Disruption of EphA2 Receptor Tyrosine Kinase Leads to Increased Susceptibility to Carcinogenesis in Mouse Skin

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Abstract

EphA2 receptor tyrosine kinase is frequently overexpressed in different human cancers, suggesting that it may promote tumor development and progression. However, evidence also exists that EphA2 may possess antitumorigenic properties, raising a critical question on the role of EphA2 kinase in tumorigenesis in vivo. We report here that deletion of EphA2 in mouse led to markedly enhanced susceptibility to 7,12-dimethylbenz(a)anthracene/12-O-tetradecanoylphorbol-13-acetate (DMBA/TPA) two-stage skin carcinogenesis. EphA2-null mice developed skin tumors with an increased frequency and shortened latency. Moreover, tumors in homozygous knockout mice grew faster and were twice as likely to show invasive malignant progression. Haploinsufficiency of EphA2 caused an intermediate phenotype in tumor development but had little effects on invasive progression. EphA2 and ephrin-A1 exhibited compartmentalized expression pattern in mouse skin that localized EphA2/ephrin-A1 interactions to the basal layer of epidermis, which was disrupted in tumors. Loss of EphA2 increased tumor cell proliferation, whereas apoptosis was not affected. In vitro, treatment of primary keratinocytes from wild-type mice with ephrin-A1 suppressed cell proliferation and inhibited extracellular signal-regulated kinase 1/2 (ERK1/2) activities. Both effects were abolished in EphA2-null keratinocytes, suggesting that loss of ERK inhibition by EphA2 may be one of the contributing mechanisms for increased tumor susceptibility. Interestingly, despite its tumor suppressive function, EphA2 was overexpressed in skin tumors compared with surrounding normal skin in wild-type mice, similar to the observations in human cancers. EphA2 overexpression may represent a compensatory feedback mechanism during tumorigenesis. Together, these results show that EphA2 is a novel tumor suppressor gene in mammalian skin.

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Introduction

Since the discovery of the first member from an erythropoietin producing hepatoma cell line nearly two decades ago (1), the number of Eph receptor protein tyrosine kinases (RPTK) has increased to 16 in vertebrate (14 in humans), making them the largest subfamily of RPTKs (2, 3). They are divided into EphA and EphB kinases according to sequence homology and ligand binding specificity of the membrane-anchored ligands called ephrins. Although EphA kinases bind to GPI-anchored ephrin-As, EphB kinases target transmembrane ephrin-Bs. Earlier studies have established a regulatory role of Eph/ephrin interactions in neural patterning in developing nervous systems, primarily through repulsive guidance of growth cones and neurons although attractive responses have also been observed in some systems (4–6). The past several years have seen explosive growth in investigation on Eph receptors and their ligands, leading to the identification of diverse cellular functions regulated by Eph/ephrin interactions, including angiogenesis, neural plasticity, brain size determination, blood clotting, epithelial morphogenesis, and viral infection (3).

In addition to their functions in normal tissues, abnormal expression of Eph receptors and/or their ligands have been reported in various human tumors (7, 8). Expression of some Eph kinases, such as EphB2 and EphB6, has been correlated with favorable prognosis in colorectal cancer and neuroblastoma, respectively (9, 10), whereas EphB4 expression has been associated with malignant progression of human breast cancer (11). More recently, Eph kinases are found to be mutated in common solid tumors in humans. Mutational analysis of tyrosine kinase domain revealed somatic mutations in the kinase domain of EphA3 in human colorectal cancer (12). Using nonsense-mediated decay microarray analysis, Huusko et al. reported frequent mutations (8%) of EphB2 kinase in human prostate cancer both in the ectodomain and the cytoplasmic tail (13). A recent study revealed that loss of EphB2 expression was found in about 80% of human colorectal cancers (14). Furthermore, EphB2 or EphB3 knockout resulted in significantly elevated colorectal tumorigenesis in APCMin/+ mice.

EphA2 was originally called epithelial cell kinase because of its wide distribution in epithelial cells in vitro and in vivo (15). Earlier studies have shown elevated expression of EphA2 in several human cancers (7, 16). This has led to the suggestion that EphA2 may be an oncogene. Supporting this notion, forced expression of EphA2 in MCF10A normal epithelial cells induced malignant transformation in one study (17). Conversely, over-expression of a dominant negative or kinase-inactive EphA2 with cytoplasmic domain deletion suppressed the growth of 4T1 breast cancer cells (18).

On the other hand, recent human genetic evidence and in vitro studies suggest that EphA2 may also possess antitumorigenic functions. EphA2 is localized on chromosome 1p36.13, a region frequently deleted in a number of human cancers, including prostate and brain tumors. Indeed, deletion of EphA2 has been reported in chordoma, a rare human tumor linked to 1p36.13 (19). In vitro, activation of endogenous EphA2 on PC3 reduced cell...
migration (20) and proliferation (21). In addition, we reported that Ras/extracellular signal-regulated kinase 1/2 (ERK1/2) signaling cascade was inhibited upon ephrin-A1 stimulation of EphA2 in several different cell types (21), an observation that was also made following activation of EphB2 kinase in neuronal cell line (22). Moreover, EphA2 activation could antagonize epidermal growth factor (EGF)– and platelet-derived growth factor (PDGF)–induced activation of Ras/ERK1/2 signaling cascade. Recently, a conditional feedback loop has been identified, whereby Ras/ERK1/2 activation promotes EphA2 expression, which in turn negatively regulates ERK1/2 activities upon ligand stimulation in human breast cell lines (23). Together, these data are more consistent with an oncogenic-suppressive role of EphA2.

Herein, we sought to directly test the role of EphA2 in malignant transformation of epithelial cells in vivo using EphA2 knockout mice. In a classic 7,12-dimethylbenz(a)anthracene/12-tetradecanoylphorbol-13-acetate (DMBA/TPA) two-stage skin carcinogenesis model, homozygous deletion of EphA2 resulted in dramatically elevated susceptibility to skin tumor development and progression. Not only were there increased tumor multiplicity and shortened latency, EphA2-null tumors also showed a significantly accelerated rate of growth and progression to malignancy. Loss of EphA2 did not seem to affect apoptosis but was associated with increased tumor cell proliferation. Treatment of primary keratinocytes from wild-type mice with ephrin-A1 inhibited ERK1/2 activities and suppressed cell growth; both effects were abolished in EphA2-null keratinocytes. Collectively, these results suggest that EphA2 is a novel tumor suppressor gene in mammalian skin.

Materials and Methods

EphA2 knockout mice and multistage skin carcinogenesis. The KST085 line of EphA2 knockout mice was generated through secretory gene trapping as described previously (24) and was kindly provided by Drs. William Skarnes (Wellcome Trust Sanger Institute, Cambridge, United Kingdom) and Mark Tessier-Lavigne (Genetech, San Francisco, CA). In this line, the secretory trapping vector was inserted at the boundary between exon 5 and intron 6, leading to the truncation of exons 6 to 17 encoding the second fibronectin type III repeat in the extracellular domain all the way to the carboxyl terminal end. The remaining ectodomain encoded by exons 1 to 5 is fused to neo and β-galactosidase (β-gal; β-geo) reporter cassette and is trapped inside the cells in secretory vesicles, presumably in inactive form (24). The mice were cared for in accordance with guidelines set forth by the American Association for Accreditation of Laboratory Animal Care and the USPS "Policy on Human Care and Use of Laboratory Animals," and all studies were approved and supervised by The Case Western Reserve University Institutional Animal Care and Use Committee. The mice on C57BL/6/J background were backcrossed for four generations to FVB/N mice, which were then bred with each other to generate cohorts of female EphA2+/-, EphA2+/+ and EphA2+/ mice that were used in subsequent studies. For two-stage chemical carcinogenesis, the backs of 8-week-old mice were shaved and treated with a single application of DMBA (25 μg in 200 μL aceton; Sigma-Aldrich, St. Louis, MO) followed by twice a week applications of TPA (200 μL of 100 mmol/L solution in aceton; Sigma-Aldrich). Mice were visually inspected weekly, and tumor number and sizes were quantitated. Sixteen weeks after DMBA initiation, mice were divided into two subgroups: one group was sacrificed, whereas another group was kept for observation of malignant conversion in the absence of further TPA treatment for additional 10 weeks. Mice were sacrificed if moribund, if any individual tumor reached a diameter of 1.5 cm, or at the termination of the experiments. Tumors were snap frozen in liquid nitrogen and stored at -80°C. Samples were homogenized in ice-cold lysis buffer containing 20 mmol/L Tris (pH 7.4), 125 mmol/L NaCl, 1% triton X-100, 0.5% DCA, 0.1% SDS, 20 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 4 μg/mL aprotinin, 4 μg/mL leupeptin, and 1 mmol/L NaVO4. Then samples were centrifuged at 20,800 × g for 10 minutes at 4°C. Protein concentrations in supernatant were measured using the bicinchoninic acid protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein extracts were resolved by SDS-PAGE and electrotransferred onto polyvinyldiene difluoride membranes (Millipore, Bedford, MA), which were then blotted with the indicated antibodies.

Proliferation and apoptotic assays. Skin tumors were fixed in 4% paraformaldehyde overnight and embedded in paraffin. To detect the proliferative cells in tumors, immunohistochemical studies were done using a polyclonal Rb-ki67-p rabbit antibody (Novoceastra Laboratories, Newcastle upon Tyne, United Kingdom). Paraffin-embedded sections (5 μm thick) were deparaffinized in xylene, hydrated in series of alcohols, and immersed in citrate buffer [10 mmol/L sodium citrate, (pH 6)] for 10 minutes at 95°C. After cooling down to room temperature, sections were blocked with 5% normal goat serum in TBS for 1 hour at room temperature to reduce nonspecific staining and then incubated with primary antibodies (1:1000) overnight at 4°C. Biotinylated secondary antibody and avidin-biotin-peroxidase system (Vector) were used. Color immunostaining was revealed using dianamobenzidine with metal enhancer (Vector). The apoptotic tumor cells on paraffin sections were detected using a terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) kit without antigen retrieval following the manufacturer’s instructions (Roche Diagnostics, Indianapolis, IN). Sections were mounted with 4,6-diamidino-2-phenylindole–containing mounting medium (Vector).

The number of positive tumor cells was enumerated independently by two researchers. Quotients were converted to density (positive cells/unit arbitrary tumor area). The ANOVA for unpaired samples was used for all statistical analyses. The post hoc analysis is Bonferroni Test.

Immunofluorescence. Frozen sections of skin or tumors were fixed with 4% paraformaldehyde. After washing with PBS, the sections were blocked with 50 mmol/L NH4Cl and permeabilized with 0.3% NP40 for 10 minutes. The sections were then incubated with goat polyclonal antibodies that recognize the ectodomain of mouse EphA2 (R&D Systems, Minneapolis, MN) and rabbit polyclonal anti-ephrin-A1 (Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 1 hour followed by detection with donkey anti-goat IgG-FITC and donkey anti-rabbit IgG-Red X (Jackson ImmunoResearch Laboratories, West Grove, PA) at room temperature for 30 minutes. Images were taken using Leica microscope.

Primary keratinocyte isolation, stimulation, immunoprecipitation, and immunoblotting. Primary keratinocytes were isolated from neonatal mouse skin as described previously (25). Briefly, the entire P1 mouse skin was dissected and incubated with 5 units/mL dispase II (Boehringer Mannheim, Mannheim, Germany) overnight at 4°C. Epidermal layer was separated from dermal layer using forceps, minced, and digested with 0.25% trypsin/0.05 mmol/L EDTA. After neutralizing trypsin by washing with serum-containing medium, cells were filtered through sterile gauze pads, spun down, and resuspended in serum-free keratinocyte culture medium (Life Technologies, Gaithersburg, MD) containing pituitary extract, 5 ng/mL EGF, 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 μg/mL amphotericin and plated on six-well plates precoated with 50 μg/mL Matrigel. Cell stimulation with ephrin-A1-Fc, immunoprecipitation, and immunoblot were carried out as described precisely (26, 27).
Results

Disruption of EphA2 led to increased susceptibility to skin carcinogenesis in a gene-dosage dependent manner. To investigate the role of EphA2 kinase in the development of mammalian skin tumors, we used the previously described EphA2 knockout mice generated by secretory trapping strategy (24). Similar to other lines of EphA2 knockout mice (28–30), the secretory trapping EphA2-null mice are fertile, develop, and grow normally. Immunoblot of postnatal day eight (P8) skin extract detected EphA2 expression in wild-type mice but not in homozygous mutant, whereas heterozygous mice showed intermediate expression (Fig. 1A). X-gal staining for β-geo under the control of EphA2 promoter revealed that EphA2 was expressed in interfollicular epidermis, hair follicles, and sebaceous glands (Fig. 1B).

A classic two-stage carcinogenesis protocol was carried out to induce skin tumors, which entails tumor initiation by topical application of DMBA followed by tumor promotion by twice a week applications of TPA. It has been well established that most skin tumors induced using this protocol harbor activating mutation of H-Ras (31). We found that disruption of EphA2 increased susceptibility to skin tumorigenesis with dramatically accelerated kinetics of tumor development (Fig. 1C and Fig. 2). In wild-type mice, benign tumors (mostly papillomas) started to appear around 8 weeks after DMBA treatment (Fig. 2A and B). By 10 weeks, about 50% of mice had developed tumors. In contrast, EphA2-null mice developed first tumors at 6 weeks, affecting 50% of mice between weeks 7 and 8, reflecting a 2- to 3-week shortening in median tumor latency relative to wild-type mice (Fig. 2B). By 10 weeks, the average number of tumors per mouse had reached 13.4, a 7-fold increase over wild-type mice (Fig. 2A). Beyond 12 weeks after DMBA initiation, tumor numbers in EphA2−/− mice were likely to be underestimated due to the fusion of adjacent tumors.

In addition to the increased tumor multiplicity and shortened latency, tumors arising in EphA2 homozygous knockout mice also displayed significantly accelerated growth rate (Fig. 2C). Tumors in knockout mice rapidly grew to ≥4 mm, which contrasted with slow-growing tumors on wild-type mice. The large tumor burden in the knockout mice necessitated the termination of the experiment at 16 weeks after DMBA initiation. Interestingly, mice heterozygous for EphA2 knockout exhibited intermediate susceptibility to skin carcinogenesis in terms of tumor numbers, incidence and growth rate. This did not seem to be due to the loss of heterozygosity, as immunoblot of tumor extracts revealed that EphA2 was expressed at the levels consistent with heterozygous deletion (Fig. 2D). Thus, EphA2 inactivation caused significantly elevated susceptibility to skin carcinogenesis in a gene dosage–dependent manner.

EphA2 dominantly suppressed invasive progression of skin tumors. A well-established function of Eph kinases is the regulation of growth cone and cell motility, primarily through repulsive mechanisms (4, 32). We reported previously that EphA2 or EphB3 activation on prostate or colorectal cancer cells inhibited cell migration (20, 27). Cell motility is associated with tumor progression toward a more invasive and metastatic phenotype. However, because skin tumors rarely metastasize, we focused on invasive progression instead. For this purpose, TPA treatment was stopped at 16 weeks, and subgroups of mice were observed for additional 10 weeks. During this time, most tumors either started to regress or grew slowly, whereas some tumors showed signs of malignant progression grossly as evidenced by inward growth and bloody appearance in tumor centers. Histopathologic analyses show that whereas most tumors comprised benign papillomas (Fig. 3A, a), a fraction of tumors had progressed to squamous cell carcinoma with clear evidence of local invasion by 26 weeks (Fig. 3A, b–e). Notably, the most aggressive tumors, such as invasive squamous cell carcinomas and spindle cell carcinomas, were more frequently found in homoyzgous knockout mice (Fig. 3A, c–e). Staining for keratin 14 keratinocyte marker confirmed the epithelial origin of the invasive spindle-like cells (Fig. 3A, f). Quantitative analyses showed that EphA2-null tumors were twice as likely to develop fully invasive phenotype compared with tumors from wild-type mice (Fig. 3B). Interestingly, tumors from heterozygous mice shared similar rate of malignant conversion as the wild-type tumors. This is in contrast with the intermediate phenotype in tumor number, incidence, and growth rate in heterozygous mice (Fig. 1C and Fig. 2). Thus, EphA2 plays a dominant role in suppressing invasive tumor progression in mammalian skin.

Deletion of EphA2 caused increased skin tumor cell proliferation. Increased tumor development in knockout mice (Fig. 1C and Fig. 2) could result from either increased cell proliferation or decreased apoptosis or both. Staining for Ki67 cell proliferation marker showed that EphA2 homozygous deletion led to increased cell proliferation compared with tumors from wild-type mice (Fig. 4A and B). Cells in heterozygous tumors also
exhibited accelerated tumor proliferation indistinguishable from homozygous tumors, which may reflect the increased heterozygous tumor growth rate at week 16 when the animals were sacrificed (Fig. 2C). Consistent with earlier studies, most proliferating cells were localized to basal layer at the stromal-tumor boundaries. Quantitative analyses across entire sections of tumors revealed significant differences in proliferation indices between wild-type and EphA2 heterozygous or homozygous tumors (Fig. 4B). Apoptosis did not seem to be a major contributing factor as TUNEL staining of tumor sections did not detect noticeable differences among different genotypes (Fig. 4C). These data suggest that homozygous deletion or haploinsufficiency of EphA2 results in increased tumor cell proliferation.

Compartmentalized expression of EphA2 and ephrin-A1 in mouse skin. Eph kinases and their membrane-anchored ligands mediate cell-cell contact signaling. To understand how EphA2 may exert its tumor suppressor function in mammalian skin, we sought to determine the expression patterns of EphA2 and one of its cognate ligands (ephrin-A1). For this purpose, we did double immunofluorescence staining with a goat anti-EphA2 and a rabbit anti-ephrin-A1 antibody. We found that EphA2 and ephrin-A1 were expressed in complementary pattern in mouse skin. In the normal epidermis adjacent to tumors (Fig. 5A and B), EphA2 was expressed in a basal to suprabasal gradient with lower expression in the basal layer and higher expression in spinous and granular layers. In contrast, ephrin-A1 expression was primarily restricted to the basal layer abutting the basement membrane (Fig. 5A and B). Such compartmentalized expression patterns were also noted in hair follicles (Fig. 5A). For example, EphA2 and ephrin-A1 were expressed in complementary gradients in outer root sheath (arrows). In hair bulb, EphA2 was expressed in hair cortex and part of matrix cells, whereas ephrin-A1 expression was located toward the top of the bulb in inner root shaft (arrowheads). The complementary EphA2/ephrin-A1 expression was observed in adult mouse skin not exposed to DMBA/TPA (Fig. 5C). Note that normal mouse skin epidermis generally is only one to two cell layers thick, in contrast with DMBA/TPA–treated skin, which has thickened epidermis with a multilayer of cells (Fig. 5A and B). The basal-restricted expression of ephrin-A1 suggests that EphA2/ephrin-A1 interactions are localized to this region, where most cell proliferation takes place (Fig. 4). Deletion of EphA2 may eliminate EphA2/ephrin-A1 interactions in this region, which may lead to increased tumor susceptibility.

EphA2 was overexpressed in skin tumors. Next, we examined EphA2/ephrin-A1 expression pattern in tumor tissues. Both X-gal staining of heterozygous tumors for β-geo reporter cassette and immunofluorescence staining of wild-type tumors for endogenous protein showed that EphA2 was abundantly expressed on tumor cells (Fig. 5D and E, left). Interestingly, despite the apparent tumor suppressor function, EphA2 expression levels in tumor parenchyma were elevated compared with surrounding normal skin either by X-gal staining for the reporter cassette (Fig. 5D, compare left and right) or by immunofluorescence staining for the endogenous protein (Fig. 5E). The increased EphA2 expression may result from Ras activation because most skin tumors induced by the DMBA/TPA harbor activating mutations in Ras (31), and Ras/ERK 1/2 activation is known to promote EphA2 expression (23).

On the other hand, ephrin-A1 expression showed significant variations between different tumors and between different regions of the same tumor compared with the relatively uniform pattern of EphA2 expression (Fig. 5F). In some well-differentiated regions, ephrin-A1 expression remained restricted to basal cell layer with cell membrane staining pattern as in adjacent normal skin (Fig. 5F, tumor 1). However, in most tumor areas, ephrin-A1 expression in the basal layer became lower or more diffuse (Fig. 5F, tumor 2, arrow). Such deregulated expression of ephrin-A1 was also detected in EphA2 knockout mice (data not shown). These results suggest that DMBA/TPA–induced skin tumor development and progression was accompanied by reduced expression and/or mislocalization of ephrin-A1 in basal cell layer. In wild-type mice, this may reduce EphA2/ephrin-A1 interactions and compromise tumor suppressive activities of EphA2 in basal cell layer. However, we were not able to directly test this possibility in the absence of ephrin-A1 knockout mice. In EphA2 knockout mice, the loss of
EphA2/ephrin-A1 interactions eliminates tumor suppressive effects of EphA2 and may predispose cells to increased cell proliferation and oncogenic transformation.

EphA2 activation inhibited ERK1/2 activities and reduced cell proliferation in wild-type but not EphA2-null keratinocytes in vitro. To investigate molecular mechanisms underlying the increased susceptibility to skin tumorigenesis, primary keratinocytes were isolated and cultured from epidermis of neonatal (P1) mice. They were stimulated with ephrin-A1 fused to the heavy chain of human IgG1 (ephrin-A1-Fc). Because ephrin-A1 interacts with most EphA kinases, we used excess ephrin-A1-Fc to precipitate EphA kinases, including EphA2, from cell lysates. The precipitated materials were blotted with an antibody that was raised against phosphorylated dityrosine peptide highly conserved in the juxtamembrane region of all Eph kinases. Tyrosine phosphorylation of this region has been correlated with Eph kinase activation (15, 17). We found that EphA2 could be readily activated by ephrin-A1 in wild-type and heterozygous primary keratinocytes (Fig. 6A). Interestingly, p-EphA/ephrin-A1 signal was undetectable in EphA2-null keratinocytes despite the fact that ephrin-A1-Fc precipitation should have brought down most EphA kinases, suggesting that EphA2 was the predominant EphA kinase expressed in primary keratinocytes.

We reported previously that EphA2 activation suppressed basal as well as growth factor–induced ERK1/2 activities and reduced cell growth in several different cell types (21). We show here that ERK1/2 activities were also significantly attenuated in wild-type keratinocytes upon ligand stimulation of EphA2 (Fig. 6B). Homozygous deletion of EphA2 completely abolished the inhibitory effects, whereas heterozygous keratinocytes showed an intermediate response. In contrast, the activities of Akt were not affected by EphA2 deletion, confirming the specificity of inhibitory effects on ERK1/2 (Fig. 6B). Although earlier studies have shown suppression of ERK1/2 MAPK activities by ephrin-A1 in other cell types, a question remains whether EphA2 is necessary to cause the effect due to the likely presence of other EphA kinases. Our results provide genetic evidence that EphA2 is necessary for ephrin-A1-induced inhibition of ERK1/2 MAPK in keratinocytes.

In a clonal growth assay, treatment with ephrin-A1-Fc suppressed the growth of wild-type and heterozygous keratinocytes (Fig. 6C). In contrast, EphA2 knockout keratinocytes became resistant to ephrin-A1-induced growth inhibition. Thus, EphA2 in keratinocytes exerts negative growth regulatory function possibly through inhibition of ERK1/2 MAPK. In vivo, EphA2/ephrin-A1 interactions are primarily localized to the basal cell layer where most cell proliferation occurs. Disruption of EphA2 eliminates the growth inhibitory effects normally imposed by EphA2, leading to increased cell proliferation and tumor susceptibility. Because EphA2 KO mice had severalfold increased tumor multiplicity, the majority of initiated cells in wild-type mouse skin were presumably prevented from ever becoming frank tumors by the tumor suppressive activities of EphA2. Conceivably, the small fractions that do develop into tumors in wild-type mice have already overcome the tumor suppressive effects of EphA2. If ERK inhibition is a major mechanism of tumor suppression, it is likely that this effect would have been overcome in wild type tumors, which could explain the lack of significant differences in ERK activities in frank tumors from EphA2+/− and EphA2−/− mice (data not shown).

Next, we tested if EphA2 expression was sufficient to mediate ephrin-A1-induced ERK1/2 MAPK inhibition. Toward this end, primary mouse embryonic fibroblasts (MEF) with different EphA2 genotypes were isolated from the same litter of E13.5 embryos. Figure 6D shows that whereas ERK1/2 MAPK activities were suppressed by ephrin-A1 in MEFs from EphA2−/− and EphA2+/− embryos, MEFs from EphA2+/− littersmates were refractory. Reintroduction of EphA2 by retrovirus-mediated gene transfer restored the inhibitory effects in a dose-dependent manner (Fig. 6E). Thus, EphA2 is sufficient to mediate ERK1/2 inhibition by ephrin-A1 in MEF cells.

Discussion

In this report, we provide genetic evidence to show that EphA2 is expressed in epidermal and follicular keratinocytes and functions as a novel tumor suppressor gene during mammalian skin
carcinogenesis. EphA2 deletion resulted in susceptibility to chemically induced skin tumorigenesis with significantly increased tumor number, shortened latency, and accelerated growth rate. Moreover, ablation of EphA2 led to significantly higher rate of malignant conversion toward invasive phenotype. In vitro, ephrin-A1 stimulation of wild-type but not EphA2-null keratinocytes attenuated ERK1/2 activities and suppressed cell proliferation. EphA2 and ephrin-A1 are expressed in a complementary pattern in epidermis, limiting their interactions to the basal layer of epidermal cells where active cell proliferation normally takes place. Consistent with these in vitro and in vivo observations, loss of EphA2 resulted in higher proliferation rate in basal layers of skin tumors. Paraffin-embedded sections were stained with an antibody against Ki67 cell proliferation marker. Bar, 40 μm. B, quantitative analysis of proliferation index. Randomly selected tumors from three different animals of each genotype were stained for Ki67-positive cells and normalized to wild-type control. The differences between EphA2+/+ and EphA2+/− or EphA2/−/− were statistically significant (P < 0.001, one-way ANOVA). C, quantitative analyses of apoptosis by TUNEL staining. No significant differences were detected among different genotypes. Figure 5. Compartmentalized expression of EphA2 and ephrin-A1 in mouse skin. A, mice were sacrificed 16 weeks after DMBA initiation. Frozen sections were obtained from tumor-free areas of DMBA/TPA–treated mouse skin and were costained with a goat polyclonal antibody that recognizes EphA2 ectodomain and a rabbit polyclonal anti-ephrin-A1 antibody. Arrows, expression of ephrin-A1 and EphA2 in opposite gradient in outer root shaft; arrowheads, compartmentalized expression of ephrin-A1 and EphA2 in hair bulb. B, insets from (A) showing basal-suprabasal gradient expression of EphA2 and basal expression of ephrin-A1 in epidermis. C, EphA2 and ephrin-A1 expression in untreated normal skin from wild-type mice. Note the thinner epidermal layer in untreated skin compared with thickened epidermis in DMBA/TPA treatment skin shown in (B). D and E, EphA2 was up-regulated during skin tumorigenesis. Skin tumors were dissected and frozen sections with normal skin attached were cut. Tumors from EphA2+/− mice were stained with X-gal (D), whereas tumors from EphA2−/− mice were stained with a goat polyclonal antibody that recognizes the ectodomain of EphA2 (E). Arrow, normal skin; arrowhead, papilloma. F, representative papillomas from wild-type mice stained for EphA2 and ephrin-A1. Arrows, tumor cells abutting the basement membrane. Bars, 40 μm.
tumor cells, which may be a contributing mechanism of tumor susceptibility. Thus, similar to EphB2 that has recently shown to be a tumor suppressor gene in colorectal cancer, our data here suggest that EphA2 is a tumor suppressor gene in mammalian skin.

EphA2 was originally called epithelial cell kinase (Eck) because of its wide distribution in epithelial cells in vitro and in vivo (15). Numerous subsequent studies have shown overexpression of EphA2 in several different types of human carcinomas, including those of the breast, lung, prostate, esophagus, and kidney (7, 16). However, the role of EphA2 kinase in epithelial tumorigenesis in vivo remains unclear. Although some in vitro studies support EphA2 as an oncogene, others are more consistent with antitumorigenic functions. In one study, forced expression of EphA2 has been reported to induce malignant transformation of MCF10A normal breast epithelial cells (36). Conversely, a dominant-negative or kinase-inactive EphA2 with cytoplasmic domain deletion suppressed the growth of a breast cancer cell line (18). On the other hand, in prostate and breast cancer cell lines, ligand stimulation of endogenous EphA2 suppresses basal and growth factor–induced ERK1/2 MAPK activation (21, 23). Moreover, ephrin-A1 treatment of prostatic epithelial cells resulted in significant growth inhibition (21). The latter studies are more consistent with antitumorigenic role of EphA2. Thus, whether EphA2 kinase is an oncprotein or tumor suppressor has remained uncertain from the existing studies. The EphA2 knockout mice allowed direct assessment on the role of EphA2 in tumor etiology and progression in mammalian epithelial cells in vivo. The dramatically increased susceptibility of EphA2 knockout mice to two-stage carcinogenesis supports a tumor suppressor role of EphA2 in mammalian epithelial cells. Deletion of EphA2 caused increased tumor multiplicity and incidence as well as shortened tumor latency. Moreover, tumors arising in homozygous knockout mice were twice as likely to progress toward an invasive phenotype.

EphA2 knockout did not affect the basal cell proliferation index in the skin compared with wild-type mice nor was there an increased rate of proliferation following short-term or long-term treatment with TPA.4 One potential explanation consistent with this observation is that the tumor suppressive effects of EphA2 in the two-stage carcinogenesis protocol may only affect the small

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4 Guo and Wang, unpublished.
number of initiated cells in paracrine fashion, preventing their expansion into tumor mass. Thus, there will not be global changes in proliferation index and hyperplasia across the entire skin.

Our results predict that loss or reduction of EphA2 expression may predispose cells to malignant transformation. Supporting this notion, EphA2 is mapped to human chromosome 1p36.13, a region frequently lost in neuroblastoma, melanoma, prostate cancer, and other tumors. Cytogenetic and molecular analyses have linked the loss of 1p36 to chordoma, a rare tumor arising from notochordal remnants in axial skeleton (35). Fine mapping narrowed the linkage to a 3,000,000-bp region near 1p36.13. Candidate gene screening identified EphA2 as a possible oncosuppressor gene (19). In keeping with the latter studies, kinked tail has been observed in one line of EphA2 knockout mice (30). This phenotype is associated with abnormal notochord development, although no chordoma has been reported in these mice.

We show here that haploinsufficiency can predispose mouse skin to chemical carcinogenesis. Further investigation is needed to characterize loss of heterozygosity of EphA2 locus or other mechanisms that may lead to reduced EphA2 expression in human tumors. Indeed, in some existing reports on EphA2 expression in human cancers, down-regulation or total lack of EphA2 expression is present in significant fractions of the specimens. For example, about 50% of human esophageal squamous cell carcinoma are negative for EphA2 expression (36). It is also interesting to note that of 29 human breast cancer cell lines examined, only eight overexpress EphA2, whereas the other 22 lines express little of the kinase (23). It will be interesting to test how loss of EphA2 expression may be correlated with tumor prognosis in different human cancers.

Why is EphA2 overexpressed in some of human cancers despite its tumor suppressive functions? A recent report by Macrae et al. provides one possible explanation (23). The authors showed that EphA2 is a direct transcription target of the Ras/Raf/MEK/ERK 1/2 signaling cascade. Expression of oncogenic Raf enhanced EphA2 expression in NIH-3T3 fibroblasts. We have similarly found that transformation of NRP152 normal rat prostatic epithelial cells with the activated Ras oncogene caused a severalfold increase in EphA2 expression.5 Because deletion of EphA2 resulted in substantially increased tumor susceptibility, we propose that the up-regulated EphA2 represents a compensatory feedback defensive mechanism against malignant transformation in cells that have the activated Ras/ERK signaling cascade. In this context, EphA2 overexpression may be more likely to be a marker of tumor progression resulting from ERK activation rather than an etiologic event underlying tumorigenesis.

However, at present, it cannot be ruled out that the overexpressed EphA2 in some human tumors may possess tumor-promoting effects (17, 18). One possibility is that the EphA2 overexpression could shutdown its tumor suppressor function. Indeed, there seems to be an inverse relationship between EphA2 and ephrin-A1 expression in breast cancer cell lines (23). Ephrin-A and EphA engagement is known to cause rapid Kuzbanian metalloprotease–mediated cleavage of ephrin-A (37). The overexpressed EphA2 may keep ligand expression at low levels by enzymatic cleavage. Alternatively or in addition, there can be lateral EphA-ephrin association that has been shown to result in inactive complexes (38). Thus, EphA2 overexpression can potentially cause latent EphA2 unable to exert its ligand-dependent tumor suppressor function. Another possibility is that the unligated EphA2 kinases may have distinct signaling pathways that facilitate tumor development. Further studies are needed to identify such mechanisms.

In 2001, we first reported that EphA2 activation potently inhibited Ras/ERK signaling cascade in fibroblasts, endothelial cells, as well as normal and transformed epithelial cells (21). Moreover, EphA2 activation could antagonize Ras/ERK activation by EGF and PDGF and reduced cell proliferation in several different cell types. However, the responses were apparently cell type dependent: activation of EphA2 in some tumor cells failed to inhibit ERK, whereas in other cell types, there was active suppression of ERK but no growth inhibition, raising the issue of whether EphA2 alone is sufficient to mediate ERK inhibition. The data reported here show that in both primary keratinocytes and MEF cells, deletion of EphA2 abolished the inhibitory effects of ephrin-A1 stimulation on ERK activities. Moreover, reexpression of EphA2 in MEF cells restored the inhibitory effects. These results show that EphA2 is necessary and sufficient to mediate ERK inhibition. Loss of responsiveness to EphA2 activation-induced ERK inhibition observed in some human tumor cells indicates that these cells may have acquired resistance to the effect of EphA2.

We found that EphA2 homozygous knockout promoted invasive progression of mammalian skin tumors. Early studies on Eph kinases have linked Eph kinase activation to repulsive guidance of growth cones and neurons (4–5, 32). Recent genetic evidence from knockout mouse shows that negative regulation of cell motility is a predominant outcome upon Eph/ephrin interactions in vivo (3). This is mainly achieved through complementary expression of Eph kinases and ephrin ligands. For example, EphB3 is expressed at the bottom of mouse intestinal crypts, whereas ephrin-B1 and ephrin-B2 are expressed in a top to bottom gradient along the crypt wall (39). Paneth cells expressing EphB3 at the bottom of the crypt become mislocalized when EphB3 is deleted, suggesting EphB3/ephrin-B interaction inhibit Paneth cell migration in vivo. We found EphA2 and ephrin-A1 expression is compartmentalized in mouse interfollicular epidermis and hair follicles. In epidermis, ephrin-A1 is expressed at the basal layer, whereas EphA2 is expressed in a basal-suprabasal gradient. Conceivably, invading tumors overexpressing EphA2 may be repulsed or stopped in track upon contact with ephrin-A1-presenting basal layer cells; loss of EphA2 may render invading cells less responsive to the repulsive effects and facilitate tumor invasion.

In sum, we have found that EphA2 is a novel tumor suppressor gene in mammalian skin. Caution may need to be exercised in therapeutic strategies designed to systemically down-regulate EphA2 from cell surfaces.

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Disruption of EphA2 Receptor Tyrosine Kinase Leads to Increased Susceptibility to Carcinogenesis in Mouse Skin


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