The role of the EP receptors for prostaglandin E_2 in skin and skin cancer

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Abstract One of the most common features of exposure of skin to ultraviolet (UV) light is the induction of inflammation, a contributor to tumorigenesis, which is characterized by the synthesis of cytokines, growth factors and arachidonic acid metabolites, including the prostaglandins (PGs). Studies on the role of the PGs in non-melanoma skin cancer (NMSC) have shown that the cyclooxygenase-2 (COX-2) isoform of the cyclooxygenases is responsible for the majority of the pathological effects of PGE_2. In mouse skin models, COX-2 deficiency significantly protects against chemical carcinogen- or UV-induced NMSC while overexpression confers endogenous tumor promoting activity. Current studies are focused on identifying which of the G protein-coupled EP receptors mediate the tumor promotion/progression activities of PGE_2 and the signaling pathways involved. As reviewed here, the EP1, EP2, and EP4 receptors, but not the EP3 receptor, contribute to NMSC development, albeit through different signaling pathways and with somewhat different outcomes. The signaling pathways activated by the specific EP receptors are context specific and likely depend on the level of PGE_2 synthesis, the differential levels of expression of the different EP receptors, as well as the levels of expression of other interacting receptors. Understanding the role and mechanisms of action of the EP receptors potentially offers new targets for the prevention or therapy of NMSCs.

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1 Prostaglandins and skin cancer

The development of cancer is a complex process during which a normal cell undergoes a progressive series of alterations resulting in the acquisition of an altered proliferative capacity, invasiveness, and metastatic potential. For non-melanoma skin cancer (NMSC), these changes are classically defined as occurring in stages: (a) initiation, involving DNA damage leading to mutations, (b) promotion, which involves proliferation and inflammation, and (c) progression, involving additional genetic mutations or structural genomic changes that result in malignancy. The mouse skin model has been extensively used to study molecular changes associated with the dysregulated signaling that occurs in the different stages of tumor development. This model is relevant to other epithelial tissues and to humans and has been intensely studied for this reason [1].

One of the most common features of tumor promotion induced by chemicals or ultraviolet light (UV) in mouse skin is the induction of inflammation, which is characterized by the synthesis of cytokines, growth factors, and arachidonic acid metabolites as well as by the infiltration of inflammatory cells such as macrophages and lymphocytes. Of all the eicosanoids synthesized in the epidermis, the prostaglandins (PGs) are the most abundant. The unstable intermediates of the arachidonic acid-derived PGs are synthesized by the cyclooxygenase (COX) enzymes, COX-1 and COX-2, and further metabolized to PGE_2, $PGF_{2\alpha}$ and PGD_2 by specific synthases. COX-1 is constitutively expressed in most tissues, and its PG products, particularly PGE_2, are involved in normal physiological functions such as maintenance of the gastric mucosa and regulation of renal blood flow. On the other hand, COX-2 expression is undetectable in most unper-

turbed adult epithelial tissues except kidney and brain, but is highly inducible by various endogenous inflammatory and mitogenic factors as well as exogenous agents such as phorbol esters and UV. As observed in many other epithelial tumors, COX-2 is constitutively upregulated in human and murine NMSC, which is accompanied by a high level of PG synthesis [1].

Both pharmacological and genetic approaches have been used to demonstrate the contribution of the PGs to murine skin cancer. Nonsteroidal anti-inflammatory drugs (NSAIDs) that target both COX-1 and COX-2, as well as selective COX-2 inhibitors have been shown to prevent tumor development elicited by either the chemical two-stage model or by UV [2–4]. To unequivocally show that COX-2 expression and activity is critical for skin carcinogenesis, COX-2 knockout mice were generated and shown to be significantly protected from tumor development in either the chemical two-stage model or UV model [5, 6].

To further assess the contribution of the PG products of COX-2, transgenic mice overexpressing COX-2 were generated using either the bovine keratin 5 (K5.COX-2 mice) or keratin 14 (K14.COX-2 mice) promoter [7, 8]. The K14.COX-2 mice on the SKH-1 background developed many more tumors in response to UV than their wild-type counterparts [8]. Treatment of the K14.COX-2 transgenic mice with the carcinogen dimethylbenz[a]anthracene (DMBA) alone (i.e., in the absence of phorbol ester promotion) also resulted in many more tumors than occurred in wild-type mice, suggesting that chronically high levels of PGs have intrinsic tumor promoting activity [5]. Subsequent studies showed that application of PGE_2 to cultured murine keratinocytes or to murine skin caused the activation of multiple signaling pathways resulting in proliferation [9]. Interestingly, these are the same signaling pathways activated by the phorbol ester tumor promoters, suggesting that phorbol ester promotion occurs via its enhancement of PGE_2 synthesis [10].

Now that it has become well-established that induction of COX-2 and its primary product, PGE_2, are important mediators of tumor promotion, attention has turned to the downstream effectors of PGE_2, as well as the other PGs. The pharmacological classification of the PG receptors is based on each receptor preferentially recognizing specific PGs, e.g., PGD_2 activates the D receptors, $PGF_{2\alpha}$ the FP receptors and PGE_2 the EP receptors [11]. PGE_2 binds and activates four G-protein-coupled E receptors referred to as EP1, EP2, E3, and EP4 [12, 13]. Like the other PG receptors, the EP receptors have seven transmembrane segments and each receptor is coupled to different $G\alpha$ subunits of the heterotrimeric G proteins. EP2 and EP4 are linked to $G\alpha s$, commonly referred to as Gs. EP1 is linked to Gq, while EP3 is couple to Gi. Ligand binding of the different EP receptors leads to activation of distinct

downstream signaling pathways (Fig. 1). It is generally accepted that the EP1 receptor activates phospholipase C (PLC), which mediates activation of protein kinase C (PKC) and elevation of cytosolic free calcium [14]. While EP2 and EP4 both activate adenylate cyclase and increase cAMP levels, EP4, but not EP2, is also coupled to the phosphatidylinositol 3-kinase (PI3K) pathway [12]. EP3 differs from the other EP receptors in that multiple variants can be generated by alternative splicing of the C-terminal tail, with several variants inhibiting adenylate cyclase [13]. The signaling pathways that are activated are also dependent in part on the level of PGE_2 that is present, due to differences in binding affinities between the receptors. The EP1 receptor binds PGE_2 with a K_d of 21 nM which is higher than the 11.2 nM K_d for EP2 and the 2.9 nM K_d for EP3 [15].

As will be discussed later, significant cross-talk can occur between the canonical signaling pathways described above and several additional pathways can be activated in some cells. One of the most important of these is the recent demonstration that the EP1, EP2, and EP4 receptors can transactivate the epidermal growth factor receptor (EGFR); EGFR-mediated signaling has been implicated in proliferation, invasion, resistance to apoptosis, angiogenesis, and metastasis, all of which are associated with tumor development [16]. In murine skin in particular, application of diverse tumor promoters induce EGFR ligands, and both EGFR and its ligands are constitutively upregulated in papillomas and squamous cell carcinomas (SCCs) [17].
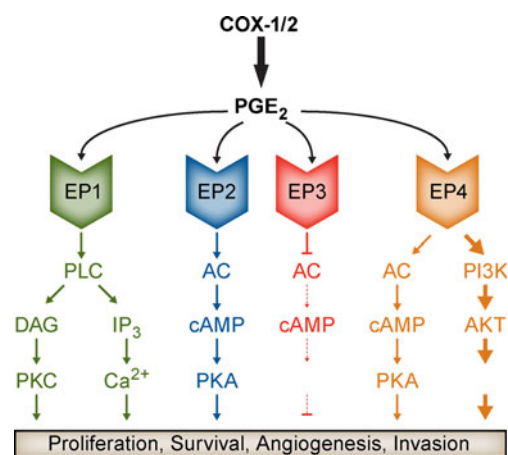

Fig. 1 Canonical signaling pathways activated by the EP receptors for PGE_2. EP1 activates phospholipase C (PLC), resulting in the production of 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG activates protein kinase C (PKC) while IP_3 mobilizes Ca^{2+}. EP2 activates adenylate cyclase (AC) which results in elevated cAMP synthesis and protein kinase A (PKA) activation. EP3 negatively regulates AC and thus cAMP levels are reduced. EP4 activates both the AC/cAMP pathway and the PI3K/AKT pathway. The activation of these signaling pathways by the EP receptors in skin results in biological changes that are associated with the promotion and progression of NMSC

This emphasizes the extensive and complex signaling networks that are activated by the EP receptors that has hindered an understanding of how each of the EP receptors elicit their multiple biological effects.

Each EP receptor subtype has distinct biochemical properties and structures. The mouse EP1, EP2, EP3 (α variant), and EP4 consists of 405, 362, 366, and 513 amino acids, respectively. The amino acid identity among the EP receptors is small, e.g., the identity of EP1 to EP2, EP3, and EP4 is 30%, 33%, and 28%, respectively. Even EP2 and EP4, which both activate adenylate cyclase, only have a 31% identity [12, 15]. The EP2 and EP3 receptors have the most compact structures. EP1 has a long third loop as does EP4, which also has the longest intracellular C-terminus [12, 15]. The long cytoplasmic domain of EP4 interacts with arrestins causing receptor internalization, something that is unique to the EP4 receptor [18].

In general, the tissue distribution, cellular localization, and level of expression are variable among tissues. EP3 and EP4 are the most widely distributed, at least in mouse, with their mRNAs expressed in almost all tissues. EP1 appears to be restricted to several tissues, including kidney, lung, stomach, colon, and skin. In many tissues, EP2 is the least abundant of the receptors [19].

Expression of the EP genes is regulated by various stimuli, both physiological and pathophysiological, resulting in variable levels of expression even in a given tissue. Among the EP receptors, only EP4 expression is regulated by a negative feedback loop [19]. Promoter analysis of the EP2 and EP4 genes identified several consensus sequences relevant to inflammatory stimuli inducing NF-IL6, NFκB, and AP-2 in both genes, while the promoter region of EP2 also contains Sp1 and MyoD sequences, as well as a putative glucocorticoid response element [20, 21].

In this review, the contribution of each of the EP receptors to NMSC will be discussed. Because PGE$_2$ is a strong endogenous tumor promoter in murine skin, elucidating the signaling pathways that are activated by the EP receptors should improve our understanding of the molecular basis for tumor promotion and also offer the possibility of improved prevention/intervention in the development of NMSC.

2 The EP1 receptor

Both human and mouse keratinocytes express all four EP receptors which has made our understanding of how PGE$_2$ functions as a tumor promoter difficult [22–24]. The EP1 and EP2 receptors are low affinity receptors, compared to EP3 and EP4. While the high affinity receptors would be expected to be activated at low levels of PGE$_2$, such as would occur in unperturbed skin, the low affinity receptors

are likely to be activated only in pathological conditions, suggesting that its signaling is more likely to be activated when COX-2 is induced and PGE$_2$ synthesis is high, conditions that are found early in skin tumor development. Initial studies on the role of EP1 in skin focused on its expression and localization. Konger et al. [25] were among the first to show that EP1 is expressed in human epidermis and that it occurs primarily in the cytoplasm of cells in the granular layer. Both plasma membrane and perinuclear/nuclear localization was observed in the basal layer and stratum spinosum [25]. Lee et al. [26] and Tober et al. [27] also reported that EP1 was found in the more suprabasal layers of mouse skin, although Neumann et al. [23] reported that EP1 expression occurred predominantly in the basal compartment. The reason for the discrepancy between laboratories is unknown but may be due to non-specific antibody reactions.

Because the EP1 receptor is more likely to function under conditions of stress or irritation, there has been greater interest in understanding whether its expression levels change after exposure of skin to UV or to chemical tumor promoters. Lee et al. [26] showed that in UV-irradiated hyperplastic skin EP1 remained predominantly suprabasal but was strongly increased. Tober et al. [27, 28] found no change in localization after either acute or chronic UV exposure, but found an increase in the mRNA for EP1 only after chronic, but not acute, UV. Acute UV was shown, however, to upregulate EP1 mRNA in cultured murine keratinocytes [29]. Our laboratory showed that EP1 mRNA was elevated after both acute and chronic UV irradiation, as well as after both single and multiple topical treatments with the tumor promoter 12-O-tetradecanoylphorbol 13-acetate (TPA) [30]. This upregulation of EP1 after UV or TPA is significant because both UV and TPA cause an upregulation of COX-2 with a concomitant increase in PGE$_2$ [1]. The increase in PGE$_2$ and in EP1 suggests that this receptor may function as a "stress" receptor.

EP1 receptor expression in murine and human NMSCs has also been investigated. In UV-induced papillomas and SCCs in mice, EP1 was highly expressed as measured by immunohistochemistry. Interestingly, EP1 was not expressed in murine basal cell carcinomas (BCCs). Similar observations were made for human actinic keratosis, SCCs and BCCs [26]. Konger et al. [31] also reported that well-differentiated SCC's had significantly greater membrane staining while BCC's and spindle cell carcinomas had notably reduced membrane staining. At the mRNA level, EP1 was found to be upregulated significantly in murine papillomas and SCCs generated either with a UV or initiation-promotion protocol by several laboratories including our own [27, 30].

A likely role for the EP1 receptor in skin carcinogenesis was suggested by the reports that EP1 null mice or use of selective EP1 antagonists resulted in reduced colon and

breast cancer development [32–34]. Tober et al. [4] investigated the effects of topical application of the EP1 antagonist ONO-8713 on UV-induced cutaneous inflammation and tumor development. Using skin thickness (vascular permeability) and neutrophil infiltration as measures of an inflammatory response, they found that the EP1 antagonist reduced these parameters as well as the level of PGE_2. More significantly, in a UV carcinogenesis experiment, the EP1 antagonist reduced tumor multiplicity by ~50% relative to wild-type controls. In the same experiment, topical application of the selective COX-2 inhibitor celecoxib reduced tumor multiplicity somewhat more, ~60%, but the combination of ONO-8713 and celecoxib had no greater effect [4]. These data offer insight into the biochemical events needed for, or contributing to, skin carcinogenesis. Celecoxib is a potent COX-2 inhibitor but does not completely stop synthesis of PGs [2, 4], likely due to continued low level synthesis of PGs from COX-1. The Tober et al. [4] observations also suggest that EP1 is responsible for a large part of the tumor promoting activity of PGE_2. The remainder of the activity is likely due to activation of the other EP receptors or possibly one or more of the peroxisome proliferator-activating receptors, which are known to be activated by eicosanoids [35].

To more definitively determine the contribution of the EP1 receptor to skin tumor development, our laboratory generated transgenic mice (BK5.EP1) that overexpress EP1, under control of a bovine keratin 5 (BK5) promoter, in the basal layer of murine epidermis [30]. Skins of these mice were histologically indistinguishable from wild-type mice and had similar levels of epidermal cell proliferation after topical treatment with TPA. Surprisingly, using a carcinogenesis protocol employing the carcinogen DMBA and the tumor promoter TPA, the BK5.EP1 mice produced significantly fewer tumors compared to wild-type mice. PKC, the receptor for TPA that mediates its tumor promoting activity [36], was found to be downregulated in the BK5.EP1 epidermis, which likely explains the refractoriness to TPA promotion [30]. However, even though tumor multiplicity was reduced in the transgenic mice, the percentage of tumors that were SCC were very high compared to wild-type mice, i.e., they had an approximately eightfold-higher papilloma to carcinoma conversion rate [37]. To circumvent the potential problem of PKC downregulation, another tumor promoter, anthralin, which generates free radicals and does not interact with PKC [38] was used in place of TPA. With this protocol, tumor multiplicity and tumor incidence were increased twofold in BK5.EP1 mice compared to wild type. More significantly, however, was the observation that the transgenic mice had a ninefold increase in SCCs. Overall, these tumor experiments indicate that the effect of EP1 overexpression is protocol- and agent-dependent with regard to

papilloma development, but consistently contributed to progression of papillomas to SCCs [30].

In a recent study, our laboratory found that a single topical application of DMBA or benzo[a]pyrene alone was sufficient to elicit SCCs in the BK5.EP1 transgenic mice, but not in wild-type mice. This difference was not due to alterations in carcinogen metabolism, DNA adduct formation, or Ras activity. Because PGE_2 signaling has been shown to be important in maintaining hematopoietic stem cell homeostasis, the effects of EP1 receptor overexpression on keratinocyte stem cell numbers was investigated as a possible mechanism for the dramatic increase in SCC's seen in the BK5.EP1 model. Using CD34 and α6 integrin as markers [39], no differences were observed between the transgenics and wild-type mice [37]. However, EP1 transgene overexpression caused COX-2 expression in the epidermis, which is not normally seen in wild-type mice. To determine the significance of this upregulated COX-2, the selective COX-2 inhibitor celecoxib was administered in the diet before DMBA application. While the DMBA-treated BK5.EP1 control mice had significant hyperplasia and inflammatory cell infiltration for at least 2 weeks, the celecoxib-fed mice had relatively normal skin architecture and very few inflammatory cells, and remained normal for the duration of the experiment. To verify that this was not an off-target effect of celecoxib, BK5.EP1 mice were crossed with COX-2 null mice. Loss of one allele of COX-2 reduced tumor development by 50% while loss of both alleles completely prevented tumor development, demonstrating a requirement for the PGs generated from COX-2 in the development of skin tumors in the BK5.EP1 model [37].

It is clear from the above tumor studies employing genetic or pharmacological approaches to up- or downregulate EP1 activity, that the EP1 receptor plays a major role in the pro-tumorigenic action of PGE_2. What remains to be determined is the mechanisms and signaling pathways by which this occurs. Konger et al. [40] showed that in human keratinocytes EP1 activation is coupled to intracellular calcium mobilization, as is found in many other cell types [12]. They further demonstrated that EP1 expression, particularly when localized to the membrane, is associated with keratinocyte differentiation, which is known to be induced by calcium mobilization [31] and is usually associated with decreased tumorigenesis. In rat osteoblasts, the EP1 receptor was shown to signal through a PLC/PKC/c-Src signaling pathway [14]. PLC activation hydrolyzes phosphatidyl inositol 4,5-bisphosphate into two second messengers, inositol 1,4,5-trisphosphate, which triggers calcium mobilization, and diacyglycerol (DAG) which activates PKC [41]. It is likely that this signaling pathway (Fig. 2) is operative in keratinocytes based on the observations that activation of EP1 causes calcium mobilization [40] and PKC is downregulated in the epidermis of
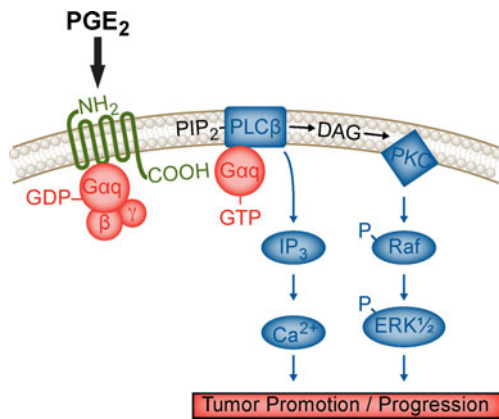
Fig. 2 EP1 signaling in keratinocytes. Ligand activation of the EP1 receptor causes conformation changes resulting in activation of the heterotrimeric G protein, Gq. Activation causes release of GDP from the Gα subunit and the binding of GTP, which is associated with release of Gβγ and activation of phospholipase C beta (PLCβ). Activated PLCβ hydrolyzes phosphatidyl inositol 4,5-bisphosphate to 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃). IP₃ raises cytoplasmic Ca^{2+} levels via movement from endoplasmic reticulum stores, leading to activation of calcium signaling pathways. DAG activates protein kinase C (PKC), which subsequently activates the MAPK pathway via Raf-1 activation. PKC can also be activated by the phorbol ester tumor promoters; chronic activation of EP1 may confer tumor promoting/progression activity through PKC

BK5.EP1 transgenic mice. Because downregulation of PKC occurs as a result of its activation, which is also something the tumor promoter TPA causes, overexpression of EP1 may lead to autonomous tumor promotion [37]. In support of this, Thompson et al. [42] reported that malignant murine keratinocytes are stimulated by PGE₂ to proliferate and that this was mediated by the EP1 receptor.

In summary, the currently available data suggest that the EP1 receptor plays a role not only in keratinocyte differentiation, but more importantly it contributes significantly to the development of NMSC. The extent to which these observations extend to other tissues is likely to be context and tissue specific. EP1 receptor deficiency significantly reduced azoxymethane-induced colon cancer in mice, suggesting that the EP1 receptor is likely to be pro-tumorigenic in self-renewing epithelium such the epidermis and gastrointestinal tract [32]. However, reduced EP1 expression increased the metastatic capacity of breast cancer cells [43], suggesting that EP1 may have anti-tumorigenic activity in certain contexts. Additional studies are needed to elucidate the mechanisms by which the EP1 receptor influences these neoplastic processes.

3 The EP2 receptor

Like the EP1 receptor, the EP2 receptor is a low affinity receptor and thus likely to be more important during disturbances of skin homeostasis and/or integrity. Like EP1, EP2 has also been shown to be localized to both the perinuclear and outer membrane of normal human keratinocytes in the basal and spinous layer. Unlike EP1, however, little EP2 was observed in the superficial stratum spinosum. The localization of EP2 (and EP1) in the perinuclear membrane is of interest because COX-1 and COX-2 localize to the nuclear membrane as well as the endoplasmic reticulum, suggesting possible nuclear second messenger signaling [25]. In mouse, the EP2 receptor was found by immunohistochemistry both on the plasma membrane and within the cytoplasm [27]. Patchy expression throughout the epidermis has also been reported [26]. However, because mice have a very thin epidermis that lacks the defined granulosa and spinous layers of differentiated human epidermis, it is difficult to assess whether the decreased expression in the more differentiated human keratinocytes also occurs in mouse.

Investigations in our laboratory showed that the level of expression of the EP2 receptor can be modulated by tumor promoting stimuli [44]. Topical application of the phorbol ester TPA upregulated EP2 mRNA more than fivefold following single or repeated applications [44]. We also found that exposure of mouse skin to UV, either once or repeatedly, also significantly elevated EP2 (unpublished data), although another study reported that EP2 was elevated only after chronic UV exposure, not after an acute exposure [27].

The expression levels and patterns of the EP2 receptor have also been examined in skin tumors resulting from either UV or chemical carcinogenesis protocols. In benign papillomas and SCCs of SKH-1 mice subjected to UV irradiation, EP2 immunohistochemical staining remained patchy. Interestingly, no expression of EP2 was observed in BCCs [26]. In a study by Tober et al. [27] on UV-induced tumors, EP2 protein was found only in the highly differentiated cells surrounding keratin pearls. While we found that UV significantly elevated EP2 mRNA levels in both papillomas and SCCs of SKH-1 mice (unpublished data), this was not observed in another study [27]. In papillomas and SCC from chemical initiation–promotion protocols, EP2 mRNA was upregulated at least 15-fold in FVB mice. This was confirmed at the protein level as well [44]. Another study, using papillomas from NMRI mice, also reported an elevation in EP2 mRNA expression [23]. Thus the majority of the studies indicate that the EP2 receptor mRNA levels are elevated in squamous carcinomas, compared to uninvolved skin.

The increased expression of EP2 following tumor promoter treatment and in skin tumors resulting from these treatments led to the question of whether EP2 contributes to the development and/or progression of tumors. To address this, we subjected mice that were null for the EP2 receptor

to a two-stage chemical carcinogenesis protocol. The EP2 null mice had significantly fewer tumors (~50%) and reduced tumor incidence compared to their wild-type counterparts. Tumor size distribution for the EP2 knockout mice was similar for small to medium size tumors (<5 mm diameters), however, the EP2 null mice produced fewer large tumors, compared to wild-type mice. Significantly, no SCCs were produced in the EP null mice whereas 20% of the wild-type mice developed SCCs [24].

To further understand the contribution of EP2 to skin tumor development, a number of parameters associated with cancer were assessed in the EP2 null mice. In untreated EP2 null mice, the extent of basal cell proliferation was slightly reduced compared to wild-type mice. Following a single treatment with TPA at a dose known to cause marked proliferation and hyperplasia, there was a significant reduction in the EP2 null mice. This suggested that the EP2 receptor may play an important role in mediating TPA-induced proliferation and that the increased PGE_2 synthesis elicited by TPA may be responsible for this. While cultures of primary keratinocytes from wild-type mice showed a large proliferative response to exogenous PGE_2, the EP2 null keratinocytes had a reduced proliferative response. This suggested that PGE_2 mediates an autonomous proliferative response in keratinocytes, i.e., PGE_2 from dermal stromal cells are not necessary [24]. This study also corroborated an early study showing that the EP2/EP4 receptors mediate PGE_2-induced proliferation in primary human keratinocytes [45].

The effect of EP2 deletion on TPA-induced inflammation and angiogenesis were also assessed. There was a significant reduction in the infiltration of macrophages into the dermis of EP2 null mice, as well as markedly reduced expression of interleukin-1α in the epidermis and a significantly reduced angiogenesis. Additionally, deficiency in EP2 receptor expression reduced the level of cAMP elicited by topical application of PGE_2 to basal levels [24].

Use of EP2 null mice, on the SKH-1 background, in UV carcinogenesis experiments produced different results than observed in the two-stage model described above. Brouxhon et al. [46] reported that acute low-dose UV exposure produced a markedly reduced proliferative response compared to wild-type mice, while with higher doses the proliferative response was the same, although the hypertrophy response remained depressed. The EP2 null SKH-1 mice produced 50% fewer tumors than the wild-type mice, similar to the two-stage chemical protocol, but their tumors were larger in size, with a more aggressive phenotype as indicated by their poor differentiation. These less keratinized tumors were also more deeply invasive than the well-differentiated tumors. To understand how deletion of the EP2 receptors could lead to a more invasive phenotype, enzymes involved in matrix remodeling were analyzed.

Matrix metalloproteinase (MMP)-2 and MMP-9 activity were dramatically increased in UV-irradiated EP2 null SKH-1 mice, which likely contributed to the enhanced SCC invasion [46].

Although the number of skin tumors was reduced in EP2 null mice in both the two-stage and UV carcinogenesis protocols, the issue that remains unanswered is why a decreased tumor aggressiveness was seen in the two-stage chemical protocol while increased aggressiveness was seen in the UV study. There are several possibilities that will require additional studies to resolve this issue. First, the types of mutations induced by the two protocols are different. The carcinogen DMBA causes mutations in the H-ras gene [10], while UV primarily causes mutations in the p53 gene [47]. The interplay of these mutations with PGE_2 signaling may be different. Second, the strain background of the mice used in these two studies is different. The SKH-1 mice carry a mutation in the hairless gene, which has been shown to be a tumor suppressor gene that is responsible for the UV susceptibility of these mice [48]. The relationship of this mutation to chemical carcinogenesis or to human NMSC is not known. Thus, targeting the EP2 receptor for prevention of skin cancer may be premature and clearly requires additional study.

While the EP2 knockout study suggests that EP2 expression is needed for a full tumorigenic response, it was unknown whether increasing EP2 above normal levels would enhance tumor development. To address this question, we generated EP2 transgenic mice in which the EP2 transgene was under the control of the BK5 promoter (BK5.EP2 mice). The EP2 receptor was primarily expressed in the basal layer of the epidermis where the keratin 5 promoter is most active. When subjected to a two-stage carcinogenesis protocol, the BK5.EP2 mice developed more papillomas than wild-type mice. More notable, however, was the marked increase (threefold) in the number of SCCs in the BK5.EP2 mice. Additionally, the BK5.EP2 mice produced much larger tumors than their wild-type counterparts. In both papillomas and SCC from the EP2 transgenic mice, the level of EP2 expression was higher than that in wild-type tumors, likely due to both endogenous EP2 and transgene EP2 expression. This was interpreted as suggesting that the EP2 receptor significantly contributes to the development of, and perhaps more importantly, to the progression of benign to malignant tumors. As was expected, the epidermis of BK5.EP2 mice produced a greater proliferative and inflammatory response to TPA. Overexpression of the EP2 receptor also caused an increase in angiogenesis, even in untreated mice, where macroscopic inspection of the dermis showed an increase in the number and size of the vessels. As opposed to the reduction in cAMP seen in EP2 receptor knockout mice, EP2 receptor overexpression significantly elevated PGE_2-

induced cAMP in the epidermis above the level of wild-type mice, which is in agreement with a model in which PGE_2 elevates cAMP levels through EP2 activation, which in turn induces genes involved in proliferation, inflammation, and angiogenesis [44, 49].

A proliferative response to EP2 activation is not restricted to murine keratinocytes. Konger et al. [45] showed that proliferation of primary adult human keratinocytes is increased following activation of EP2 and subsequent production of cAMP. They later showed that in immortalized human keratinocytes (HaCaT cells) loss of EP2 receptor expression was associated with increased invasiveness and decreased expression of paxillin, a component of focal adhesion complexes [50]. Several possible explanations were offered for this observation, including defects in post-translational modification, much reduced expression of COX-2 and reduced synthesis of PGE_2. As the authors suggested, the normally reduced PG production may contribute to the normally non-invasive phenotype of HaCaT cells [50].

Given the generally pro-tumorigenic function of the EP2 receptor, there is considerable interest in elucidating the mechanisms and signaling pathways involved. Although both EP2 and EP4 activate adenylate cyclase, Fujino et al. [51] reported that the stimulation of cAMP in EP4-expressing cells is significantly less than in EP2-expressing cells at equal levels of receptor expression. Additionally, EP4 receptors, but not EP2, undergo rapid agonist-induced desensitization and internalization. EP4, but not EP2, was also found to activate the PI3K/AKT pathway. In a later study, Fujino et al. [52] reported that PGE_2 stimulation of cells expressing either the EP2 or EP4 receptor results in phosphorylation of $CREB^{ser133}$. However, inhibition of PKA reduced this phosphorylation in EP2 expressing cells, but not in EP4 expressing cells. They provided evidence that activation of the EP4 receptor, but not EP2, results in activation of a PI3K pathway that inhibits the activity of PKA. It was concluded that although both EP2 and EP4 cause phosphorylation/activation of CREB, they do so through different signaling mechanisms [52]. On the other hand, Castellone et al. [53] reported that EP2 receptor stimulation of proliferation in colon cancer cells involved both the PI3K/AKT and axin/β-catenin pathways. They showed that the free G protein β/γ subunit activated PI3K/AKT while the Gαs subunit associated with the "regulator of G protein" domain of axin, leading to activation of β-catenin and proliferation of DLD-1 cells that carry a mutation in the *APC* gene. Contrary to expectations, PKA activation was not involved in the EP2-mediated proliferation [53]. Thus, it appears that the signaling pathways activated by the EP2 receptor that lead to proliferation are likely to be cell-type and/or context specific with regard to other signaling alterations or mutations.

With regard to keratinocytes, the Langenbach laboratory has been particularly interested in understanding how COX-2-generated PGs protect the skin from UV-induced apoptosis and the relationship between the reduced apoptosis and tumor development [54–56]. They compared the protein levels of all the EP receptors following UV exposure of COX-2 null and wild-type mice and found that although EP1, EP2, and EP4 were induced in wild-type mice, EP2 and EP4 levels were 50% lower in the COX-2 null mice. The levels of activated PKA and AKT were also reduced in the COX-2 nulls, as were the phosphorylated forms of the anti-apoptotic protein Bad, $pBad^{ser136}$ and $pBad^{ser155}$, which are downstream products of activated AKT and PKA, respectively [55]. These findings suggested that both EP2 and EP4 are responsible for the anti-apoptotic effects of PGE_2 in the setting of UV-induced COX-2. To confirm this suggestion, COX-2 null mice were exposed to UV to induce apoptosis, followed by topical application of EP2 and EP4 agonists, each of which reduced epidermal apoptosis by 50% in the COX-2 null mice. It was proposed that the remaining apoptosis was independent of EP2/EP4 and likely occurred through a PG-independent pathway [56].

Our laboratory has also been interested in a possible feedback relationship between COX-2, PGE_2, and EP2. Maldve and Fischer [57] showed that in primary cultures of murine keratinocytes, PGE_2 could induce COX-2. Subsequently, we reported that EP2 null mice had a reduced induction of COX-2 following TPA treatment [49]. Conversely, primary keratinocyte cultures from EP2 transgenic mice had increased COX-2 expression after either TPA or PGE_2 treatment which was blocked by an adenylate cyclase inhibitor. PGE_2 treatment also caused greater phosphorylation of CREB in keratinocytes from EP2 transgenic mice, compared to wild-type mice; phosphorylation was reduced when a PKA inhibitor was included. Conversely, there was no CREB phosphorylation in EP2 null mice following PGE_2 treatment and this correlated with a lack of a proliferative response to PGE_2 [49]. These events are indicative of a positive feedback loop between COX-2 and EP2 and it has been suggested that this could result in the continuous overexpression of COX-2 that is observed in many epithelial tumors [56].

To further elucidate the contribution of EP2 to papilloma development in the mouse skin initiation-promotion model, Langenbach's laboratory looked at the effects of inhibiting PKA and EGFR, each of which reduced tumor multiplicity by ~50% [58]. To assess EP2's contributions to the activation of these pathways, mice were treated with an EP2 agonist concomitantly with the NSAID indomethacin to inhibit TPA induction of endogenous PGE_2 synthesis. The agonist restored tumor development that was inhibited by indomethacin and increased cAMP and PKA activity. The agonist also increased c-Src and EGFR activation [58].

This suggests that signaling from EP2 is more complex than a linear cAMP-PKA-pCREB pathway, which was described earlier. Chun et al. [58] in fact found that EP2 could be immunoprecipitated as a complex with β-arrestin1 and p-Src^{Tyr416}. Thus, the contribution of EP2 to skin tumor development is likely via activation of both the cAMP/PKA and EGFR pathways. What remains unclear, however, is the mechanism by which the EP2 receptor activates EGFR. While c-Src mediated activation has been reported in murine keratinocytes [58], a study on human epidermoid A431 and SCC-9 cells showed that EP2-mediated EGFR transactivation can also occur via PKA activation [59], again indicating cell or context specificity.

The Langenbach lab has also explored the possibility that the β-arrestin1-Src complex contributes to EP2-mediated signaling in murine keratinocytes [58]. The EP2 agonist butaprost induced complex formation with subsequent c-Src and EGFR activation, which was dependent on the presence of β-arrestin1 (Fig. 3). Butaprost also induced the activation of AKT, ERK1/2, and
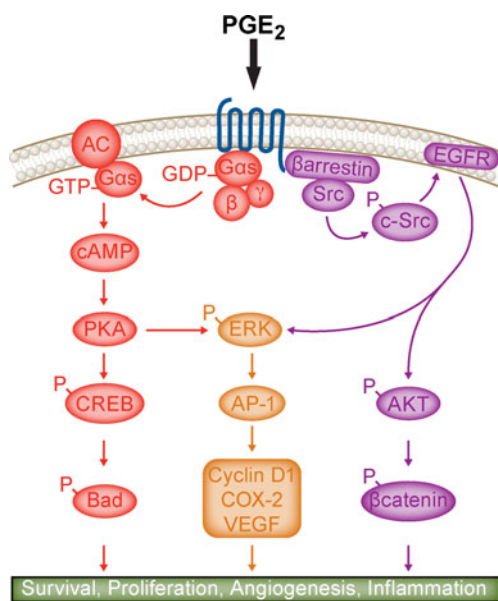

Fig. 3 EP2 signaling in keratinocytes. Ligand activation of the EP2 receptor causes the activation of at least several signaling pathways. Receptor activation results in conformational changes causing a loss of GDP from the α subunit of the Gs protein and binding of GTP, which leads to activation of membrane bound adenylate cyclase (*AC*). Activation of AC results in the conversion of adenosine triphosphate into cyclic adenosine monophosphate (*cAMP*), which activates protein kinase A (*PKA*). PKA is responsible for activating, via phosphorylation, the transcription factor CREB and the signaling factor ERK1/2. Activation of EP2 can also lead to the formation of a β-arrestin1-Src complex, with subsequent activation of the epidermal growth factor receptor (*EGFR*). Activation of EGFR causes activation of both the ERK1/2 (MAPK) and AKT pathways. The activation of downstream components of these 3 pathways leads to increased survival (*p-Bad*), increased proliferation (*Cyclin D1*), angiogenesis (*VEGF*) and inflammation (*COX-2*), all of which contribute to tumorigenesis

STAT3, all of which were inhibited by β-arrestin defi-ciency or EGFR inhibition. Butaprost also increased PKA activation leading to phosphorylation of CREB, as expected, but inhibition of PKA did not affect butaprost-induced activation of EGFR. This observation, plus the finding that β-arrestin1 deficiency did not affect butaprost-induced PKA activation, indicated that there are independent EP2-mediated signaling pathways, both of which contribute to murine keratinocyte proliferation [58].

To further investigate the mechanism by which EP2 mediates keratinocyte survival, Chun and Langenbach [60] explored the possible role of survivin, a regulator of cell survival. They reported that exposure to UV upregulated survivin and pSTAT3, a transcription factor that regulates survivin expression. Mice deficient in either COX-2 or EP2 had a reduced ability to increase the expression of survivin or pSTAT3 after UV irradiation. When wild-type mice were treated with indomethacin, survivin levels were also reduced but could be restored by treatment with PGE$_2$ or butaprost, the EP2 agonist. Additionally, inhibition of EGFR activity blocked the effect of butaprost, indicating that COX-2 synthesis of PGE$_2$ regulates survivin expres-sion in keratinocytes, in part, through an EP2-mediated EGFR/STAT3 pathway. This in turn suggests that EP2/survivin may be an efficacious target for chemoprevention or therapy of skin cancer [60]. However, as discussed earlier, the observation by Brouxhon et al. [46] that while EP2 null mice develop fewer tumors than wild-type mice, the tumors that do arise are more aggressive.

The EP2 receptor has also been implicated in the progression of epidermal lesions from dysplastic to SCCs. Brouxhon et al. [61] showed a sequential loss of E-cadherin-mediated cell-cell contact with tumor progression due to decreased E-cadherin levels. Use of EP2 agonists or EP2 deletion indicated that EP2 mediates the mobilization of E-cadherin for proteosomal degradation [61], although the molecular mechanisms by which this occurs are currently unknown.

Thus a clear picture is emerging that EP2 receptor activation results in altered keratinocyte behavior that is associated with malignancy, including survival, prolifera-tion, angiogenesis and invasion. Similar findings with other epithelial tissues suggest that this may be a ubiquitous phenomenon for most, if not all, epithelial tissues. For example, in the APC^{Δ716} mouse model of intestinal polyps, EP2 receptor activation results in induction of vascular epithelial growth factor (VEGF) that causes tumor angio-genesis [62]. Similarly, in mouse mammary tumor cells, activation of the EP2 receptor by PGE$_2$ induced VEGF though a cAMP/PKA pathway that did not involve hypoxia inducible factor (HIF)-1α, MAPK or PI3K/AKT [63]. The EP2 receptor also stimulates proliferation of colon cancer cells, albeit through pathways involving PI3K/AKT and β-

catenin [53]. The common theme appears to be that EP2 mediates proliferation in many cells; the confounder is that the signaling pathways and mechanisms are cell-type dependent. Future work may show that at least some of these pathways are interconnected, which would provide a more universal picture of the mechanism of action of the EP2 receptor.

4 The EP3 receptor

Of the four EP receptors, EP3 is the most complex and also the least studied receptor. EP3 differs from the other EP receptors in that multiple variants can be generated by alternative splicing of the C-terminal tail. In mouse, there are three splice isoforms of EP3, α, β, and γ, containing C-terminal tails of 26–30 amino acids that do not have structural motifs or hydrophobic features in common. The α isoform has a hydrophilic tail while the beta isoform has a hydrophobic tail. Although they both have the same ligand binding properties, because of differences in their C-terminal tails the different isoforms couple to different G proteins, resulting in differences in signaling [19, 64].

The EP3 and EP4 receptor have binding constants in the sub-nanomolar-range, with EP3 having the highest affinity for PGE_2 of all the EP receptors. This suggests that EP3 likely has a maintenance function in the epidermis where it would respond primarily to COX-1 elicited PGE_2 due to the lack of expression of COX-2 in naive skin. However, determining the function of EP3 is complicated by the fact that the splice variants encode different functional proteins, which elicit numerous intracellular signaling pathways. Depending on the cell type, EP3 has been shown to inhibit adenylate cyclase, stimulate adenylate cyclase, activate PLC, activate nitric-oxide-cyclic GMP pathways and activate Rho-dependent pathways [65].

Although human keratinocytes express all four EP receptors, the level of expression of EP2 and EP3 is considerably higher than expression of EP1 or EP4 [66]. In naive mouse skin, however, the EP3 receptor has been reported to be expressed at moderate levels throughout the epidermis [26], although Tober et al. [27] found that, unlike the EP1 or EP2 receptor, EP3 localized to the proliferative basal compartment of the epidermis. This was recently corroborated by Honda et al. [67], who found colocalization with keratin 5. EP3 receptor expression was reported to be localized to the cytoplasm and perinuclear region of keratinocytes, although it was localized to plasma membrane in dermal sebocytes [27]. Perinuclear localization of EP3 has been noted in other tissues as well, including human kidney, porcine brain endothelial cells, and rat liver [68], suggesting the ubiquitous nature of this localization. In chronically UV-irradiated skin of SKH-1 mice, EP3 receptor expression was undetectable by immunohisto-chemistry [26], although in another study using a single exposure to UV, EP3 receptor expression was observed throughout all layers of the epidermis by 48 h [27]. The reason for the conflicting data on the protein expression of EP3 is unknown. Interestingly, inhibition of the EP1 receptor with ONO-8713 prevented UV-induced EP3 expression in the suprabasal keratinocytes, suggesting a regulatory interaction between the EP1 and EP3 receptors [27].

Our laboratory measured EP3 mRNA levels in FVB mice and found that they were upregulated by both single and multiple treatments with TPA (unpublished data). However, Tober et al. [27] found that acute UV exposure reduced expression of EP3γ and EP3β, compared to unirradiated skin, although in chronically irradiated skin only EP3β was significantly downregulated. With regard to human keratinocytes, Konger et al. [40] showed that three splice variants, EP3A1, EP3C, and EP3D mRNAs, were expressed in primary cultures. Analysis of protein expression by Western blotting (the three isoforms have similar molecular weights) showed that expression of the EP3 receptor was not affected by the extent of confluency or state of calcium-induced differentiation. Immunohistochemical localization in intact human skin showed positive staining throughout the epidermis, with staining most prominent in the basal layer where cytoplasmic, plasma membrane, and perinuclear staining patterns were observed. It was postulated that the perinuclear/nuclear localization is related to an autocrine function of EP3 because cyclo-oxygenases and microsomal PGE synthase are also localized there. On the other hand, the plasma membrane localization is consistent with a role for EP3 in responding to paracrine signaling [40]. Neumann et al. [23] reported that EP3 protein was elevated by 20% in their COX-2 transgenic mice. Overall, these studies indicate the inducible nature of this receptor.

In tumors arising from chronic UV exposure, very little expression of EP3 protein or mRNA was seen by Lee et al. [26] in either benign papillomas or SCC. Tober et al. [27] reported that in tumors from chronically UV-irradiated mice, EP3α and β were significantly repressed while EPγ remained unchanged. Neumann et al. [23] also reported that in papillomas from a two-stage experiment in NMRI mice that the mRNA for EP3 (splice variants were not measured) appeared to decrease, although this was not rigorously evaluated. It is difficult to compare the results of the above studies because different tumor-inducing protocols were used and only one study [27] assessed expression at the isoform level. However, there appears to be some, if variable, decrease in EP3 expression in murine skin tumors.

In an early study focused on understanding the contribution of each EP receptor to the proliferation of human keratinocytes, Konger et al. [45] reported that growth

stimulation occurs via EP2/EP4 receptor adenylate cyclase-mediated responses while activation of EP3 by sulprostone, which lacks EP2 or EP4 agonist activity, inhibited proliferation. There was no measurable effect of EP3 activation on cAMP levels, indicating that growth inhibition was independent of adenylate cyclase pathway signaling. The growth inhibitory affect of EP3 is not restricted to the epidermis as this has been observed in colonic epithelium as well, and EP3 expression is downregulated in colon cancer [69]. A subsequent study by Konger et al. [40] explored the relationship between the lipid second messengers DAG and ceramide, which are known to inhibit growth, and the activity of EP3. They found that the EP3 agonist sulprostone, which inhibits growth, quickly induces DAG and ceramide production and this was completely blocked by an antagonist of the EP1-3 receptors. Sulprostone was also shown to induce the cell-cycle inhibitory protein p21^{WAF1} [40].

The growth-promoting activity of EP2 and growth inhibitory activity of EP3 suggests that their overall effect on keratinocyte proliferation depends on ligand concentration as well as the level of receptor expression. As discussed by Konger et al. [40], the low-affinity EP4 receptor requires a 100-fold greater concentration of PGE$_2$ for maximal activation than the EP3 receptor. Stimuli that induce COX-2 and thus raise PGE$_2$ levels, or exogenous PGE$_2$, have been reported to increase keratinocyte proliferation above that of basal conditions where PGE$_2$ levels are low [9, 49]. Additionally, stimuli such as tumor promoters or wounding can increase EP2 expression, thus shifting the homeostatic balance between proliferation and differentiation that occurs in the normal epidermis, to a more proliferative state. This may be the reason for our finding that the EP3 receptor does not appear to play a significant role in skin tumor development. Our laboratory subjected EP3 null mice to the two-stage DMBA/TPA protocol and observed no differences in either tumor multiplicity or tumor incidence, compared to wild-type mice. The tumor size distribution was also almost the same as wild-type mice [24]. Similar studies on EP3 knockout mice were performed by Shoji et al. [70] in which they observed an enhanced latency in skin tumor development in the EP3 null mice early in the experiment, although by the end of the study tumor multiplicity and incidence were identical. Interestingly, they found no SCC in the EP3 null mice while ~12% of the wild-type mice developed SCCs [70]. The overall lack of a role for the EP3 receptor in skin cancer is consistent with similar observations in other epithelial cancers, including colon and breast [71, 72].

A possible role for EP3 in cutaneous inflammation has also been studied, based on the known pro-inflammatory effects of PGE$_2$, however, the outcomes of these studies are not consistent. A study by Goulet et al. [73] showed that EP3 mediated the pro-inflammatory action of PGE$_2$, measured by the edema response to topical arachidonic acid. Using mice that were deficient in each of the EP receptors, only the absence of the EP3 receptor resulted in a significant reduction in edema and vascular permeability compared to wild-type mice [73]. As discussed by Goulet et al. [73] there are a number of reasons for expecting EP3 to mediate edema. The second messenger systems to which EP3 is linked would be expected to result in vasoconstriction instead of vasodilatation and this was previously observed by others [74]. Other studies showed that pharmacological agents that preferentially activate EP3 receptors potentiated bradykinin-induced inflammation in rabbit skin [75]. Additionally, in atopic dermatitis, PGE$_2$ promotes the innervation of skin lesions through the activation of the EP3 receptor in keratinocytes, which subsequently induces neurotrophin-4. The signaling pathway utilized in this case is the phosphoinositol-specific PLC/PKCα/ERK pathway [76]. Thus, there are several examples of a pro-inflammatory role for the EP3 receptor in skin, although the signaling pathways utilized may depend on the etiological agent and/or isoforms that are activated.

There are other studies, however, in which EP3 receptor activation has anti-inflammatory activity. Using selective EP receptor agonists, Ahluwalia and Perretti [77] reported that EP3 has anti-inflammatory effects against edema elicited by exogenous agents. Kanda et al. [66] analyzed the contribution of each EP receptor to PGE$_2$-induced inhibition of NFκB activity and expression of the skin-homing T cell chemokine CCL27. Antagonists for EP3 or EP1/EP2 each partially blocked the PGE$_2$-induced inhibition of CCL27, while together they completely blocked the response [66]. In another study on the role of EP3 in allergic skin inflammation in mice, use of an EP3 agonist, but not agonists of the other EP receptors, reduced the extent of edema and inflammatory cell infiltration. Microarray analysis of keratinocytes from mice treated with the EP3 agonist ONO-AE-248 showed that expression of neutrophil-recruiting chemokines, especially CXCL1 and CXCL2, were suppressed, compared to control mice, which correlated with reduced neutrophil infiltration. Additionally, in cultured keratinocytes, the EP3 agonist suppressed CXCL1 expression and production induced by TNFα, indicating that EP3 is anti-inflammatory through altering the expression of genes in keratinocytes that are responsible for the dermal infiltration of inflammatory cells. Conversely, a deficiency in EP3 expression enhanced the edema associated with allergic inflammation in skin [67].

The anti-inflammatory effect of EP3 in allergic skin reactions was postulated to be due to EP3 coupled with Gi because the cAMP decrease mediated by Gi occurs at lower agonist concentrations than Gs or Gq signaling [67]. In general, Gi activation by the murine EP3 receptor isoforms

are the most investigated and likely the most common. There are, however, several novel associations of EP3 other than in Gi/Gs signaling, although the extent to which this occurs in epidermal cells has not been well studied [65]. In CHO cells, activation of the three mouse EP3 isoforms causes intracellular Ca^{2+} mobilization through the $G\beta\gamma$ subunit of the Gi protein. This may augment cAMP synthesis resulting from activation of Gs by other receptors, including EP2 [65]. This contributes to the complexity of understanding the function of the EP3 isoforms.

The reason for the discrepancy between the various studies on the role of the EP3 receptor in inflammation is not clear although differences in models (pharmacological vs. genetic), isoform expression and methods of eliciting inflammation likely play a role. The lack of a role for the EP3 receptor in skin tumorigenesis [24, 70], in which inflammation is generally a driving force, suggests that EP3 is not strongly enough anti-inflammatory or anti-tumorigenic to counteract the pro-tumorigenic activity of the other EP receptors.

5 The EP4 receptor

The EP4 receptor has several structurally unique features that suggest that its activation may result in distinctive biological responses. This receptor has a much longer cytoplasmic tail and has an insertion of 25 amino acids in the third intracellular loop, both of which are involved in coupling to G proteins. Additionally, unlike the other EP receptors, EP4 is rapidly internalized in response to PGE_2 [22]. The deduced transcription initiation site contains motifs usually associated with activation by proinflammatory cytokines such as CCAAT boxes, Sp1, and AP-2 [78]. Understanding the mechanisms and outcomes of EP4 activation are thus of interest in a number of tissues, including skin, and physiological processes.

In localization studies on the EP receptors in mouse skin, although Lee et al. [26] found that the EP4 receptor was virtually undetectable by immunohistochemistry, Tober et al. [27] found EP4 protein expression in the majority of murine epidermal keratinocytes and dermal leukocytes in naive skin. Neumann et al. [23] also found low but detectable EP4 protein by Western blotting. In UV-irradiated skin EP4 protein expression was reported to be undetectable in one report [26], but Tober et al. [27] found acute and chronic UV induced relocalization to the plasma membrane in the more highly differentiated cells of the stratum granulosum, although diffuse cytoplasmic staining was observed throughout the rest of the epidermis.

With regard to a role for EP4 in NMSC, in human SCCs, EP4 mRNA was reported to be significantly upregulated, although it was downregulated in BCCs, compared to normal skin. In murine skin tumors resulting from chronic UV exposure, Lee et al. [26] also observed increased levels of EP4 protein in both papillomas and SCC, although the mRNA levels were not significantly increased. This was corroborated by Tober et al. [27] in UV-elicited murine tumors and by Neumann et al. [23] in papillomas from a DMBA/TPA protocol. When our laboratory evaluated EP receptor expression in mice, we found that both in TPA-treated skin and in papillomas and SCCs, EP4 mRNA was reduced, compared to wild-type skin [79, 80]. It is not clear at this time whether the differences in EP4 mRNA expression reported by others [23, 27] and our laboratory [79, 80] are due to differential activities of UV and TPA. As described in Section 3 above, the Langenbach laboratory has been interested in elucidating the involvement of specific EP receptors in UV-induced apoptosis and tumor development [54–56]. They found that both EP2 and EP4 were responsible for the anti-apoptotic effects of PGE_2 via regulating pBad levels, following UV irradiation [56]. However, differences were seen in other downstream events. EP2 receptor activation was found to upregulate survivin expression via an EGFR/STAT3 pathway that likely contributes to, or is responsible for, the anti-apoptotic activity of EP2. Activation of EP4, however, did not increase STAT3 activation or survivin levels [60]. Although the mechanism/pathway by which EP4 exerts its anti-apoptotic activity has not yet been elucidated in keratinocytes, the EP4 receptor has strong anti-apoptotic effects in glomerular epithelial cells via activation of AKT [81]; it is likely that the AKT survival pathway is operative in the epidermis as well [26].

Studies in other organ systems led to the suggestion that EP4 may actively contribute to skin cancer. Mutoh et al. [82] reported that EP4 null mice treated with the carcinogen azoxymethane reduced the development of aberrant crypt foci to 56% of the wild-type level. Additionally, administration of the EP4 receptor antagonist ONO-AE2-227 also reduced formation of aberrant crypts as well as intestinal polyps in the Min mouse [82]. EP4 receptor antagonists have also been used to show that EP4 inhibits experimental metastasis of murine mammary tumor cells [83].

Based on these reports, our laboratory has been investigating the effect of EP4 overexpression on murine skin tumor development. Transgenic mice that overexpress EP4 under the control of the BK5 promoter (BK5.EP4 mice) were generated on the FVB background. At the macroscopic level, the skins of these mice are essentially identical to wild-type skin, although the hair cycles are slightly different. When subjected to the two-stage DMBA/TPA protocol, the BK5.EP4 mice develop many more tumors than wild-type mice. Additionally most of the tumors in the transgenic mice were SCC while in wild-type mice the majority were benign papillomas. This

pattern of tumor development resembles that seen in the EP1 transgenic mice [30] except that there was a greater latency to tumor development. Because TPA treatment of the BK5.EP4 and wild-type mice did not produce pronounced differences in epidermal proliferation or apoptosis, which are events usually associated with tumor promotion, the effect of DMBA alone was investigated. Using a single application of DMBA, a significant number of SCCs developed in the BK5.EP4 mice but not wild-type mice. Macroscopically, it was apparent that DMBA caused sloughing of the epidermis of the transgenic but not wild-type mice, and this was associated with an increase in apoptosis. This was followed by a significant increase in keratinocyte proliferation in the transgenic skin as well as a robust angiogenic response, consistent with regenerative hyperplasia. Studies on expression of enzymes involved in metabolizing DMBA, CYP1A1 and CYP1B1, and stem cell numbers (CD34 and α6 integrin positive cells) showed no difference in these parameters. Additionally, ongoing studies are showing that UV exposure also produces a sloughing and angiogenic response in the transgenic, but not wild-type, mice (unpublished data), suggesting that the EP4 transgenic epidermis has a much lower apoptosis threshold than wild-type mice. The basis for this is currently under study. It is clear, however, that the EP4 receptor contributes to skin tumor development and appears to be particularly important in the development of malignant (SCC) rather than benign (papillomas) tumors [79, 80] (and unpublished data).

The involvement of EP4 in UV-induced dermal inflammation has also been studied using genetic and pharmacological approaches. Kabashima et al. [84] reported that edema, inflammatory cell infiltration, and local blood flow were reduced in both EP2 and EP4 null mice. Interestingly, EP4 mediates an anti-inflammatory response in macrophages and does so through an EP4 (but not EP2) interacting protein, EP4 receptor-associated protein (EPRAP); the complex also interacts with NFκB [85]. Whether EPRAP is expressed in other cell types is unknown. Recently, however, EP4 receptor activity in skin was shown to mediate UV-induced systemic immunosuppression; EP4 antagonist treatment downregulated UV-induced expression of receptor-activator-of-NFκB-ligand in keratinocytes which elevates the number of regulatory T cells [86].

It is of interest that of the events elicited by the EP2 and EP4 receptors, some are the same while others are significantly different. Both EP2 and EP4 have anti-apoptotic activity against UV exposure [56] and overexpression of each promotes skin tumor development [44, 79, 80]. In human adipocytes and breast cancer cells, both EP2 and EP4 upregulate aromatase expression and do so through activation of the cAMP/PKA/CREB pathway [87]. However, EP2,

but not EP4, induces survivin in keratinocytes [60]. It is likely that the similarities between EP2 and EP4 are due to both receptors signaling via the cAMP-PKA pathway; the differences are likely due to observations that EP4, but not EP2, also activates the PI3K pathway that can lead to AKT activation as well as ERK activation (Fig. 4) [88]. It has also been shown that under equal expression levels, EP4 activation results in significantly less cAMP production than EP2 activation [51]. Both the EP2 and EP4 receptors couple to the stimulatory guanine tripeptide Gαs and via this linkage activate adenylate cyclase. The weaker stimulation of cAMP production by the EP4 receptor could also be due to its rapid desensitization, but signaling via PI3K is robust, suggesting that other factors are involved [22]. In this regard, EP4, but not EP2, can couple with a pertussis toxin-sensitive inhibitory G-protein (Gαi) that leads to activation of PI3K signaling [89]. Furthermore, EP4 activation of PI3K results in inhibition of PKA, which is activated by EP2. Additionally, although the transcription factor CREB is phosphorylated on serine 133 following either EP2 or EP4 activation, this phosphorylation is mediated through different signaling pathways, i.e., via PKA for EP2 and via AKT for EP4 [52]. The extent to which these signaling pathways are differentially activated will also depend on the level of PGE2 because the affinity of EP2 for PGE2 is considerably lower than that of EP4 [65]. Clearly, the biological outcomes of EP2 and EP4 activation are very context dependent, involving levels of expression of the EP (and other interacting) receptors, ligand, as well as cross-talk among the EP and other receptors.

As described in Section 3, we showed that the EP2 receptor induces COX-2 in murine keratinocytes in a cAMP
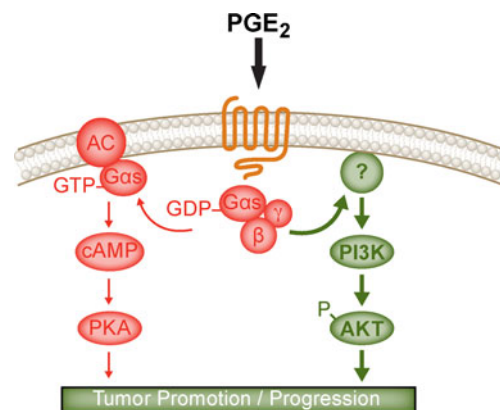

Fig. 4 EP4 signaling in keratinocytes. Similar to the EP2 receptor, ligand activation of EP4 also leads to activation of adenylate cyclase (AC) with subsequent formation of cAMP and activation of the transcription factor CREB, although the extent of cAMP formation is significantly less than that resulting from EP2 activation. Although the mechanisms are not understood, activation of the EP4 receptor causes activation of the PI3K and AKT pathway. Thus, the biological outcomes of EP2 vs. EP4 activation are somewhat different, although both contribute to the development of skin cancer

dependent manner [49]. Regan [22] reported that EP4 activation could also induce COX-2, in this case through a PI3K/AKT pathway leading to an inhibitory phosphorylation of GSK-3α which is also inhibited when PKA is activated, followed by translocation of cytosolic β-catenin into the nucleus. In the nucleus, β-catenin interacts with the Tcf/Lef family of transcription factors that results in induction of COX-2. Thus, both EP2 and EP4 signaling induce COX-2, although different mechanisms are involved [22]; as has been previously mentioned, it is possible that the constitutive upregulation of COX-2 observed in many epithelial tumors is due to a positive feedback loop involving COX-2 and the EP2/EP4 receptors [49, 56].

PI3K activation by EP4 also leads to the phosphorylation (activation) of ERK1/2, which induces early growth response factor-1 (EGR-1). EGR-1 regulates at least several genes involved in proliferation and inflammation, including TNF-α, PGE$_2$ synthase and cyclin D1, supporting a role for the EP4 receptor in neoplastic processes [88].

Interestingly, EP4 receptor expression was shown to be downregulated by a number of cancer chemopreventive agents, including troglitazone, sulindac sulfide and indomethacin in human glioblastoma cell lines [90]. In melanoma cells, the anti-carcinogenic alkaloid berberine was reported to repress expression of EP2, EP4, and COX-2 through inhibition of NFκB activation [91]. These recent studies indicate that new mechanisms of action may be ascribed to a number of chemopreventive agents. Future studies in this area may identify additional agents that are effective in either repressing EP4 expression or blocking its activity. Given the pro-tumorigenic role for EP4, this could offer new approaches to the prevention of NMSC.

6 Conclusions

In this review, we provide evidence from several laboratories that the EP1, EP2, and EP4 receptors mediate the tumor promoting activity of PGE$_2$ in NMSC. This information offers possibilities for preventing NMSC development and for potentially intervening in the progression of these tumors. Elucidation of the signaling pathways activated by these receptors also illuminates possible targets for prevention/intervention. Further studies are needed to validate this approach because these signaling pathways are utilized in normal keratinocyte proliferation, differentiation and cell-cell interactions. However, it has been well-demonstrated that inhibiting PI3K/AKT, MAPK or c-Src signaling pathways early in epithelial neoplasia results in reduced keratinocyte proliferation and survival, leading to decreased tumor formation [92].

The interaction of the EP1, EP2 and EP4 receptors with EGFR raises some interesting questions and options, and is very significant because EGFR-mediated signaling has also been implicated in proliferation, survival, invasion, angiogenesis and metastases, all of which are associated with tumor development [16]. Because of similarities between EGFR and other growth factor receptor tyrosine kinases (RTKs), one or more of the EP receptors may interact with other RTKs. While this muddies our understanding of the pathways by which the EP receptors carry out their function, it also supports the hypothesis that tumor promotion is characterized by, and is due to, a chronic state of aberrant signaling through numerous pathways [10, 49].

The approaches taken so far in inhibiting EP receptor activity have lacked cell-type specificity, i.e., whole animal knockouts or treatment of skin with inhibitors have been used. Because this is likely to affect other cell types such as stromal, immune, or inflammatory cells, it confounds our understanding of the role of the EP receptors in keratinocytes, in terms of both normal function and NMSC development. Studies using targeted ablation *in vivo* are needed to definitively establish the function of each EP receptor in keratinocyte behavior.

Additional studies are also needed on understanding how the EP receptors are regulated. As previously described, tumor promoting agents such as phorbol esters and UV can upregulate several of the EP receptors. Elucidating the signaling pathways and transcription factors involved in this upregulation would be potentially useful in designing approaches to regulating keratinocyte behavior and in understanding how important the level of expression of each EP receptor is to normal and pathological keratinocyte physiology.

The significance of elucidating the regulation and function of the EP receptors in NMSC lies in the recognition that NMSC is the most common cancer affecting humans—NMSC accounts for 40% of all newly diagnosed cancers and the incidence is rising [93]. New strategies and targets are needed to prevent or intervene in NMSC; inhibition of the EP receptors has great potential as an effective new approach to prevention of NMSC as well as other epithelial cancers.

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