC-SOD), which results in increased protection against oxidative stress (Yamamoto et al., 2000). In addition, a device containing HS to deliver FGF2 enhanced FGF2's antioxidative property (Galderisi

et al., 2013). Accordingly, one could argue that Epac1 has antioxidative stress effects via the modification of HS-FGF2 signaling. Indeed, CM of SK-Mel-2 cells inhibited H_2O_2-induced apoptosis of WM1552C cells (data not shown). This antiapoptotic effect of the CM may modify the survival of WM1552C cells coinoculated with SK-Mel-2 cells *in vivo* (Figure 5), whereas rigorous examination for the protection against antioxidative stress should be performed to obtain conclusive evidence.

In summary, this study for the first time demonstrated Epac1-mediated cell/cell communication by modification of FGF2–HS interaction. Our findings may lead to a new strategy for the melanoma therapy targeting a certain population of melanoma cells, that is, Epac1-rich melanoma cells. Future research should attempt to examine the effect of Epac1-specific inhibitors on melanoma progression.

Methods

Reagents and cell lines

HEMA-LP was purchased from Invitrogen (Carlsbad, CA, USA), HUVEC was from VEC Technologies. WM1552C was from Dr. Meenhard Herlyn, Wistar Institute. C8161 cell line was provided by Dr. Mary JC Hendrix. SK-Mel-2 cells (ATCC) were maintained in MEM containing 10% FBS, 1% penicillin/streptomycin. WM1552C and C8161 cells were maintained in RPMI with 10% FBS, 1% penicillin/streptomycin. HEMA-LP and HUVEC cells were maintained in EndoGRO medium (EMD Millipore, Billerica, MA, USA) containing 5% FBS. Antibodies against Epac1, FGF2, and FGFR-1 were from Cell Signaling, anti-NDST-1 antibody was from Abnova and anti-α-tubulin antibody was purchased from Abcam (Cambridge, MA, USA).

Short hairpin RNA transduction

Short hairpin RNA (shRNA) transductions with lentivirus (Santa Cruz Biotechnology) were performed as we previously described (Baljinnyam et al., 2010). C8161 cells were incubated with 8 μg/ml of Polybrene and lentiviral particles harboring shRNA were selected with puromycin dihydrochloride for 1 week. Fresh puromycin-containing medium was replaced every 3–4 days. Established cell lines are as follows: C8161 cells with control shRNA (C8161/control), C8161 cells with Epac1 shRNA [C8161/Epac1(−)].

Migration assay

Migration assay was performed using the 24-well Boyden chambers (8 μm pores, BD Biosciences, San Jose, CA, USA) as we previously described (Baljinnyam et al., 2009). The cells were plated at a density of 1×10^6 cells/100 μl of medium in the inserts and incubated for 16 h at 37°C in the conditioned media. The insert membranes were stained using the Diff-Quick kit (Dade Behring). Pictures were taken and migrated cells were counted with Image J software using 10 randomly chosen fields.

Purification of human perlecan

About 2 L of conditioned medium for 72 h by confluent cultures of human melanoma cells was purified by DEAE–Sepharose chromatography (Whitelock et al., 1999) (100 ml bed volume, flow rate 1 ml/min) which had been equilibrated with 250 mM NaCl (20 mM Tris, 10 mM Methylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, pH 7.5). The column was washed extensively with the buffer, and bound proteins were eluted using 1 M NaCl, 20 mM Tris, 10 mM Methylenediaminetetraacetic acid. The presence of HS-bound perlecan was monitored in column fractions using antibodies to HS (10E4) in an enzyme-linked immunosorbent assay (ELISA). The protein concentration was measured using the Coomassie Plus assay (Pierce), and aliquots were stored at −70°C until used for further Western blot analyses.

Western blot analysis

Western blot analysis was performed as previously described (Iwatsubo et al., 2003, 2004). Briefly, cells were lysed and sonicated in RIPA lysis buffer. Equal amounts of protein were subjected to SDS-PAGE, were transferred to Millipore Immobilon-P membrane, and immunoblotting with respective antibodies was performed.

Tube formation assay

Human umbilical vein endothelial cells under seven passages were used in all experiments. *In vitro* angiogenesis tube formation assay was performed as we previously described with some modifications (De Lorenzo et al., 2004; Movafagh et al., 2006). HUVEC (5000/well) were seeded in 24-well plates coated with Matrigel (Biosciences Discovery), incubated in CM for 4 h at 37°C. The tube formation was quantified by counting the number of connecting branches between two discrete endothelial cells.

Immunoprecipitation

Dynabeads-Protein G for immunoprecipitation (Life Technologies, Carlsbad, CA, USA) were incubated with the primary antibodies and added to the soluble cell lysate fraction. These antibody-coated Dynabeads™, Life Technologies, Carlsbad, CA, USA bound to the target proteins were separated by the magnet and after repeated washing three times, the isolated protein complexes were subjected to SDS-PAGE and immunoblotting with respective antibodies.

FGF2-binding assay

FGF2-binding assay was performed as previously described (Reiland and Rapraeger, 1993). Briefly, HUVEC cells were plated in 24-well plate with 1.5×10^5 cells

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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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.

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 $G12/13\alpha$. $G_s\alpha$ 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.

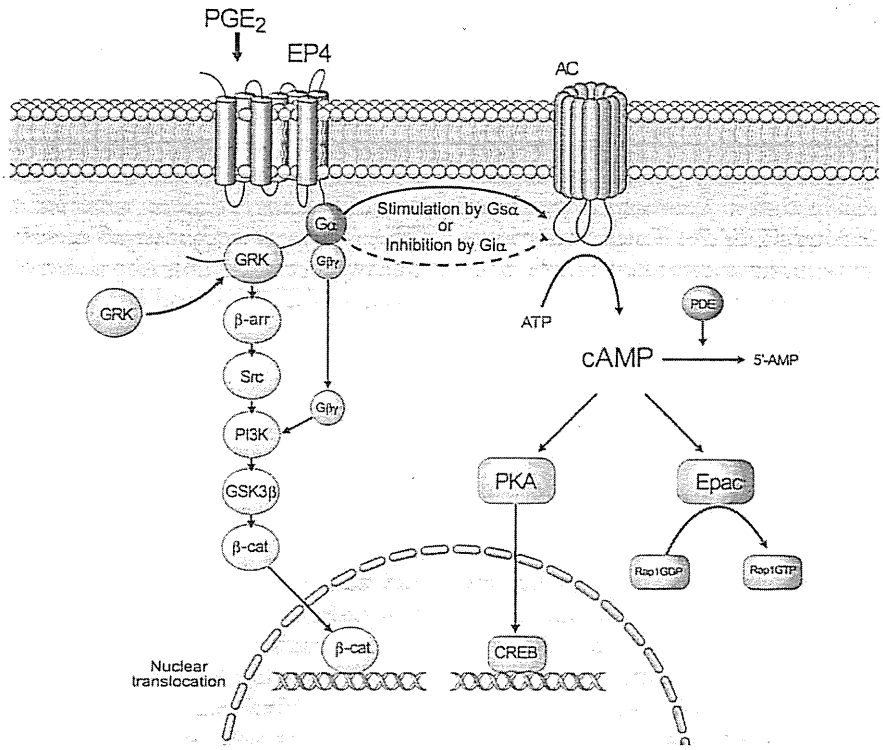

Fig. 1. Upon activation of the EP4 receptor by PGE_2, the $G\alpha$ subunit is dissociated from the receptor and $G\beta\gamma$. Conventionally, EP4, similar to EP2, activates $G_s\alpha$, leading to increased AC catalytic activity and thus cAMP production. In some cases, however, EP4, unlike EP2, may 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 $G\beta\gamma$ dissociated from $G\alpha$ 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.

$G12/13\alpha$ 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 $G\beta\gamma$ 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