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The role of EP1 receptor for prostaglandin E_2 in mouse skin carcinogenesis

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The role of EP1 receptor for prostaglandin E_2 in mouse skin carcinogenesis

by

In Ok Surh, B.S.; M.S.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

May 2009

Acknowledgements

I would like to take this opportunity to thank the people who have helped me in the completion of this work. I could not have done it without them.

I want to acknowledge my appreciation especially to Dr. Susan M. Fischer for her guidance, advice, support and encouragement. I would also like to extend appreciation to my committee members, Dr. Andrew P. Butler, Dr. Shawn B. Bratton, Dr. Edward M. Mills and Dr. Andrea C. Gore for serving on my committee and for their valuable guidance throughout my entire graduate study.

I also would like to express my sincere appreciation to Dr. Joyce E. Rundhaug and Dr. Melissa Simper for their kind assistance and fruitful discussion. I thank all my past and present labmates including Amy Pavone, Carol Mikulec, Jennifer Colby, Eun Jung Kim, Guobin He, You Me Sung and thank all my fellow co-workers in the University of Texas M.D. Anderson Cancer Center-Science Park Research Division for their assistance and for being such wonderful friends.

Finally, I would like to extend my gratitude to all my relatives and friends for their constant concern and support.

The role of EP1 receptor for prostaglandin E_2 in mouse skin carcinogenesis

Publication No._____

In Ok Surh, Ph.D.

The University of Texas at Austin, 2009

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Prostaglandin E_2 (PGE_2), the most abundant prostaglandin in mouse skin, has been shown to promote skin tumor development. EP1 is one of four PGE_2 receptors. EP1 mRNA levels analyzed by a quantitative real-time polymerase chain reaction were increased after treatments of 12-O-tetradecanoylphorbol 13-acetate (TPA) or ultraviolet light on skin as well as in 7,12 dimethylbenz[a]anthracene (DMBA)/TPA or UV-induced skin tumors. To determine whether the EP1 receptor levels affect skin tumor development, we generated BK5.EP1 transgenic mice which overexpress EP1 in the basal layer of the epidermis. The skins of these mice are histologically indistinguishable from wild type mice. To determine the role of EP1 in skin tumor development, a DMBA/TPA skin carcinogenesis protocol was used. EP1 transgenic mice had a reduced tumor multiplicity and a reduced tumor incidence compared to wild type mice, but had a higher papilloma to carcinoma conversion rate. In a DMBA-only skin carcinogenesis protocol, EP1 transgenic mice developed more tumors than wild type mice. The effect of EP1 on cell proliferation was measured in vivo. After TPA treatment, cell proliferation

was induced in both EP1 transgenic mice and wild type mice to a similar extent. However, 5 days after DMBA treatment, there were about 2-fold more proliferating cells in the basal layer of the epidermis of EP1 transgenic mouse skin than in wild type mice. To confirm that the enhanced tumor formation in transgenic mice is in fact PGE_2 dependent, EP1 transgenic mice were administered the selective cyclooxygenase-2 inhibitor Celecoxib or a control diet starting 1 week before DMBA treatment. Surprisingly, there was no lesion development on mice that were fed Celecoxib. Histological sections of skin from Celecoxib-fed mice showed a fairly normal skin histology 2 weeks after DMBA treatment compared to the pronounced pseudocarcinomatous hyperplasia observed in control diet mice. Therefore, it can be concluded that EP1 signaling increases PGE_2 production through COX-2 induction and promotes tumor development.

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List of Abbreviations

5-bromo-2'-deoxyuridine	BrdU
7,12-dimethylbenz[a]anthracene	DMBA
12-O-tetradecanoylphorbol 13-acetate	TPA
15-hydroxyprostaglandin dehydrogenase	PGDH
Azoxymethane	AOM
benzo[a]pyrene	B[a]P
Bovine keratin	BK
cAMP-dependent protein kinase	PKA
cAMP-response element binding protein	CREB
Cyclooxygenase	COX
Cytochrome P450	CYP
Diacylglycerol	DAG
Epidermal growth factor	EGF
Epidermal growth factor receptor	EGFR
Glutathione	GSH
Granulocyte/macrophage – colony stimulating factor	GM-CSF
Inositol 1,4,5-*tris*phosphate	IP3
Intercellular adhesion molecule	ICAM
Interleukin	IL
Keratin	K
Matrix metalloproteinases	MMPs
Neuronal apoptosis inhibitory protein	NAIP
Nitric oxide	NO

Nitric oxide synthase	NOS
Non-steroidal anti-inflammatory drugs	NSAIDs
Proliferating cell nuclear antigen	PCNA
Phospholipase C	PLC
Platelet derived growth factor	PDGF
Prostaglandin	PG
Prostaglandin E_2 synthases	PGESs
Protein kinase C	PKC
Signal transducers and activators of transcription	STAT
Squamous cell carcinoma	SCC
Thromboxane A_2	TXA_2
Transforming growth factor-β	TGF-β
Tumor necrosis factor-α	TNF-α
Ultraviolet	UV
Vascular endothelial growth factor	VEGF

Chapter 1 Introduction and literature review

1.1 SKIN CARCINOGENESIS

1.1.1 Skin

The skin is the largest organ in the body, forming a protective barrier against the environment in terms of ultraviolet (UV) rays, scratches, wounds and microbes and dehydration. The skin is separated into two parts by the basement membrane under which is the dermis, the connective tissue. Hair follicles, sweat glands, sebaceous glands and blood vessels are located in the dermis (Figure 1-1). Above the basement membrane is the epidermis which is composed primarily of keratinocytes (95%). Melanocytes, Langerhans cells (dendritic cells) and Merkel cells (sensory receptors) are also present in the epidermis (Janes, Lowell et al. 2002). The bottom layer of the epidermis is called the basal layer which is the only layer that proliferates and expresses keratin 5 (K5) and K14 (Figure 1-2) (Fuchs and Cleveland 1998). K1 and K10 are expressed in the spinous layer and also used as early differentiation markers. Cytoskeletal keratin filaments are strengthened to provide mechanical strength against physical trauma (Segre 2006). Cells in the granular layer express involucrin, loricrin, filaggrin and keratinocyte transglutaminase and contain lamella granules which are filled with lipids that extrude to the cornified envelop (Yuspa 1994). These produce a water-impermeable seal that prevents the escape of fluid (Alonso and Fuchs 2003). Filaggrin aggregates keratin filaments into tight bundles and promotes collapse of the cell into a flat shape. The cornified layer is composed of terminally differentiated dead, flat and anucleated keratinocytes (corneocytes) (Candi, Schmidt et al. 2005). In the cornified envelope, involucrin is crosslinked with other proteins and also forms an exterior surface with

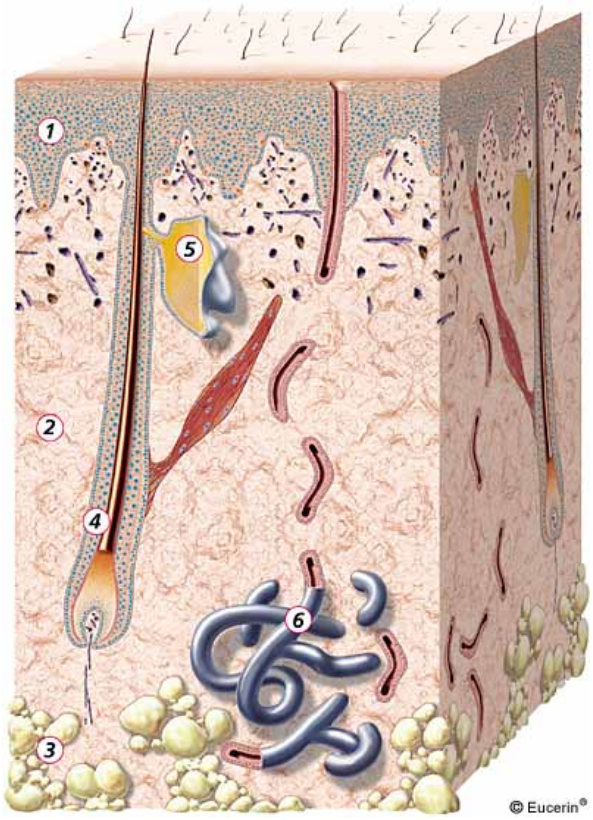

Figure 1-1. Human skin structure. Epidermis (1) and Dermis (2) are separated by the basement membrane. Epidermis is mostly keratinocytes. In the dermis, hair follicle (4), sebaceous gland (5), sweat gland (6) and blood vessels are present. [Fig taken from http://www.nmsl.chem.ccu.edu.tw/tea/SKIN_910721.htm]

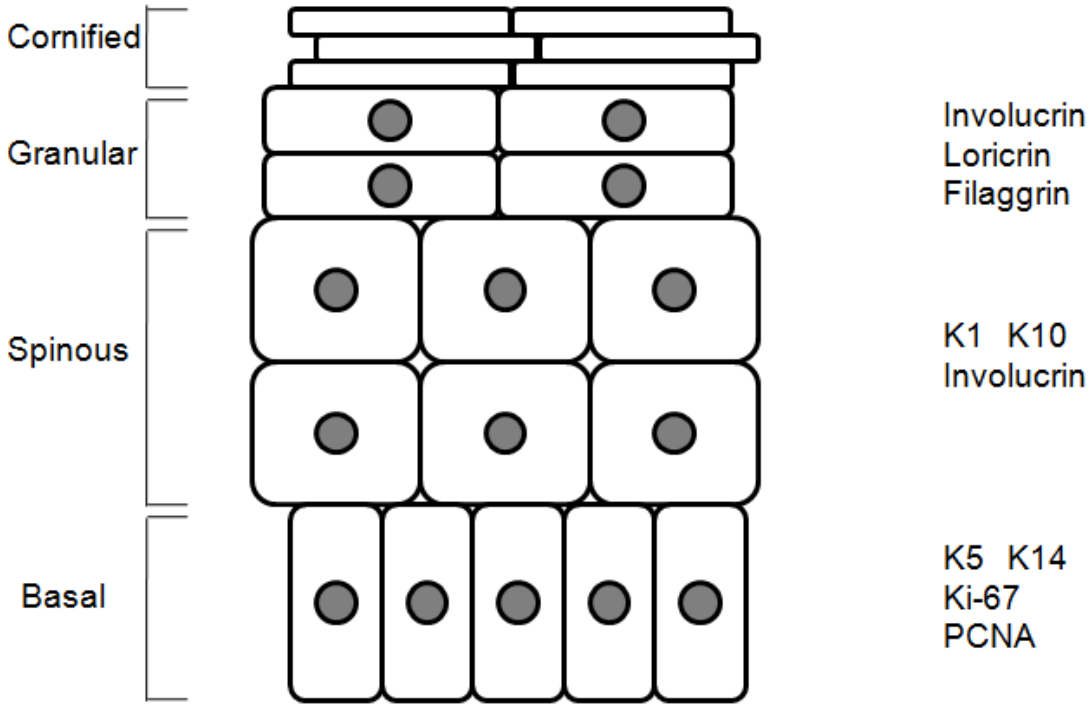

Figure 1-2. Human skin epidermis. Name of each layer and specific marker are shown. The bottom layer of the epidermis is the basal layer which expresses K5 and K14 and the only layer that proliferates. Ki-67 and PCNA (proliferating cell nuclear antigen) are proliferation markers. As keratinocytes move outerward, they form a cornified envelop and become flattened.

externalized lipids, mostly ceramides. Loricrin is a main structural protein in the cornified envelope (70~85 % of the total protein). In the cornified cells, fillagrin is degraded and hydrophilic amino acids contributes to retention of water and flexibility (Candi, Schmidt et al. 2005). Figure 3 shows human skin, but in mouse skin there are no sweat glands in the dermis and the epidermis is normally only two layers of cells and the suprabasal cells are relatively flat (Janes, Lowell et al. 2002).

In normal human skin, cyclooxygenase -1 (COX-1) is expressed throughout the entire epidermis while COX-2 is slightly expressed only in suprabasal layers. In normal mouse skin, COX-2 is not expressed. COX-2 is important in normal skin physiology. In COX-2 deficient mouse epidermis, the proliferation index is about 30% reduced as compared to wild type mice (Tiano, Loftin et al. 2002). COX-2 deficient mice show accelerated epidermal differentiation. Keratin 5-driven COX-2 transgenic mice have sparse hair due to delayed hair follicle development, hyperplastic epidermis and sebaceous gland, and impaired differentiation (Lee, Mukhtar et al. 2003).

1.1.2 Two-stage mouse skin carcinogenesis

Multistage skin carcinogenesis is divided into three stages; initiation, promotion and progression (Figure 1-3). Initiation is achieved by a subcarcinogenic dose of a carcinogen. There is no morphological change in the epidermis following initiation. During initiation, a genetic mutation occurs following interaction of a chemical carcinogen (7,12-dimethylbenz[a]anthracene (DMBA), 3-methylcholanthrene, etc) or physical carcinogen (UV, X-rays, etc) and DNA in epidermal cells. To fix the mutation, DNA synthesis is required (Pitot and Dragan 1991). Therefore, basal cells and epidermal stem cells in hair follicles are targets for initiation. This initiation is irreversible.

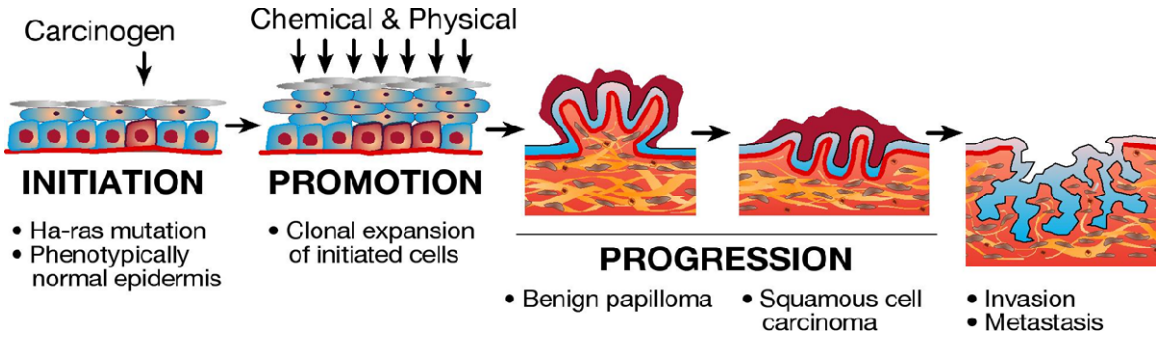

Figure 1-3. Multistage skin carcinogenesis model. In the initiation, genetic mutation occurs. In the promotion, initiated cells undergo clonal expansion. Because tumor promotion is a reversible step, repetitive application of a promoter is required. In the progression stage, benign papillomas undergo additional genetic changes and become squamous cell carcinomas (SCCs) which are invasive and metastatic.

In skin, the two most common experimental initiating agents are chemical carcinogens and UV light. DMBA is metabolized by CYP1B1 to reactive intermediates (Buters, Sakai et al. 1999). More than 90% of tumors initiated with DMBA have A182→ T transversion at codon 61 of c-Ha-ras (Quintanilla, Brown et al. 1986). This mutation causes Gln → Leu (Sekiya, Fushimi et al. 1984) and the mutation increases the transforming activity of c-H-ras (Der, Finkel et al. 1986).

UV rays are divided into three layers based on wavelength. UVC is 200-280nm and absorbed by the ozone layer. UVA is 320-400nm and 90-99% of the sunlight that reaches the earth. UVB is 280-320nm and is 1-10% of sunlight. Both UVA and UVB cause cyclobutane-pyrimidine dimers but only UVB induces pyrimidine (6-4) pyrimidones (Cadet, Sage et al. 2005). These photoproducts cause mutations by C → T transversion. UV radiation induce C→T transversion in p53, the tumor suppressor gene (Ziegler, Leffell et al. 1993). These photoproducts can be repaired by nucleotide excision repair (NER). As a result, Xeroderma pigmentosum patients who are genetically deficient in one of the genes in NER are sensitive to UV and develop multiple skin cancers (Cleaver 2005). Both UVA and UVB generate oxidative stress and cause 8-oxo-7,8-dihydroxyguanine formation which causes G → T transversion (Cadet, Sage et al. 2005).

Initiated cells undergo clonal expansion during promotion. Tumor promoters are not mutagenic but act via epigenetic mechanisms, inducing inflammation (edema, erythema, leukocyte infiltration of dermis), producing free radicals from polymorphonuclear leukocytes, increasing mitotic activity in the basal layer of the epidermis, producing more prostaglandin (PG) synthesis, decreasing the activities of superoxide dismutase and catalase and inducing ornithine decarboxylase, the rate-limiting enzyme in polyamine biosynthesis needed for DNA synthesis. This stage is a reversible step early on and requires repetitive application to develop a tumor but a tumor promoter

alone does not induce a tumor. In the skin, 12-O-tetradecanoylphorbol 13-acetate (TPA), fatty acid methyl esters, anthrones, retinoic acids and UV work as tumor promoters (DiGiovanni 1992).

Protein kinase C (PKC) is a serine/threonine kinase and receptor for the tumor promoter TPA. PKC isoforms are classified into three groups; classical cPKC (α, βI, βII, and γ) are calcium-sensitive and need diacylglycerol (DAG) or phorbol esters for activation, novel nPKC (δ, ε, η, θ), are calcium-independent and need DAG or phorbol esters for activation, while atypical aPKC (ζ and ι/λ) isoforms are calcium independent and are not activated by TPA. All isomers require phosphatidylserine for activation. In response to TPA, PKC is activated and translocated from the cytosol to the membrane. Also, TPA or UV treatment releases the intramolecular inhibitory pseudosubstrate domain of PKC and leaves an active PKC catalytic domain (Breitkreutz, Braiman-Wiksman et al. 2007).

PKCα is expressed abundantly in the skin. Transgenic mice that overexpress PKCα have increased inflammatory responses including increased epidermal thickness, edema, inflammatory cytokines and chemokines. However, PKCα does not affect tumor promotion (Wang and Smart 1999). PKCδ is expressed in basal cells and is involved in early onset of differentiation. Interestingly, transgenic mice which overexpress PKCδ are resistant to DMBA/TPA-induced skin cancer development (Reddig, Dreckschmidt et al. 1999) but not resistant to UV-induced skin cancer development (Aziz, Wheeler et al. 2006). PKCη is expressed in the suprabasal layer. Overexpression of PKCη in human keratinocytes induces differentiation markers (involucrin and transglutaminase-1) (Ueda, Ohno et al. 1996). PKCη deficient mice are more susceptible to skin tumor formation (Chida, Hara et al. 2003). PKCε is expressed in the basal keratinocytes. Transgenic mice which overexpress PKCε show epidermal

hyperproliferation. Interestingly, these mice produce fewer papillomas but the progression rate to squamous cell carcinoma (SCC) was increased (Reddig, Dreckschmidt et al. 2000).

Benign papillomas are not invasive. These benign tumors undergo additional genetic changes during progression and become SCCs which break through the basement membrane, grow rapidly, are invasive and metastatic and have a blood supply into the tumor (DiGiovanni 1992).

1.1.3 Inflammation and cancer

Inflammation is a tissue change in response to injury. Characteristics of inflammation are vasodilation, edema, clotting, migration of innate immune cells and swelling of tissue cells (Guyton 1996). Inflammation is one of the defense mechanisms used to protect tissue from damage. However, if the inflammation is not resolved completely and chronic inflammation takes place, it is no longer beneficial to the host (Katzung 1995). Chronic inflammation increases risk of cancer development in bladder, stomach, liver, colon and ovary (Moore, Owens et al. 1999). Reactive oxygen species generation during inflammation is important in initiation. During tumor promotion and tumor progression, cell proliferation, apoptosis and angiogenesis are affected by inflammation (Kundu and Surh 2008). Inflammatory molecules that are important in the tumor development are cytokines, nitric oxide, NF-κB and COX-2.

In the skin, interleukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α) are important cytokines. IL-1α mRNA was increased in skin after application of TPA on skin (Lee, Fischer et al. 1993). Intradermal injection of IL-1α increased vascular permeability, cell proliferation and inflammatory cell infiltration in the dermis. Injection of antibody aginst IL-1α prevented TPA induced vascular permeability, cell proliferation and

inflammatory cell infiltration in the dermis (Lee, Lockniskar et al. 1994). TNF-α is one of the important cytokines in tumorigenesis. Thus, TNF-α null mice and TNF-α receptor null mice are resistant to skin tumor development (Moore, Owens et al. 1999; Arnott, Scott et al. 2004). IL-1 and/or TNF- α can induce a variety of cytokines including IL-6, IL-8/neutrophil activating protein (NAP)-1, granulocyte/macrophage – colony stimulating factor (GM-CSF), G-CSF, and monocyte chemotactic and activating factor in the skin (Kupper 1990). Chemokines are induced by inflammatory cytokines and attract leukocytes to the inflammation site. When melanoma cells which overexpress chemokines (CXCL-1, CXCL-2 or CXCL-3) were injected subcutaneously into nude mice, more than 90% efficiency of tumor formation were observed compared with 7% in controls (Owen, Strieter et al. 1997). CXCL-12 increased colorectal tumor cell migration (Kollmar, Rupertus et al. 2007).

Nitric oxide (NO) is produced by nitric oxide synthase (NOS). NO is important in vascular tone, neurotransmission and platelet aggregation. NO contributes to tumor development by stimulating cell proliferation and inducing DNA damage. Inducible NOS (iNOS) increased in human breast, prostate and gynecological cancers (Thomsen, Lawton et al. 1994; Thomsen, Miles et al. 1995; Aaltoma, Lipponen et al. 2001). In mouse skin, TPA treatment induces iNOS (Chun, Cha et al. 2004). In prostate cancer, iNOS expression level positively correlates with cell proliferation (Aaltoma, Lipponen et al. 2001).

NF-κB is a transcription factor and induces inflammatory (COX-2, iNOS, and TNF-α), anti-apoptotic (XIAP, Bcl-2), cell cycle regulatory (cyclin D1) and proangiogenic (vascular endothelial growth factor (VEGF) and angiopoietin) factors or inhibits apoptosis-inducing genes (p53 and Bad) (Kundu and Surh 2008). Deletion of

IKKβ, a kinase that release free NF- κB, in intestinal epithelial cells decreased tumor formation significantly (Greten, Eckmann et al. 2004).

1.2 COX

1.2.1 COX

Cyclooxygenases are 72 kDa enzymes that convert arachidonic acid to PGG_2 then to PGH_2 (Howe, Subbaramaiah et al. 2001). PGH_2 is further metabolized to produce various prostaglandin isomers (PGD_2, PGE_2, PGI_2, thromboxane A_2 (TXA_2) and $PGF_{2\alpha}$) by specific isomerases (Figure 1-4) (Simmons 2004).

There are two isoforms of COX. The COX-1 gene is on chromosome 9. COX-1, a constitutive COX, is expressed in most cells and tissues and maintains normal physiological functions. In the stomach, prostaglandins have protective roles by decreasing gastric secretion and increasing mucus production and by increasing bicarbonate in the duodenum. In the kidneys, vasodilator prostaglandins (PGD_2, PGE_2, and PGI_2) regulate renal blood flow and renal vascular beds (Sanghi, MacLaughlin et al. 2006). COX-1 is also expressed in neurons in the brain and in the uterus during pregnancy. Because platelets do not have nuclei, COX-2 cannot be induced. Therefore, COX-1 is important in platelets and leads to synthesis of TXA_2 that induces platelet aggregation, vasoconstriction and vascular proliferation (DuBois, Abramson et al. 1998; Furstenberger, Krieg et al. 2006).

COX-2, the inducible COX, is an immediate-early response gene that is not expressed in most normal tissues but is induced by mitogens, hypoxia, growth factors (epidermal growth factor (EGF), platelet derived growth factor (PDGF)), hormones, tumor promoters, polysaccharides, PGs and inflammatory stimuli, such as endotoxin,

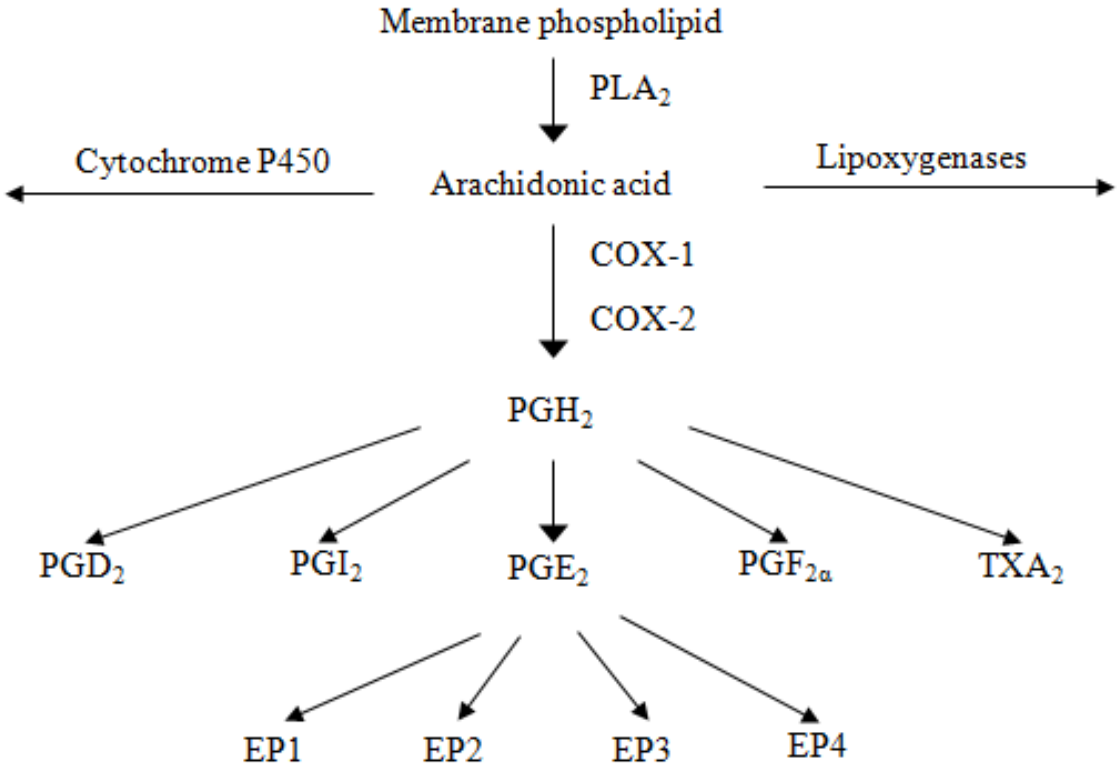

Figure 1-4. Arachidonic acid metabolism. Arachidonic acid is cleaved from phospholipids by phospholipase A_2 (PLA_2) and metabolized by cytochrome P450, lipoxygenases and cyclooxygenases. Cyclooxygenases convert arachidonic acid into PGH2 which is further metabolized to PGD_2, PGI_2, PGE_2, $PGF_{2\alpha}$ and TXA_2 by specific isomerases. PGE_2 binds four G protein coupled receptors (EP1, EP2, EP3, and EP4)

cytokines (transforming growth factor-β (TGF-β), IL-1β, IL-2, interferon-γ, and TNF-α) (Breyer, Bagdassarian et al. 2001; Wang, Mann et al. 2005; Meric, Rottey et al. 2006). The COX-2 gene is on chromosome 1. COX-2 is important in inflammation, ovulation, kidney function, wound healing etc. In several types of tissues, COX-2 is also constitutively expressed; brain, kidney, bones, pancreatic β cells, spinal cord, ovaries, uterus, tracheal epithelial cells, small intestine and testicles (Sanghi, MacLaughlin et al. 2006; Iezzi, Ferri et al. 2007).

COX-2 is important in maintaining vascular function. PGI_2 has both a vasodilatory function and an anti-platelet aggregation function (Sanghi, MacLaughlin et al. 2006). By comparison, COX-1 produces PGI_2 for physiological homeostasis, but stress-induced COX-2 produces PGI_2 to protect vessel walls from injury (Iezzi, Ferri et al. 2007). COX-2 is important in cardioprotection. PGI_2 is the predominant COX-2 product in endothelium. PGI_2 inhibits platelet aggregation, induces vasodilation and inhibits proliferation of vascular smooth muscle cells (Fitzgerald 2004). Thus, it opposes the action of TXA_2, which is produced by platelets.

In the kidneys, prostaglandin levels are 1,000 fold higher than that of circulating levels. Prostaglandins are important in kidney physiology and modulate glomerular hemodynamics, tubular reabsorption of salt and water and rennin secretion (Sanghi, MacLaughlin et al. 2006). COX-2 is also important in the female reproductive system; specifically, in the rupture of follicle and implantation of the embryo (DuBois, Abramson et al. 1998). Therefore, COX-2 deficient mice die early due to renal problems and experience several difficulties in the female reproductive system: ovulation, fertilization and implantation (Dinchuk, Car et al. 1995; Morham, Langenbach et al. 1995; Lim, Paria et al. 1997). COX-2 is also important with regard to pain. COX-2 inhibitors reduce

inflammation and pain-related behavior in chemically-induced arthritis and inflammation in rats (Seibert, Zhang et al. 1994; Laird, Herrero et al. 1997).

COX-2 is important in inflammatory responses. Proinflammatory agents (IL-1, TNF-α, lipopolysaccharide) induce COX-2 while anti-inflammatory cytokines (IL-4, and IL-13) decrease COX-2 levels (DuBois, Abramson et al. 1998). In a clinical study, celecoxib, a COX-2 inhibitor, improved rheumatoid arthritis conditions including morning stiffness and painful and tender joints (Lipsky and Isakson 1997).

1.2.2 Importance of COX in tumor development

There are extensive epidemiological studies that show that non-steroidal anti-inflammatory drugs (NSAIDs), which are COX inhibitors, reduce risk of several types of cancer including colorectal (Brown 2005), esophageal, lung, breast, bladder and prostate (Pereg 2005; Rigas and Kashfi 2005). COX-2 is highly expressed in human cancers of the colon, gastric (Ristimaki, Honkanen et al. 1997), cervix, liver, pancreas (Howe, Subbaramaiah et al. 2001), esophagus, breast, bladder, lung and skin (Kagoura 2001; Subbaramaiah 2003).

The importance of COX-2 in cancer has been studied with experimental animal models as well as in clinical studies. When $APC^{\Delta 716}$ mice or Min mice, models for human familial adenomatous polyposis, were crossed with COX-2 knockout mice, the number of polyps and size of the polyps were significantly reduced (Oshima, Dinchuk et al. 1996; Chulada, Thompson et al. 2000). In a clinical study with familial adenoma polyposis patients, the administration of celecoxib for six months significantly reduced the number of colorectal polyps (Steinbach, Lynch et al. 2000). In three clinical studies with patients who had adenomas removed, celecoxib or rofecoxib reduced new adenoma formation (Arber, Eagle et al. 2006; Baron, Sandler et al. 2006; Bertagnolli, Eagle et al. 2006). In

breast cancer and lung cancer, COX-2 expression levels correlate with poor prognosis (Ristimaki, Sivula et al. 2002; Boland, Butt et al. 2004; Su, Shih et al. 2004). Administration of the NSAIDs aspirin, indomethacin or celecoxb reduced mammary tumor formation in rats (Howe, Subbaramaiah et al. 2001). Transgenic mice that overexpress COX-2 in mammary glands, developed mammary tumors (Liu, Chang et al. 2001). In the skin, COX-2 is induced in UVB-exposed human keratinocytes, UVB irradiated human skin, human SCC, UVB irradiated mouse skin, UVB induced mouse papillomas and SCC, and DMBA/TPA induced mouse papillomas and SCC (Buckman, Gresham et al. 1998; Muller-Decker, Kopp-Schneider et al. 1998; Athar, An et al. 2001; An, Athar et al. 2002). In a DMBA/TPA skin carcinogenesis model, both COX-1 null mice and COX-2 null mice had reduced skin tumor development (Tiano, Loftin et al. 2002). Application of COX inhibitors on skin reduced DMBA/TPA induced tumor formation as well as UV-induced tumor formation (Muller-Decker, Kopp-Schneider et al. 1998; Fischer, Lo et al. 1999; Pentland, Schoggins et al. 1999). Consistent with these findings, transgenic mice that overexpress COX-2 in the epidermis develop more skin tumors than wild type mice (Muller-Decker, Neufang et al. 2002; Rundhaug, Pavone et al. 2007). Celecoxib, a COX-2 inhibitor, significantly suppressed lung metastasis when colon cancer cells were injected into the paws of mice and reduced fibroblast growth factor-2 induced neovascularization in the rat cornea (Masferrer, Leahy et al. 2000).

1.2.3 Cyclooxygenase inhibitors

Cyclooxygenase inhibitors are used to control pain, fever, and inflammation. The first NSAID to be developed was acetylsalicylic acid (Aspirin, Bayer Corp.). These NSAIDs have side effects on gastrointestinal tissues, inducing hemorrhagic gastric erosions and gastric ulcers, inducing or exacerbating duodenal ulcers, and damaging

distal regions of the small intestine. The causes of these side effects are systemic suppression of prostaglandin synthesis and topical irritation on the epithelium. Acidic NSAIDs migrate to epithelial cells and trap hydrogen ion, reducing hydrophobicity of the gastric mucus, thus allowing gastric acid and pepsin to damage the surface epithelium (Wallace 1997; Wolfe, Lichtenstein et al. 1999). The importance of reduced prostaglandin levels for gastrointestinal side effects of NSAIDs has been well documented. Parenteral or rectal NSAIDs still induced ulcers and PGE_2 administration prevented indomethacin–mediated reduction of gastric blood flow (Wolfe, Lichtenstein et al. 1999). In addition, inhibition of COX-1, not COX-2, is responsible for these side effects. COX-1 is expressed in normal gastrointestinal tissue and endothelial cells. Gastric ulcers in mice had increased COX-2 mRNA and PG but no change in COX-1 mRNA (Wallace 1997). Indomethacin (COX-1/COX-2 inhibitor) reduced PG synthesis but NS-398 (COX-2 specific inhibitor) did not reduce PG synthesis in gastrointestinal tissues. Thus, COX-1 is important in the cytoprotection of gastrointestinal tissue (Kargman, Charleson et al. 1996). The active site of COX-2 is about 20% larger than COX-1 (Smith, DeWitt et al. 2000). Therefore, COX-2 selective inhibitors were developed to reduce gastrointestinal side effects while retaining the same effect as NSAIDs on pain, fever, and inflammation.

The COX-2 selective inhibitors, celecoxib and rofecoxib, had similar effects on rheumatoid arthritis inflammation and pain when compared to traditional NSAIDs (ibuprofen, diclofenac and naproxen) but celecoxib- and rofecoxib- treated patients had less gastrointestinal toxicity (Emery, Zeidler et al. 1999; Bombardier, Laine et al. 2000; Silverstein, Faich et al. 2000). Because COX-2 is upregulated in many tumors, these COX-2 specific inhibitors were studied for chemopreventive efficacy. In mouse models, COX-2 inhibitors showed effective reduction in tumor formation in colon, esophagus,

stomach, pancreas, prostate, tongue, skin and mammary (Oshima, Dinchuk et al. 1996; Fischer, Lo et al. 1999; Harris, Alshafie et al. 2000; Nakatsugi, Ohta et al. 2000; Buttar, Wang et al. 2002; Furukawa, Nishikawa et al. 2003; Rahme, Barkun et al. 2003; Wei, Morimura et al. 2003; Yamamoto, Kitayama et al. 2003; Bardou, Barkun et al. 2004; Hu, Yu et al. 2004; Narayanan, Narayanan et al. 2004). As previously mentioned, familial adenomatous polyposis patients who received celecoxib or rofecoxib had reduced number of colorectal polyps (Steinbach, Lynch et al. 2000; Higuchi, Iwama et al. 2003), however, they also had a higher incidence of cardiovascular side effects (Solomon, McMurray et al. 2005; Baron, Sandler et al. 2006). A possible explanation is a shift in the balance between PGI_2 and TXA_2. Rofecoxib and celecoxib suppressed PGI_2 formation which is the predominant COX-2 product in endothelium. Since TXA_2 is a COX-1 product in platelets, inhibition of COX-2 may increase the activity of TXA_2 by removal of counteracting PGI_2, without altering TXA_2 levels. Thus, platelet aggregation and blood pressure might be increased, which could lead to increased risk of cardiovascular disease (Fitzgerald 2004). Therefore, it is likely that all COX-2 selective inhibitors might have similar cardiovascular side effects.

1.3 PROSTAGLANDIN E_2

1.3.1 Prostaglandin E_2 synthases (PGESs)

PGESs convert PGH_2 to PGE_2. There are three isoforms of prostaglandin E_2 synthases. Cytosolic PGES, cPGES, is 23kDa and glutathione (GSH) is a cofactor for this enzyme. cPGES is constitutively expressed in several tissues and not induced by proinflammatory stimuli. mPGES-1 is a membrane bound form and also needs GSH as a cofactor. Proinflammatory stimuli induce mPGES-1, often along with induction of COX-

2. Increased mPGES-1 expression is observed in several types of cancers. The third isoform, mPGES-2, does not require GSH as a cofactor. mPGES-2 is expressed in the brain, heart, skeletal muscle and kidneys. Unlike mPGES-1, mPGES-2 is expressed in several tissues and not induced during inflammation (Murakami and Kudo 2006). mPGES-1 and mPGES-2 are expressed in the tail skin, back skin, and in DMBA/TPA induced papillomas of NMRI mice (Neumann, Dulsner et al. 2007).

1.3.2 Importance of PGE_2 in tumor development

Among the COX products, PGE_2 levels have been found to be increased in human colon cancer (Rigas, Goldman et al. 1993) and human gastric cancer (Uefuji 2000). Also, PGE_2 is the major arachidonic acid metabolite in cultured mouse skin epidermal cells (Fischer, Baldwin et al. 1988), human normal colorectal mucosa and human colorectal adenoma from familial adenomatous polyposis patients (Yang, Shields et al. 1998) and mouse mammary tissue (Chang 2004). In human keratinocytes, the PGE_2 level is increased after UVB exposure (Buckman, Gresham et al. 1998). There are several studies that show that PGE_2 is important in tumorigenesis. Celecoxib, a COX-2 selective inhibitor, inhibited human head and neck xenograft tumor growth with concomitant inhibition of PGE_2 production by the tumor (Zweifel, Davis et al. 2002). Administration of PGE_2 reversed NSAID-mediated suppression of intestinal tumor formation in Min mice (Hansen-Petrik 2002) and enhanced azoxymethane-induced colon cancer formation in rats (Kawamori, Uchiya et al. 2003). Injection of an antibody against PGE_2 decreased intestinal tumor formation in Min mice (Hansen-Petrik 2002) and tumor growth in a head and neck squamous cell carcinoma xenograft model (Zweifel, Davis et al. 2002). PGE_2 abolished indomethacin–mediated growth inhibition in malignant keratinocytes (Thompson, Gupta et al. 2001).

PGE_2 is known to exert its tumor promoting action through increasing proliferation, decreasing apoptosis, inducing angiogenesis, increasing invasion, and modulating immunosuppression (Wendum 2004; Wang, Mann et al. 2005). In colorectal carcinoma cells, PGE_2 increased cell proliferation and cell motility via phosphatidylinositol-3-kinase (PI3K) (Sheng, Shao et al. 2001). In some tissues, PGE_2 increases cell proliferation through epidermal growth factor receptor (EGFR) via induction of EGFR ligand or shedding of EGFR ligand (Pai, Soreghan et al. 2002; Shao, Lee et al. 2003). In human colon cells, PGE_2 treatment was found to reduce apoptosis through induction of Bcl-2 expression (Sheng, Shao et al. 1998). Treatment with PGE_2 reversed COX-2 inhibitor mediated suppression of capillary-like structure formation of rat aortic endothelial cells (Jones, Wang et al. 1999). PGE_2 increased vascular endothelial growth factor and basic fibroblast growth factor in several different cells (Pai, Szabo et al. 2001; Trompezinski, Pernet et al. 2001; Chang, Liu et al. 2004; Spinella, Rosano et al. 2004; Bradbury, Clarke et al. 2005; Taylor, Kim et al. 2008). Also, PGE_2 promotes endothelial cell adhesion and spreading (Dormond, Bezzi et al. 2002). PGE_2 promotes tumor growth by suppressing the immune system through inhibition of immune regulatory lymphokines, T- and B-cell proliferation and cytotoxic activity of natural killer cells and inducing IL-10 (Dempke, Rie et al. 2001).

1.3.3 Metabolism of PGE_2

NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase (PGDH) oxidizes the 15-hydroxyl group of the prostaglandin molecule into an inactive keto group (Tai, Ensor et al. 2002). PGDH deficient mouse pups have about 2-fold higher PGE_2 levels while TXA_2 levels are similar, compared to wild type mouse pups (Coggins, Latour et al. 2002). As bladder cancer progresses, expression of PGDH decreases and expression of

COX-2 increases, which would raise the level of PGE_2 (Gee, Montoya et al. 2003). Expression of PGDH decreases in Min mouse adenomas, human breast cancer, lung carcinomas, and colon cancer relative to normal tissue (Backlund, Mann et al. 2005).

1.3.4 PGE_2 receptors

PGE_2 binds four subtypes of membrane receptors (EP1, EP2, EP3 and EP4) which are members of the family of seven transmembrane G-protein coupled receptors. Distribution of these receptors is tissue specific (Ushikubi, Hirata et al. 1995; Breyer, Bagdassarian et al. 2001).

EP2 is coupled to Gαs and increases cAMP formation via activation of adenylate cyclase. cAMP binds to regulatory subunits of cAMP-dependent protein kinase (PKA), then the catalytic subunit is released and phosphorylates PKA substrates (Taylor, Kim et al. 2008). PKA phosphorylates cAMP-response element binding protein (CREB) on Ser 133. Increased levels of cAMP translocate CREB co-activators (transducers of regulated CREB activity, TORC) from cytosol to nucleus. Together with Ser133 phosphorylated CREB, TORC facilitates recruitment of transcriptional coactivator CBP or p300 to CREB and increases transcription (Sands and Palmer 2008). PKA also phosphorylates GSK-3 and activates β-catenin (Fujino, West et al. 2002). The binding affinity for EP2 is PGE_1 > PGE_2 > 16,16-dimethyl-PGE_2 (synthetic PGE_2 analog) > 11-deoxy PGE_1 (synthetic PGE_1 analog) >> 1-hydroxy PGE_1 > Butaprost (EP2 selective agonist) > AH-6809 (EP2 antagonist) (Kiriyama, Ushikubi et al. 1997). EP2 is important in the female reproductive system in that female EP2 deficient mice have a reduced number of ovulations and a lower fertilization rate (Hizaki, Segi et al. 1999). EP2 is expressed in the lung (Ushikubi, Hirata et al. 1995) where it mediates the bronchodilating action of PGE_2 (Sheller, Mitchell et al. 2000). In EP2 deficient mice salt-sensitive hypertension develops

(Kennedy, Zhang et al. 1999) and depression of blood pressure by PGE_2 is impaired (Zhang, Guan et al. 2000).

The importance of EP2 in cancer development has been shown in several different studies. When EP2 deficient mice were crossed with $APC^{\Delta 716}$ mice, the number and size of intestinal polyp formation decreased (Sonoshita, Takaku et al. 2001). EP2 expression increased in TPA-treated mouse skin, DMBA/TPA-induced papillomas and carcinomas (Sung, He et al. 2006), chronic UV-irradiated mouse skin, UV-induced papillomas and carcinomas in SKH mice, human SCC (Lee, Kim et al. 2005), and human endometrial adenocarcinoma (Jabbour, Milne et al. 2001). EP2 deficient mice produced fewer tumors in the DMBA/TPA skin carcinogenesis model and EP2 overexpression produced more tumors in the DMBA/TPA skin carcinogenesis model with a concomitant effect on cell proliferation (Sung, He et al. 2005; Sung, He et al. 2006). When EP2 deficient mice were crossed with MMTV-COX-2 transgenic mice, which produce spontaneous mammary tumors, mammary hyperplasia and amphiregulin expression were reduced (Chang, Ai et al. 2005).

There are three isoforms (EP3α, EP3β and EP3γ) of EP3 in the mouse, and seven isoforms in human. All mouse EP3 are coupled to Gαi and inhibit adenylate cyclase and decrease cAMP levels. Interestingly, EP3γ is also coupled to Gαs. Activation of EP3 also increases intracellular calcium levels by activation of phospholipase C (PLC) through the Gβγ subunit (Hatae, Sugimoto et al. 2002). The binding affinity of EP3 is sulprostone (PGE_2 analog, EP3 agonist)> PGE_2 > PGE_1 > 11-deoxy PGE_1 > 16,16-dimethyl PGE_2 > 17-phenyl PGE_2 (EP1, EP3 agonist) (Kiriyama, Ushikubi et al. 1997). In EP3 deficient mice, PGE_2 failed to induce fever (Ushikubi, Segi et al. 1998), acid did not induce bicarbonate ion secretion in the duodenum (Takeuchi, Ukawa et al. 1999), PGE_2 reduced

blood pressure more effectively (Audoly, Tilley et al. 1999), and indomethacin-mediated concentration of urine was reduced (Fleming, Athirakul et al. 1998).

There are several literature reports that show EP3 is not important in carcinogenesis. When EP3 deficient mice were crossed with $APC^{\Delta 716}$ mice, there was no difference in intestinal polyp formation (Sonoshita, Takaku et al. 2001). In chronic UVB-irradiated mouse skin, EP3 expression is not detectable. In UVB induced mouse papillomas and SCC, EP3 expression was very low (Lee, Kim et al. 2005). In human skin SCC, EP3 expression was not changed compared to adjacent tissue (Lee, Kim et al. 2005). EP3 deficient mice produced a similar number of DMBA/TPA-induced skin tumors with similar incidence to wild type mice (Sung, He et al. 2005). In contrast, EP3 deficient mice were found to produce less VEGF and implanted sarcoma growth was also reduced (Amano, Hayashi et al. 2003).

EP4 is also coupled to Gαs and increases cAMP formation. Similar to EP2, activation of EP4 induces activation of PKA, resulting in phosphorylation of CREB, GSK-3 and activation of β-catenin. In addition, EP4 is coupled to Gαi which decreases cAMP and activates PI3K. PI3K can activate Akt which phosphorylates CREB and GSK-3. Also, PI3K can activate Erk (Regan 2003; Fujino, Salvi et al. 2005). The binding affinity of EP4 is PGE_2 > PGE_1 >> 11-deoxy-PGE_1 > 16,16-dimethyl PGE_2 > > 1-hydroxy PGE_1 (Kiriyama, Ushikubi et al. 1997). EP4 is expressed in human and mouse heart, lung, thymus, spleen, ileum and skin (Ushikubi, Hirata et al. 1995; Lee, Kim et al. 2005). In EP4 deficient mice, ductus arteriosus, an artery that allows for bypassing the fetal pulmonary system, fails to close. As a result, 95% of EP4 deficient newborn mice die after birth (Nguyen, Camenisch et al. 1997). In mice, PGE_2 enhances bone resorption through EP4 (Miyaura, Inada et al. 2000).

The role of EP4 in cancer has also been studied. EP4 deficient mice were found to produce fewer azoxymethane (AOM)-induced aberrant crypt foci. The EP4 antagonist ONO-AE2-227 reduced AOM-induced aberrant crypt foci formation and intestinal polyp formation in Min mice (Mutoh, Watanabe et al. 2002). The EP4 agonist 1-hydroxy PGE_1 abolished indomethacin-mediated growth inhibition in a mouse adenocarcinoma cell line (Pozzi, Yan et al. 2004). The administration of EP4 antagonist ONO-AE3-208 reduced Lewis lung carcinoma cell metastasis in mouse (Yang, Huang et al. 2006). Mouse mammary tumor cell lines treated with the EP4 antagonists AH23848 and ONO-AE3-208 resulted in a reduction in lung metastasis (Ma, Kundu et al. 2006).

1.4 EP1 RECEPTOR FOR PGE_2

1.4.1 EP1

EP1 is composed of 405 amino acids with an estimated molecular weight of 43kDa. At the N terminal, 3 possible N-glycosylation sites are present. There are two possible phosphorylation sites for PKA in the first and third intracellular loop and one possible phosphorylation site for protein kinase C (PKC) in the third intracellular loop of the receptor. The dissociation constant for PGE_2 is 21 nM. The binding affinity for EP1 is in the following order: 17-phenyl PGE_2 > PGE_2 > iloprost (a stable prostacyclin analogue) = sulprostone > PGE_1 (Watabe, Sugimoto et al. 1993).

EP1 is expressed in the mouse lung, kidney, brain, bladder, blood vessel, sebaceous gland and in the epidermis of the skin (stratum corneum, stratum granulosum and basal layer) and human skin (Watabe, Sugimoto et al. 1993; Ushikubi, Hirata et al. 1995; Lee, Kim et al. 2005; Matsuoka, Furuyashiki et al. 2005; Tober, Wilgus et al. 2006; Neumann, Dulsner et al. 2007; Wang, Momota et al. 2008). In normal human epidermis,

EP1 is located in the plasma membrane and cytosol in the basal layer and spinous layers of the skin as well as in primary cultures of human keratinocytes (Konger, Billings et al. 2005).

EP1 is a G-protein coupled rhodopsin-type receptor as are other PGE_2 receptors. However, the G protein is pertussis toxin-insensitive. After EP1 activation, extracellular calcium influxes through the voltage-independent, Mn^{2+} impermeable calcium channel. Calcium is also released from intracellular stores in a PLC-dependent manner (Figure 1-5). Phosphorylation of EP1 by PKC reduced PGE_2 binding to EP1 and PGE_2-mediated intracellular calcium increase. Thus, PKC phosphorylation desensitizes EP1 by dissociation of EP1 with G-protein. In EP1 expressing Chinese hamster ovary cells, EP1 mRNA expression was reduced one day after treatment with TPA, a potent PKC activator (Katoh, Watabe et al. 1995). However, in human erythroleukemia cells, EP1 mRNA is induced two days after TPA treatment (Funk, Furci et al. 1993).

In osteoblast cells, EP1 activated PKCα and Src, resulting in increased fibronectin expression (Tang, Yang et al. 2005). In human cholangiocarcinoma cells, PGE_2 promoted cell growth and invasion through EP1 receptor activated Src/EGFR/Stat3/Akt signaling (Han and Wu 2005; Han, Demetris et al. 2006). EP1-mediated Erk activation was shown in non-small cell lung cancer cells (Krysan, Reckamp et al. 2005).

EP1 agonists also induce COX-2 with concomitant PGE_2 increases in mouse osteoblastic cells. EP1 also increased c-fos and c-jun mRNA. However, antisense c-fos or c-jun did not affect 17-phenyl-2 trinor PGE_2-mediated COX-2 mRNA induction (Suda, Tanaka et al. 2000).

Interestingly, EP1 is very important in the brain. EP1 antagonist SC51089 protected against mouse brain injury induced by middle cerebral artery occlusion (Abe, Kunz et al. 2008; Zhou, Qian et al. 2008). EP1 deficient mice show increased

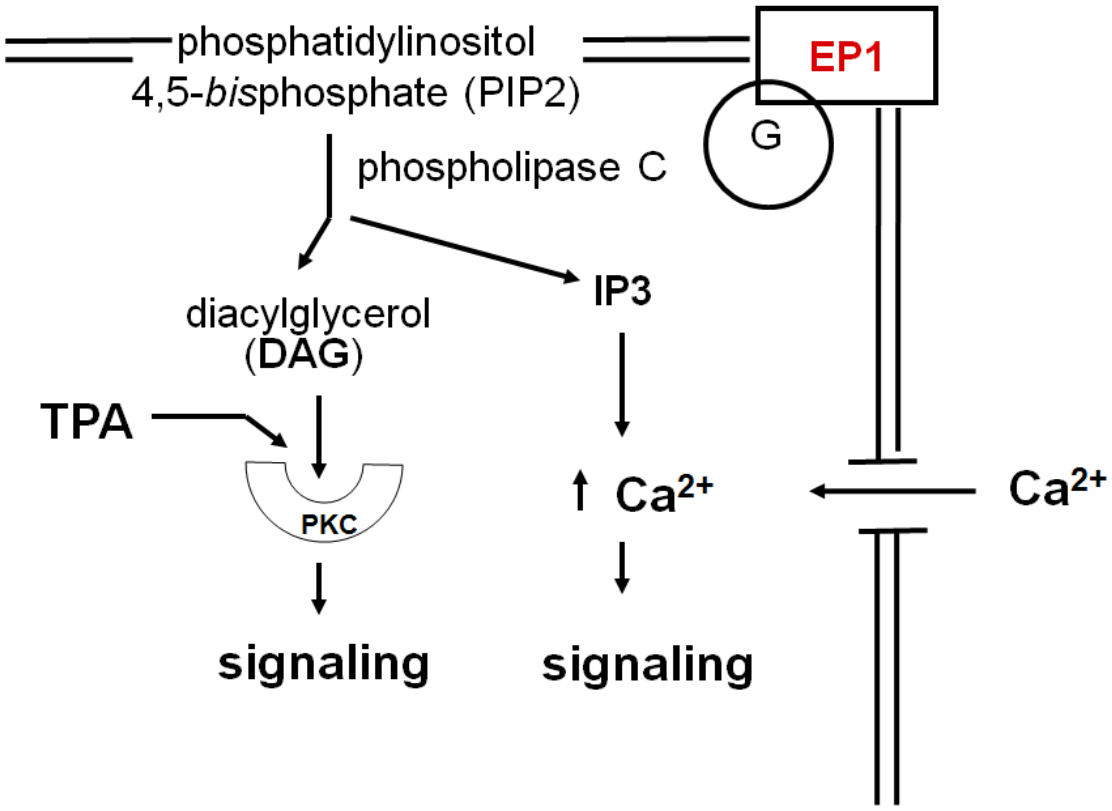

Figure 1-5. Second messenger system of EP1 receptor. After EP1 activation extracellular calcium influxs through calcium channel. Activation of phospholipase C release IP3 (inositol 1,4,5-*tris*phosphate) which releases calcium from intracellular stores. Diacylglycerol activates PKC. TPA, tumor promoter activates PKC.

aggressiveness when they are stressed, along with reduced social interaction (Matsuoka, Furuyashiki et al. 2005). In EP1 deficient mice, PGE_2 failed to induce bicarbonate ion secretion in the stomach (Takeuchi, Aihara et al. 2006). In the bladder, EP1 mediates micturition in mice (Wang, Momota et al. 2008). EP1 is also important in inflammation. In Leydig cell progenitors, EP1 mediates IL-1β expression (Walch, Clavarino et al. 2003). EP1 deficient mice are insensitive to pain and EP1 receptor antagonist, ONO-8711, suppressed mechanical hyperalgesia (Omote, Yamamoto et al. 2002). In EP1 deficient mice, systolic blood pressure is also reduced (Stock, Shinjo et al. 2001).

1.4.2 Importance of EP1 in tumor development

The importance of EP1 receptors in carcinogenesis has been studied using diverse models. EP1-deficient mice developed fewer aberrant crypt foci, preneoplastic lesions, after AOM treatment. EP1 antagonists, ONO-8711 and ONO-8713, in the diet reduced aberrant crypt foci formation in mouse. In Min mice, ONO-8711 administration reduced intestinal polyp development (Watanabe, Kawamori et al. 1999; Watanabe, Kawamori et al. 2000). In EP1 deficient mice, tumor incidence, tumor multiplicity and tumor volume were decreased in the AOM-induced colon carcinogenesis model. In these tumors, a lower cell proliferation index and higher apoptotic index were observed (Kawamori, Kitamura et al. 2005). After UV treatment of mice, the number of EP1 positive cells was increased in skin (Tober, Wilgus et al. 2006). Thirty weeks of UV exposure increased EP1 expression extensively. In UVB-induced mouse papillomas and SCCs and human SCCs, EP1 mRNA expression and protein levels were increased 2 ~ 10 fold (Lee, Kim et al. 2005; Tober, Thomas-Ahner et al. 2007). Topical application of ONO-8713, an EP1 antagonist, reduced UV-induced skin tumor formation and UV-induced inflammatory index, such as skin thickness, myeloperoxidase activity, PGE_2 content and the number of

p53 positive cells (Tober, Wilgus et al. 2006). Also 17-phenyl trinor PGE_2 (EP1/EP3 agonist) abolished indomethacin–mediated growth inhibition in malignant keratinocytes that do not express EP3 (Thompson, Gupta et al. 2001). The EP1 antagonists, SC51089 and AH6809, suppressed cell proliferation about 30~50% in human glioma cells. SC51089 injected i.p. suppressed human glioma cell xenograft growth by about 50% (Matsuo, Yoshida et al. 2004). EP1 is not expressed in normal mouse mammary gland but highly expressed in mammary tumors. ONO-8711 administration inhibited 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine induced mammary tumor development (Kawamori, Uchiya et al. 2001). In rat hepatocytes, treatment with 17-phenyl-trinor-PGE_2 and sulprostone, EP1 agonists, increased DNA synthesis and the number of nuclei in a PLC-dependent manner. (Kimura, Osumi et al. 2001).

EP1 is also important in lymphangiogenesis. In human lung cancer cells, 17-phenyl trinor PGE_2, an EP1/EP3 agonist, significantly induced VEGF-C mRNA and protein, a lymphangiogenic factor. SC19220, an EP1 antagonist or an EP1 antisense oligonucleotide effectively inhibited PGE_2-mediated VEGF-C mRNA and protein induction. EP1-mediated VEGF-C induction in these cells was mediated through Src and Her2/Neu activation (Su, Shih et al. 2004). In human breast cancer cells, EP1 receptor antagonist treatment significantly suppressed VEGF-C protein production (Timoshenko, Chakraborty et al. 2006).

Tumor cells evade the immune system by upregulation of Fas ligand which is associated with apoptosis of lymphocytes. Inhibition of EP1 by siRNA successfully inhibited PGE_2-mediated upregulation of Fas ligand in colon tumor cells (O'Callaghan, Kelly et al. 2008).

1.5 HYPOTHESIS AND THE SPECIFIC AIMS

Cancer was the second leading cause (27%) of death in the U.S.A. in 2005. As mentioned above, NSAIDs reduce the risk of several types of cancer. The gastro-intestinal side effect of NSAIDs led to the development of COX-2 specific inhibitors. However, COX-2 inhibitors increase the risk of cardiovascular disease. Thus, the downstream effectors of COX-2 could be the next targets for chemoprevention. PGE_2 is an important mediator of COX-2-mediated cancer promoting action and EP1 is one of the PGE_2 receptors. EP1 is expressed in mouse skin epidermis as well as in human skin epidermis (Konger, Billings et al. 2005; Lee, Kim et al. 2005). EP1 expression increases in chronically UV-irradiated mouse skin and UVB-induced papilloma and SCC. Also, EP1 is the most highly expressed PGE_2 receptor in mouse and human nonmelanoma skin cancer (Lee, Kim et al. 2005). Topical application of an EP1 antagonist reduces UV-induced skin tumor development (Tober, Wilgus et al. 2006). The importance of EP1 in carcinogenesis has been studied in the colon, tongue, and mammary in vivo tumor models. Therefore, it is hypothesized that **activation of EP1 contributes to skin tumor development by mediating the tumor promoting action of PGE_2 in skin carcinogenesis**. Following are the specific aims designed to test the hypothesis.

1. **Determine whether changes in EP1 expression and activation alter skin tumor development.**

2. **Determine the mechanism by which EP1 alters skin tumor development.**

Chapter 2 Materials and methods

2.1 MATERIALS

2.1.1 Materials

TRI-reagent (Molecular Research Center,Inc., Cincinnati, OH), RETROscript kit (Ambion, Austin, TX), RNeasy Mini kit (Qiagen, Valencia, CA), universal master mix (Applied Biosystems, Foster City, CA), EP1 specific primers and probes (Applied Biosystems), 1Kb DNA ladder (Invitrogen Corporation, Carlsbad, CA), QIAEX II Gel Extraction kit (Qiagen), PerfectHyb plus solution (Sigma Aldrich, St Louis, MO), [γ-^{32}P]d-CTP (Perkin Elmer, Waltham, MA), Random Primed DNA Labeling Kit (Roche Applied Science, Indianapolis, IN), SDS (Bio-Rad Laboratories, Hercules, CA), sodium chloride (Fisher Scientific), sodium citrate (Sigma Aldrich), Signal Transduction Pathway Finder PCR Array (SABiosciences, Frederick, MD), acetone (Fisher Scientific, Pittsburgh, PA), magnesium chloride (Sigma Aldrich), EGTA (Sigma Aldrich), glycerol (Fisher Scientific), Triton X-100 (Sigma Aldrich), Mouse TNF–α Quantikine (R &D Systems, Minneapolis, MD), 5-bromo-2'-deoxyuridine (BrdU) (Sigma Aldrich), anti-BrdU antibody (BD Biosciences, San Jose, CA) biotinylated rabbit anti-mouse IgG (Accurate Chemical Company, Westbury, NY), streptavidin peroxidase (BioGenex, San Ramon, CA), Ki-67 antibody (DAKO, Carpinteria, CA), DMBA (Sigma Aldrich), TPA (LC Laboratories, Woburn, MA), dithranol (Sigma Aldrich), EDTA (Sigma Aldrich), Tris (Fisher Scientific), Tween 20 (Sigma Aldrich), protease inhibitor cocktail tablet (Roche Applied Science), polyvinylidene fluoride (PVDF) membrane (Thermo-Fisher, Waltham, MA), BCA kit (Bio-Rad, Richmond CA), 30% acrylamide (ProtoGel, National Diagnostics USA, Atlanta, GA), Benchmark prestained ladder (Invitrogen Corporation), enhanced chemiluminescence (ECL) Plus (GE Healthcare, Piscataway, NJ), Blue Lite

Autorad film (ISC Bioexpress, Kaysville, UT), COX-2 antibody (Cayman Chemical Co., Ann Arbor, MI), PKC-α antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), β-actin antibody (Santa Cruz Biotechnology, Inc.), EGFR antibody (Cell Signaling Technology, Danvers, MA), p-EGFR antibody (Cell Signaling Technology), Stat3 antibody (Cell Signaling Technology), p-Stat3 antibody (Cell Signaling Technology), CYP1B1 (Santa Cruz Bio Technology), benzo[a]pyrene (B[a]P) (Sigma Aldrich), ^{3}H B[a]P (American Radiolabeled Chemicals, Inc., St. Louis, MO), phenol (Fisher Scientific), chloroform (Fisher Scientific), isoamyl alcohol (Sigma Aldrich) and ras-activation assay kit (Upstate Inc., Waltham, MA) were used.

2.1.2 Animals

Wild type female FVB mice and SKH mice at the age of 3-4 weeks were purchased from Harlan (Indianapolis, IN). EP1 knockout mice on a C57BL/6 background were obtained from Dr. Shuh Narumiya, Kyoto University, Japan and backcrossed to FVB and SKH more than five times. For experiments, female mice at 6-9 weeks of age were used. Mice were maintained at Science Park and housed in an air conditioned (22 ± 1° C at 50% humidity) facility that is accredited by the Association for Assessment and Accreditation of Lab Animal Care Accredited.

2.2 METHODS

2.2.1 Quantitative real-time polymerase chain reaction analysis

Epidermal scrapes were collected from three mice at each time point after the last treatment. Total RNA was extracted from epidermal scrapes, papillomas and carcinomas with TRI-reagent (Molecular Research Center, Inc.) and purified through RNeasy

minicolumns (Qiagen). One microgram of total RNA was reverse transcribed with random decamers using RETROscript kit (Ambion) in 20 µl reactions, then diluted to 40 µl with tRNA. For PCR reactions, 2 µl (50 ng starting RNA) were used per assay and each sample was analyzed in triplicate. PCR was carried out in a 25 µl volume with 1X universal master mix (Applied Biosystems). Proprietary 20X EP1 specific primers and probes (Applied Biosystems) were used. C-Ha-ras was used as a control gene to compare expression; forward primer (300 nM, 5'-CTTAGACACAGCAGGTCAAGAAGAGT-3'), reverse primer (300 nM, 5'-TTGATGGCAAATACACAGAGGAA-3') and probe (100 nM, 5'FAM-CCAGTACATGCGCACAG-MGB). Reaction condition was 50° C for 2 min, 95° C for 10 min, followed by 40 cycles of 95° C for 15 s and 60° C for 1 min. To verify lack of contaminating DNA in samples, control samples were processed without reverse transcriptase. ABI 7700 (Applied Biosystem) was used for quantitative real time PCR analysis. Relative quantification method was used to calculate relative fold changes.

2.2.2 Production of BK5.EP1 transgenic mice

Murine EP1 cDNA was excised from pBluescript-EP1 (obtained from Dr. Yukihiko Sugimoto, Kyoto University, Japan) by digestion with EcoR I enzyme. Klenow DNA polymerase was used to blunt end the cDNA. The pBK5.EP1 construct was made by ligating the mouse EP1 cDNA into pBK5, which contains the bovine keratin 5 promoter, a β-globin intron and SV40 poly A. The orientation of the construct was checked by a series of diagnostic restriction digestions. The BK5.EP1 transgene construct was excised from the pBK5.EP1 plasmid by digestion with Kpn I enzyme and separated in 0.7% agarose gel. The 8.2 kb fragment was isolated from the gel with a QIAEX II Gel Extraction kit (Qiagen). The BK5.EP1 transgene was microinjected into the pronucleus of fertilized FVB embryos. After overnight incubation, those embryos were transferred to

pseudopregnant mice. The injection procedures were carried out by the Science Park Transgenic Animal Facility Core. Transgenic mice were genotyped by Southern blot using the mouse EP1 cDNA.

2.2.3 Southern blot analysis

Genomic DNA was isolated from tail and 5 μg DNA was digested with AlwNI enzyme. Digested DNA was separated on 0.7% agarose gel then transferred onto nylon membrane. EP1 cDNA was labeled with [γ-^{32}P]d-CTP using Random Primed DNA Labeling Kit (Roche Applied Science, Indianapolis, IN) and hybridized with the blot overnight. The blot was washed twice with low stringency wash solution (0.1% SDS/0.3 M NaCl/30 mM sodium citrate) for 15 min each at room temperature and high stringency wash solution (0.1 % SDS/15 mM NaCl/0.15 mM sodium citrate) twice for 30 min at 60° C. The blot was exposed to X-ray film at -80° C.

2.2.4 Northern blot analysis

Total RNA was extracted from the epidermis of BK5.EP1 transgenic mice and wild type mice with TRI-reagent (Molecular Research Center, Inc.). Ten microgram of total RNA was separated on a 1% agarose gel containing formaldehyde and transferred onto nylon membrane. EP1 cDNA was labeled with [γ-^{32}P]d-CTP using Random Primed DNA Labeling Kit (Roche Applied Science). The nylon membrane was incubated with the labeled EP1 cDNA at 65° C overnight. Unincorporated probe was removed by incubation of the blot with low stringency wash solution (0.1% SDS/0.3 M NaCl/30 mM sodium citrate) twice for 15 min each at room temperature and with high stringency wash

solution (0.1% SDS/15 mM NaCl/0.15 mM sodium citrate) twice for 30 min at 60° C. The blot was exposed to X-ray film at -80° C .

2.2.5 Microarray

Epidermal scrapes were collected from 3 mice for each group. Total RNA was extracted with TRI-reagent (Molecular Research Center, Inc.) and purified through RNeasy minicolumns (Qiagen). RNA from three mice was pulled together. Signal Transduction Pathway Finder PCR Array (SABiosciences) was used according to the manufacturer's instructions.

2.2.6 TNF – α

Total protein was extracted from epidermis 2.5 hr after acetone (200 µl) or TPA (2.5 µg/200 µl acetone) treatment with lysis buffer (50 mM HEPES, 150 mM sodium chloride, 1.5 mM magnesium chloride, 1 mM EGTA, 10% glycerol, 1% Triton X-100). TNF–α level was determined by quantitative sandwich enzyme immunoassay technique according to the manufacturer's instructions (Quantikine Mouse Immunoassay, R & D Systems).

2.2.7 Bromodeoxyuridine (BrdU) incorporation

Groups of 3 mice were used. Dorsal hair was shaved 2 days prior to treatment. BrdU (0.1 mg/g body weight) was injected intraperitoneally 17 hours after the last treatment. One hour after BrdU injection, the mice were euthanized, then dorsal skin was removed and fixed in 10% formalin. The Histology and Tissue Processing Core at the

University of Texas M. D. Anderson Cancer Center embedded the formalin fixed samples in paraffin and immunostained for BrdU.

Briefly, 4 μm thick paraffin sections were deparaffinized and gradually hydrated. To block endogenous peroxidase activity, the sections were incubated with 0.3% H_2O_2 for 30 min. After three washes with phosphate buffered saline (PBS), sections were incubated with 10 mM citrate buffer, pH 6.0 for 15 min in a microwave oven to retrieve antigen. Following three washes with PBS, sections were incubated with 10% rabbit serum for 30 min, followed by incubation for 1.5 hr with a monoclonal mouse anti-BrdU antibody (BD Biosciences) at 1:500 dilution. After washing with PBS, sections were incubated with biotinylated rabbit anti-mouse IgG (Accurate Chemical Company) at 1:250 dilution for 15 min and followed by washing with PBS. Sections were incubated with streptavidin peroxidase (BioGenex) for 30 min. 3,3'-Diaminobenzidine tetrahydrochloride was used as a chromogen and sections were counterstained with hematoxylin. BrdU positive and negative basal cells in the interfollicular region of the epidermis were counted under a microscope (200 X). At least 1500 total basal cells were counted for each sample.

2.2.8 Measurement of epidermal thickness

Groups of 3 mice were used. Dorsal hair was shaved 2 days prior to treatment. Eighteen hours following the last treatment, dorsal skin was removed and fixed in 10% formalin. The Histology and Tissue Processing Core at the University of Texas M. D. Anderson Cancer Center embedded the formalin fixed samples in paraffin and performed hematoxylin and eosin (H&E) staining. An imaging system (Nikon ACT-1, Nikon, Melville, NY) was used to measure epidermal thickness. The on-screen thicknesses of the

epidermis were obtained for at least 50 points of interfollicular region per sample. A magnification factor (X650) was used to calculate the actual thickness of epidermis.

2.2.9 DMBA/TPA skin carcinogenesis protocol

Groups of 28 female BK5.EP1 transgenic mice and 28 wild type mice at 6-9 weeks of age were used. Dorsal hair of the mice was shaved 2 days prior to initiation. Initiation was achieved by a single topical application of DMBA (100 μg/200 μl acetone). Two weeks after initiation, TPA (2.5 μg/200 μl acetone) was applied topically twice weekly for 20 weeks. Tumors were counted weekly to calculate tumor incidence (percentage of mice bearing tumors) and multiplicity (average number of tumors per mouse).

2.2.10 DMBA/Anthralin skin carcinogenesis protocol

Groups of 32 BK5.EP1 transgenic mice and 32 wild type mice were used. Dorsal hair of the mice was shaved 2 days prior to initiation. Initiation was achieved by a single application of DMBA (100 μg/200 μl acetone). Two weeks after initiation, anthralin (100 nM/200 μl acetone) was applied once weekly. Tumors were counted every week to calculate tumor incidence and multiplicity.

2.2.11 UV skin carcinogenesis protocol

Groups of 26 female BK5.EP1 transgenic mice and wild type mice at 6-9 weeks of age were used. An UV apparatus (Fischer, Conti et al. 2003) providing 80% UVB and 20% UVA was used. An established UV protocol (Noonan, Otsuka et al. 2000) was employed with modification. Dorsal hair of the mice was shaved just prior to UV

treatment. For the first 4 weeks, mice were treated with 220 mJ/cm^2 three times a week. The dose was increased every two weeks to 260 mJ/cm^2, 320 mJ/cm^2, 340 mJ/cm^2, and maintained at 360 mJ/cm^2 for 24 weeks. Tumors were counted every week to calculate tumor incidence and multiplicity.

Groups of 25 female EP1 deficient mice on SKH background and wild type SKH mice at 6-9 weeks of age were used. The above described apparatus providing 80% UVB and 20% UVA was used. For the first week, mice were treated with 90 mJ/cm^2 three times a week. Every week, the dose was increased 10% up to 175 mJ/cm^2, then maintained at the 175mJ/cm^2 dose. Tumors were counted every week to calculate tumor incidence and multiplicity.

2.2.12 DMBA-only skin carcinogenesis protocol

Groups of 32 female BK5.EP1 transgenic mice and 32 female wild type mice were used. Dorsal hair of the mice was shaved 2 days prior to initiation. Initiation was achieved by application of DMBA (400 μg/200 μl acetone). Tumors were counted every week to calculate tumor incidence and multiplicity.

2.2.13 Western blot analysis

At specified times after treatment, total protein was extracted from epidermis with modified RIPA buffer (150 mM NaCl, 0.005% Triton X-100, 0.2 mM EDTA (pH 8.0) in 50 mM Tris, pH 7.5). Fifty microgram of protein was denatured by incubation at 95° C for 10 min and separated on 7-10% SDS-polyacrylamide gel by electrophoresis and transferred onto polyvinylidene difluoride membranes. The blot was incubated for 1 hr in 5% non-fat milk solution in Tris-buffered saline to block nonspecific binding. A specific

primary antibody was incubated for 1 hr at room temperature. The blot was washed three times with 0.1% Tween 20 in Tris-buffered saline for 5 min each. Horseradish peroxidase-conjugated secondary antibody was incubated for 1 hr at room temperature. The blot was washed with 0.1% Tween 20 in Tris-buffered saline for 5 min each and incubated with chemiluminescent substrate and exposed to autoradiography film.

2.2.14 B[a]P-DNA adduct formation

Groups of 4 mice were used. Dorsal hair was shaved 2 days prior to treatment. 200 nmol, 200 μCi of ^{3}H-B[a]P in 200 μl of acetone was applied to each mouse back. Fifteen hours after the ^{3}H-B[a]P treatment, epidermis was scraped into 0.75 M guanidine isothiocyanate and homogenized by passing through a syringe 10 times. The epidermal scrape was dialyzed in 0.15 M sodium chloride, 0.01 M sodium citrate for 2 days. To remove protein, proteinase K was incubated with sample at 55° C for 3 hours. Sample DNA was extracted with water-saturated phenol. To remove RNA, 10 μl of RNase A was incubated with sample at 37° C for 30 min. DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1), then chloroform:isoamyl alcohol (24:1). DNA was precipitated with ethanol then incubated -80° C for 5 min. Precipitated DNA was washed with cold 75% ethanol. DNA pellets were dissolved in 0.01 M Tris, 0.01 M $MgCl_2$ solution, then the amount of DNA was quantitated by ultraviolet spectrophotometer. Twenty microgram of DNA aliquots were used to measure B[a]P-DNA adduct in a scintillation counter.

2.2.15 Ras activation assay

Ras activity was measured by a ras activation assay kit (Upstate Inc.). Briefly, total protein was extracted from epidermis with magnesium lysis buffer. Ten microgram of the Ras assay reagent was incubated with 500 μg of protein extract overnight at 4° C. After centrifugation, beads were washed 3 times with the magnesium lysis buffer then resuspended in sample buffer and boiled for 5 min. After centrifugation, supernatant was loaded onto a 12% SDS-polyacrylamide gel, separated by electrophoresis, then transferred onto polyvinylidene difluoride membranes. The blot was incubated for 2 hr in 3% non-fat milk solution in Tris-buffered saline to block nonspecific binding. Anti-ras antibody was incubated overnight at 4° C. The blot was washed three times with 0.05% Tween 20 in Tris-buffered saline for 5 min each. Horseradish peroxidase conjugated anti-mouse antibody was incubated for 1 hr at room temperature. The blot was washed with 0.05% Tween 20 in Tris-buffered saline for 5min each and incubated with chemiluminescent substrate and exposed to autoradiography film.

2.2.16 Statistical analysis

Data are shown as the mean ± standard deviation. To determine statistical differences between means, independent t-test, independent proportions test, two-way analysis of variance (ANOVA), Poisson regression, Fisher exact test were used where applicable.

Chapter 3 Results

3.1 EP1 mRNA EXPRESSION AFTER TUMOR PROMOTER TREATMENT AND IN TUMORS

EP1 mRNA levels in the mouse epidermis and skin tumors were measured with a quantitative real-time PCR method. After a single application of TPA, the EP1 mRNA level was increased about 2-fold after 6 hours and returned to slightly below normal levels after 18 hours. Four treatments of TPA produced similar patterns as one TPA treatment. In papillomas and carcinomas from a DMBA/TPA protocol, EP1 mRNA also increased 11-fold and 7-fold respectively (Figure 3-1A).

After a single exposure to UV, EP1 mRNA levels increased about 2-fold after 6-hours and this elevated level was maintained for 18 hours. Twenty-four hours after UV treatment, the EP1 mRNA level returned to the control level. Seven exposures to UV increased EP1 mRNA level by 18 hours and this elevated level returned to control level after 48 hours. In UV-elicited papillomas and carcinomas, EP1 mRNA increased 3-fold and 4-fold respectively (Figure 3-1B). Thus, increased EP1 mRNA levels in tumor promoter-treated epidermis and tumors imply increased EP1 signaling. Therefore, EP1 could be one of the important PGE_2 receptors contributing to skin carcinogenesis.

3.2 EFFECT OF EP1 DEFICIENCY ON DMBA/TPA SKIN CARCINOGENESIS

To examine the effect of EP1 deficiency on skin tumor development, a DMBA/TPA skin carcinogenesis protocol was used with EP1 deficient mice and wild type mice. Both wild type mice and EP1 deficient mice produced the first tumor at 8 weeks of promotion. Starting at week 8 of promotion, the EP1 deficient mice produced more tumors (average 5.5 for EP1 deficient mice versus 2.9 for wild type mice) with

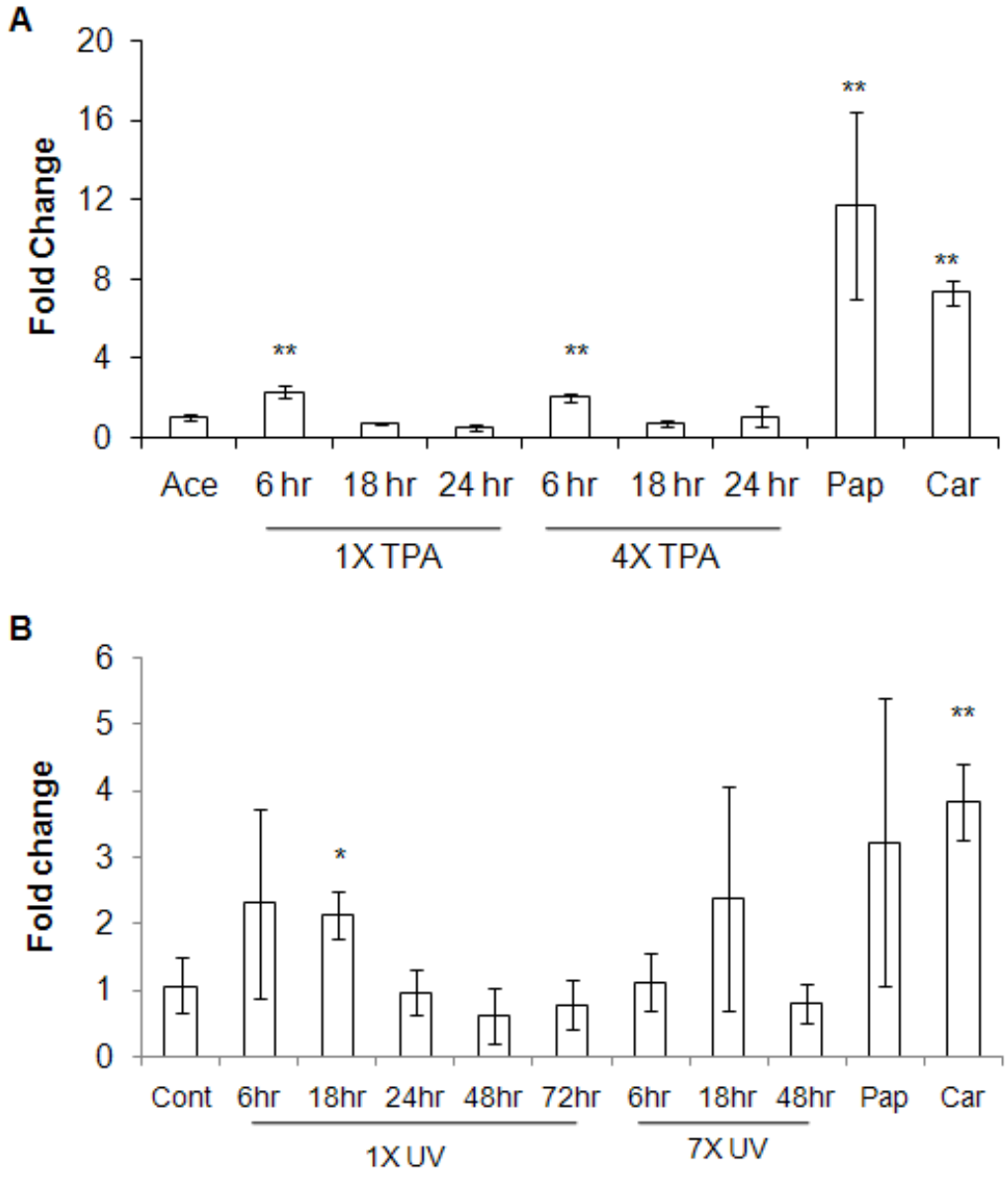
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Figure 3-1. EP1 mRNA expression after TPA or UV treatment and in tumors. A. EP1 mRNA levels in TPA-treated epidermis and DMBA/TPA-induced tumors. Dorsal hair of FVB mice was shaved at least two days before TPA (2.5 μg/200 μl acetone) treatment. Epidermal scrapes were collected at the time points shown after the last TPA treatment. The EP1 mRNA level was determined by quantitative real-time PCR. Ace, acetone (200 μl) treatement; 1X TPA, one TPA (2.5 μg/200 μl acetone) treatment; 4X TPA, mice were treated with TPA (2.5 μg/200 μl acetone) every three days for 4 treatments; Pap, papilloma from DMBA-TPA tumor study of FVB mice; Car, carcinoma from DMBA-TPA tumor study of FVB mice. Three mice were used at each time point and three papilloma and five carcinoma samples were used. Data are expressed as the fold change in EP1 mRNA levels relative to acetone-treated epidermal levels. ** $p < 0.01$, independent t test, compared to acetone treated group. B. EP1 mRNA levels in UV-exposed epidermis and UV-induced tumors. SKH mice were exposed to UV. Epidermal scrapes were collected at the time points shown after the last UV exposure. The EP1 mRNA level was determined by quantitative real-time PCR. Cont, control, non-irradiated epidermis; 1X UV, one UV (120mJ/cm^2) exposure; 7X UV, 7 exposures to UV, 3 times per week at 90 mJ/cm^2 three times, 120 mJ/cm^2 three times, and one treatment of 140 mJ/cm^2 ; Pap, papilloma from UV tumor study of SKH mice; Car, carcinoma from UV tumor study of SKH mice. Three mice were used at each time point and three papilloma and four carcinoma samples were used. Data are expressed as the fold change in EP1 mRNA levels relative to non-irradiated epidermal levels. * $p < 0.05$, independent t test, compared to control. ** $p < 0.01$, independent t test, compared to control.

slightly higher tumor incidence (88% for EP1 deficient mice versus 66% for wild type mice) compared to wild type mice (Figure 3-2A and B). At the end of the experiments, the size of tumors was measured with calipers. EP1 deficient mice produced a similar ratio of medium-size tumors (58% for EP1 deficient mice versus 62% for wild type mice) but more small tumors (26% for EP1 deficient mice versus 16% for wild type mice) with fewer large tumors (17% for EP1 deficient mice versus 22% for wild type mice) (Figure 3-2C). These data show that EP1 deficiency increases tumor development but decreases tumor size in the DMBA/TPA carcinogenesis protocol.

3.3 EFFECT OF EP1 DEFICIENCY ON CELL PROLIFERATION

In the absence of treatment, BrdU incorporation was similar in the epidermis of wild type and EP1 deficient mice. After one or four vehicle treatments, there was no change in BrdU incorporation in both wild type and EP1 deficient mice. Both one TPA (2.5 μg) and four treatments of TPA increased BrdU incorporation to the same extent in wild type and EP1 deficient mice (Figure 3-3). These data show that deficiency of EP1 did not affect TPA-induced epidermal cell proliferation.

3.4 EFFECT OF EP1 DEFICIENCY ON UV SKIN CARCINOGENESIS

To examine the effect of EP1 deficiency on skin tumor development, a UV skin carcinogenesis protocol was used with EP1 deficient mice and wild type mice. For this experiment, EP1 deficient mice were backcrossed to the SKH strain background 5 times. While wild type mice produced the first tumor at 14 weeks of UV treatment, EP1 deficient mice produced the first tumor at 13 weeks of UV treatment. Starting at week 23 of UV treatment, wild type mice produced more tumors (average 2.7 for wild type mice

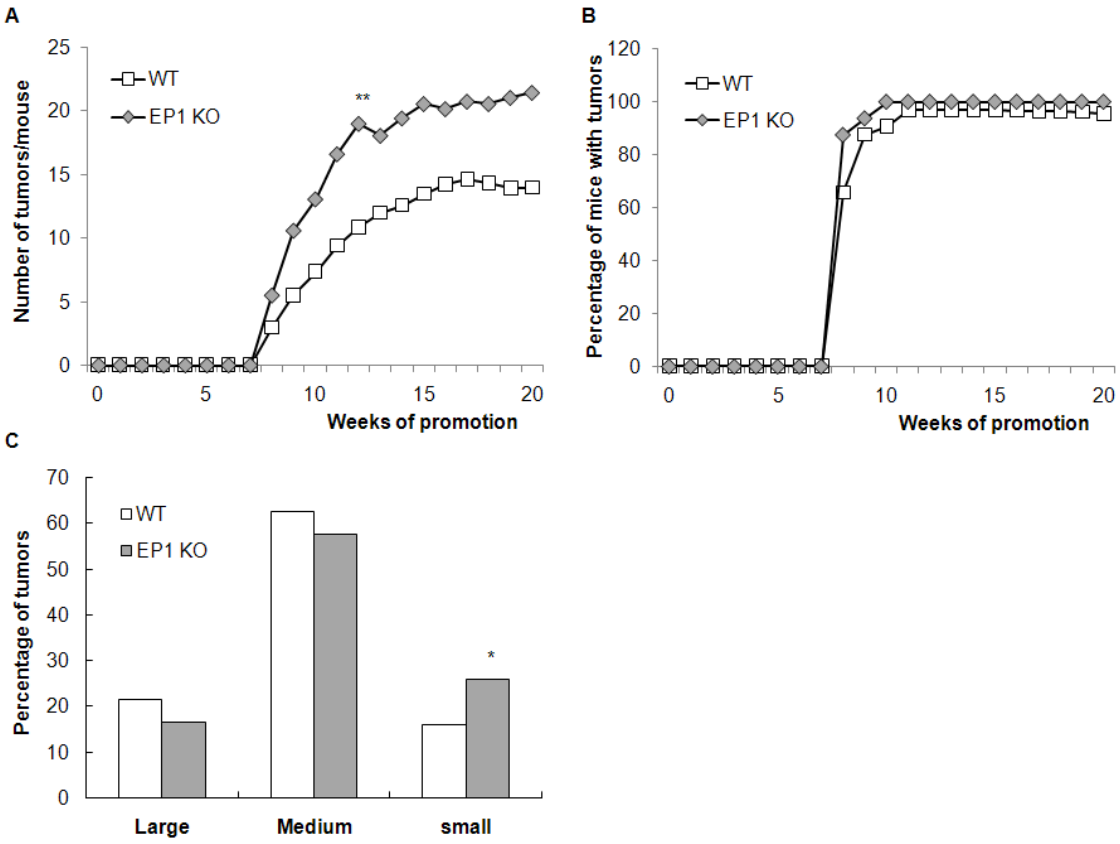


Figure 3-2. The effect of EP1 deficiency in DMBA/TPA–induced skin tumor development. DMBA/TPA skin carcinogenesis protocol was used with 32 EP1 deficient mice and 32 wild type mice. A, Tumor multiplicity; data are expressed as the average number of tumors per mouse. **p<0.01, Poisson regression. B, Tumor incidence; data are expressed as the percentage of mice bearing tumors. *Open squares* represent wild type mice. *Closed triangles* represent EP1 deficient mice. C, Tumor size ; Percentage of skin tumors by diameter size : Small < 2 mm ; Medium 2 – 5 mm; Large > 5 mm. *Open bars* represent skin tumors from wild type mice. *Closed bars* represent skin tumors from knockout mice. * p<0.05, 2-sample test for equality of proportions.

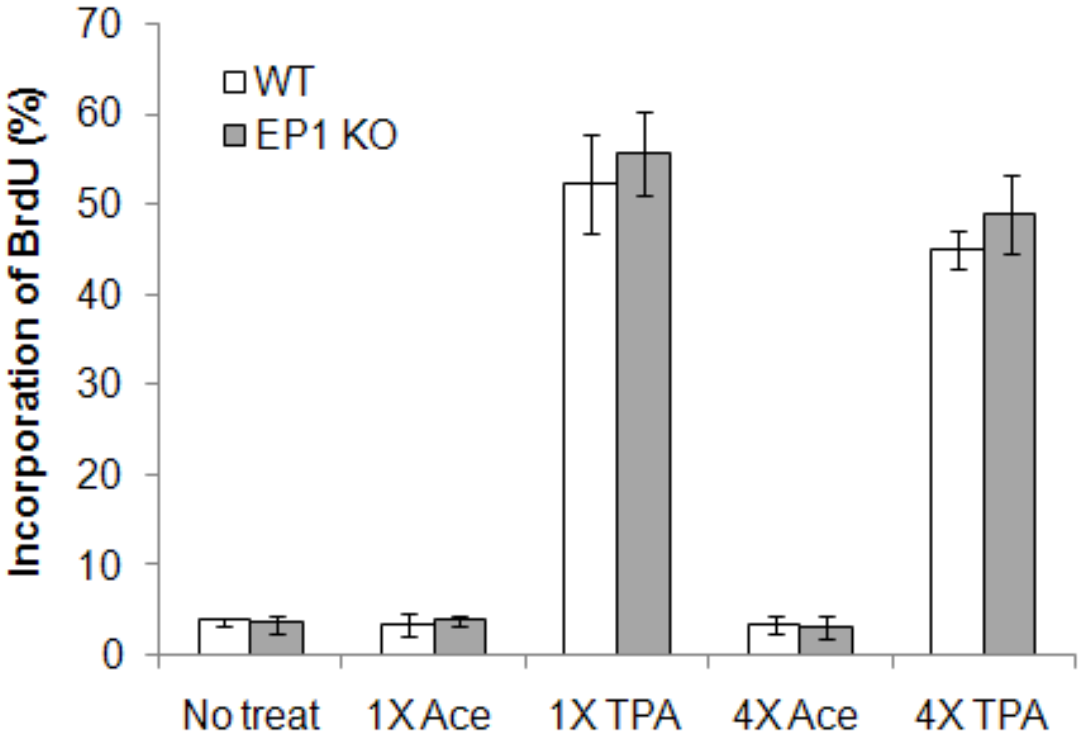


Figure 3-3. DNA synthesis in the epidermis of EP1 deficient and wild type mice. Mice were topically treated with TPA (2.5 µg/200 µl) or acetone (200 µl). Seventeen hours after last treatment, mice were injected with BrdU. One hour later, dorsal skin was removed, fixed in formalin, and immunostained for BrdU. The data are shown as percentage of BrdU positive basal cells.

versus 1.8 for EP1 deficient mice) compared to wild type mice (Figure 3-4A). However, there was no difference statistically. Also, tumor incidence was very similar in wild type mice and EP1 deficient mice. (Figure 3-4B). These data show that EP1 deficiency does not affect skin tumor development in the UV carcinogenesis protocol.

3.5 GENERATION OF BK5.EP1 CONSTRUCT

Because PGE_2 can inhibit the immune system, targeted overexpression of EP1 in the skin can serve as a useful model to study the role of EP1 in skin carcinogenesis. Therefore, BK5.EP1 transgenic mice which overexpress EP1 in the basal layer of the epidermis under the control of a bovine keratin 5 promoter were generated.

To make BK5.EP1 transgenic mice, a BK5.EP1 construct had to be made first. Mouse EP1 cDNA (in pBluescript plasmid, pBluescript EP1) was a deeply appreciated gift from Dr. Sugimoto of Kyoto University, Japan (Figure 3-5). The strategy for making the BK5.EP1 construct is shown in Figure 3-6. The pBluesctipt EP1 plasmid was checked for fidelity by diagnostic restriction digests. EcoRI digestion produced the correct fragment sizes of 1312 bp and 2958 bp fragments. SmaI digestion produced the anticipated fragment sizes of 977 bp and 3293 bp. AlwNI digestion also produced the expected fragment sizes of 612 bp, 1065 bp and 2593 bp (Figure 3-7A). The EP1 cDNA was cut from pBluescript EP1 by digestion with EcoRI enzyme (Figure 3-7B). The pBK5 vector (Figure 3-8) was also digested with SnaBI (Figure 3-7B). After the ligation reaction of EP1 cDNA into the pBK5 vector, E. coli were transformed and 13 colonies were picked and cultured. To determine that the plasmid had the correct orientation of EP1 in the pBK5 vector, a series of diagnostic restriction digestions were performed. By NheI and SmaI digestion, sense-ligated plasmid should produce 10.246 kb and 0.966 kb fragments but anti-sense-ligated plasmid should produce 10.866 kb and 0.347 kb

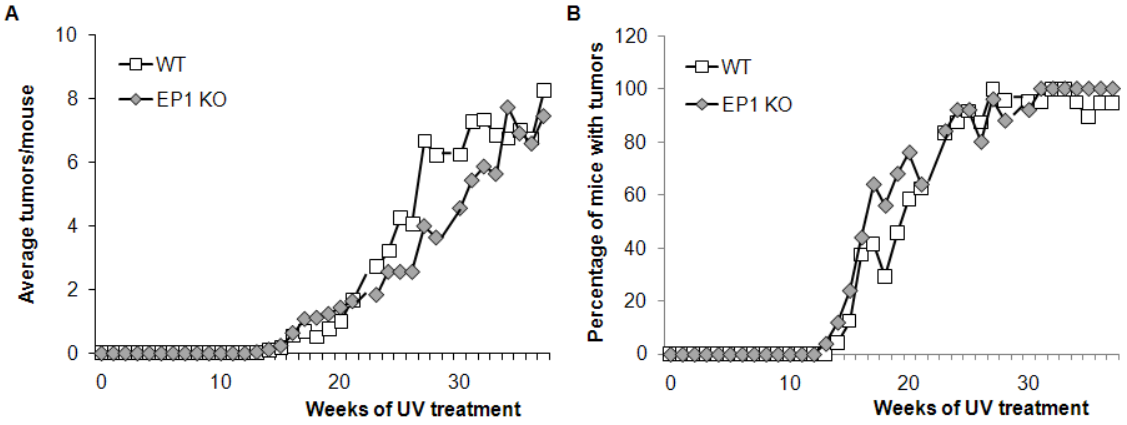


Figure 3-4. The effect of EP1 deficiency in UV-induced skin tumor development. A UV skin carcinogenesis protocol was used with 25 EP1 deficient mice and 25 wild type mice. A, Tumor multiplicity; Data are expressed as the average number of tumors per mouse. B, Tumor incidence; data are expressed as the percentage of mice bearing tumors. *Open squares,* wild type mice (WT); *Closed diamonds,* EP1 deficient mice (EP1 KO).

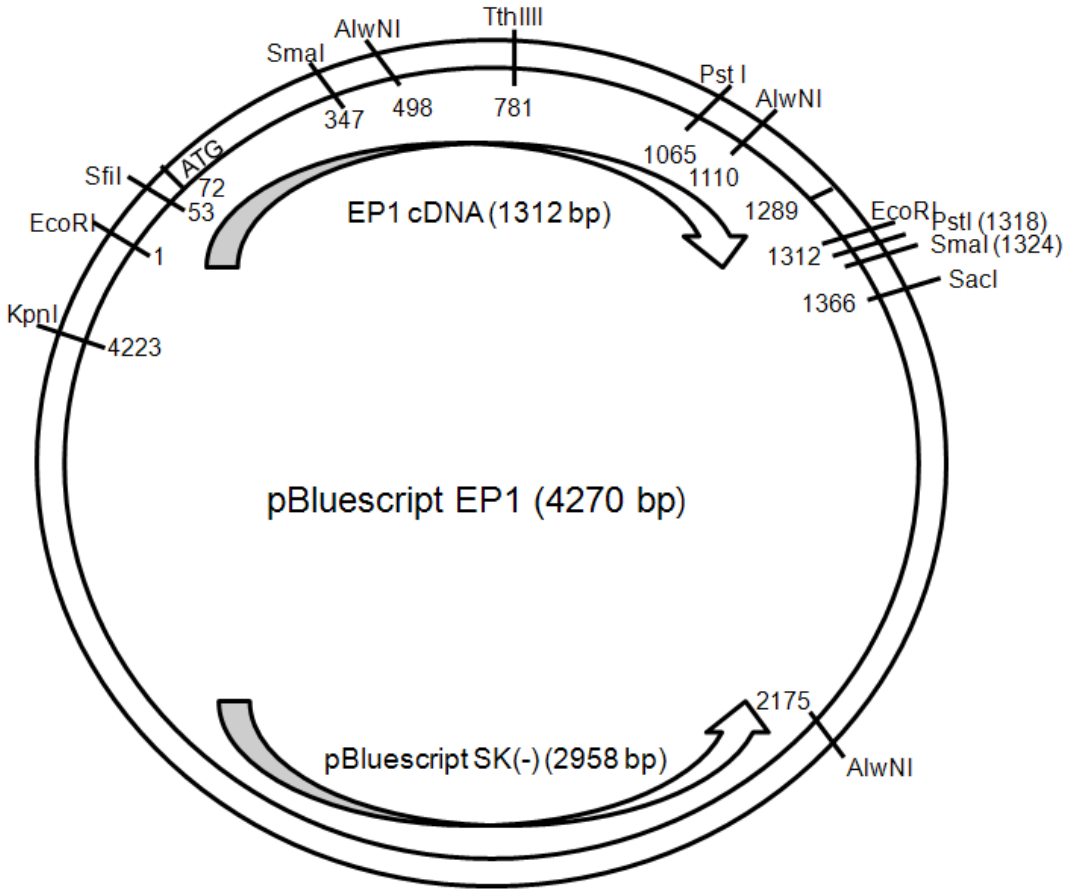


Figure 3-5. pBluescript EP1 plasmid. Mouse EP1 cDNA (1312 bp) was inserted into pBluescriptSK(-) (4270 bp). Coding region: 72-1289 bp. The pBLuescript EP1 plasmid was a generous gift from Dr. Sugimoto at Kyoto University, Japan.

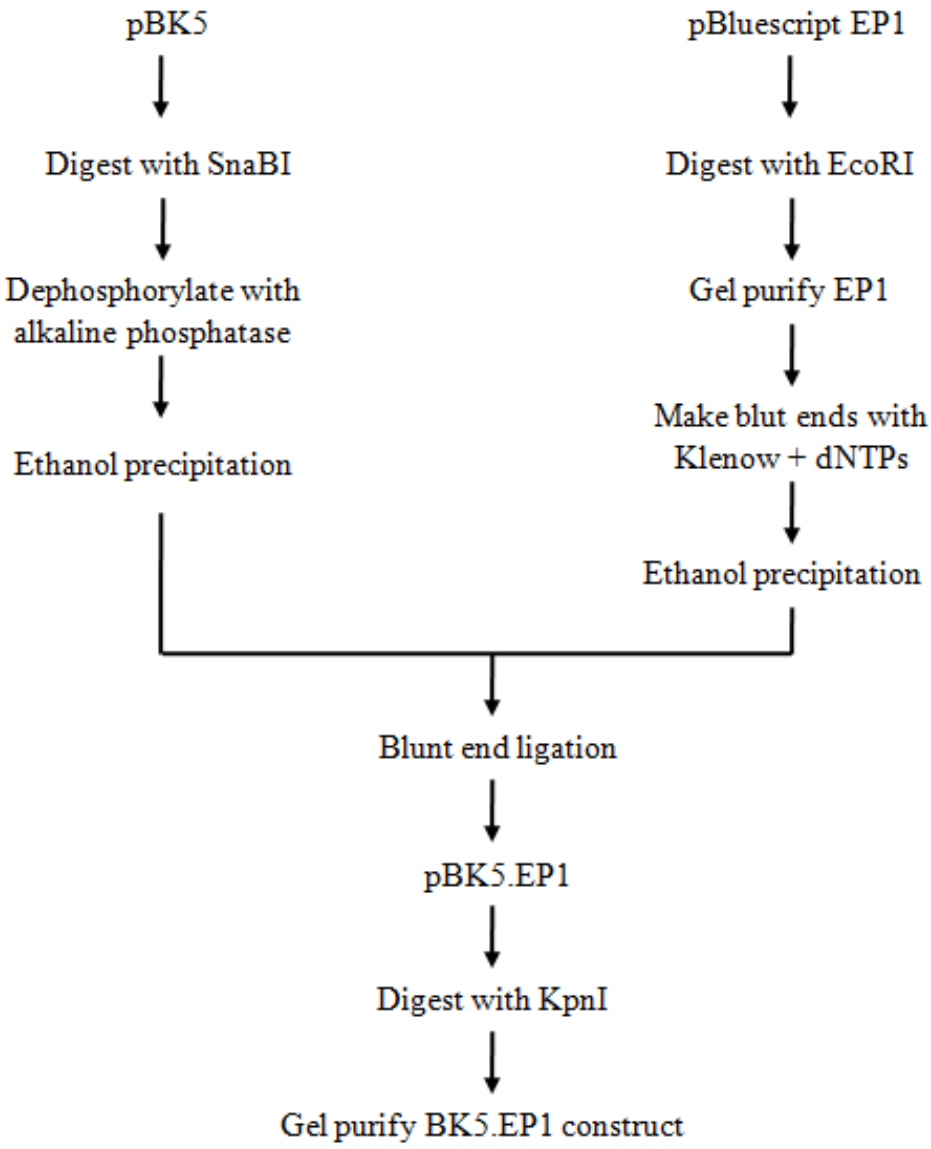


Figure 3-6. Strategy to make pBK5.EP1

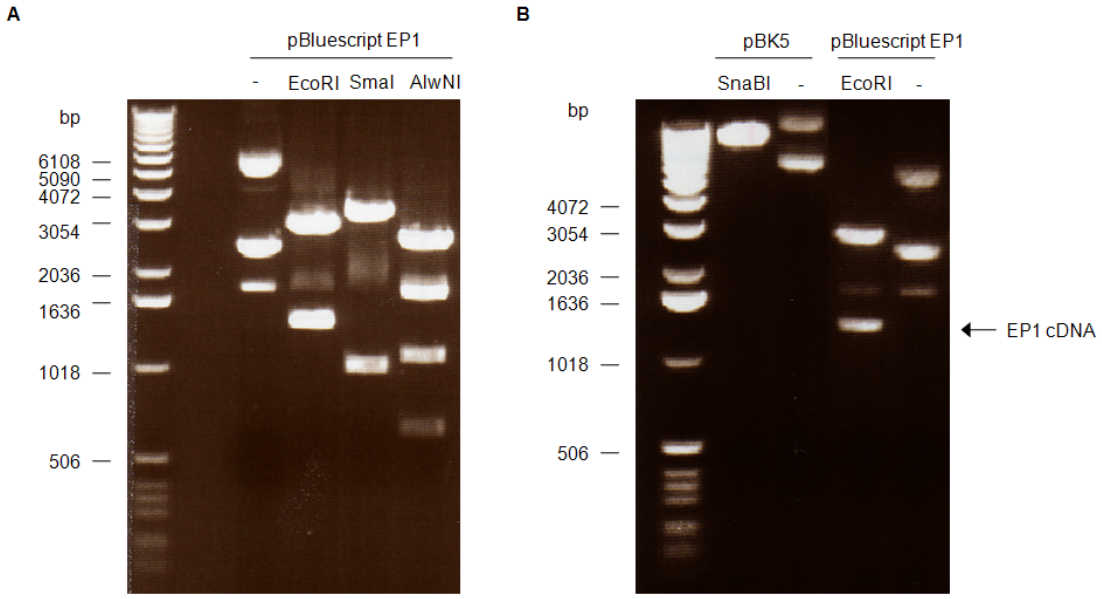


Figure 3-7. Diagnostic digestion of EP1 and digestion of pBK5 and EP1 cDNA. A, diagnostic restriction digest of pBluescriptEP1. To check the fidelity of pBluesctiot EP1 plasmid, the pBluescriptEP1 plasmid was digested with EcoRI, SmaI and AlwNI. B, Digestion of pBK5 and EP1 cDNA. pBK5 plasmid was cut by SnaBI. EP1 cDNA was cut by EcoRI digestion of pBluescriptEP1.

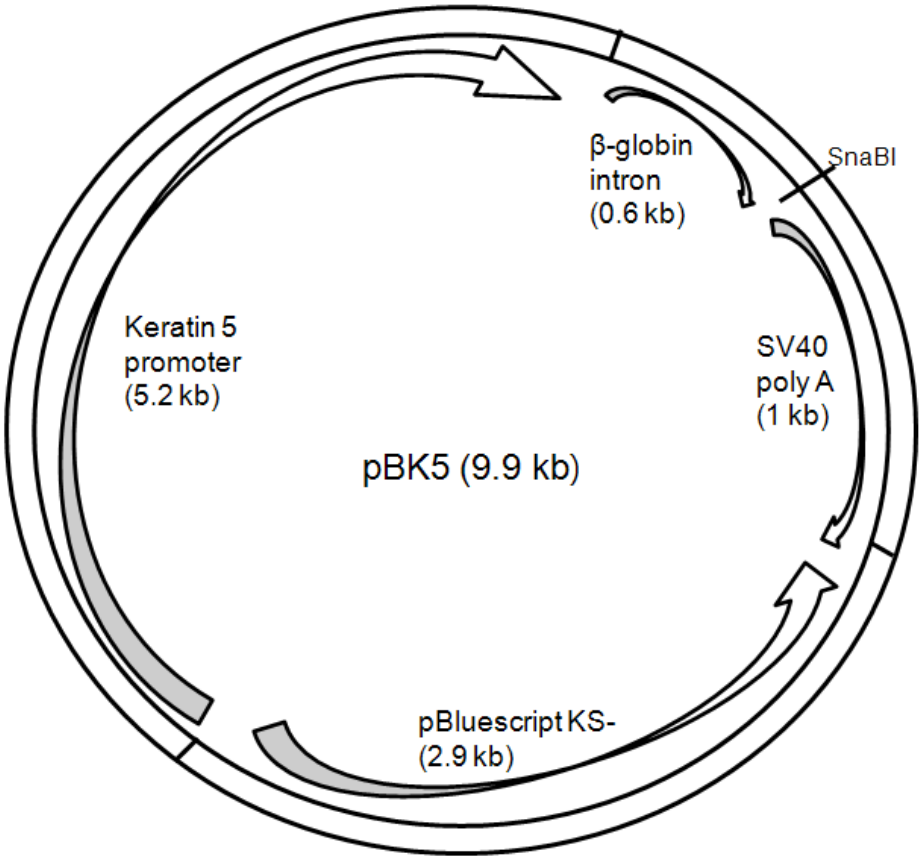


Figure 3-8. pBK5 plasmid. Keratin 5 promoter is followed by β-globin intron and SV 40 poly A signal.

fragments. With NheI and SfiI digestion, sense-ligated plasmid should produce 9.952 kb and 1.260 kb fragments but anti-sense ligated plasmid should produce 11.16 kb and 0.052 kb fragments (Figure 3-9A). Plasmid #1, and #12 had the correct orientation (Figure 3-10A). Colony #12 was cultured and checked by a series of diagnostic digestions – NheI and SmaI, NheI and SfiI, then KpnI and SmaI. KpnI and SmaI digestion should produce 6.046 kb, 3 kb, and 2.166 kb fragments (Figure 3-10B). The BK5.EP1 DNA construct (Figure 3-9B) was cut by KpnI digestion (Figure 3-10C) and injected into fertilized eggs by the Science Park Transgenic Mouse Facility Core.

3.6 GENERATION OF BK5.EP1 TRANSGENIC MICE

Four BK5.EP1 transgenic founders (# 8, 9, 10 and 15) were identified by Southern blot analysis (Figure 3-11A). One founder (#10) did not produce any offspring. To examine the expression level of EP1 in epidermis, EP1 mRNA levels in epidermis from heterozygous offspring of each founder were measured by northern blot analysis. All three lines of BK5.EP1 transgenic mice showed significantly increased levels of EP1 expression compared to wild type mice (Figure 3-11B). Line 8 and line 9 express about two-fold higher levels of EP1 mRNA compared to line 15. Therefore, line 8 heterozygous BK5.EP1 transgenic mice were used for all subsequent experiments.

The BK5.EP1 transgenic mice had macroscopically normal skin and did not show any signs of health problems. When examined under a microscope, the epidermis of BK5.EP1 transgenic mice was slightly thicker than that of wild type mice. Other than that, there was no histological difference between wild type and BK5.EP1 transgenic mice (Figure 3-13).

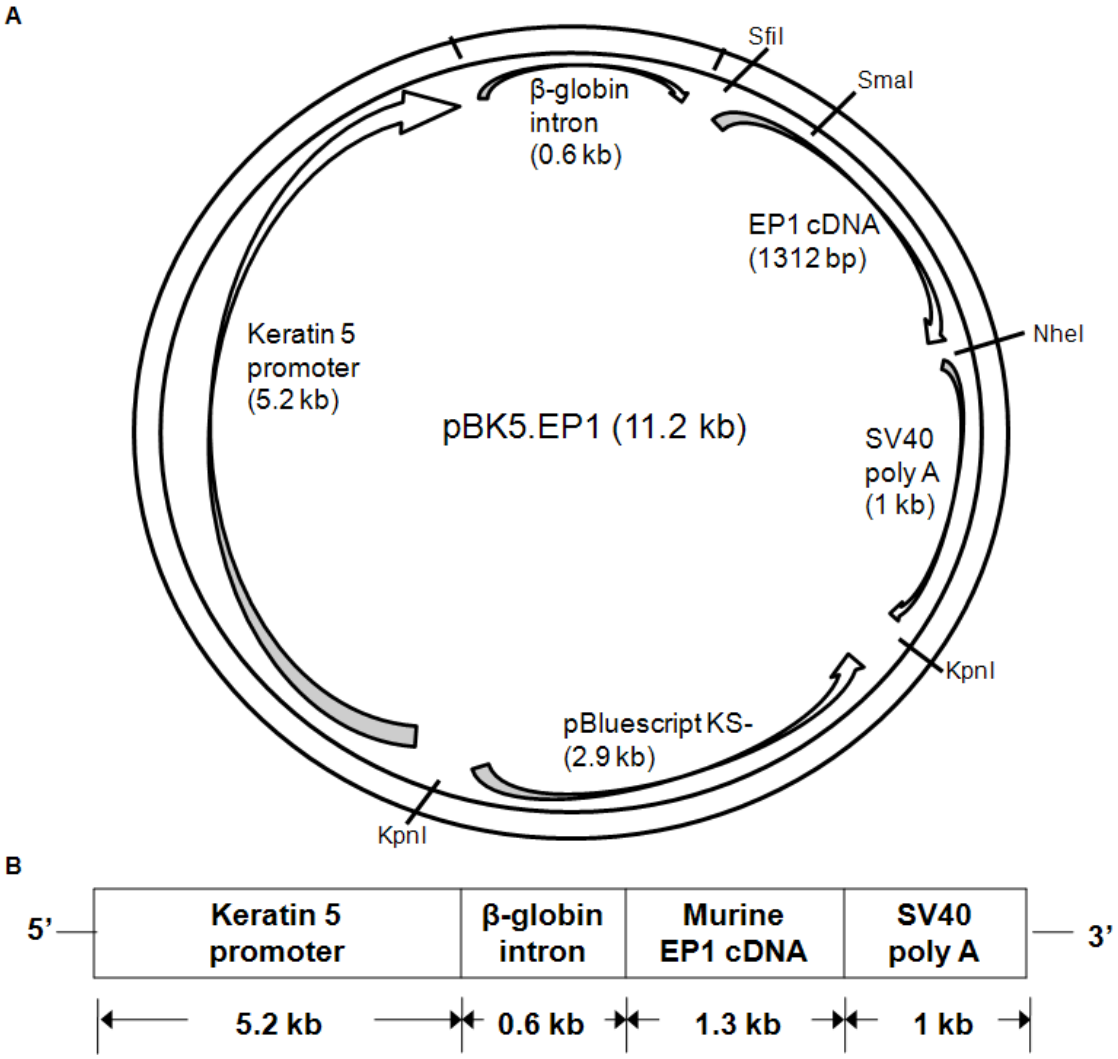
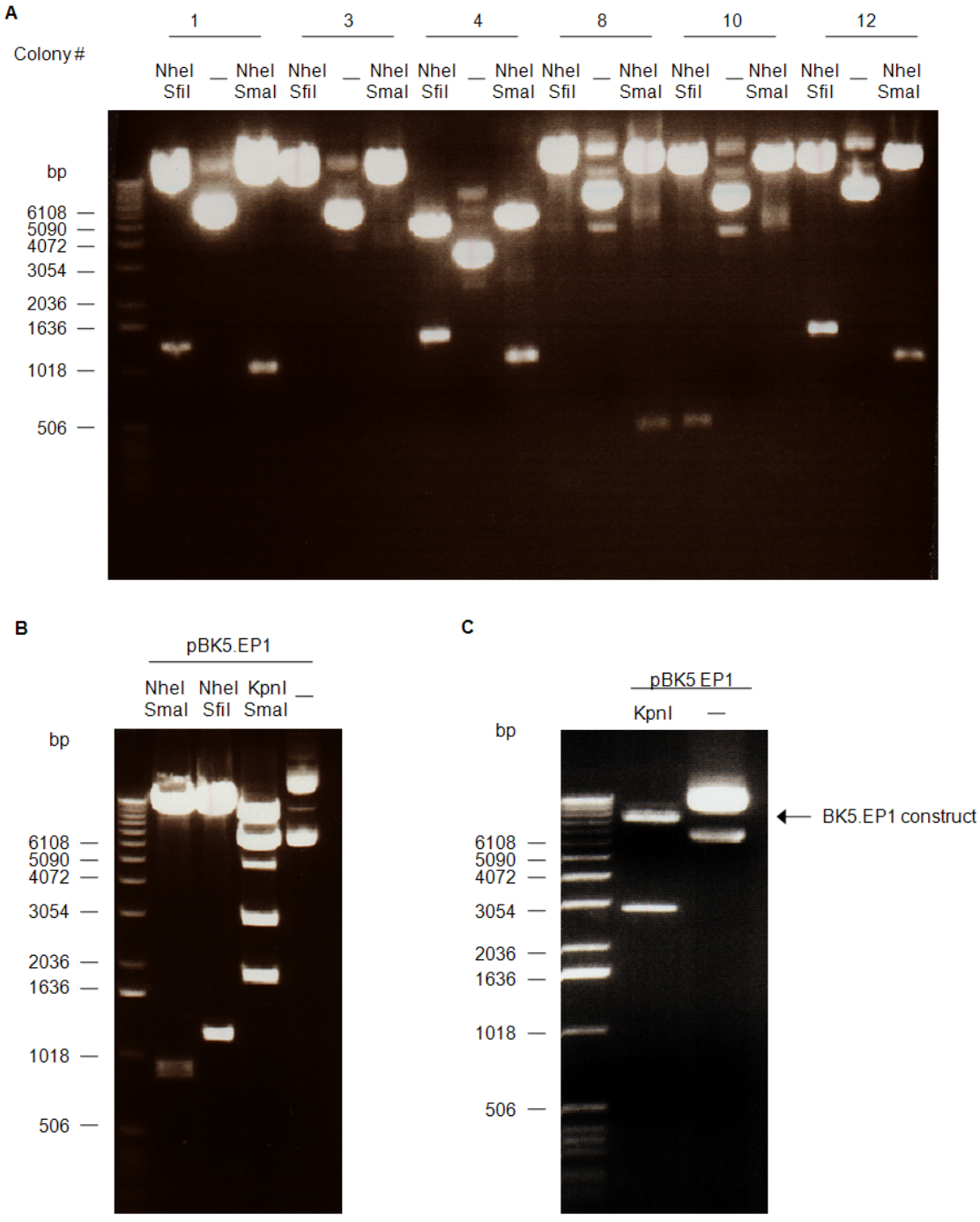


Figure 3-9. pBK5.EP1 plasmid and BK5.EP1 construct. A. pBK5.EP1 plasmid. Murine EP1 cDNA was inserted into pBK5. B. Diagram of the BK5.EP1 DNA construct.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1018
506
B
pBK5.EP1
NheI SmaI
NheI SfiI
KpnI SmaI
C
pBK5 EP1
KpnI
BK5.EP1 construct

Figure 3-10. Characterization of the pBK5.EP1 plasmid. A. Diagnostic restriction digestion to determine correct orientation of EP1 in the pBK5 vector. Plasmid was digested with NheI and SfiI or Nhe and SmaI. B. Diagnostic restriction digestion of pBK5.EP1 to check fidelity. Plasmid was digested with NheI and SmaI, Nhe and SfiI and KpnI and SmaI. C. The BK5.EP1 construct was cut from pBK5.EP1 by KpnI digestion for injection into fertilized eggs.

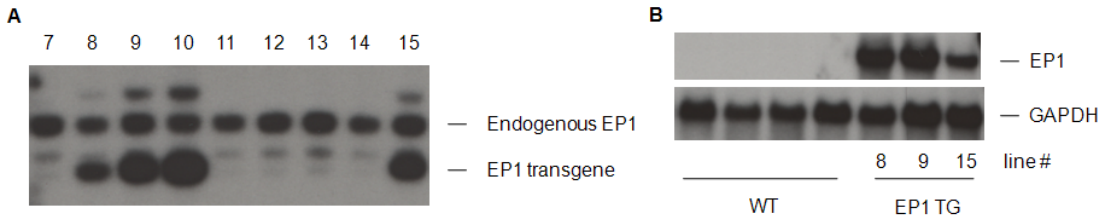


Figure 3-11. Generation of BK5.EP1 transgenic mouse. A, Identification of founder BK5.EP1 transgenic mice. Genomic DNA was isolated from tails and used for Southern blot analysis. B, EP1 mRNA levels in epidermis from BK5.EP1 transgenic mice and wild type mice were analyzed by northern blot with specific EP1 probe.

3.7 EFFECT OF EP1 OVEREXPRESSION ON GENE EXPRESSION RELATED TO SIGNAL TRANSDUCTION PATHWAY

To examine the effect of EP1 overexpression on gene expression, the mRNA expression level of 84 genes related to signal transduction in the epidermis of wild type and BK5.EP1 transgenic mice was analyzed by PCR array. The genes that are expressed more than 2-fold or less than 2-fold in BK5.EP1 transgenic mice compared to wild type mice are shown (Table 3-1). To confirm the increased level of tumor necrosis factor-α in the BK5.EP1 transgenic mice, the expression of TNF-α was measured by enzyme immunoassay. With vehicle treatment and TPA treatment, there was no difference in TNF-α levels in wild type mice and in BK5.EP1 transgenic mice (Table 3-2). Although the tumor necrosis factor-α mRNA level was increased in the untreated BK5.EP1 transgenic mice, the tumor necrosis factor-α protein level was not increased in the untreated BK5.EP1 transgenic mice compared to the wild type mice. After TPA treatment, both the wild type and the BK5.EP1 transgenic mice induced similar levels of tumor necrosis factor-α protein.

3.8 EFFECT OF EP1 OVEREXPRESSION ON DMBA/TPA SKIN CARCINOGENESIS

To examine the effect of EP1 overexpression on skin tumor development, a DMBA/TPA skin carcinogenesis protocol was carried out with BK5.EP1 transgenic mice and wild type mice. In BK5.EP1 transgenic mice, the first tumor appeared 2 weeks after initiation, that is, prior to application of the promoter. By contrast, the first tumor appeared at 7 weeks after starting promotion in wild type mice. By week 7 of promotion, BK5.EP1 transgenic mice had more tumors and a greater tumor incidence than wild type mice. However, starting at 9 weeks of promotion, wild type mice produced more tumors

Gene	Fold change	Function
NAIP	2.32	Anti-apoptotic
Wnt 1	2.71	oncogene
Wnt 2	2.35	
aromatase	11.25	Estrogen synthesis
Matrix metalloproteinase 10	4.97	Invasion
Chemokine ligand 20	13.65	Proinflammatory
Chemokine ligand 1	5.72	
Colony stimulating factor2	9.35	
Interleukin 2 receptor α chain	2.56	
COX-2	6.18	
Tumor necrosis factor α	2	
ICAM 1	2.08	Adhesion
Engrailed 1	0.41	Transcription factor
Leptin	0.26	Regulation of body weight

Table 3-1. Representative genes that are expressed more than 2-fold or less than 2-fold in BK5.EP1 transgenic mice compared to wild type mice. Epidermal scrapes were collected from 3 mice for each group. Signal Transduction Pathway Finder PCR Array (SABiosciences) was used.

	Ace	TPA
WT	0.15 ± 0.23	11.60 ± 0.81
EP1 TG	0.17± 0.13	11.89 ± 1.04

Table 3-2. Effect of EP1 expression on TNF- α level in skin epidermis. Level of TNF-α (pg/100 μg protein) was measured 2.5 hours after 200 μl of acetone (Ace) or 2.5 μg/200 μl TPA treatment of wild type and BK5.EP1 transgenic mice by enzyme immune assay. Groups of 3 mice were used.

(average of 7.5 for wild type mice versus 3.1 for BK5.EP1 transgenic mice) with a higher tumor incidence (93%) compared to 81% for BK5.EP1 transgenic mice (Figure 3-12A and B). Interestingly, in BK5.EP1 transgenic mice the first carcinoma was observed at 5 weeks of promotion but in the wild type mice the first carcinoma did not appear until 14 weeks of promotion. By week 20 of promotion, BK5.EP1 transgenic mice produced many more carcinomas than wild type mice (26 for BK5.EP1 transgenic mice versus 3 for wild type mice) (Figure 3-12C). At the end of the experiment, tumors larger than 2 mm were collected and histologically examined. From BK5.EP1 transgenic mice, 24 squamous cell carcinomas and 62 papillomas were collected. By contrast, wild type mice produced only 4 squamous cell carcinomas with 114 papillomas. Thus, BK5.EP1 transgenic mice were found to have a higher papilloma to carcinoma conversion rate (28.0% v.s. 3.4%, $p<0.001$, Fisher's exact test). The high rate of carcinoma production in BK5.EP1 transgenic mouse implies that EP1 is important in tumor progression.

3.9 EFFECT OF EP1 OVEREXPRESSION ON TPA-INDUCED CELL PROLIFERATION

In malignant keratinocytes, PGE_2 and an EP1 agonist prevented NSAID-induced cell growth inhibition (Thompson, Gupta et al. 2001). Therefore, the effect of EP1 overexpression in basal cells on cell proliferation was examined. Without any treatment, BrdU incorporation was similar in the epidermis of wild type and BK5.EP1 transgenic mice. In BK5.EP1 transgenic mice, BrdU incorporation was increased 2-fold compared to wild type mice treated once or four times with vehicle. However, one TPA (2.5 μg) or four treatments of TPA increased BrdU incorporation to the same extent in BK5.EP1 transgenic mice and wild type mice (Figure 3-13A). Measurements of epidermal

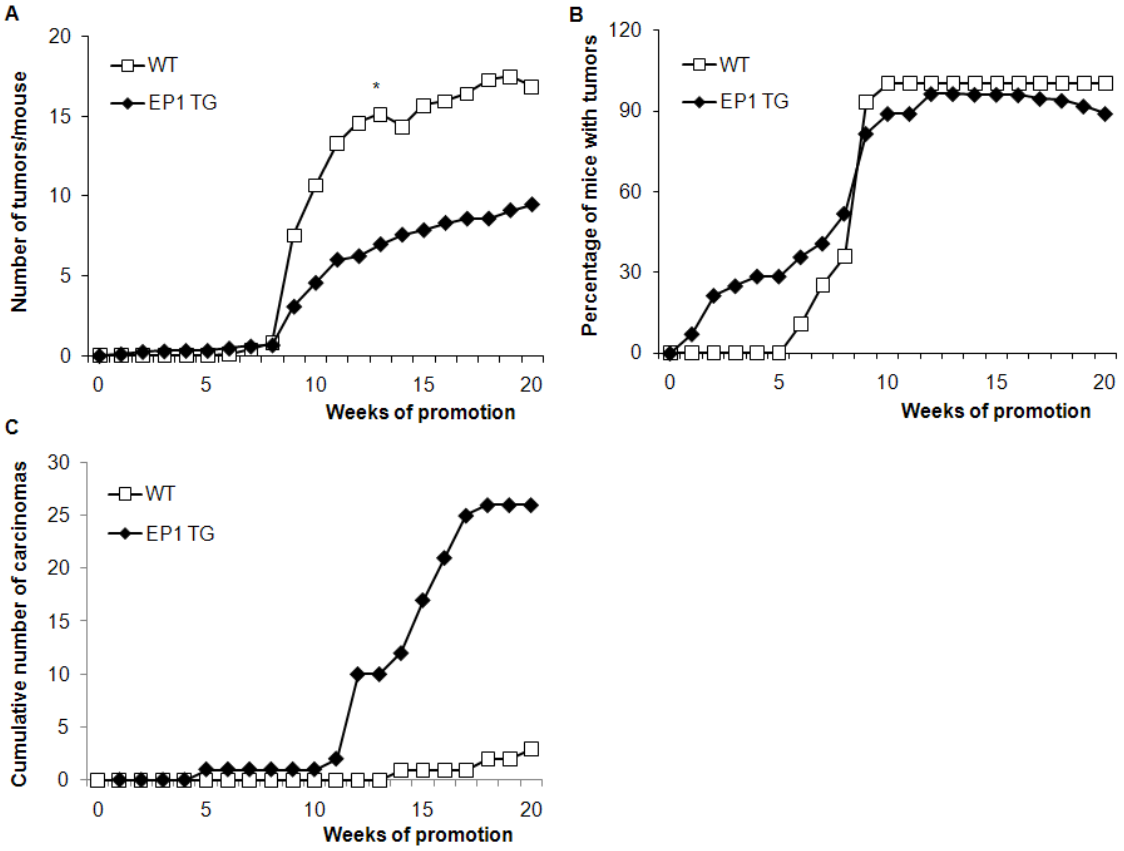
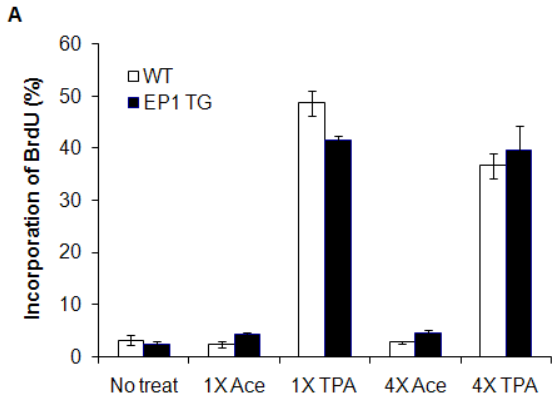
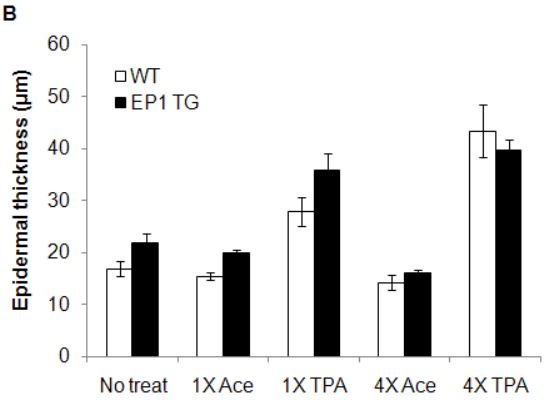
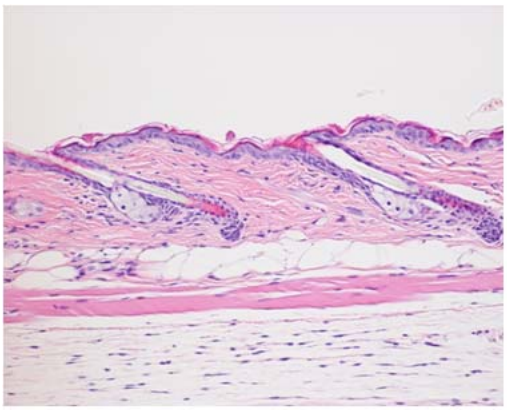


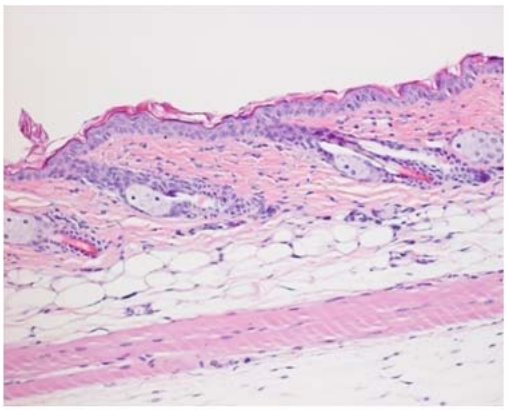
Figure 3-12. The effect of EP1 overexpression on DMBA/TPA–induced skin tumor development. A DMBA/TPA skin carcinogenesis protocol was used with 28 BK5.EP1 transgenic mice and 28 wild type mice. A, Tumor multiplicity; data are expressed as the average number of tumors per mouse. B, Tumor incidence; data are expressed as the percentage of mice bearing tumors. C, Carcinoma multiplicity; data are expressed as the cumulative number of carcinomas. *Open squares* represent wild type mice. *Closed diamonds* represent BK5.EP1 transgenic mice. * $p<0.001$, Poisson regression



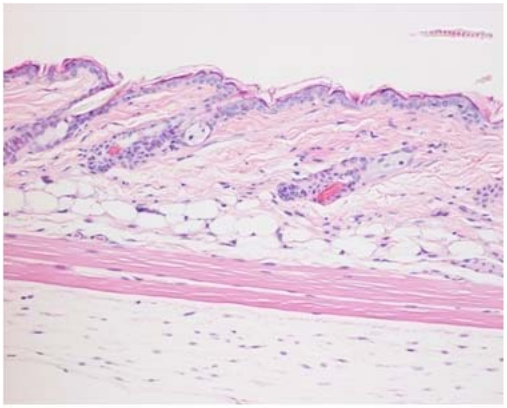




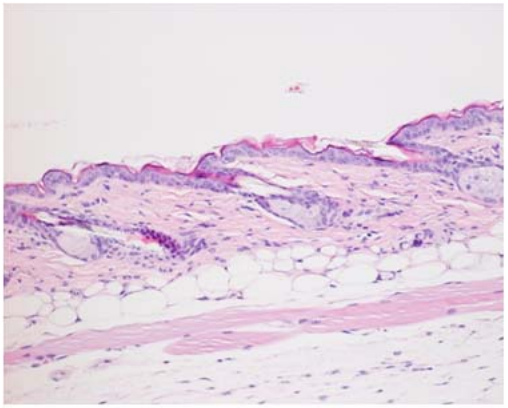
WT – No treatment



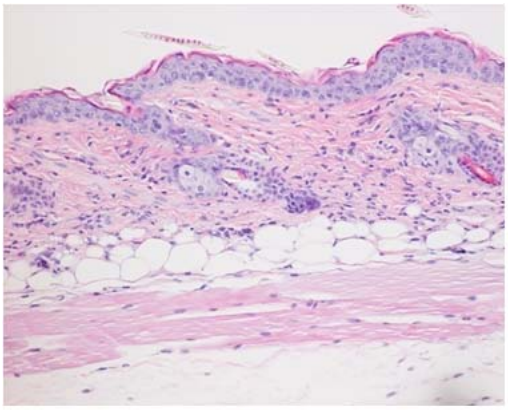
TG – No treatment



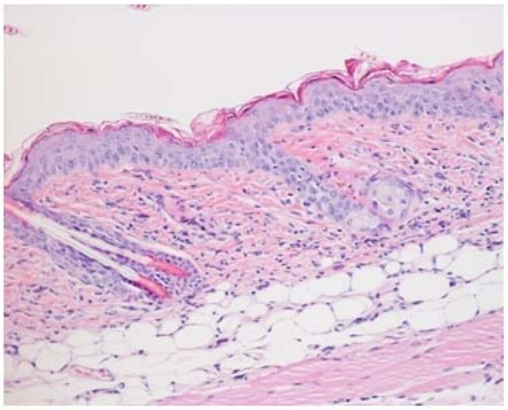
WT – 1X Acetone



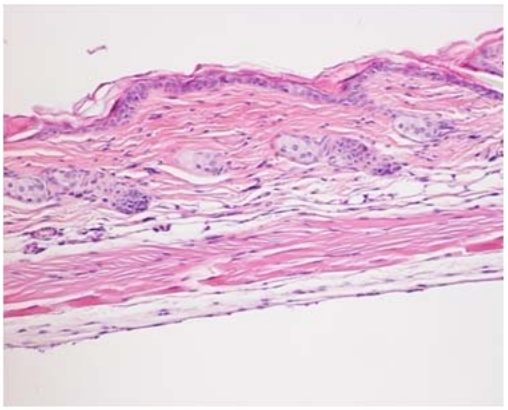
TG – 1X Acetone



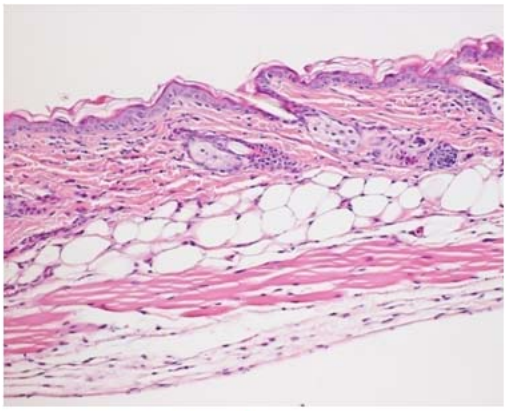
WT – 1X TPA



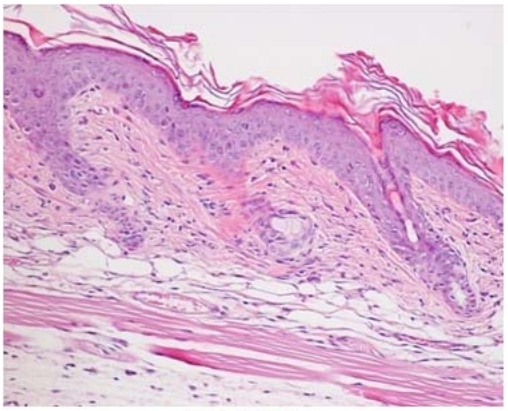
TG – 1X TPA



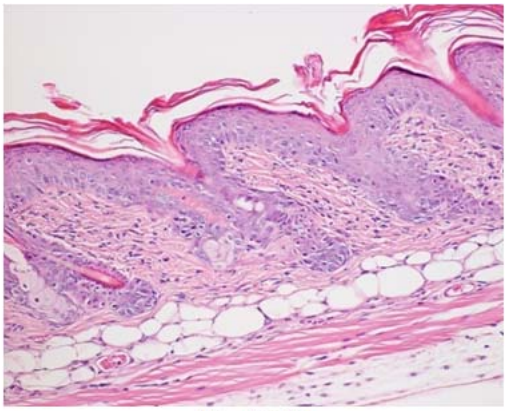
WT – 4X Acetone



TG – 4X Acetone



WT – 4X TPA



TG – 4X TPA

Figure 3-13. Epidermal cell proliferation in BK5.EP1 transgenic and wild type mice. A, BrdU incorporation. Mice were topically treated with TPA (2.5 µg/200 µl) or acetone (200 µl). Seventeen hours after the last treatment, mice were injected with BrdU. One hour later, dorsal skin was removed, fixed in formalin, and immunostained for BrdU. The data are shown as percentage of BrdU positive basal cells. B, Epidermal thickness. Dorsal skin was collected 18 hours after the last acetone (200 µl) or TPA (2.5 µg/200 µl acetone) treatment and stained with H&E. Epidermal thickness was measured under the microscope. 1X Ace, one acetone (200 µl) treatment; 1X TPA, one TPA (2.5 µg/200 µl acetone) treatment; 4X Ace, acetone (200 µl) treatment every three days for 4 treatments, 4X TPA, TPA (2.5 µg/200 µl acetone) treatment every three days for 4 treatments.

thickness revealed that the epidermis was slightly thicker in BK5.EP1 transgenic mice without any treatment or after one vehicle treatment. Eighteen hours after one TPA treatment, the epidermis of BK5.EP1 transgenic mice was still slightly thicker than that of wild type mice. However, 18 hours after four applications of vehicle or TPA, the epidermal thickness of wild type and BK5.EP1 transgenic mice was very similar (Figure 3-13B). These data showed that overexpression of EP1 does not affect TPA-induced proliferation. Because deficiency of EP1 also did not affect TPA induced epidermal cell proliferation, it is concluded that the level of EP1 expression does not affect TPA induced epidermal cell proliferation.

3.10 EFFECT OF EP1 EXPRESSION LEVEL ON TPA-INDUCED SIGNALING

It has been shown that an EP1 agonist induces COX-2 in mouse osteoblastic cells (Suda, Tanaka et al. 2000). Therefore, the effect of EP1 overexpression on TPA-induced COX-2 expression was examined by western blot. Without any treatment, there was no COX-2 protein expression in the skin epidermis of wild type mice. However, in wild type mice COX-2 expression was induced 3 hours after 2.5 μg of TPA treatment, with a maximum induction occurring at 6 hours after TPA treatment. In the BK5.EP1 transgenic mice, however, COX-2 was expressed in the absence of TPA. Interestingly, the COX-2 expression level was not changed by TPA treatment in the BK5.EP1 transgenic mice. That is, at 6 hours after TPA treatment wild type mice expressed more COX-2 than the BK5.EP1 transgenic mice (Figure 3-14A). The effect of EP1 deficiency on TPA-induced COX-2 was determined by western blot. At 3 hours after TPA treatment, both wild type and EP1 deficient mice had a moderate level of COX-2 induction. At 6 hours after TPA treatment, in EP1 deficient mice COX-2 induction was similar to that of wild type mice (Figure 3-14B). That is, overexpression of EP1 induced COX-2 without TPA application

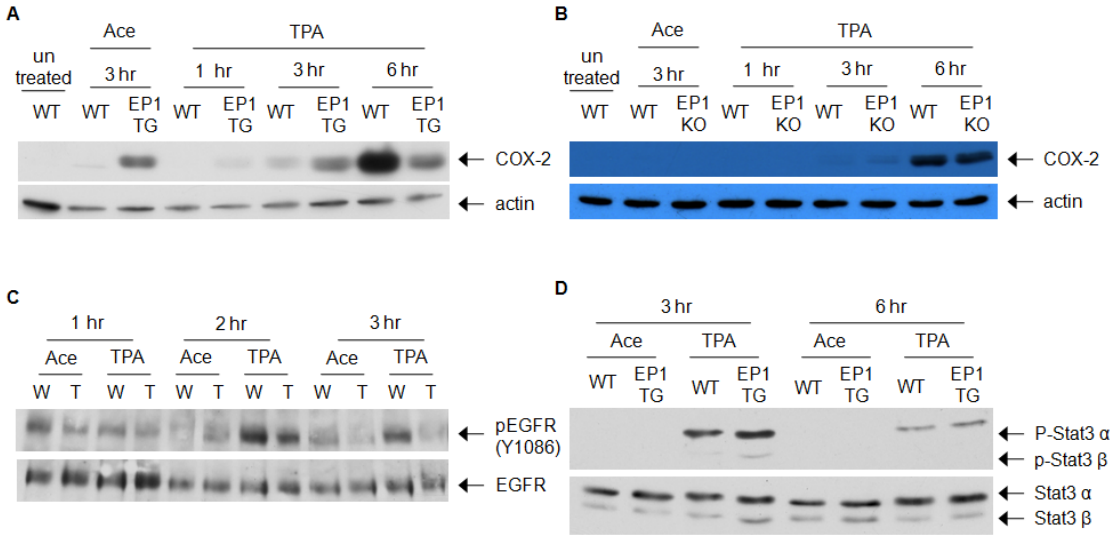


Figure 3-14. Effect of EP1 overexpression on TPA-induced signaling. A. Protein was extracted from epidermis at specified times after acetone (Ace) or TPA treatment of wild type (WT) and BK5.EP1 transgenic mice (EP1 TG). COX-2 protein level was determined by western blot. Actin protein levels were used as loading controls. B. Protein was extracted from epidermis at specified times after acetone (Ace) or TPA treatment of wild type (WT) and EP1 deficient mice (EP1 KO). COX-2 protein level was determined by western blot. Actin protein levels were used as loading controls. C. Protein was extracted from epidermis at specified times after acetone (Ace) or TPA treatment of wild type (W) and BK5.EP1 transgenic mice (T). pEGFR (Y1086) protein level was determined by western blot. The same blot was striped and probed with an antibody that recognizes EGFR. D. Protein was extracted from epidermis of untreated wild type (WT) and BK5.EP1 transgenic mice (EP1 TG). pStat 3 protein level was determined by western blot. The same blot was striped and probed with an antibody that recognizes total Stat 3.

but TPA did not further induce COX-2. These data could be a clue for determining the underlying mechanism of why BK5.EP1 transgenic mice produced fewer tumors than wild type mice in response to TPA.

It has been shown that EP1 transactivates EGFR in human cholangiocarcinoma cells (Han and Wu 2005). Therefore, the effect of EP1 overexpression on TPA-induced EGFR phosphorylation was also examined by western blot. TPA induced maximum phosphorylation of EGFR at 2 hours after TPA treatment in wild type mice. In BK5.EP1 transgenic mice, TPA also induced phosphorylation of EGFR at 2 hours but to a much lower extent than observed in wild type mice. At 3 hours after TPA treatment, the level of pEGFR was slightly reduced but still higher than vehicle-treated wild type mice. However, at 3 hours after TPA treatment, the level of pEGFR was completely back to normal level in BK5.EP1 transgenic mice (Figure 3-14C). Interestingly, overexpression of EP1 induced less pEGFR after TPA treatment and that is possibly one of the reasons why the BK5.EP1 transgenic mice produced fewer tumors than wild type mice in the DMBA/TPA induced skin carcinogenesis protocol.

An EP1 antagonist pretreatment effectively blocked PGE_2 induced Stat3 phosphorylation in human cholangiocarcinoma cells (Han, Demetris et al. 2006). Therefore, the effect of EP1 overexpression on TPA-induced Stat3 phosphorylation was also examined by western blot. With vehicle treatment, there was no phosphorylated Stat3 detectable in either the BK5.EP1 transgenic or the wild type mice. Both wild type and BK5.EP1 transgenic mice induced a similar level of phosphorylated Stat3 at 3 hours after TPA treatment. By 6 hours after TPA treatment, the phosphorylated Stat3 level was reduced but still higher than normal level in both the wild type and the BK5.EP1 transgenic mice. There was no change in total Stat3 expression (Figure 3-14D).

Phosphorylated Stat3 level was not affected at all by overexpression of EP1. That is, the Stat3 pathway seems not important in EP1-mediated signaling.

3.11 EFFECT OF EP1 OVEREXPRESSION ON DMBA/ANTHRALIN SKIN CARCINOGENESIS

Since one of the downstream signaling molecules of the EP1 receptor is PKC (Krysan, Reckamp et al. 2005), the expression level of PKC in the BK5.EP1 transgenic mice was examined. PKC protein expression level decreased in the BK5.EP1 transgenic mice compared to wild type mice (Figure 3-15A). Therefore, another tumor promoter, anthralin, which generates free radicals and does not interact with PKC was used (Battalora, Johnston et al. 1995; Bol, Rowley et al. 2002). The first tumor that developed in BK5.EP1 transgenic mice was observed at 2 weeks after initiation whereas in wild type mice the first tumor formed at 29 weeks after promotion. From week 33 of promotion, tumor multiplicity and tumor incidence were increased 2-fold in BK5.EP1 transgenic mice but there was no difference statistically (Figure 3-15B, C). By week 57 of promotion, BK5.EP1 transgenic mice produced 9 carcinomas while wild type mice produced only one carcinoma. In this experiment, anthralin produced fewer papillomas compared TPA. While the BK5.EP1 transgenic mice produced fewer tumors with TPA tumor promotion, the BK5.EP1 transgenic mice produced slightly more tumors with anthralin tumor promotion. Notably, the BK5.EP1 transgenic mice produced more carcinomas than wild type mice in both TPA tumor promotion and anthralin tumor promotion. These data imply that EP1 signaling pathway is more important in progression than promotion.

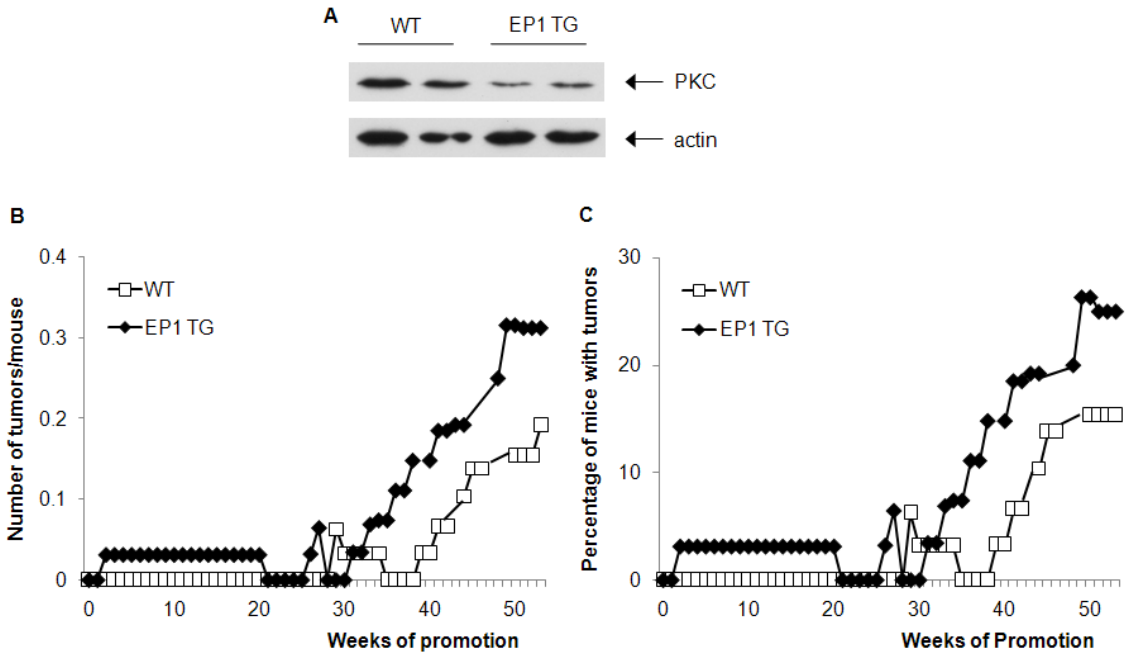


Figure 3-15. The effect of EP1 overexpression on DMBA/anthralin–induced skin tumor development. A. Protein was extracted from epidermis of untreated wild type (WT) and BK5.EP1 transgenic mice (EP1 TG). PKC protein level was determined by western blot. Actin protein levels were used as loading controls. B-C, DMBA/anthralin skin carcinogenesis protocol was used with 32 BK5.EP1 transgenic mice and 32 wild type mice. B, Tumor multiplicity; data are expressed as the average number of tumors per mouse. C, Tumor incidence; data are expressed as the percentage of mice bearing tumors. *Open squares* represent wild type mice. *Closed diamonds* represent BK5.EP1 transgenic mice.

3.12 EFFECT OF EP1 OVEREXPRESSION ON UV SKIN CARCINOGENESIS MODEL

A UV carcinogenesis model was employed in that it is more relevant to humans than other carcinogenesis models used. The first tumor developed at 4 weeks of UV exposure in BK5.EP1 transgenic mice, whereas the first tumor was observed at 21 weeks of UV exposure in wild type mice. At 41 weeks, BK5.EP1 transgenic mice had an average of 1 tumor /mouse, whereas wild type mice produced an average of 0.58 tumors/mouse (Figure 3-16A). However, there was no difference between wild type and the BK5.EP1 transgenic mice statistically. The first carcinoma appeared at 29 weeks after UV treatment in both groups. While BK5.EP1 transgenic mice produced 14 carcinomas by week 50, wild type mice produced 8 carcinomas (Figure 3-16B). Thus, while overexpression of EP1 produces only slightly more tumors than wild type mice in the UV induced carcinogenesis protocol, the BK5.EP1 transgenic mice produced significantly more carcinomas than wild type mice.

3.13 EFFECTS OF EP1 OVEREXPRESSION ON DMBA-ONLY SKIN CARCINOGENESIS

Since tumor formation was observed in EP1 transgenic mice prior to application of the tumor promoter TPA, a DMBA-only experimental protocol was used. However, the dose of DMBA (400 μg) was higher than in the DMBA/TPA protocol (100 μg) to ensure tumor formation in wild type mice. More tumors and a 100% incidence were observed at 3 weeks after DMBA treatment in BK5.EP1 transgenic mice (average 7 tumors/mouse) than wild type mice (average 1 tumor/mouse) (Figure 3-17A and B). There are two reasons for the declining tumor multiplicity seen after week 4. Tumors formed fairly close together and coalesced. Mice with larger than 10 mm carcinoma were sacrificed in the middle of the experiment for humane reasons. The first carcinoma

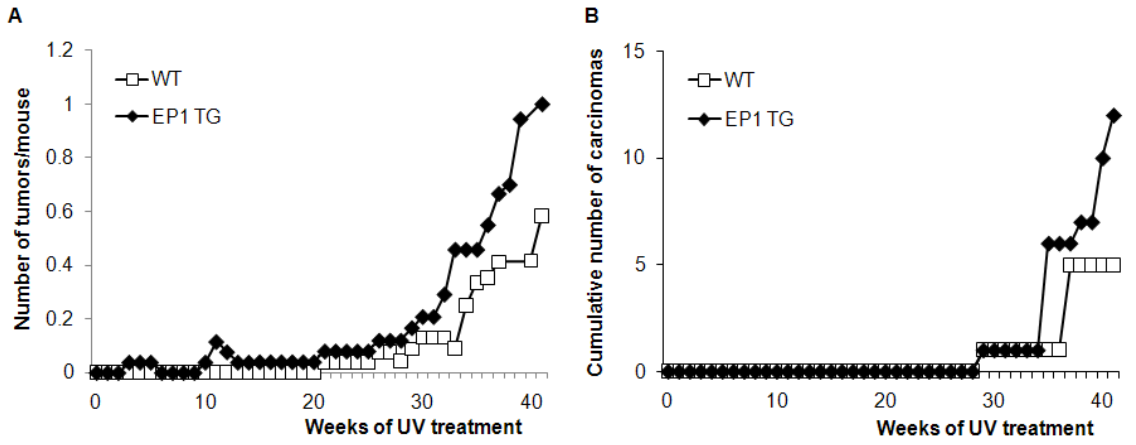


Figure 3-16. The effect of EP1 overexpression in UV-induced skin tumor development. A UV skin carcinogenesis protocol was used with 26 BK5.EP1 transgenic mice and 26 wild type mice. A, Tumor multiplicity; data are expressed as the average number of tumors per mouse. B, Cumulative number of carcinoma; data are expressed as cumulative number of carcinomas. *Open squares,* wild type mice (WT); *Closed diamonds,* BK5.EP1 transgenic mice (EP1 Tg).

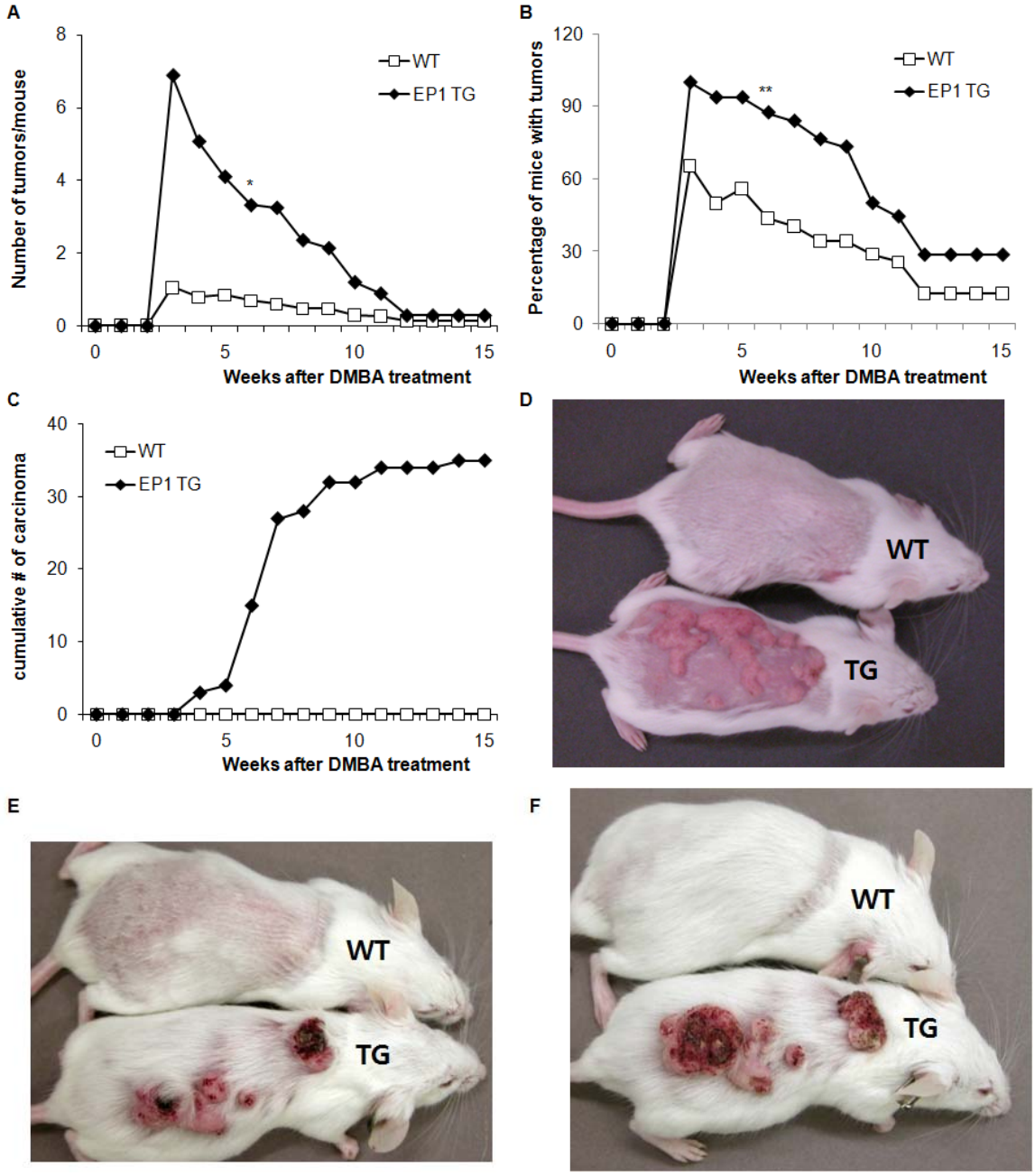

A
Number of tumors/mouse
WT
EP1 TG
*
Weeks after DMBA treatment
B
Percentage of mice with tumors
WT
EP1 TG
**
Weeks after DMBA treatment
C
cumulative # of carcinoma
WT
EP1 TG
Weeks after DMBA treatment
D
WT
TG
E
WT
TG
F
WT
TG

Figure 3-17. The effect of EP1 overexpression in DMBA-only skin tumor development. A DMBA-only skin carcinogenesis protocol was used with 32 BK5.EP1 transgenic mice and 32 wild type mice. A, Tumor multiplicity; data are expressed as the average number of tumors per mouse. * $p<0.001$, Poisson regression. B, Tumor incidence; data are expressed as the percentage of mice bearing tumors. * $p<0.001$, Fisher exact test. C, Cumulative number of carcinomas; data are expressed as cumulative number of carcinomas. Open squares, wild type mice (WT); Closed diamonds, BK5.EP1 transgenic mice (EP1 Tg). D, Two weeks after DMBA treatment; E, Four weeks after DMBA treatment; F, Six weeks after DMBA treatment; Wt, wild type; Tg, BK5.EP1 transgenic.

formed at 4 weeks after DMBA treatment in BK5.EP1 transgenic mice, whereas no carcinoma formation was observed in wild type mice by week 49 after DMBA treatment (Figure 3-17C). The photograph taken from another set of mice 2 weeks after application of DMBA (400 μg), clearly shows that the BK5.EP1 transgenic mice develop lesions while the wild type mice have macroscopically normal skin (Figure 3-17D). Four weeks after DMBA treatment, the BK5.EP1 transgenic mouse had some papillomas but the wild type mice had no tumors (Figure 3-17E). At 6 weeks after DMBA treatment, the BK5.EP1 transgenic mouse had 4 papillomas and a carcinoma whereas the wild type mice developed no tumors (Figure 3-17F). These data clearly show that overexpression of EP1 enhanced malignant tumor formation in the DMBA-only skin carcinogenesis protocol. Thus, overexpression of EP1 alone has tumor promoting activity and enhances progression.

3.14 EFFECT OF EP1 OVEREXPRESSION ON TUMOR INITIATION

The effect of EP1 overexpression on skin tumor initiation was examined by measuring B[a]P-DNA adduct formation to find the underlying mechanism of how EP1 overexpression increased DMBA-induced tumor development. ^{3}H-DMBA was not practically available. B[a]P and DMBA are polycyclic aromatic hydrocarbons and are metabolized by the CYP1 enzyme to reactive forms (DiGiovanni 1992). Therefore, ^{3}H-B[a]P was used. Fifteen hours after 200 nmol, 200 μCi of ^{3}H-B[a]P treatment, wild type mice had an average of 7.4 pmol adduct/mg DNA while EP1 transgenic mice produced 6.5 pmol adduct/mg DNA. The effect of EP1 overexpression on CYP1B1, the enzyme that metabolizes DMBA to reactive intermediates, expression was also examined by western blot. After vehicle treatment or TPA treatment, there was no change in the CYP1B1 expression level (Figure 3-18A). Activation of EP1 possibly activates Ras

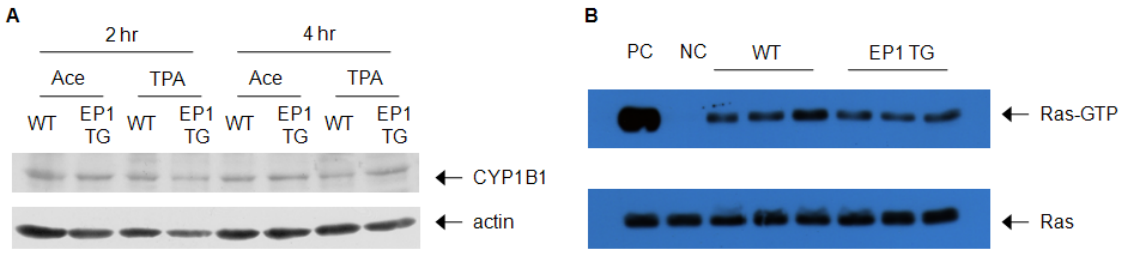


Figure 3-18. Effect of EP1 overexpression on initiation. A. Protein was extracted from epidermis at specified times after acetone (Ace) or TPA treatment of wild type (WT) and BK5.EP1 transgenic mice (TG). CYP1B1 protein level was determined by western blot. Actin protein levels were used as loading controls. B. Protein was extracted from epidermis of untreated wild type (WT) and BK5.EP1 transgenic mice (TG). Ras-GTP protein level was determined by pull down assay. PC, positive control, untreated wild type protein treated with GTPγS before pull down; NC, negative control, untreated wild type protein treated with GDP before pull down. Total Ras protein level was determined by western blot.

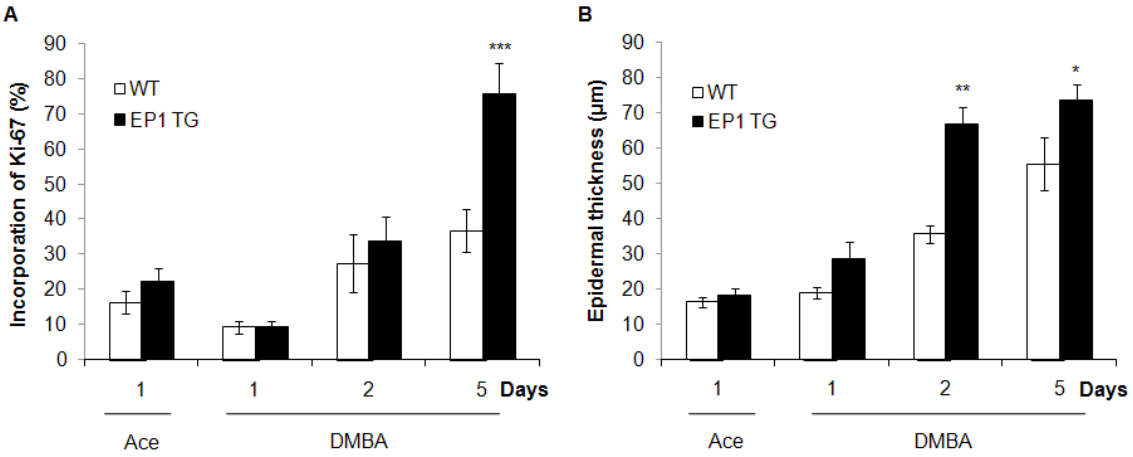
through activation of PLC (Dhanasekaran, Tsim et al. 1998). Therefore, the level of Ras activity was measured by a pull-down assay. Untreated BK5.EP1 transgenic mice had a similar level of epidermal ras activity as wild type mice (Figure 3-18B). These data show that overexpression of EP1 does not affect tumor initiation.

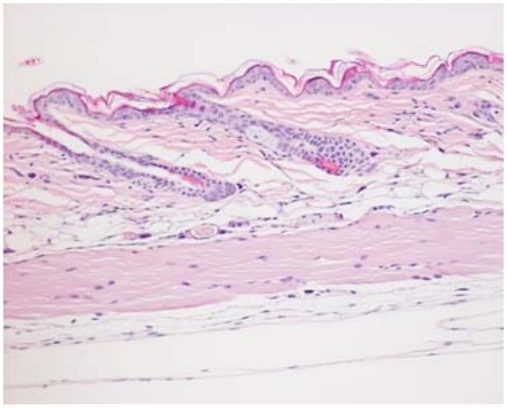
3.15 EFFECT OF EP1 OVEREXPRESSION ON DMBA– INDUCED CELL PROLIFERATION

Because overexpression of EP1 did not affect tumor initiation, the effect of EP1 overexpression on DMBA-induced epidermal cell proliferation was examined by assessing Ki-67 incorporation and epidermal thickness. With vehicle (acetone, 200 μl) treatment, Ki-67 incorporation and epidermal thickness was similar in wild type and BK5.EP1 transgenic mice (Figure 3-19). Five days after 400 μg of DMBA treatment, BK5.EP1 transgenic mice showed an increase in Ki-67 incorporation that was about twice that of the wild type mice (75.6% for BK5.EP1 transgenic mice versus 36.8% for wild type mice) (Figure 3-19A). Two days after DMBA treatment, epidermal thickness in BK5.EP1 transgenic mice showed an increase twice that of wild type mice (66.6 μm for BK5.EP1 transgenic mice versus 35.7 μm for wild type mice). Five days after DMBA treatment, the epidermis of BK5.EP1 transgenic mice was still thicker than that of the wild type mice (73.5 μm for BK5.EP1 transgenic mice versus 55.4 μm for wild type mice) (Figure 3-19B). These data clearly show that overexpression of EP1 increases DMBA-induced epidermal cell proliferation.

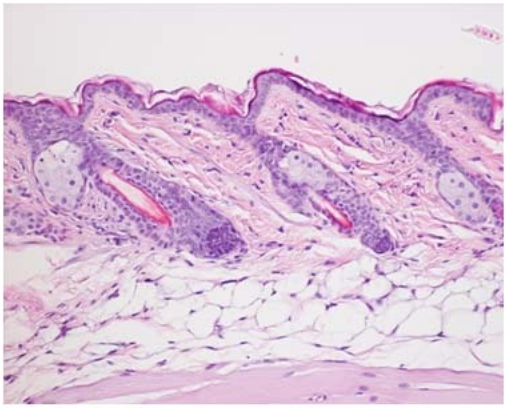
3.16 THE EFFECT OF EP1 OVEREXPRESSION ON SKIN CARCINOGENESIS IS COX-2 DEPENDENT

Previous research reported that the EP1 agonist 17-phenyl-2-trinor PGE_2 induces COX-2 mRNA and increases PGE_2 production in mouse osteoblastic cells

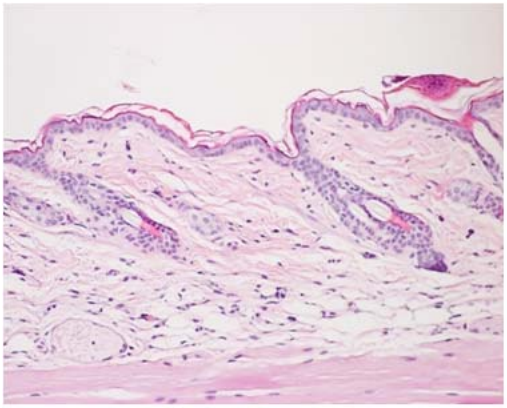




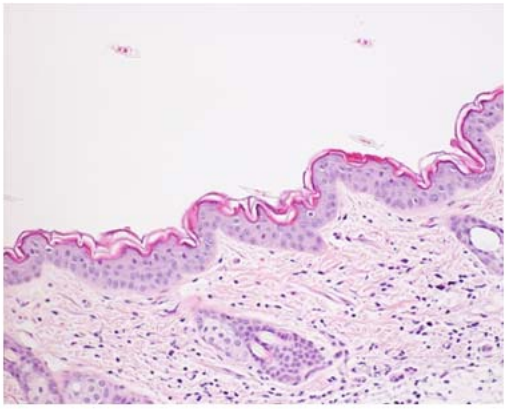
WT – 1X Ace 1 Day



TG – 1X Ace 1 Day



WT – 1X DMBA 1 Day



TG – 1X DMBA 1 Day

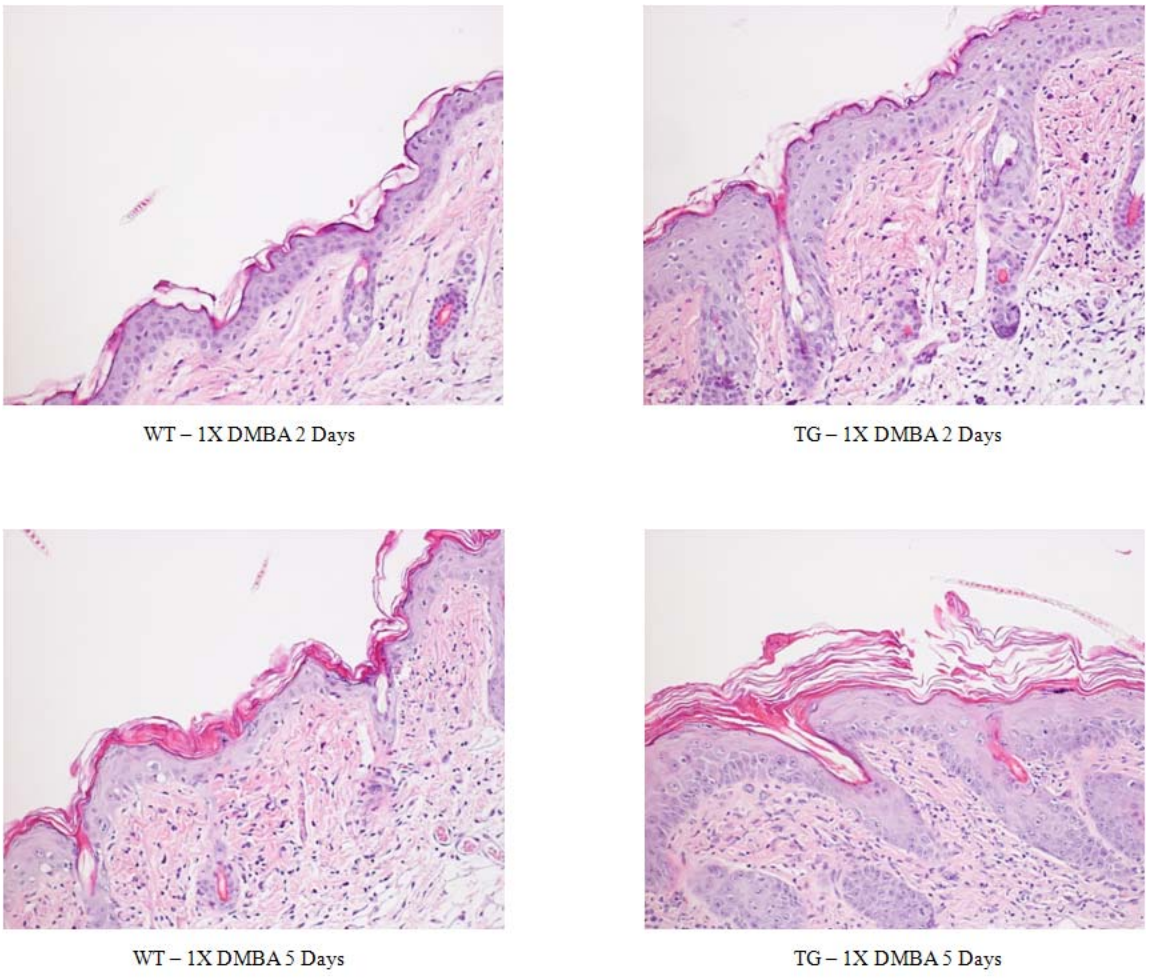


Figure 3-19. Effect of DMBA on epidermal cell proliferation and hyperplasia in BK5.EP1 transgenic and wild type mice. A, Ki-67 incorporation. Mice were topically treated with DMBA (400 µg/200 µl) or acetone (200 µl). At specified times after treatment, dorsal skin was removed, fixed in formalin, and immunostained for Ki-67. The data are shown as percentage of Ki-67 positive basal cells. B, Epidermal thickness. Dorsal skin was collected at specified times after acetone (200 µl) or DMBA (400 µg/200 µl acetone) treatment and stained with H&E. Epidermal thickness was measured under a microscope. Ace, acetone (200 µl) treatment; DMBA, DMBA (400 µg/200 µl acetone) treatment. * $p < 0.05$, independent T test, compared to WT, ** $p < 0.01$, independent T test, compared to WT, *** $p < 0.001$, independent proportions test, compared to WT.

(Suda, Tanaka et al. 1998). In addition, application of the EP1 antagonist ONO-8713 on mouse skin effectively prevents UV-mediated induction of PGE_2 (Tober, Wilgus et al. 2006). As previously reported (Athar, An et al. 2001; Bol, Rowley et al. 2002), there was no COX-2 protein expression in wild type mouse epidermis, whereas the COX-2 protein was clearly induced without any treatment in BK5.EP1 transgenic mice (Figure 3-20A). To determine the significance of upregulated COX-2 in BK5.EP1 transgenic mouse, Celecoxib (1000 ppm in the diet) was administered starting 1 week before DMBA treatment. The BK5.EP1 transgenic mice that were fed AIN control diet produced an average of four skin lesions at 2 weeks after DMBA treatment. As expected, the BK5.EP1 transgenic mice that were fed Celecoxib did not produce any lesions or tumors after DMBA treatment (Figure 3-20B). Two weeks after DMBA treatment, hyperplasia of the epidermis and more inflammatory cell infiltration in the dermis were observed in BK5.EP1 transgenic mice fed control diet. However, celecoxib fed BK5.EP1 transgenic mice showed a fairly normal skin architecture (Figure 3-20C and D). Induction of COX-2 increases PGE_2 levels, the ligand that binds the EP1 receptor. Therefore, EP1 signaling will be enhanced. These data show that expression of COX-2 in the BK5.EP1 transgenic mice is very important in the increased tumor development of these mice in the DMBA-induced carcinogenesis model.

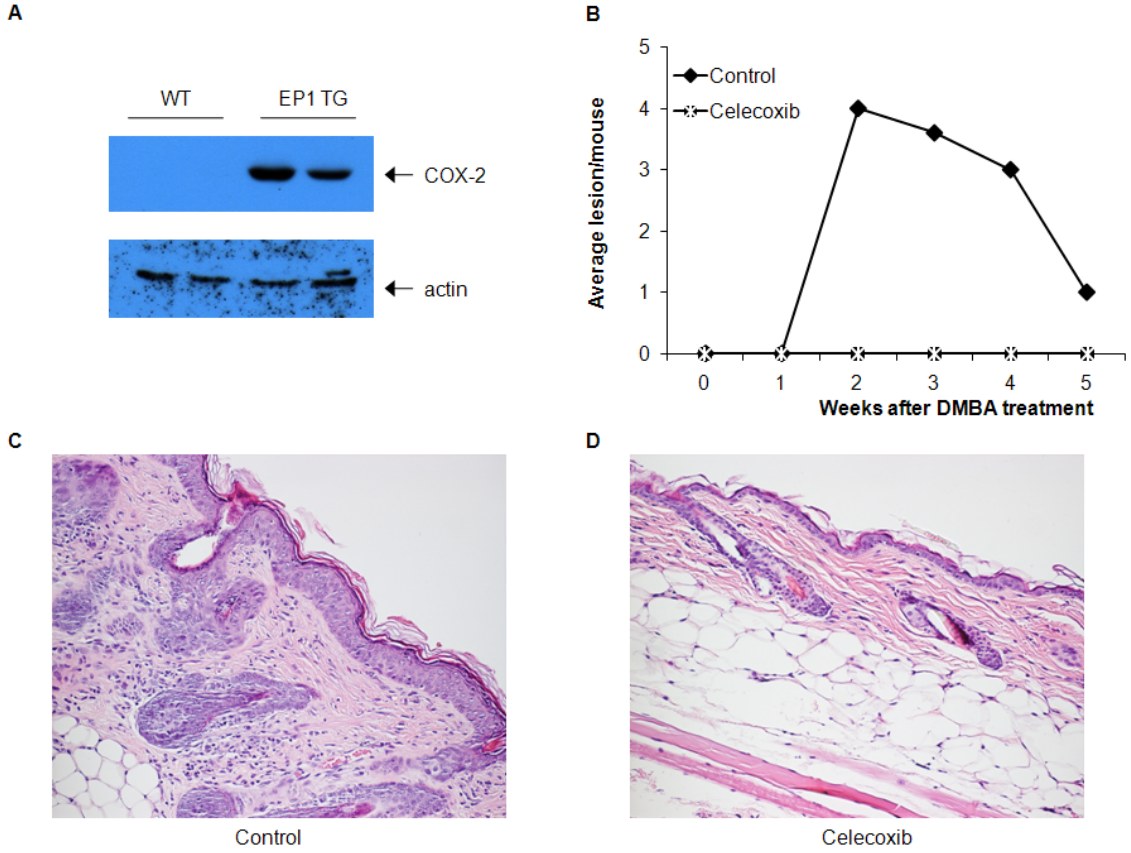


Figure 3-20. The effect of EP1 overexpression on DMBA-induced skin carcinogenesis is COX-2 dependent. A. Protein was extracted from epidermis of untreated wild type (WT) and BK5.EP1 transgenic mice (TG). COX-2 protein level was determined by western blot. Actin protein levels were used as loading controls. B. Groups of six mice were fed AIN-control diet or AIN diet containing Celecoxib (1000 ppm) starting one week before a single DMBA (400 µg) application. C-D. Two weeks after DMBA treatment, a skin sample of one mouse from each group was collected, fixed in formalin and stained with H&E. C. Skin of AIN-control diet fed mice. D. Skin of Celecoxib fed mice.

Chapter 4 Discussion

EP1 is expressed in normal human epidermis as well as in normal mouse epidermis (Lee, Kim et al. 2005). Increased EP1 mRNA levels in tumor promoter (TPA and UV) treated epidermis and DMBA/TPA or UV-induced tumors in this study imply increased EP1 signaling after application of tumor promoter and in tumors. These data are in agreement with Lee's publication (Lee, Kim et al. 2005) which showed that EP1 mRNA level was increased in UV treated mouse skin, UV-induced papilloma and squamous cell carcinoma (SCC) in mouse and human (Lee, Kim et al. 2005) and in 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine-induced mammary tumors (Kawamori, Uchiya et al. 2001). According to Lee's publication (Lee, Kim et al. 2005), all UV-induced mouse papillomas and SCC expressed EP1 and more than 90% of human actinic keratosis samples as well as 75% of human SCC expressed EP1. Since EP1 is upregulated in tumor promoter treated skin and tumors, overexpression of EP1 in the skin could be a useful system to study the role of EP1 in skin carcinogenesis in vivo. Therefore, transgenic mice which overexpress EP1 in the basal layer of the skin were made.

The BK5.EP1 transgenic mice did not develop any health problems. Body size of the BK5.EP1 transgenic mice was similar to that of wild type mice. There were no reproductive problems in breeding of the BK5.EP1 transgenic mice. Also, the BK5.EP1 mice had macroscopically normal hair coat and skin.

In untreated BK5.EP1 transgenic mice, no changes in cell proliferation (BrdU incorporation and epidermal thickness) were observed as compared to wild type mice. However, a slight increase in inflammatory cells in the dermis of the BK5.EP1 transgenic mice led us to assess the level of pro-inflammatory gene expression. As expected,

microarray data show that there are some changes in gene expression in untreated BK5.EP1 transgenic mice skin compared to wild type skin. Anti-apoptotic protein NAIP (neuronal apoptosis inhibitory protein) is increased in the BK5.EP1 transgenic mice. NAIP does not inhibit caspases directly (Roy, Deveraux et al. 1997) but inhibits apoptosis (Gotz, Karch et al. 2000). Thus, overexpression of EP1 might affect apoptosis through induction of NAIP. Overexpression of EP1 also increased Wnt expression. After Wnt ligand binds the Frizzled receptor, GSK-3β is inactivated and β-catenin is released. Then the free β-catenin moves into the nucleus and associates with Tcf and Lef, which induces target gene expression such as c-myc, c-jun, cyclin D1, VEGF and COX-2 (Bhatia and Spiegelman 2005; Telliez, Furman et al. 2006; Eisinger, Prescott et al. 2007). COX-2 induction in BK5.EP1 transgenic mice might be through this induction of Wnt.

There was extensive induction of aromatase in BK5.EP1 transgenic mice. Aromatase is an enzyme that converts androgen into estrogen and is expressed in reproductive systems such as placenta, ovary, as well as in other tissues such as adipose tissue, liver, intestine, brain and skin fibroblasts (Simpson, Mahendroo et al. 1994). In accordance with our data, human keratinocytes also express aromatase (Hughes, Robinson et al. 1997). There are several reports in the literature that show EP1 affects aromatase expression. In human adenocortical carcinoma cell line, EP1 antagonist inhibited EGF-induced aromatase upregulation (Watanabe, Noda et al. 2006). In human adipose stromal cells, the EP1 agonist 17-phenyl trinor PGE_2 increased aromatase expression and activity (Richards and Brueggemeier 2003). In primary human breast stromal cells, the EP1 agonist 17-phenyl trinor PGE_2 increased aromatase activity and an EP1 antagonist prevented induction of aromatase activity by PGE_2 (Brueggemeier, Richards et al. 2001). The role of estrogen in the skin has been shown in several literature reports. 17β-Estradiol administration increased epidermal thickness (Azzi, El-Alfy et al.

2005). 17β-Estradiol increased cell proliferation through cyclin D2 as well as increasing GM-CSF secretion in normal human keratinocytes (Kanda and Watanabe 2004). Also, both estrogen receptor (ER) alpha and beta are expressed in human keratinocytes (Ohnemus, Uenalan et al. 2006).

Matrix metalloproteinases (MMPs) are proteolytic enzymes and activate typsin- or plasmin-activated procollagenase-I and other MMPs by cleavage of the prodomain. MMPs are important in tumor progression for enhancing angiogenesis and breaking down extracellular matrix and basement membrane. Thus MMPs enhance tumor growth and metastasis (Kerkela, Ala-aho et al. 2001). In the skin, MMP-10 (Stromelysin-2) is induced by wounding, TNF-alpha, TGF-alpha, and EGF treatment (Windsor, Grenett et al. 1993; Madlener, Mauch et al. 1996). Enhanced tumor progression in the BK5.EP1 transgenic mice could be partly due to increased MMP-10 expression.

In the BK5.EP1 transgenic mice, there was an increase of several proinflammatory factors such as CCL1, CXCL20, CSF2, and COX-2. Inflammation is a tissue change in response to injury. Characteristics of inflammation are vasodilation, edema, clotting, migration of innate immune cells and swelling of tissue cells (Guyton 1996). Chronic inflammation increases risk of cancer development in bladder, stomach, liver, colon and ovary (Moore, Owens et al. 1999). Cancers are rich in inflammatory cytokines, chemokines, and inflammatory enzymes. These inflammatory cytokines can affect tumor growth, invasion and angiogenesis directly and/or indirectly (Mantovani 2005).

ICAM-1 is a transmembrane glycoprotein and binds to integrins, fibrinogen and extracellular matrix factor hyaluronan that mediates cell-cell adhesion. In ICAM-1 deficient mice, leukocyte migration to inflammatory sites is decreased, reducing the inflammatory reaction (van de Stolpe and van der Saag 1996). UVA induces ICAM-1

mRNA and protein in normal keratinocytes (Krutmann 2000). Increased ICAM-1 expression in keratinocytes is observed in inflammatory dermatoses and allergic skin reactions (van de Stolpe and van der Saag 1996). Chemokine ligand 1 (CXCL1) is expressed in normal human epidermis and induced during wound healing and psoriatic skin (Payne and Cornelius 2002; Zaja-Milatovic and Richmond 2008). CXCL1 recruits neutrophils. PKC-alpha transgenic mice induced more CXCL1 after TPA treatment compared to wild type mice in a NF-κB-dependent manner (Cataisson, Pearson et al. 2006). In human colorectal cancer cells, PGE_2 induces CXCL1 (Wang, Wang et al. 2006). CCL20 (macrophage inflammatory protein-3α) attracts dendritic cell and Langerhans cell precursors (Zhou, Krueger et al. 2003). In human primary keratinocytes, IL-1beta, TNF-alpha, and S100A8/A9 treatment induced CCL20 (Dieu-Nosjean, Massacrier et al. 2000) (Nukui, Ehama et al. 2008). In GM-CSF deficient mice, wound healing was delayed (Fang, Gong et al. 2007). UVA treatment induced GM-CSF in cultured human keratinocytes (Imokawa, Yada et al. 1996). Epidermal keratinocytes produce GM-CSF under normal conditions and GM-CSF is induced in response to TPA, lipopolysaccharides, and UV (Gallo, Grabbe et al. 1992). In lung carcinoma cell lines, a COX-2 inhibitor suppressed GM-CSF production suggesting that PGE_2 is involved in GM-CSF induction (Nakata, Uemura et al. 2003). In mast cells, PGE_2 enhanced GM-CSF level (Gomi, Zhu et al. 2000). The upregulation of inflammatory mediators suggests that EP1-induced inflammation likely contributes to enhanced tumor progression, These changes needed to validated at the protein level and their functional significance determined.

While untreated wild type mice do not express COX-2 in the epidermis, in the untreated BK5.EP1 transgenic epidermis, COX-2 protein levels are elevated. In agreement with our data, an EP1 agonist was reported to induce COX-2 in mouse

osteoblastic cells (Suda, Tanaka et al. 1998). Thus, elevated levels of prostaglandin are expected in the BK5.EP1 transgenic mice. Along with overexpression of EP1 in the BK5.EP1 transgenic mice, EP1 signaling will be more potentiated due to high level of the agonist, PGE_2. Thus, a possible EP1 → COX-2 → PGE_2 → EP1 positive feedback loop may exist. Due to increased levels of proinflammatory factors, inflammation and a proliferative epidermis would be expected in the BK5.EP1 transgenic mice. Compared to wild type mice, BK5.EP1 transgenic mice have a mild increase in inflammatory cells in the dermis without any treatment, with TPA treatment and after DMBA treatment. Therefore, more detailed research on the effect of EP1 on recruitment of inflammatory cells needs to be carried out in the future.

There are several reports in the literature that show the importance of EP1 in cell proliferation (Kimura, Osumi et al. 2001; Thompson, Gupta et al. 2001; Kawamori, Kitamura et al. 2005; Niho, Mutoh et al. 2005). In this study, both the BK5.EP1 transgenic mice and EP1 deficient mice produced similar levels of epidermal proliferation after TPA treatment. Thus, EP1 expression level did not affect TPA induced epidermal proliferation.

In response to TPA, wild type mice induced COX-2 protein maximally at 6 hours. However, in the BK5.EP1 transgenic mice, COX-2 expression levels were not affected by TPA treatment. Thus, at 6 hours after TPA treatment, wild type mice expressed more COX-2 than the BK5.EP1 transgenic mice. Therefore, it would be expected that wild type mice produce more prostaglandin than the BK5.EP1 transgenic mice after TPA treatment. Because COX-2 induction is an important feature in DMBA/TPA skin carcinogenesis model (Muller-Decker, Kopp-Schneider et al. 1998; Tiano, Loftin et al. 2002), this could explain why the BK5.EP1 transgenic mice produced fewer tumors in the DMBA/TPA

skin carcinogenesis model. However, EP1 deficient mice induced similar levels of COX-2 at 6 hours after TPA treatment.

Interestingly, in the DMBA/TPA skin carcinogenesis model, the BK5.EP1 transgenic mice produced fewer tumors than wild type mice while the EP1 deficient mice produced more tumors than wild type mice. Thus, EP1 level inversely correlated with tumor formation in the DMBA/TPA skin carcinogenesis model. Similarly, skin cancer development was substantially reduced in transgenic mice overexpressing COX-2 and in transgenic mice overexpressing PKCε compared to wild type mice in the DMBA/TPA skin carcinogenesis model (Reddig, Dreckschmidt et al. 2000; Bol, Rowley et al. 2002; Muller-Decker, Neufang et al. 2002; Rundhaug, Mikulec et al. 2007). This effect was dependent on the type of tumor promoter used. When anthralin, UV, or no promoters were used, BK5.EP1 transgenic mice produced similar or a greater number of tumors than wild type mice. This suggests that there is an interaction between EP1 signaling and TPA signaling. PKC down-regulation in the BK5.EP1 transgenic mice can be one possible reason why TPA promotion does not produce more tumors in the BK5.EP1 transgenic mice than in wild type mice while other promoters (UV, anthralin, or no promoter) produced more tumors in the BK5.EP1 transgenic mice than in wild type mice. Although the total tumor numbers were reduced in BK5.EP1 transgenic mice, many more carcinomas were produced, starting much earlier and with a greater papilloma to carcinoma conversion rate compared to wild type. This is similar to what was reported in PKCε transgenic mice (Jansen, Verwiebe et al. 2001).

EGFR is subclass Ⅰ of the receptor tyrosine kinase family, which has four members: EGFR (ErbB1) HER2/neu (ErbB2) HER3 (ErbB3) HER4 (ErbB4); skin expresses ErbB1, ErbB2 and ErbB3 (Stoll, Kansra et al. 2001). EGF is induced in the TPA treated skin and after TPA treatment, in tumors, expression levels of EGFR as well

as EGFR ligands are high (Woodburn 1999; Bol, Rowley et al. 2002; Hynes and Lane 2005; Maubec, Duvillard et al. 2005). Overexpression of TGF-α, an EGFR ligand, in the epidermis induced hyperplasia and spontaneous papilloma formation (Wang, Greenhalgh et al. 1994). V-ras transduced, EGFR-deficient keratinocytes, as compared to wild type keratinocytes, produced a reduced tumor volume when injected onto nude mice (Dlugosz, Hansen et al. 1997). Activation of EP1 has been shown to induce transactivation of EGFR in hepatocytes, lung cancer cells and cholangiocarcinoma cells (Kimura, Osumi et al. 2001; Su, Shih et al. 2004; Han and Wu 2005). The BK5.EP1 transgenic mice also had a higher level of phosphorylation of EGFR than did wild type mice. However, this response is weak and transient in the BK5.EP1 transgenic mice.

Signal transducers and activators of transcription (STATs) are transcription factors. An EGFR inhibitor reduced TPA-induced Stat3 activation (Chan, Carbajal et al. 2004). In the skin, Stat3 is important in both the initiation and promotion stage of skin carcinogenesis (Kataoka, Kim et al. 2008). Due to transient phosphorylation of EGFR after TPA treatment in the BK5.EP1 transgenic mice, it might be expected that BK5.EP1 transgenic mice have reduced activation of Stat3 in response to TPA. However, both wild type and BK5.EP1 transgenic mice induced a similar level of phospho-Stat3 after TPA treatment.

The importance of EP1 in UV-induced skin carcinogenesis has been suggested by several studies. EP1 expression was increased in human SCC as well as UV-induced papilloma and SCC in mouse skin (Lee, Kim et al. 2005). In this study, increased expression of EP1 was also observed in UV-exposed skin and UV-induced tumors. Also, topical treatment with an EP1 antagonist reduced UV-induced skin carcinogenesis (Tober, Wilgus et al. 2006). In agreement with these studies, BK5.EP1 transgenic mice produced more tumors and EP1 deficient mice produced fewer tumors in the UV-

induced skin carcinogenesis model. Thus, EP1 contributes to UV-induced skin carcinogenesis.

Papilloma formation before TPA application led us to suspect that EP1 has the ability to endogenously promote tumors. Therefore, a DMBA-only skin carcinogenesis protocol was used to test the tumor promoting action of EP1 signaling. BK5.EP1 transgenic mice increased tumor development compared to wild type in the DMBA-only carcinogenesis protocol. Again, this observation is similar to PKCε transgenic mice and COX-2 transgenic mice, since PKCε transgenic mice show increased carcinoma development in a DMBA/acetone protocol and COX-2 transgenic mice produce more tumors with a DMBA-only protocol (Reddig, Dreckschmidt et al. 2000; Jansen, Verwiebe et al. 2001; Rundhaug, Pavone et al. 2007).

To determine the mechanism by which EP1 overexpression increases tumor formation in DMBA-only skin carcinogenesis model, B[a]P-DNA adduct formation, CYP1B1 level and Ras activation levels were determined. The level of expression of CYP1B1, a cytochrome P450 enzyme that metabolizes DMBA to its active carcinogenic form, did not change in the BK5.EP1 transgenic mice compared to wild type mice. B[a]P-DNA adduct formation was also similar in the BK5.EP1 transgenic mice compared to wild type mice. In addition, the amount of the active form of Ras protein level was also not changed in the BK5.EP1 transgenic mice compared to wild type mice. Thus, EP1 overexpression did not affect these aspects of the initiation stage of skin carcinogenesis.

However, BK5.EP1 transgenic mice showed a greater proliferative response and inflammatory cell recruitment after DMBA treatment. These features likely contribute to the tumor promoting activity of EP1.

Administration of Celecoxib, a COX-2 selective inhibitor, completely blocked DMBA-induced tumor formation in the BK5.EP1 transgenic mice. Thus, COX-2

overexpression is a very important downstream event in EP1 signaling in the DMBA – induced skin carcinogenesis model. In the mouse mammary epithelial cell line, COX-2 expression level was inversely correlated with smad3 expression (Neil, Johnson et al. 2008). In DMBA/TPA induced skin SCC, smad3 expression level was reported to be significantly reduced (He, Cao et al. 2001). When v-ras transduced smad3 deficent keratinocytes were injected onto nude mice, papilloma formation was reduced but carcinoma formation was greatly enhanced compared to wild type keratinocytes (Vijayachandra, Lee et al. 2003). Therefore, it is possible that increased expression of COX-2 in the BK5.EP1 transgenic mice affects smad signaling and thus affects tumor progression.

In all four skin carcinogenesis models used, BK5.EP1 transgenic mice produced more carcinomas than wild type mice. These data strongly suggest that EP1 is important in the progression stage of tumor development. One of characteristics of the tumor progression stage is invasion. Therefore, to understand how EP1 overexpression affects tumor progression, the role of EP1 overexpression in invasion needs to be studied in the future.

In summary, EP1 expression was increased in tumor promoter treated mouse skin and mouse skin tumors. BK5.EP1 transgenic mice produced more SCC than did wild type mice. BK5.EP1 transgenic mice had a similar proliferative response after TPA treatment but a greater poliferative response after DMBA treatment than did wild type mice. COX-2 was induced in the untreated skin of the BK5.EP1 transgenic mice. BK5.EP1 transgenic mice produced fewer tumors than did wild type mice, and EP1 deficient mice produced more tumors, than did wild type mice in the DMBA/TPA skin carcinogenesis protocol. Reduced PKC expression in the BK5.EP1 transgenic mice, reduced induction of COX-2 after TPA treatment in the BK5.EP1 transgenic mice, and

reduced and more transient induction of phosphor-EGFR are possible reasons why BK5.EP1 transgenic mice produced fewer tumors in the DMBA/TPA skin carcinogenesis protocol. Clearly, COX-2 is a very important down-stream molecule of EP1 signaling, especially for inducing tumors in the DMBA-only skin carcinogenesis protocol.

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Vita

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